EFFECTS OF OCEAN ACIDIFICATION COMBINED WITH MULTIPLE STRESSORS ON EARLY LIFE STAGES OF THE PACIFIC PURPLE SEA URCHIN

By

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OCAD, Seneca College, 2001

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE
in
ENVIRONMENT AND MANAGEMENT

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March 2014

Abstract

Decreases in ocean pH through ocean acidification has shown to have direct negative impacts on the early life stages of the Pacific purple sea urchin, *Strongylocentrotus purpuratus.* Research has suggested that multiple stressors could exacerbate, cancel, or even alleviate the impacts of ocean acidification on echinoderms. This study assessed the combined effects of changes in pCO_2 concentrations (390, 800, 1500 ppm), salinities (28, 31, 34 ppt) and temperatures (12, 15, 18°C) on fertilization and larval development in *S. purpuratus*. Increased pCO_2 was the predominant stressor, with additive and antagonistic effects from temperature changes, and no effect from salinity changes. Stressor combinations significantly decreased the rate of normal larval development by 28 – 68%, whereas fertilization and larval survival were unaffected. The strong impact on normal larval development likely indicates that later development stages could be detrimentally affected and could influence the population dynamics of *S. purpuratus.*

Keywords
Ocean acidification, Pacific purple sea urchin, *Strongylocentrotus purpuratus*, fertilization, larval development, temperature, pH, salinity, multiple stressors

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Acknowledgements

I would like to acknowledge the following people, which, without their help, I would likely still be deliberating which topic to research. First, thank you to Dr. Karen Kohfeld for all of her guidance and assistance through this process and providing laboratory space to allow these experiments to become a reality. A special thank-you to Mrs. Janet Pickard and Dr. Curtis Eickhoff at Maxxam Analytics for their generosity in providing some much needed equipment and a holding space for the culture of sea urchins. Thanks to Carolyn Duckham for sharing her experience and providing guidance through the intricacies that are ocean acidification work, and making the overall process that much easier. Thank you to the members of the SFU COPE lab for providing a positive and welcoming atmosphere, specifically, Hafsa Salihue, who helped with some of the never-ending chemical analyses. Thank you to Dr. Chris Harley at UBC for generously providing necessary instrumentation for my DIC analyses, and to the number of graduate students in the Harley lab for their help. Finally, I would like to acknowledge my family and friends who have contributed to this thesis research through their undying support, love, and encouragement. I could not have done this without you.

Introduction

Absorption of anthropogenic atmospheric carbon dioxide (CO_2) by the oceans over the last 200 years has decreased the pH of the ocean, a phenomenon known as ocean acidification. Laboratory studies suggest that ocean acidification can have a direct and negative impact on the early life stages of many calcifying marine organisms in coastal water ecosystems, such as the Pacific purple sea urchin, *Strongylocentrotus purpuratus*. Larval development is delayed, growth and survival rates are reduced (Byrne, 2012; Doney, Fabry, Feely, & Kleypas, 2009; Kroeker, Kordas, Crim & Singh, 2010; Yu et al., 2011) along with their ability to feed, putting sea urchins at risk of increased predation (Byrne & Przeslawski, 2013). Predictions of an additional pH decrease of 0.3 to 0.4 units by the year 2100 (Caldeira & Wickett, 2005; Orr et al., 2005; Solomon et al., 2007), suggest ocean acidification is a global concern, which will continue to affect coastal ecosystems as atmospheric CO_2 levels continue to increase (Caldeira & Wickett, 2003; Solomon et al., 2007).

The majority of ocean acidification measurement and modelling studies have focused on the impacts of this phenomenon in open ocean (Doney et al., 2009; Feely et al., 2004; Feely et al., 2012; Zeebe & Westbroek, 2003). However, an increasing number of studies suggest that coastal areas and the organisms within those habitats will have greater detrimental effects caused by ocean acidification (Feely, Sabine, Hernandez-Ayon, Ianson & Hales, 2008; Feely et al, 2010; Johannessen & Macdonald, 2009; Wooton, Pfister & Forester, 2008). Coastal areas experience much higher variability in environmental conditions due to upwelled waters that are higher in pCO_2 concentration and lower in pH (Feely et al., 2008; Feely et al., 2010b). The

impacts of acidification are amplified in coastal areas, potentially increasing the impacts of multiple stressors.

The Pacific purple sea urchin is prevalent along the Pacific Coast of North America and is an important keystone species. Numerous studies have identified that the development of this urchin is negatively affected by high CO_2/low pH conditions (Dupont, Ortega-Martinez & Thorndyke, 2010b; O'Donnell, Hammond & Hofmann, 2009), while others have modeled the potential for the urchin to adapt to these future conditions (Kelly, Padilla-Gamino & Hofmann, 2013; Yu et al., 2011). However, few studies have examined whether these urchins will be able to develop normally or adapt to these changes in ocean chemistry when coupled with changes in other key parameters, such as temperature and salinity. As the ocean continues to respond to the continual increase in anthropogenic atmospheric CO_2 (R. Byrne, Mecking, Feely, & Liu, 2010) could the populations of Pacific purple sea urchins along the Pacific Coast become endangered before they are able to adapt to an ever-changing ocean? This study investigates the effects of a combination of multiple stressors (temperature, salinity, and CO_2) on two early life stages (fertilization and larval development) of the Pacific Purple sea urchin, *S. purpuratus*.

Background

The absorption of atmospheric CO_2 into the ocean is driven by air-sea gas exchange processes that are controlled, in part, by the difference in air-sea gas concentrations. Thus, as atmospheric CO_2 increases, it is more readily absorbed in seawater, altering seawater chemistry. The increase in aqueous CO_2 results in an increase of hydrogen ions (H^+), lowering the pH of the water, creating an acidic environment (Figure 1). This reaction reduces the

amount of available carbonate ions (CO_3^{-2}) available for calcium carbonate ($CaCO_3$) formation, which is required for calcifying organisms, such as the Pacific purple sea urchin, to form their shells, tests, and skeletons (Dickson et al., 2007). This reduction in ocean carbonate ion concentrations will continue as the level of anthropogenic CO_2 continues to increase and the sea surface continues to work towards maintaining equilibrium with CO_2 levels in the atmosphere (Doney et al., 2009; Solomon et al., 2007).

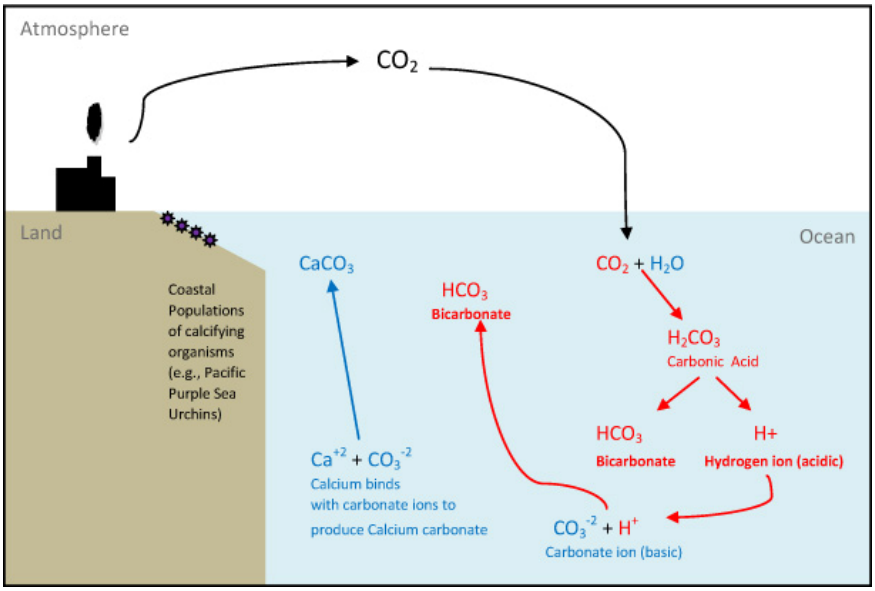

Figure 1. Diagram of Ocean Acidification Reaction.

Single stressor studies. Numerous studies show that ocean acidification resulting from the increase in anthropogenic atmospheric CO_2 concentration has detrimental effects on calcifying species in coastal regions. These studies include the effects of increased CO_2 concentration on the Pacific Purple sea urchin, *S. purpuratus* (Dupont et al., 2010b; O'Donnell, Hammond & Hofmann, 2009) as well as on various other species of sea urchins (Kurihara &

Shirayama, 2004; Reuter, Lotterhos, Crim, Thompson, & Harley, 2011; Yu, Matson, Martz, & Hofmann, 2011), abalone (Crim, Sunday, & Harley, 2011), bivalve molluscs (Gaylord et al., 2011; Gazeau et al., 2007; Gazeau et al., 2011), and coral (Kurihara, 2008). In contrast, some non-calcifying species are predicted to potentially thrive in future acidified environments due to their ability to adapt to the changes in the ocean environment (Somero, 2010), such as the keystone predator sea star, *Pisaster ochraceus*, shown to grow at a faster rate in acidified environments (Gooding, Harley & Tang, 2009). Varied responses to ocean acidification (Kroeker et al., 2010; Ries, Cohen & McCorkle, 2009) could further complicate predator-prey interactions. Specifically for *S. purpuratus*, delayed larval development in acidified environments combined with their reduced ability to feed and an increase in competition for food and carbonate (Allen, 2008) puts sea urchins at risk to increased predation (Byrne & Przeslawski, 2013; Pechenik, 1999).

A number of single-stressor temperature studies have assessed that the early larval development on *S. purpuratus* is negatively affected by an increase in temperature (Diaz-Perez & Carpizo-Ituarte, 2011; Runcie et al., 2012). Further, Allen and Pechenik (2010) found that a decrease in seawater salinity could reduce the fertilization success of the echinoderm, *Echinarachnius parma.* In fact, some studies suggest that increases in ambient temperature (i.e., due to ocean warming) may have a greater potential to affect some species more so than the predicted decrease in pH due to acidification (Byrne, 2012; Ericson et al., 2012).

Multiple stressor studies. Oceanic events along the North American Pacific coast, such as upwelling (Feely et al., 2008), temperature increase (Baumann & Doherty, 2013;

Johannessen & Macdonald, 2009), and coastal pH fluctuations (Wooton, Pfister & Forester, 2008; Yu et al., 2011), may have the potential to have compounding effects on marine organisms already affected by climate change and ocean acidification (Byrne, 2012; Feely, Doney & Cooley, 2009). The combination of multiple stressors in acidified environments has been proposed to amplify effects on organisms within coastal ecosystems (Byrne & Przeslawski, 2013; Feely et al., 2009; Harley, 2011; Harley et al., 2006; Wooton et al., 2008). However, the extent of those effects is relatively unknown. The investigation of cumulative or interactive effects of multiple stressors is growing in importance (Byrne, 2012; Crain, Kroeker, & Halpern, 2008; Harley et al., 2006), as is the recognition that the combination of stressors can produce additive, synergistic or antagonistic responses (Byrne& Przeslawski, 2013; Crain et al., 2008). Various studies of the effects of multiple ocean acidification stressors have been published (Byrne et al., 2009; Byrne & Przeslawki, 2013; Ericson et al., 2012). However, the effects of multiple stressors on sea urchins, specifically the Pacific Purple sea urchin, *S. purpuratus*, remain poorly constrained.

Life stage sensitivities. The fertilization life stage of some groups of marine invertebrates may not be as vulnerable to acidification conditions as the larval development life stage (Byrne, Soars, Selvakumaraswamy, Dworjanyn & Davis, 2010; Kroeker et al., 2010; Ross, Parker, O'Connor & Bailey, 2011). Therefore, analyzing the effects on each early life stage with an understanding of the potential for a "developmental domino effect" (Ericson et al., 2012) is necessary. However, multiple-generation studies on Pacific Purple sea urchins are not as feasible, given the long timeframe before purple urchins reach sexual maturity (Byrne &

Przeslawki, 2013). Examining physiological responses and gene expression of sea urchins (*S. purpuratus*, *Lytichinus pictus*) exposed to high CO_2/low pH conditions may allow scientists to discover whether these species may be able to adapt to predicted future conditions (Hofmann & Todgham, 2010; O'Donnell et al., 2010; Todgham & Hofmann, 2009; Yu et al., 2011). The effects of ocean acidification on individual species and on ocean ecosystems is complex and therefore, must be examined with a holistic ecosystem approach incorporating effects of multiple stressors acting at once, rather than using a species-specific, single-stressor approach.

Description of Study

This goal of this research is to examine the effects of multiple environmental stressors (temperature, salinity, and CO_2) on two integral early life stages (fertilization and larval development) of the Pacific purple sea urchin, *S. purpuratus*, a sea urchin indigenous to the Pacific coast of North America. Unfertilized eggs, sperm, and developing embryos have different sensitivities and, therefore, an investigation of these two early life stages may indicate if, and when, the Pacific purple sea urchin would be most sensitive, or more robust, to changes in their natural environment in a future acidified ocean.

The range of stressors used was chosen to reflect current and projected variability in temperature, salinity, and CO_2 along the Pacific coast of North America. Specifically, stressors were modelled after the variables found along the Southern British Columbia coast (Environment Canada, 2011; Feely et al., 2010; Ocean Networks Canada, 2013) compared to those found in the Southern California coast (McClatchie et al., 2009; Yu et al., 2011). For these experiments, natural seawater was manipulated to represent three salinities (28, 31, and 34

ppt), three temperatures (12°C, 15°C, and 18°C), and three CO_2 concentrations representing present day ambient concentrations (390 ppm), and two future predicted conditions (~900 and 1500 ppm). These manipulations resulted in a total of 27 treatments that were used to examine the effects of multiple stressors on two early life stages (fertilization and larval development) of *S. purpuratus*. These results may provide insight into the resilience of the populations of *S. purpuratus* to physical and chemical environmental changes expected to occur a long the North American Pacific coast in the future.

Methods

Two sets of 27 treatments were set up to assess the combined effect of multiple stressors (I.e., CO_2, temperature, and salinity) on two early life stages (I.e., fertilization and larval development) of Pacific purple sea urchins, *S. purpuratus*. In the urchin fertilization tests, urchin sperm and eggs were exposed to the 27 treatments at specific time intervals to determine the effects of the treatments on the fertilization success. In the urchin larval development tests, newly fertilized urchin embryos were exposed to the 27 treatments to determine the effects on the ability of the larvae to survive and develop normally.

Experiments were performed utilizing laboratory space in the Climate, Oceans, and Paleo-Environment (COPE) lab and in the Biology cold lab at Simon Fraser University (SFU), Burnaby, BC. Methods were adapted from Environment Canada (2011), US Environmental Protection Agency (1995) and American Society for Testing and Materials (1998) guidance for echinoid tests.

Experimental Setup

Treatments were prepared by manipulating the salinity, temperature, and CO_2 concentrations of natural seawater. Treatments consisted of a combination of three salinities (28, 31, and 34 ppt), three temperatures (12, 15, and 18°C), and three CO_2 concentrations (390, ~900, and 1500 ppm), to achieve a total of 27 treatments (Table 1). Each treatment was named according to its combination of manipulations. Shortened codes were used to indicate whether the salinity level in the treatment was low (LS), medium (MS), or high (HS). Similarly, codes

were used to indicate if the CO_2 concentration was low (LC), medium (MC), or high (HC). Therefore, a treatment with a combination of low salinity (28 ppt) and low CO_2 (390 ppm) was called "LS/LC". The medium salinity and low CO_2 combination was called "MS/LC" and so on (Table 1).

Table 1

Summary of Test Treatments

Treatment #	Treatment Name	Nominal Temperature (°C)	Nominal Salinity (ppt)	Nominal CO_2 Concentration (ppm)
1	LS/LC	15	28	390
2	MS/LC	15	31	390
3	HS/LC	15	34	390
4	LS/MC	15	28	800
5	MS/MC	15	31	800
6	HS/MC	15	34	800
7	LS/HC	15	28	1500
8	MS/HC	15	31	1500
9	HS/HC	15	34	1500
10	LS/LC	18	28	390
11	MS/LC	18	31	390
12	HS/LC	18	34	390
13	LS/MC	18	28	800
14	MS/MC	18	31	800
15	HS/MC	18	34	800
16	LS/HC	18	28	1500
17	MS/HC	18	31	1500
18	HS/HC	18	34	1500
19	LS/LC	12	28	390
20	MS/LC	12	31	390
21	HS/LC	12	34	390
22	LS/MC	12	28	800
23	MS/MC	12	31	800
24	HS/MC	12	34	800
25	LS/HC	12	28	1500
26	MS/HC	12	31	1500
27	HS/HC	12	34	1500

The low (390 ppm) and high (1500 ppm) CO_2 gas concentrations were balanced with air, pre-mixed and certified by Praxair. The medium CO_2 concentration was prepared by creating a mixture of both the high and low CO_2 gases, fed through a mixing bottle (Figure 3). A concentration of approximately 900 ppm was intended; actual CO_2 concentrations (calculated from dissolved inorganic carbon (DIC) measurements on day 4) ranged between 650 and 990 µatm (Table G1).

Due to space constraints, the 12, 15, and 18°C tests were performed on different dates. Therefore, one set of fertilization and larval development tests for each test temperature were conducted at three different times; the nine combinations of salinity and CO_2 were the same, but the temperature of the test treatments was varied (Figure 3, Table 2). Water baths (Julabo SW-20C and Thermo Scientific Precision 2841) were used to create and maintain the desired test temperature (within ±2°C). For the first set of experiments, the water baths were set at 15°C. For the second set of experiments, they were set at 18°C, and for the final set of experiments, they were set to 12°C (Figure 3). The fertilization and the larval development tests for a given test temperature were set up in the same day.

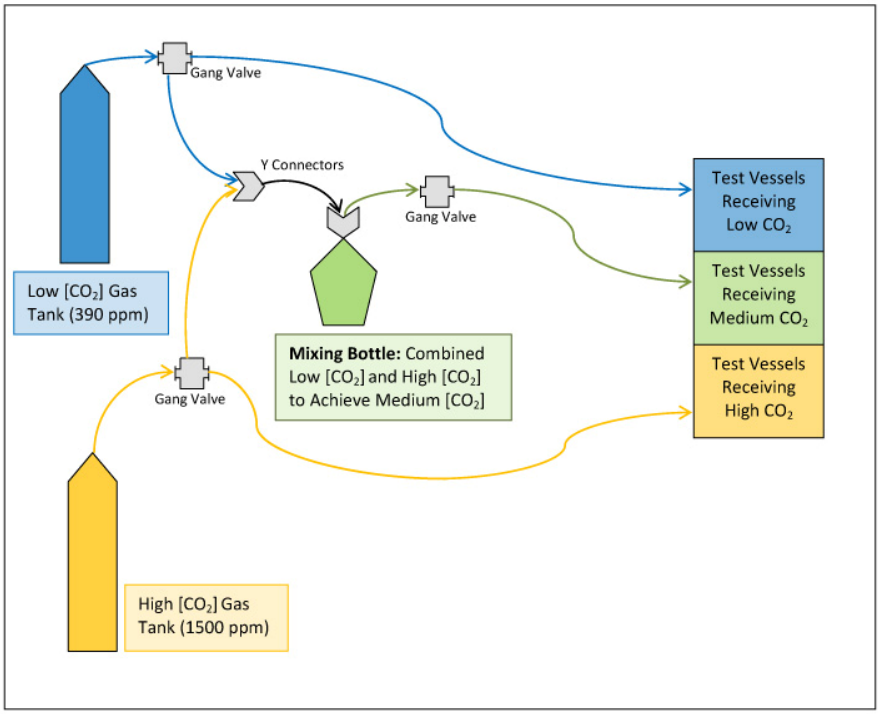

Figure 2. CO_2 tank and mixing bottle schematic. The medium CO_2 concentration was prepared by mixing the high and low CO_2 gases through a mixing bottle.

Table 2

Timeline and Duration of Tests

Experimental Procedure	Temperature (°C)	Test Initiation (Date & Time)	Test Completion (Date & Time)	Total Test Duration
Larval Development	15	2013 February 26 19:38	2013 March 02 20:20	96.7 hours
Fertilization	15	2013 February 26 20:00	2013 February 26 20:20	20 minutes
Larval Development	18	2013 March 11 18:48	2013 March 15 20:25	97.6 hours
Fertilization	18	2013 March 11 19:15	2013 March 11 19:35	20 minutes
Larval Development	12	2013 March 18 19:30	2013 March 22 20:16	96.8 hours
Fertilization	12	2013 March 18 19:40	2013 March 18 20:00	20 minutes

OECD principles of Good Laboratory Practice (OECD, 2003) were utilized for the performance of the study, whenever possible and applicable. A detailed record of the procedures used, including the materials and reagents and corresponding lot numbers or serial numbers, were documented. This approach was used to allow for traceability and replication of the study, as well as ensuring a level of quality and transparency for the analysis of the results. A brief timeline of tasks performed for each set of tests is included in Appendix A, and a summary of test conditions for the fertilization and larval development tests is included in Appendix B.

Treatment Preparation. Approximately 60L of seawater (sourced from the Vancouver Aquarium) were obtained (on-tap) from the SFU Biology cold room, filtered through a 1 µm filter, and then allowed to aerate overnight with oil-free compressed air at room temperature (14.6 ± 1.5°C, Appendix C) before use.

Physicochemical measurements for water quality including temperature, dissolved oxygen, salinity, and pH, were performed on the seawater prior to and after the preparation of treatments, and frequently monitored throughout the test. Dissolved oxygen and temperature readings were measured using a YSI 52 CE Dissolved Oxygen Meter with 0.01 mg/L dissolved oxygen precision, and 0.1°C temperature precision. Salinity readings were measured using a WTW LF330 conductivity meter with 0.1 ppt precision reading. The pH readings were taken using an Accumet Basic pH meter with ±0.01 precision. All instruments were calibrated prior to use during the experiments.

Three 8 L aliquots of seawater were adjusted to the desired test temperature by use of submerged ice packs or a heat stick. The salinity of the seawater in each of the three aliquots was adjusted to the desired salinity (28, 31, and 34 ppt) using 90‰ hypersaline brine (Environment Canada, 2011; USEPA, 1995), or deionized water. A calculation to determine the volume of 90‰ hypersaline brine required to adjust an 8 L batch of seawater to the desired salinity was performed using Equation 1.

$$\text{Volume of brine required} = \frac{(\text{Desired salinity} - \text{Actual salinity}) \times (\text{Total volume})}{(\text{Brine salinity} - \text{Desired salinity})} \quad (1)$$

Each of the three salinity treatments was then further divided and transferred into three 2 L glass jars. Each of these nine 2 L treatment jars was then aerated with either the low, medium, or the high CO_2 concentrations. CO_2 was delivered to each preparation jar through borosilicate glass pipettes, regulated by gang valves to an aeration rate of ~44 ml/min (Figure 2). Each treatment preparation jar was sealed with Parafilm® and aerated overnight in water baths to allow the seawater treatments to equilibrate at the chosen test temperature (Figure 3).

(a) 15°C Test (Round 1)

CO_2 Treatments	*28 ppt*	*31 ppt*	*34 ppt*
390 ppm	LS/LC	MS/LC	HS/LC
900 ppm	LS/MC	MS/ MC	HS/MC
1500 ppm	LS/HC	MS/HC	HS/HC

(b) 18°C Test (Round 2)

CO_2 Treatments	*28 ppt*	*31 ppt*	*34 ppt*
390 ppm	LS/LC	MS/LC	HS/LC
900 ppm	LS/MC	MS/ MC	HS/MC
1500 ppm	LS/HC	MS/HC	HS/HC

(c) 12°C Test (Round 3)

CO_2 Treatments	*28 ppt*	*31 ppt*	*34 ppt*
390 ppm	LS/LC	MS/LC	HS/LC
900 ppm	LS/MC	MS/ MC	HS/MC
1500 ppm	LS/HC	MS/HC	HS/HC

Figure 3. Test Treatment Schematic**.** Nine treatments consisting of combinations of salinity and CO_2 stressors were set up at (a) 15°C (Round 1), (b) 18°C (Round 2) and (c) 12°C (Round 3), for a total of 27 treatments.

Following the 24-hour aeration period, the nine treatment solutions (e.g., Figure 3a) were siphoned out of the treatment preparation jars, using Teflon tubing submerged into the middle of the water column, and divided between test vessels and bottles for chemical analysis (Appendix D). After the first approximately 50 - 100 ml was discarded, the next ~950 ml of test solution was allowed to run into three 350 ml glass jars for the larval development tests (and aliquots for fertilization test), with the remaining ~950 ml used for chemical analyses and water quality (temperature, dissolved oxygen, salinity, and pH) (Appendix D).

For chemical analyses, the solution was allowed to run into one 100 ml glass jar for initial water quality measurements, one 300 ml glass biological oxygen demand (BOD) bottle

and one 40 ml amber glass vial for DIC analyses (for backup readings, if needed), and one 100 ml raw plastic bottle for total phosphate (TP) and total silicate (TS) analyses. The DIC subsamples were immediately preserved with a 5% aqueous solution of Mercuric chloride ($HgCl_2$) (Dickson, Sabine, and Christian, 2007) and refrigerated for future measurement in the Harley Laboratory for the Study of Coastal Marine Ecology and Impacts of Climate Change (University of British Columbia).

Organisms, Spawning, and Gamete Collection

Specimens of the Pacific purple sea urchin *S. purpuratus* were obtained from an organism supplier in San Marcos, California where they were field collected from Mission Bay, California. Upon arrival at the Maxxam Analytics Laboratory, Burnaby, BC, urchins were acclimated in a 12°C temperature controlled room with 16 hr light : 8 hr dark photoperiod. The sea urchins were held in 35 L tanks containing filtered natural seawater, fitted with a circulating pump filter. The urchins were gradually acclimated to artificial salt water, received frequent water renewals, and were fed a steady diet of carrots and seaweed. A selection of approximately 12 urchins were transported in coolers to the Biology cold laboratory at Simon Fraser University on test initiation days (Day 0) and held below 13 °C until needed for testing, to ensure the urchins would not spawn during the holding time.

Approximately 12 urchins were placed aboral side down on 100 ml jars filled to the brim with filtered, unadjusted seawater, and allowed to equilibrate. Urchins were induced to spawn using the wet-spawning method (Environment Canada, 2011). Each urchin was injected with 1 ml of 0.5M Potassium chloride (KCl) using a syringe into the coelom, through the peristomial

membrane beside aristotle's lantern, and angled toward the outer shell (Environment Canada, 2011). Once they began spawning, they were sorted based upon their sex.

Gamete quality was assessed and screened before use in tests. Small aliquots of the gametes shed from each urchin was examined at 40x magnification by placing one drop of the suspension onto one of two Petri dishes, one for males, one for females. Eggs were screened by observing shape and maturity. Sperm were assessed by motility, based upon comparison of motility to each other. The eggs were also screened for their ability to fertilize normally by adding one drop of sperm to each of the drops of eggs. Any females whose eggs showed abnormalities or slow fertilization were not used in the test. Gametes of good quality were pooled; eggs were pooled in a 25 ml glass graduated cylinder and sperm were pooled into a separate 100 ml glass graduated cylinder. The two cylinders of pooled gametes were aerated very gently through borosilicate glass pipettes, to preserve viability until use.

Experiment 1: Fertilization test

Fertilization test replicate preparation. The nine treatments (E.g., Figure 3a) used for the fertilization tests were the same as those used for the larval development tests. For each of the treatments prepared, five 5 ml aliquots were transferred into five replicate 20 ml borosilicate glass test tubes. Test tubes were organized in test tube racks in groups based upon CO_2 treatment and covered with Parafilm®. An additional airline from the appropriate CO_2 treatment was inserted into the air space between the test tubes and the Parafilm®, in an effort to create a CO_2 atmosphere and reduce the potential of the CO_2 treatments to outgas back to

ambient concentration. The test tube racks were placed in a water bath set to the desired test temperature prior to and for the duration of the 20-minute fertilization test.

Gamete preparation. An aliquot of the pooled eggs was transferred to a new 25 ml graduated cylinder and diluted with filtered, unadjusted seawater to obtain a density of approximately 200 eggs/10 µl. Four 10 µl aliquots of the new egg suspension were counted microscopically to estimate the average density.

To determine the density of the concentrated pooled sperm, a 1:100 sperm suspension was prepared using deionized water[1]. Two 10 µl aliquots of the sperm suspension were counted microscopically using a Haemocytomer, and sperm density was estimated using Equation 2 (Environment Canada, 2011). The estimated sperm density was used to calculate the volume needed to achieve the required sperm:egg ratio for the fertilization test.

$$\#\text{sperm} \cdot \text{ml}^{-1} = \frac{(\text{dilution})(\text{count})(\text{haemocytometer conversion factor})(\text{mm}^3 \cdot \text{ml}^{-1})}{\text{Number of squares counted}} \quad (2)$$

Pre-test fertilization trials. Prior to initiating the 20-minute fertilization test, a fertilization trial was performed in which ten sperm dilutions producing sperm:egg ratios between 1.2:1 to 600:1 were tested to determine which sperm density concentration would achieve >80% and <98% fertilization (Environment Canada, 2011). Counts of the first 100 eggs

[1] Guidance for the preparation of a 1:100 sperm suspension for determining initial density describes using 10% Glacial Acetic Acid; this modification to the method substituted deionized water for glacial acetic acid, and was tested prior to these tests with favourable results. The sperm were immobilized to facilitate counting without the use of a chemical.

for each dilution determined that a sperm: egg ratio of 75:1 provided the optimum density, and this ratio was used for all the fertilization tests conducted in the 27 treatments (Figure 3).

Test initiation and completion. A new sperm suspension was prepared by transferring an aliquot of concentrated sperm to a clean graduated cylinder, diluting with filtered seawater, and transferring the thoroughly mixed suspension to a 20 ml scintillation vial. The sperm suspension was mixed gently throughout the seeding process to ensure homogeneous density before filling the pipette tip. The sperm suspension was pipetted into each test tube (five replicate test tubes per treatment) in 50 µl aliquots using an Eppendorf Repeater® Plus pipette. After all test tubes were seeded, they were swirled gently to mix the sperm into the treatment and allowed to stand for 10 minutes. Then, 50 µl aliquots of the egg suspension were added to each test tube in the same order and at the same rate that the sperm was added, resulting in an egg density of 170 – 181 ml/test tube. Test tubes were swirled once again to allow the sperm and eggs to mix and allowed to stand and fertilize for an additional 10 minutes. At the 20-minute time point, each test tube was preserved with 1 ml of 10% neutral buffered formalin in the same order and at the same rate as the sperm and the eggs. Each test tube was swirled gently to ensure complete preservation. Test tubes were covered and stored at 4 °C until microscopic examination. The materials providing the CO_2 atmosphere over the test tubes were removed to facilitate the seeding process but were returned during standing times.

Experiment 2: Larval Development test

Development test replicate preparation. The development test vessels (350 ml glass jars) containing prepared treatments were placed into the water baths and aerated prior to test

initiation and until test completion at a reduced rate of approximately <100 bubbles/min (USEPA, 1995), by visual inspection. This gentle aeration was maintained through micro-bore airlines that were fed into the test vessels through a small hole in the 58 mm lids. In order to facilitate the CO_2 aeration into each of the development test treatments, test vessels were placed in practical groupings and a randomization pattern was not used. Two of the three treatment replicates (replicates A and B) were designated for the final analysis of larval survival and development; the last of the three replicates (replicate C) was designated for water quality and monitoring of test progress, so as not to compromise the growth and development of the larvae in the first two replicates.

Gamete preparation. Embryos were prepared for the larval development test by adding an aliquot of the concentrated, pooled sperm to an aliquot of the pooled eggs. After the confirmation of fertilization, the eggs were aerated gently and allowed to develop further into the 2-cell to 8-cell embryo stage (ASTM, 1998). The density of the embryo suspension was adjusted to approximately 60-80 embryos/10 µl aliquot. The embryo suspension was gently re-suspended with a Plexiglas plunger immediately prior to seeding to ensure consistent embryo density due to rapid embryo settling. Each 350 ml development test vessel was seeded with 1 ml of embryo suspension using an Eppendorf Repeater® Plus pipette, resulting in an embryo density of 22 – 24 embryos/ml per test vessel. Test vessels were covered with lids, micro-bore airlines replaced, and aeration briefly monitored to confirm even CO_2 flow rates. The embryos were not fed during the ~ 96-hour test, and no water renewals were performed.

To estimate the initial density of embryos in each test vessel at test initiation, and to calculate embryo survival at test completion, two extra test vessels containing unadjusted, filtered seawater were prepared and seeded in the same manner as the treatment test vessels. After seeding, seawater was mixed gently to ensure a homogenous density of embryos throughout the water column, and three 10 ml aliquots were removed from each of the test vessels and transferred into 20 ml borosilicate glass test tubes. Each test tube was immediately preserved with 2 ml of 10% neutral buffered formalin. The total number of embryos in each replicate was counted microscopically, and the mean number of embryos per replicate was calculated.

Daily maintenance. The larval development tests were monitored daily to maintain consistent water bath temperature and consistent flow rates of CO_2 into each test vessel. The pH in each test treatment was monitored daily (Appendix E) in one designated replicate (replicate C). Further, the development of the embryos was monitored by both visual inspection and microscopic observations of selected treatments, in at least the low and the high CO_2 treatments. Embryo development was assessed as normal or abnormal according to their known development stages (ASTM, 1998).

Test completion. The tests were concluded near the 96-hour time point (Table 2). Three 10 ml aliquots were removed from two replicates in each treatment and transferred to preservation vials (for a total of six vials per treatment). Each preservation vial was preserved with 2 ml of 10% neutral buffered formalin, sealed with a lid, and stored at 4°C.

A final set of water quality measurements (I.e., temperature, salinity, pH, and dissolved oxygen concentrations) was taken from the third test replicate jar (replicate C; Appendix E). Final chemical analyses (i.e., DIC, TP, TS) were measured in aliquots obtained from the two test replicate jars (replicates A and B). For TP and TS, the subsamples collected at the end of the 12°C and 18°C tests were poured through 100 µm mesh to remove embryos from the test solution to reduce possible interference in the spectrophotometer readings.

Biological Endpoint Measurements and Statistical Analyses

Fertilization tests. At the conclusion of the fertilization tests, percent fertilization success was estimated as the total number of fertilized eggs out of the first 100 eggs counted from a 1 ml aliquot taken from each treatment. Eggs were examined in a Sedgewick-Rafter cell using an inverted microscope at 40X magnification. Eggs with a complete or partially raised fertilization membrane were scored as fertilized (Figure 4a). If no membrane was present, the egg was scored as not fertilized (Figure 4b). A total of five replicates per treatment were counted and the means and standard deviations calculated.

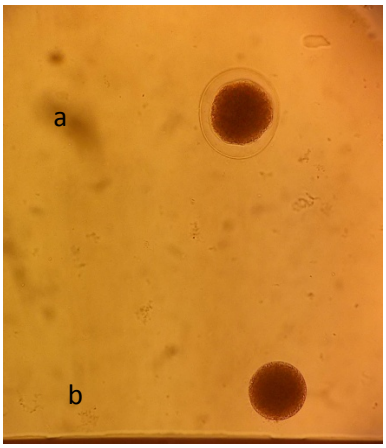

Figure 4. Photograph of Pacific purple sea urchin eggs. Microscopic image of (a) fertilized egg with raised fertilization membrane; (b) unfertilized egg.

Larval development tests. For the larval development tests, the percent normal larval development and percent survival of embryos from each treatment were estimated microscopically using an inverted microscope at 40X magnification. Embryos that had reached the 4-armed pluteus stage with four well defined arms or with two well defined arms and the second pair beginning to develop (PSEP, 1995) were considered to have developed normally (Figures 5d). Embryos that had developed only to the gastrulation (Figure 5c) or prism stage or showed obvious evidence of deformation, were considered abnormal. Any unfertilized eggs were excluded from analyses and the total count (USEPA, 1995). The total number of embryos in each replicate at test completion was scored. A total of six replicates per treatment were counted to determine means and standard deviations. The mean percent survival was calculated by dividing the total number of embryos counted (normal and abnormal combined) by the estimated number of larvae added to each test vessel at test initiation (preserved counts from Day 0).

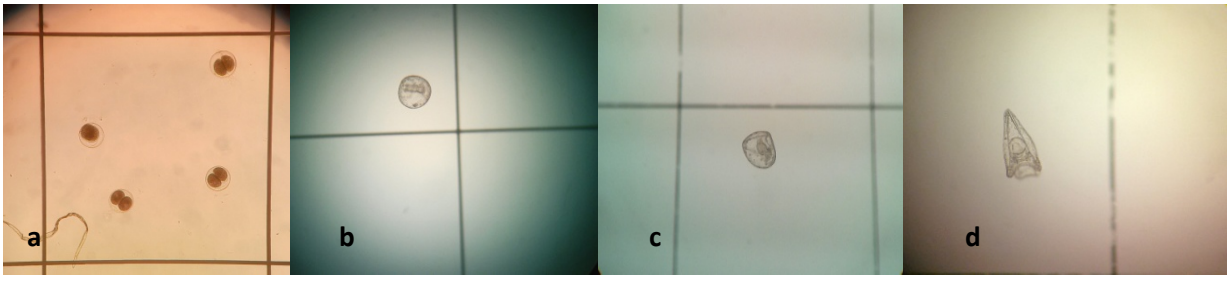

Figure 5. Photographs of stages of larval development. Microscopic images of early life stages of Pacific Purple sea urchin larval development (a) Fertilized sea urchin eggs and early stage of development (cell division); (b) Early stage of normal embryo development (blastulation); (c) Early stage of normal embryo development (gastrulation); (d) Normal embryo development (4-armed pluteus).

Data were assessed for normality and for homogeneity of variance prior to testing for analysis of variation (ANOVA). Parametric analyses of data against the LS/LC treatments were performed using Dunnett's Multiple Comparison Test. Lastly, biological results (fertilization, larval development, and larval survival) were plotted graphically against measured and calculated chemical parameters to demonstrate the relationship between them (Figures 6 to 10).

Chemical Analyses

Data obtained from chemical analyses were compiled into Excel spreadsheets (Appendix F). Methods for chemical analyses were adapted from Hansen and Koroleff (1999) and Dickson et al., (2007). To analyze samples for TP and TS, an aliquot of each treatment (25 ml) was transferred into a pre-weighed falcon tube, processed using various reagents, and allowed to develop. Each processed treatment was then analyzed for absorbance using a Hach DR 5000™ UV-Vis spectrophotometer against a salinity blank (NaCl solution at 31 ppt salinity). The treatments processed for phosphate analyses were read using a wavelength of 880 nm, whereas the treatments processed for silicate analyses were read using a wavelength of 810 nm. The concentration of TP and TS in each sample was calculated by comparing the absorbance readings against calibration curves created from known standard concentrations.

The DIC analyses were performed using the Apollo SciTech Dissolved Inorganic Carbon Analyzer (Model AS-C3) instrument with LI-7000 solid-state infrared CO_2 detector, at the Harley Laboratory (UBC). A small volume (0.75 ml) of each preserved DIC subsample from each treatment was combined with 1.0 ml of DIC acid (10% v/v phosphoric acid containing 10% w/v NaCl) inside the instrument while medical grade nitrogen gas flowed through the instrument to maintain a flow rate of approximately 302 ml/min. A minimum of three replicate readings were performed for each treatment and the resulting values for area under the curve produced by the instrument program were averaged. The concentration of DIC in each treatment was calculated by comparing the average area under the curve against a calibration curve created using a seawater standard with known DIC concentration, obtained from the Marine Physical Laboratory at the Scripps Institution of Oceanography, La Jolla, California.

Measured values of TP, TS, and DIC, salinity, temperature, and pH were used to calculate the state of the carbonate system in the seawater treatments. Specifically, estimates of total carbonate ion concentration ($[CO_3^{-2}]$), CO_2 partial pressure (pCO_2), and saturation state of aragonite ($\mathbf{\Omega}_{Ar}$) were calculated using the executable Microsoft Excel file, CO2SYS (Pierrot, Lewis, & Wallace, 2006). Calculated results for day 0 (fertilization) and day 4 (development) are outlined in Appendix G.

Results

Fertilization Success

In the 12°C and 18°C test experiments, little or no differences in mean percent fertilization rates were observed in treatments across the full range of salinity and CO_2 concentrations (Table 3). However, in the 15°C test the mean percent fertilization rate (±SD) in all of the high CO_2 (HC) treatments were substantially lower than the mean fertilization rate of 93.4% (±2.3) measured for the 15°C LC/LS treatment (Table 3). The difference between the 15°C LS/LC and all of the 15°C HC treatments was statistically significant (p = < .0001). In addition, the high salinity treatment combined with both medium CO_2 (HS/MC, p = .0013) and low CO_2 (HS/LC, p = .0433) were statistically different from the LS/LC treatment. There was no statistically significant difference between the LS/LC and the remaining treatments at 15°C.

Overall, the mean percent fertilization was highest in the 12°C test. A slight decrease in mean percent fertilization was seen in the 18°C test, with the lowest mean percent fertilization for all treatments tested at the 15°C temperature (Figures 6a, 7a).

Table 3

Summary of Mean Percent Fertilization Success (±SD)

Salinity Treatment	Carbon Treatment		
	Low Carbon (~390 ppm)	Medium Carbon (~800 ppm)	High Carbon (~1500 ppm)
12°C			
Low Salinity (28 ppt)	94.8 (1.6)	96.8 (0.8)	95.2 (2.9)
Medium Salinity (31 ppt)	97.6 (0.5)	97.2 (1.9)	95.0 (1.2)
High Salinity (34 ppt)	95.8 (1.3)	95.6 (2.5)	94.2 (4.0)
15°C [1,2]			
Low Salinity (28 ppt)	93.4 (2.3)	92.6 (2.3)	16.6 (7.1)***
Medium Salinity (31 ppt)	91.2 (2.6)	86.4 (9.0)	56.6 (10.3)***
High Salinity (34 ppt)	82.8 (6.0)*	75.8 (1.3)**	61.6 (20.5)***
18°C			
Low Salinity (28 ppt)	96.4 (1.8)	93.2 (3.3)	90.8 (3.8)**
Medium Salinity (31 ppt)	93.8 (1.9)	94.5 (1.7)	94.2 (2.2)
High Salinity (34 ppt)	93.6 (2.3)	93.8 (2.2)	92.2 (4.0)*

Note: Result values were compared to LS/LC values for the same temperature, using Dunnett's Multiple Comparison Test

*$p < .05$, **$p < .01$, ***$p < .001$

[1] Results did not meet the assumptions of normality using Shapiro-Wilk's Normality Test

[2] Results did not meet the assumptions of homogeneity using Bartlett Equality of Variance Test

Larval Development and Survival

Normality. The mean percent normality (i.e., the percentage of larvae showing normal development to the 4-armed pluteus stage at test completion) was distinctly lower in the MC and HC treatments compared to the LS/LC treatment for each test temperature (12, 15, and 18°C) (Table 5, Figure 9). The greatest overall differences in normality between the HC and LC treatments was seen in the 18°C test in which average percent normality in the HC treatments (LS/HC, MS/HC, and HS/HC) was 67.7% lower than the average percent normality in the LC treatments (LS/LC, MS/LC, HS/LC). For comparison, the average percent normality in the HC

treatments at 12°C and 15°C were 45.3% and 40.7% lower, respectively, than the average percent normality in the LC treatments at the same temperatures. The MC and HC treatments at each test temperature showed statistically significant difference to the LS/LC at each temperature (p = < .0001, and p = .0006 for HS/MC at 12°C), with the exception of the LS/MC treatment at 15°C. Overall, the percent normality across all treatments was lowest in the 15°C test; however, temperature did not appear to cause a trending effect on normality (Figure 7b). No relationship was found between normality and salinity (Figure 6b).

Table 4

Summary of Mean Percent Normal Larval Development (±SD)

Salinity Treatment	Carbon Treatment		
	Low Carbon (~390 ppm)	Medium Carbon (~800 ppm)	High Carbon (~1500 ppm)
12°C			
Low Salinity (28 ppt)	68.2 (9.7)	34.3 (5.8)***	22.5 (3.4)***
Medium Salinity (31 ppt)	69.8 (5.4)	37.3 (6.0)***	24.7 (3.8)***
High Salinity (34 ppt)	71.3 (5.0)	53.9 (5.1)***	26.3 (7.8)***
15°C [1]			
Low Salinity (28 ppt)	52.3 (4.4)	51.0 (4.0)	12.0 (3.8)***
Medium Salinity (31 ppt)	47.3 (3.0)	40.6 (5.3)***	11.9 (2.6)***
High Salinity (34 ppt)	49.5 (3.2)	36.4 (6.3)***	3.2 (1.0)***
18°C			
Low Salinity (28 ppt)	81.3 (5.7)	42.7 (8.1)***	6.1 (1.7)***
Medium Salinity (31 ppt)	74.8 (3.9)	56.7 (4.9)***	8.9 (1.9)***
High Salinity (34 ppt)	77.0 (3.5)	34.0 (5.2)***	14.9 (4.6)***

Note: Result values were compared to LS/LC values for the same temperature, using Dunnett's Multiple Comparison Test

*p < .05, **p < .01, ***p <.001

[1] Results did not meet the assumptions of normality using Shapiro-Wilk's Normality Test

Larval Survival. There was no distinct relationship between larval survival and salinity or CO_2 concentrations, although there was a distinct difference in overall survival between test

temperatures (Table 5). The 15°C test yielded the highest percentage of larval survival (Figure 7c, 10b) despite having the lowest overall normal development (Table 4), compared to the other test temperatures.

There was no statistical difference in survival throughout the 12°C test, and the majority of treatments in the 15°C and 18°C tests. In the 18°C test, the LS/MC (p = .0002), HS/MC (p = .0396), and HS/HC (p = .0473) treatments were all statistically different than the 18°C LS/LC with 17.2%, 9.7%, and 9.4% lower survival, respectively.

Table 5

Summary of Mean Percent Larval Survival (±SD)

Salinity Treatment	Carbon Treatment		
	Low Carbon (~390 ppm)	Medium Carbon (~800 ppm)	High Carbon (~1500 ppm)
12°C			
Low Salinity (28 ppt)	59.5 (5.8)	64.5 (6.1)	69.5 (7.3)
Medium Salinity (31 ppt)	61.7 (3.0)	66.2 (7.1)	71.6 (4.7)
High Salinity (34 ppt)	63.9 (8.7)	65.2 (6.6)	61.4 (7.4)
15°C			
Low Salinity (28 ppt)	86.9 (4.2)	82.7 (6.2)	85.6 (5.5)
Medium Salinity (31 ppt)	86.5 (3.6)	82.2 (2.2)	83.3 (3.9)
High Salinity (34 ppt)	77.5 (3.9)**	84.9 (4.8)	83.3 (2.8)
18°C			
Low Salinity (28 ppt)	66.2 (3.0)	49.0 (9.7)***	70.2 (10.0)
Medium Salinity (31 ppt)	73.5 (7.3)	60.1 (3.6)	68.3 (7.0)
High Salinity (34 ppt)	68.3 (4.1)	56.5 (6.1)*	56.8 (4.4)*

Note: Result values were compared to LS/LC values for the same temperature, using Dunnett's Multiple Comparison Test

*p < .05, **p < .01, ***p <.001

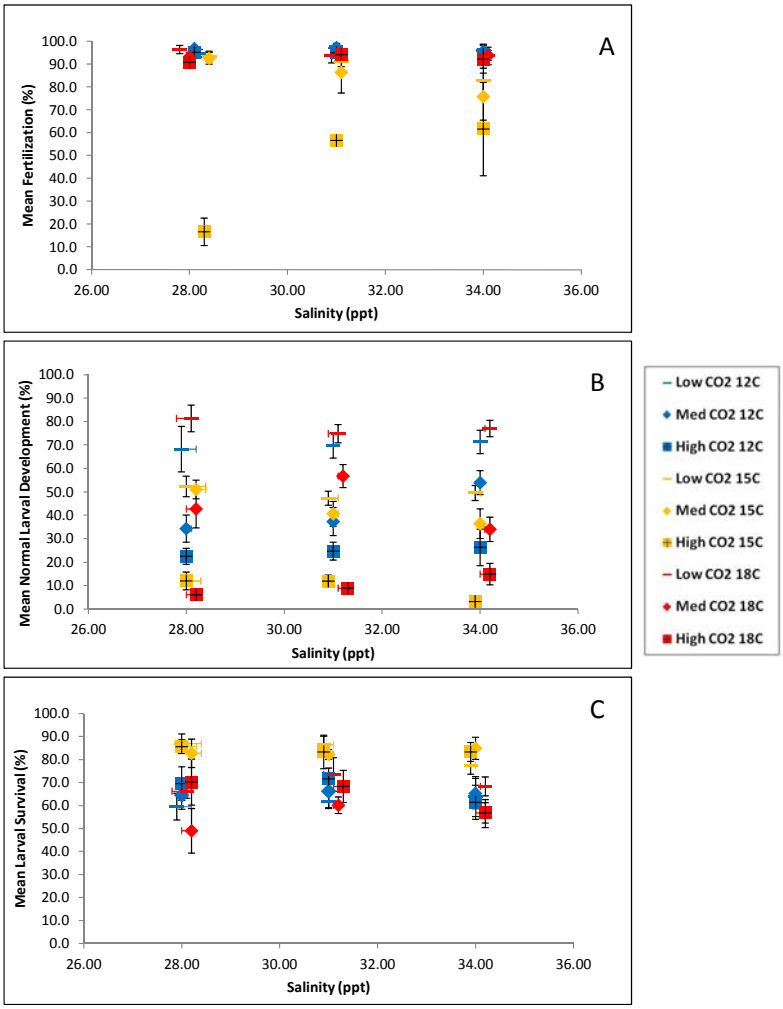

Figure 6. Biological endpoints vs. salinity for each treatment. Colours represent test temperatures, symbols represent CO_2 concentrations. (a) fertilization vs. day 0 salinity, (b) normal larval development vs. day 4 salinity, (c) larval survival vs. day 4 salinity. Vertical error bars represent standard deviations. The horizontal, one-sided error bars on Figures 6b and 6c represent the range in salinity values experienced throughout the experiment between day 0 and day 4, with the farthest end of the bar representing salinity on day 0.

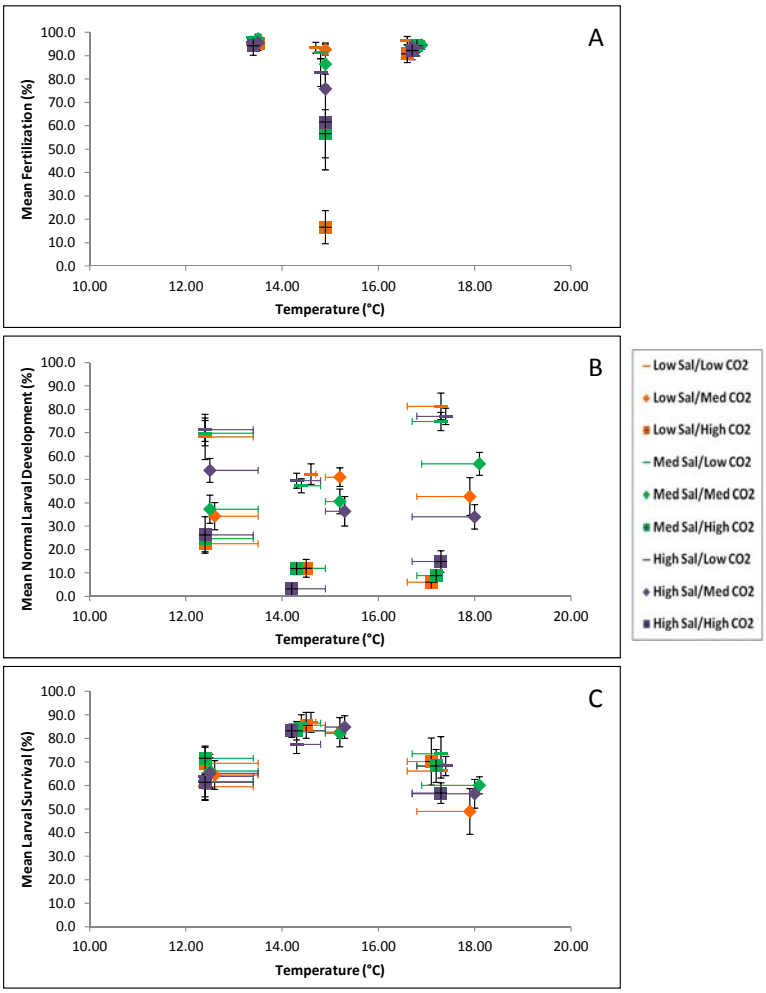

Figure 7. Biological endpoints vs. temperature for each treatment. Colours represent salinity, symbols represent CO_2 concentrations. (a) fertilization vs. day 0 temperature, (b) normal larval development vs. day 4 temperature, (c) larval survival vs. day 4 temperature. Vertical error bars represent standard deviations. The one-sided horizontal error bars on Figures 7b and 6c represent the range in temperatures experienced throughout the experiment between day 0 and day 4, with the farthest end of the bar representing temperatures on day 0.

Relationship Between Fertilization Rates and Carbonate Chemistry.

Graphical analysis of the fertilization results for each treatment against carbonate chemistry parameters (pH, pCO_2, CO_3^{-2}, ΩAr), did not demonstrate any strong relationships (Figure 8a-d). Fertilization tests were performed on Day 0 of the larval development test and lasted for a total of 20 minutes. Therefore, fertilization results were plotted against the day 0 parameters (Tables 8 to 10).

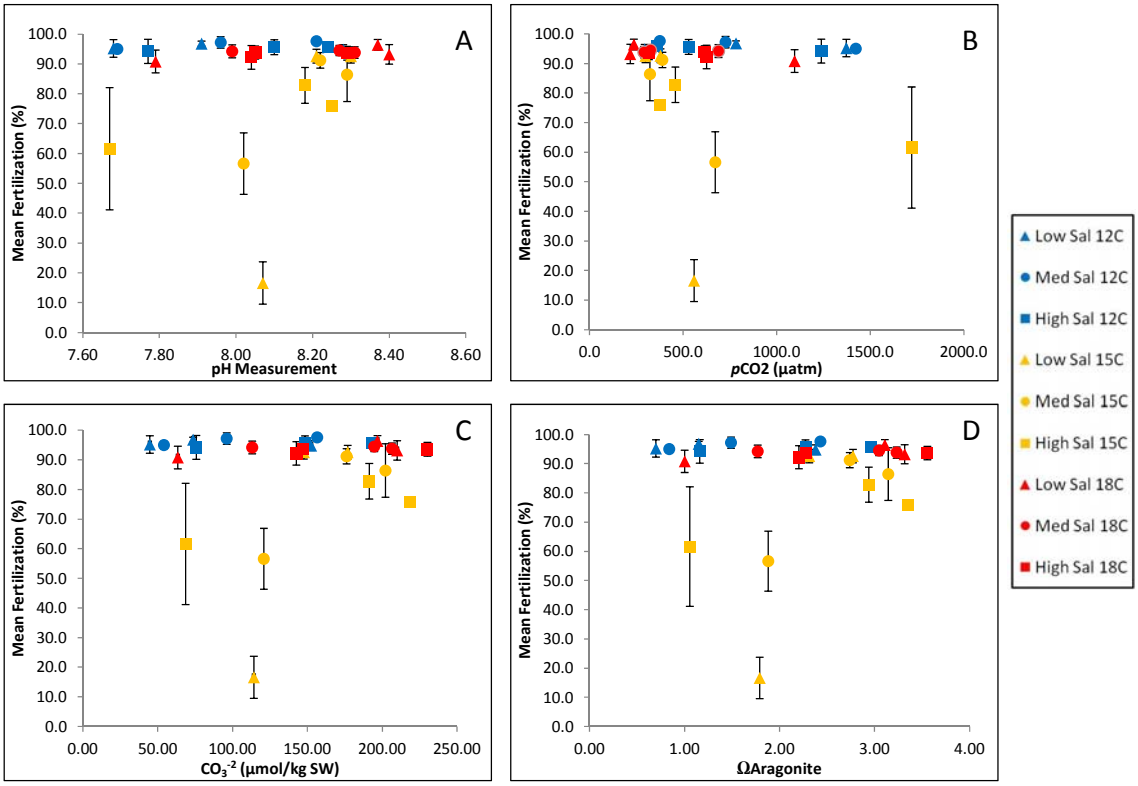

Figure 8. Mean percent fertilization vs. carbonate chemistry (Day 0 measurements) for each treatment (a) against pH, (b) against pCO_2, (c) against CO_3^{-2}, (d) against Ωaragonite. Error bars represent standard deviations (*n* = 5).

Relationship between Larval Development and Carbonate Chemistry.

Larval development results were assessed on day 4, and were therefore plotted against the day 4 parameters. However, there was some variation in the carbonate chemistry values between day 0 and day 4 (Tables 6 – 8), likely due to the constant aeration of test vessels for the duration of the experiments. For example, the 15°C and 18°C tests showed the most significant changes in pH, pCO_2, CO_3^{-2} and Ωaragonite between day 0 and 4. The *p*CO2 concentrations in the HC treatments on day 0 were much lower than the target of 1500 µatm, but had increased to the target by day 4 (Tables 7 & 8, Figure 9b, 10b). The average pH decrease from day 0 to day 4 for all test treatments was 0.18 (± 0.09). The greatest average pH decrease was measured in the 18°C test (0.25 ± .05), whereas the least average pH decrease was measured in the 12°C test (0.09 ± 0.06) (Table E2, Figure 7a, 9a). Similarly, the saturation of aragonite was generally higher on day 0 compared to day 4 (Tables 6 to 8, Figure 9d). One-sided horizontal error bars were added to graphical analyses to illustrate the range of conditions experienced by the larvae throughout the 96-hour exposure period.

Graphical analyses of the larval development results for each treatment against measured and calculated carbonate chemistry parameters (pH, pCO_2, CO_3^{-2}, ΩAr) demonstrated a significant relationship between the parameters and the development results (Figure 9). An approximately linear relationship was established between increasing pCO_2 concentrations and decreasing normal larval development (Figure 9b), formed by the results of the MC treatments. The linear relationship does not identify a particular tipping point; rather, it suggests a consistent and continual decline in normality, as the pCO_2 concentration continues to increase.

This is also the case for decreasing pH, CO_3^{-2} concentration, and aragonite saturation (Figures 9a, c, d). The average normal larval development in the MC treatments ranged from 28% to 33% lower than average ambient CO_2 in the 12°C and 18°C tests, corresponding to a 0.1 – 0.3 decrease from ambient pH, and pCO_2 concentrations in the range of 735– 982 µatm (Table G2).

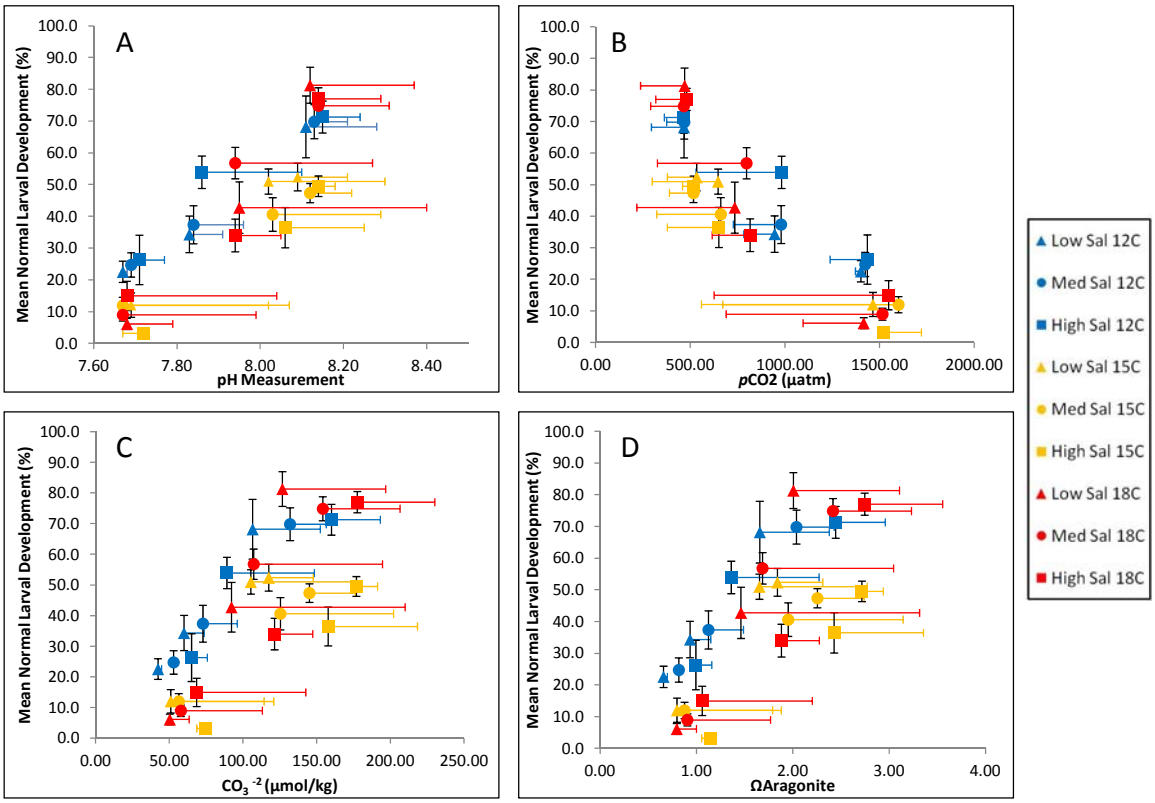

Figure 9. Mean percent normal larval development vs. carbonate chemistry (Day 4 measurements) for all treatments (a) against pH, (b) against pCO_2, (c) against CO_3^{-2}, (d) against Ωaragonite. The one-sided horizontal error bars represent the range in chemistry values experienced between day 0 and day 4. Vertical error bars represent standard deviations for larval development (n = 6).

Relationship between Larval Survival and Carbonate Chemistry.

Graphical analyses of the larval survival results for each treatment against measured and calculated carbonate chemistry parameters (pH, pCO_2, CO_3^{-2}, ΩAr) did not demonstrate a significant relationship, contrary to the larval development results (Figure 10). The graphical analyses for survival results were plotted against day 4 carbonate chemistry values and adjusted in the same manner as the larval development results, where horizontal error bars represent the day 0 parameters (Tables 6 to 8).

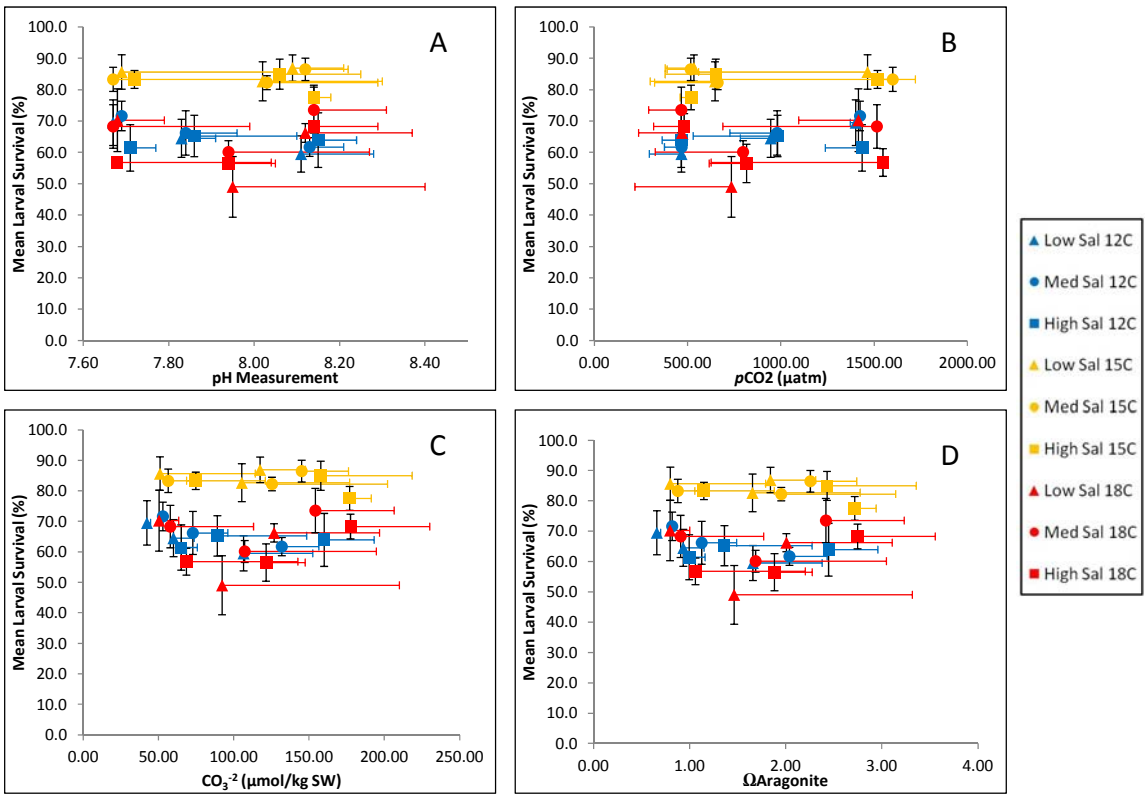

Figure 10. Mean percent larval survival vs. carbonate chemistry (Day 4 measurements) for all treatments (a) against pH, (b) against pCO_2, (c) against CO_3^{-2}, (d) against Ωaragonite. The one-sided horizontal error bars represent the range in chemistry values measured between day 0 and day 4. The plotted points (symbols) represent day 4; the farthest end of the bar represents conditions on day 0. Vertical error bars represent standard deviations for larval survival (n = 6).

Table 6

Summary of Measured and Calculated Parameters for 12°C Test Treatments

Parameters	Low CO_2 Treatments			Medium CO_2 Treatments			High CO_2 Treatments		
	LS/LC	MS/LC	HS/LC	LS/MC	MS/MC	HS/MC	LS/HC	MS/HC	HS/HC
				Day 0					
Temperature (°C)	13.4	13.4	13.4	13.5	13.5	13.5	13.5	13.4	13.4
Salinity (‰)	28.2	31.0	34.0	28.1	31.0	34.0	28.1	31.0	34.0
pH	8.28	8.21	8.24	7.91	7.96	8.10	7.68	7.69	7.77
Phosphate (µmol/kg)	0.31	0.27	0.36	0.18	0.22	0.52	0.13	0.35	0.40
Silicate (µmol/kg)	44.76	53.60	59.45	45.61	55.14	61.39	47.34	55.35	62.66
DIC (µmol/kg)	1950.6	2157.3	2310.2	2126.6	2284.6	2390.3	2186.9	2384.6	2557.4
pCO_2 (µatm)	294.5	375.4	362.8	782.9	726.8	529.6	1372.4	1423.1	1238.7
Alkalinity (µmol/kg)	2160.4	2368.2	2572.9	2201.5	2394.0	2582.3	2196.1	2405.7	2617.0
CO_3^{-2}µmol/kg)	152.39	156.52	193.16	73.59	96.05	148.32	44.73	54.12	75.73
HCO_3^- (µmol/kg)	1786.2	1985.7	2102.7	2021.2	2159.5	2221.2	2086.4	2273.4	2432.9
ΩAr	2.38	2.43	2.96	1.15	1.49	2.27	0.70	0.84	1.16
ΩCa	3.78	3.82	4.63	1.83	2.35	3.56	1.11	1.32	1.82
				Day 4					
Temperature (°C)	12.4	12.4	12.4	12.6	12.5	12.5	12.4	12.4	12.4
Salinity (‰)	27.9	31.0	34.0	28.0	31.0	34.0	28.0	31.0	34.0
pH	8.11	8.13	8.15	7.83	7.84	7.86	7.67	7.69	7.71
Phosphate (µmol/kg)	0.32	0.41	0.45	0.13	0.45	0.40	0.18	0.22	0.37
Silicate (µmol/kg)	42.24	59.89	60.60	44.76	53.87	61.26	45.17	55.35	63.23
DIC (µmol/kg)	2055.7	2234.3	2396.5	2148.9	2347.4	2528.3	2207.7	2413.8	2608.3
pCO_2 (µatm)	466.5	467.9	465.1	945.5	979.7	982.4	1401.4	1424.2	1435.4
Alkalinity (µmol/kg)	2190.6	2405.3	2606.8	2196.5	2412.2	2616.2	2210.2	2431.1	2643.0
CO_3^{-2}µmol/kg)	106.51	131.92	160.00	59.97	72.82	89.09	42.34	52.93	65.09
HCO_3^- (µmol/kg)	1929.5	2083.1	2217.6	2049.4	2234.2	2399.4	2106.4	2301.9	2484.8
ΩAr	1.66	2.04	2.45	0.93	1.13	1.36	0.66	0.82	1.00
ΩCa	2.65	3.22	3.84	1.49	1.78	2.14	1.05	1.29	1.56

Table 7

Summary of Measured and Calculated Parameters for 15°C Test Treatments

Parameters	Low CO_2 Treatments			Medium CO_2 Treatments			High CO_2 Treatments		
	LS/LC	MS/LC	HS/LC	LS/MC	MS/MC	HS/MC	LS/HC	MS/HC	HS/HC
				Day 0					
Temperature (°C)	14.7	14.8	14.8	14.9	14.9	14.9	14.9	14.9	14.9
Salinity (‰)	28.4	31.1	34.0	28.4	31.1	34.0	28.3	31.0	34.0
pH	8.21	8.22	8.18	8.30	8.29	8.25	8.07	8.02	7.67
Phosphate (µmol/kg)	0.19	0.27	0.31	0.19	0.32	0.31	0.19	0.26	0.40
Silicate (µmol/kg)	39.81	46.77	53.29	38.68	46.44	55.79	39.88	46.63	55.52
DIC (µmol/kg)	2093.7	2265.7	2487.5	2056.5	2233.9	2439.3	2190.7	2401.7	2771.9
pCO_2 (µatm)	379.0	389.2	458.9	298.6	322.8	378.7	558.4	671.8	1721.4
Alkalinity (µmol/kg)	2289.4	2500.0	2738.4	2296.5	2507.2	2731.3	2331.0	2546.2	2803.1
CO_3^{-2}µmol/kg)	147.56	176.07	191.21	176.81	202.05	218.27	114.18	120.83	68.64
HCO_3^- (µmol/kg)	1931.3	2074.7	2279.0	1868.1	2019.5	2206.7	2054.9	2255.2	2638.5
ΩAr	2.31	2.74	2.94	2.77	3.14	3.36	1.79	1.88	1.06
ΩCa	3.66	4.30	4.59	4.39	4.94	5.24	2.84	2.95	1.65
				Day 4					
Temperature (°C)	14.6	14.4	14.3	15.2	15.2	15.3	14.5	14.3	14.2
Salinity (‰)	28.0	30.9	33.9	28.2	31.0	34.0	28.0	30.9	33.9
pH	8.09	8.12	8.14	8.02	8.03	8.06	7.69	7.67	7.72
Phosphate (µmol/kg)	0.22	1.49	1.06	0.94	0.85	0.98	0.73	0.84	0.74
Silicate (µmol/kg)	16.21	38.76	41.58	23.75	7.35	31.53	21.19	21.05	28.36
DIC (µmol/kg)	2196.1	2359.8	2555.1	2242.1	2413.9	2619.9	2359.9	2532.7	2764.0
pCO_2 (µatm)	533.7	516.7	518.0	646.7	661.2	651.0	1464.9	1600.1	1518.2
Alkalinity (µmol/kg)	2340.8	2545.1	2782.9	2366.3	2564.6	2815.3	2374.6	2550.3	2810.1
CO_3^{-2}µmol/kg)	117.45	145.10	176.95	105.36	125.36	157.80	50.98	56.44	74.79
HCO_3^- (µmol/kg)	2057.7	2194.6	2358.3	2111.8	2263.4	2437.9	2251.2	2413.8	2630.7
ΩAr	1.84	2.26	2.72	1.65	1.95	2.43	0.80	0.88	1.15
ΩCa	2.92	3.55	4.25	2.62	3.07	3.79	1.27	1.38	1.80

Table 8

Summary of Measured and Calculated Parameters for 18°C Test Treatments

Parameters	Low CO_2 Treatments			Medium CO_2 Treatments			High CO_2 Treatments		
	LS/LC	MS/LC	HS/LC	LS/MC	MS/MC	HS/MC	LS/HC	MS/HC	HS/HC
				Day 0					
Temperature (°C)	16.6	16.7	16.8	16.8	16.9	16.7	16.6	16.8	16.7
Salinity (‰)	27.8	30.9	34.1	28.0	31.1	34.1	28.0	31.1	34.0
pH	8.37	8.31	8.29	8.40	8.27	8.05	7.79	7.99	8.04
Phosphate (µmol/kg)	0.27	0.41	0.74	0.32	0.59	0.71	0.31	0.46	0.70
Silicate (µmol/kg)	11.83	12.62	18.11	11.49	- [a]	18.88	11.61	14.61	18.97
DIC (µmol/kg)	1904.6	2081.0	2226.9	1888.2	2109.4	2385.5	2174.3	2252.4	2365.4
pCO_2 (µatm)	237.0	291.0	317.5	217.5	325.9	615.2	1095.8	689.2	625.4
Alkalinity (µmol/kg)	2177.0	2364.8	2543.7	2180.5	2375.5	2573.1	2224.5	2388.9	2546.4
CO_3^{-2}µmol/kg)	196.69	206.36	230.08	209.84	194.54	147.29	63.41	113.05	142.53
HCO_3^- (µmol/kg)	1699.1	1864.1	1985.6	1670.4	1903.2	2216.3	2070.4	2114.4	2200.5
ΩAr	3.11	3.23	3.56	3.32	3.05	2.28	1.00	1.77	2.20
ΩCa	4.92	5.06	5.52	5.24	4.76	3.54	1.58	2.77	3.42
				Day 4					
Temperature (°C)	17.3	17.3	17.4	17.9	18.1	18.0	17.1	17.2	17.3
Salinity (‰)	28.1	31.1	34.2	28.2	31.2	34.2	28.2	31.3	34.2
pH	8.12	8.14	8.14	7.95	7.94	7.94	7.68	7.67	7.68
Phosphate (µmol/kg)	3.52	0.48	0.76	0.49	0.57	0.85	0.38	0.47	0.75
Silicate (µmol/kg)	10.72	14.49	17.99	11.48	13.35	16.91	3.90	5.52	17.49
DIC (µmol/kg)	2028.4	2178.2	2310.2	2100.1	2284.7	2398.3	2171.0	2330.0	2486.3
pCO_2 (µatm)	469.8	465.9	481.0	734.9	797.3	815.8	1416.0	1515.1	1547.7
Alkalinity (µmol/kg)	2196.2	2381.5	2546.5	2206.0	2409.5	2543.5	2191.8	2359.0	2530.4
CO_3^{-2}µmol/kg)	126.74	154.16	177.53	92.21	107.27	121.42	50.33	57.85	68.54
HCO_3^- (µmol/kg)	1884.6	2007.4	2115.9	1981.7	2149.7	2248.9	2069.1	2218.1	2363.6
ΩAr	2.01	2.42	2.75	1.46	1.69	1.88	0.80	0.91	1.06
ΩCa	3.17	3.78	4.26	2.30	2.63	2.92	1.26	1.42	1.64

[a] Value could not be measured for MS/MC at 18°C on Day 0.

Discussion

Larval Development Experiment

Normal Larval Development Endpoint. The results from these experiments suggest changes in normal larval development were predominantly driven by an increase in pCO_2 concentration, rather than by changes in salinity or temperature. Although temperature was not the predominant factor driving changes in larval development, the 15°C and 18°C temperatures appeared to show lower percentages of normal larval development when compared with the 12°C conditions (Figure 9). This difference is most apparent in comparisons with estimated carbonate ion concentrations (Figure 9c) and aragonite saturation levels (Figure 9d). This result suggests that higher temperatures may combine with acidified conditions to reduce normal larval development.

The result from this work is somewhat different from studies that have not explicitly found changes in larval development rates, but rather changes in the rate of development depending on pH and temperature conditions. For example, Stumpp, Wren, Melzner, Thorndyke, and Dupont (2011b) found that *S. purpuratus* larvae suffer a developmental delay of ~8% when exposed to high CO_2/low pH conditions (1318 µatm, pH 7.7) at 14°C. Similarly, Yu et al. (2011) found that larvae were smaller in high pCO_2/low pH conditions (1000 µatm, pH 7.67 and 1450 µtam, pH 7.53) at 15°C, but did not display differences in development compared to ambient conditions. In contrast to Stumpp et al. (2011b,)Padilla-Gamino, Kelly, Evans, and Hofmann (2013) found that higher temperature (18°C vs. 13°C) increased (rather than delayed) the pace of development in *S. purpuratus*, and that the addition of high pCO_2

(1100 µatm) did not affect this development rate. This increased pace of development could be a possible explanation for the higher normality rates observed in the 18°C LC treatments.

Survival Endpoint. Contrary to the larval development results, larval survival appears relatively unaffected by the changes in ambient pH, pCO_2, CO_3^{-2}, and aragonite saturation (Figures 10a-d). Similar studies that have looked at the impact of acidified conditions on larval survival have also shown little or no effect on survival (or mortality). Stumpp, Dupont, Thorndyke, & Melzner (2011a) also found that there was little to no effect on mortality of *S. purpuratus* larvae in high CO_2/low pH conditions (1318 µatm, pH 7.7). Larval survival of the Antarctic sea urchin, *S. neumayeri*, is minimally affected by elevated temperature and elevated pCO_2 combined (Ericson et al., 2012). Lastly, Clark, Lamare, and Barker (2009) found that survival in a temperate sea urchin species (*Pseudechinus huttoni*) was also unaffected up to pCO_2 concentrations resulting in a pH ≥7.0. Taken together, these studies suggest that at the early stages, larval survival is not strongly impacted by acidified conditions.

However, the seemingly unaffected survival rates in response to higher CO_2 concentrations are likely misleading. The larval survival endpoint at 96 hours is less indicative of the future of the larvae, as the larvae with abnormalities would likely die off in the near future for lack of normal arm development when feeding becomes a necessity to continued growth (M. Smith, Smith, Cameron, & Urry, 2008). Stumpp et al. (2011b) suggest that *S. purpuratus* larvae are impacted by ocean acidification more so in the feeding stages than the non-feeding stages causing developmental delay. Furthermore, Dupont, Dorey, and Thorndyke (2010a) suggest that mortality can increase significantly due to predation when larval growth rate is delayed due to ocean acidification. Thus, although survival rates are not strongly impacted by

acidified conditions, the strong impact on normal larval development likely indicates that later development stages will be detrimentally affected and could influence the population dynamics of *S. purpuratus.*

Fertilization Experiment

Changes in fertilization success with seawater carbonate chemistry were only seen in the 15°C test (Figure 8a-d), in that lower fertilization rates were observed at higher values of pCO_2 (Figure 8b) and lower values of pH, CO_3^{-2} concentration, and aragonite saturation (Figure 8a, c, d). In contrast, fertilization rates in both the 12°C and 18°C tests remained high at all combinations of temperature, CO_2, and salinity stressors (Figures 6a, 7a, 8a-d). A similar high tolerance to CO_2 stressors was noted in experiments with a subtropical sea urchin species, *Heliocidaris erythrogramma*, in which fertilization rates were heavily influenced by sperm density and not by temperature or lower pH conditions (M. Byrne et al., 2010). Findings by Place and Smith (2012) indicate that fertilization rate was reduced in *S. purpuratus* embryos reared in pH conditions as low as 7.0. However, this study measured fertilization success by rate of cleavage, a larval stage beyond the scope of my study where fertilization success was measured by the formation of a fertilization membrane in pH conditions that did not decrease below 7.7. Thus, while the findings of Place and Smith (2012) suggest that some impacts on fertilization success might be observed at extreme pH levels of 7.0, this study suggests that the fertilization stage is not heavily influenced by pH values in the range of 7.6 to 8.4.

Increases in ambient temperatures have been suggested to have an impact on fertilization success through its impact on lowered sperm motility, in particular for an Antarctic species *Sterechinus neumayeri* (Ericson et al., 2011). However, the effect of temperature

increase on sperm motility was not apparent in this study. Furthermore, while fertilization in some echinoderm species such as the sand dollar*, Echinarachnius parma*, can be affected by changes in salinity (Allen & Pechenik, 2010), this did not appear to be the case in my fertilization experiments (Figure 6a). Havenhand, Buttler, Thorndyke, & Williamson (2008) found that increased pCO_2 (~1000 ppm) and resulting low pH (7.7) narcotizes sperm in *H. erythrogramma*, suggesting that fertilization could be impaired due to acidification. A compilation of echinoderm fertilization results in Byrne & Przeslawski (2013) showed that the interactive effects of warming and acidification may counter each other. Given that the results of my fertilization experiments do not show a consistent pattern between fertilization success and carbonate chemistry (Figures 6a, 7a, 8a-d), it is likely that some other factor may have been influencing the 15°C fertilization results.

Effects of Multiple Stressors

The normality endpoint results of the larval development experiment may support the idea that stressors work together to create additive, antagonistic, or synergistic effects. Specifically, the possible stimulatory effect on larval development seen in the 18°C LC treatments, the potentially antagonistic effect seen for the 12, 15, and 18°C MC treatments, and the potentially additive or antagonistic effects seen in the 15°C and 18°C HC treatments.

Byrne & Przeslawski (2013) outlined these varying effects in twenty-six species of marine invertebrates and found 70% of species studied were significantly affected by the combination of ocean acidification and ocean warming, explaining that additive negative effects were the most common, and synergistic effects were the least common. Studies where antagonistic effects were present were due to higher temperatures reducing some of the

negative effects of ocean acidification. Therefore, an antagonistic effect would be expected in the 18°C HC treatments. However, as the larval development results in the 18°C HC treatments were very similar to the results of 15°C HC treatments (Figures 9a-d), it is difficult to determine if the effects seen in the 18°C HC treatment were antagonistic or additive. One scenario could demonstrate that both the 15°C and 18°C HC treatments had additive effects, when compared to the 12°C HC treatment. A second scenario could demonstrate that the 18°C HC treatment had antagonistic effects, when compared to the 15°C and 12°C HC treatments. Although, it appears that MC treatments for all test temperatures caused antagonistic effects, despite having a wider pCO_2 concentration range on day 4 than anticipated, showing consistent larval development results between all test temperatures. Further tests would be needed to support this suggestion, and to confirm the effects seen in this test are representative of the intended pCO_2 concentrations for the MC and HC treatments. However, whether additive or antagonistic effects occurred in these treatments, the overall negative effects on larval development in the presence of increased pCO_2 are significant and should continue to be a consideration for future research.

The tolerance to multiple stressors seen at the fertilization stage in this study did not translate into a tolerance in the larval development stage. This result is similar to findings by M. Byrne et al. (2010) that indicate that vulnerabilities may lie across life histories and different life stages, suggesting that larval development may by more sensitive to the same stressors than fertilization. Therefore, the larval development results may be more indicative of the long-term outcome than the survival results in this study. However, if the duration of this study was extended, the longer-term survival rates may decline.

Comparing the impact of these combinations of stressors with those of other studies is difficult as no studies were found to have investigated these particular combinations of stressors on *S. purpuratus*. Multiple stressor studies vary, utilizing different CO_2 concentrations, temperatures and salinities. Furthermore, the studies are performed using different species of echinoderms that are found around the world, which may have different ambient conditions and different sensitivities to future ocean acidification conditions. In addition, biological test methods vary to suit the specific focal point of the study, adding to the difficulty of comparing results. As suggested by Byrne (2012), results from studies of this nature would be more comparable if researchers used test protocols with greater similarities in the test conditions and endpoints employed.

Study Limitations

The twenty-seven treatments were tested in three groupings over three different test initiation days. Every effort was made to replicate conditions between each set of tests, including the density and quality of gametes used in the tests. However, natural variation between tests is to be expected. To increase confidence in results of future studies, all treatments should be tested at the same time utilizing the same batch of gametes.

Gravid urchins were site collected near Mission Bay, California, and acclimated to 12°C prior to testing to prevent premature spawning. It is unknown if the performance of the gametes in the 12°C HC treatments of the larval development experiment could be attributed to their acclimation to the same temperature, or their phenotypic plasticity due to frequent and extreme pH fluctuations in their natural environment (Hofmann et al., 2014; Kelly et al., 2013; Yu et al., 2011). The adults used in these studies were not pre-acclimated to treat treatments,

and therefore, the results are not representative of adaptation potential in adults or gametes (Kelly et al., 2013).

Two separate standardized methods for fertilization and larval development were used for these experiments. The larval development test uses eggs that are pre-fertilized in ideal conditions prior to the start of the test, which forces an assumption that fertilization will not be affected. Combining the fertilization stage with the larval development stages in one test would provide better insight into the effects of ocean acidification across multiple life stages. Furthermore, continuing the experiment beyond 96-hour larval development to include additional sub-lethal effects such as reduced size would be beneficial.

The optimal test temperature for echinoderm fertilization and larval development tests performed with *S. purpuratus* (ASTM, 1998; Environment Canada, 2011) is 15°C, and so the factor for the especially low rate of normal larval development in the LC treatments is unknown. In addition, the significant effect seen on fertilization at 15°C fertilization test indicates a possibility that other factors were at play.

The day 0 carbonate chemistry results possibly indicate that the pCO_2 concentrations were approaching, but not quite in equilibrium. The development and fertilization test treatments were aerated with their CO_2 treatment until test initiation time, whereas, the aliquots for carbonate chemistry analyses were taken much earlier in the day for logistical purposes. Future studies should ensure that aliquots are representative of the test treatments at test initiation time. Furthermore, I recommend analyzing carbonate chemistry values for each day of the development test exposure.

These laboratory experiments were tightly controlled to ensure variables are constantly maintained in an effort to isolate cause and effects. However, confident extrapolation of the effects of multiple stressors by may be confounded by other unaccounted for stressors in the natural environment. Therefore, the results of these experiments, and any other such experiments must be put into perspective when considering the future effects on the target species, and the environment around them.

Implications for Future Populations

The implications of the negative effects of ocean acidification on various echinoderms have been detailed in terms of limitations on feeding, delayed fertilization and development, and reduced growth and (Asnaghi et al., 2013; Byrne & Przeslawski, 2013; Kelly et al., 2013; Place & Smith, 2012; Stumpp et al., 2011b). Slowed development of *S. purpuratus* puts this important species at risk of increased predation, increased vulnerability to disease and parasites (Lenihan, Micheli, Shelton, & Peterson, 1999) and at risk of population decline. Furthermore, a decline in the *S. purpuratus* population along the Pacific coast could have significant cascading effects on other species, potentially altering the balance of the ecosystem along the Pacific coast (Byrne, 2012; Estes, Tinker, Williams, & Doak, 1998; Fabry, Seibel, Feely, & Orr, 2008; Gaylord et al., 2011; Harley et al., 2006; Harley, 2011). However, studies continue to suggest that sea urchins will likely have the ability to adapt to changing ocean conditions (Kelly et al., 2013; Pespeni et al., 2013; Yu et al., 2011). Byrne & Przeslawski (2013) found that *S. droebachiensis* are able to pass on their tolerance when acclimated to an elevated pCO_2 for prolonged periods of time. Yu et al. (2011) suggest that the urchin's ability to adapt may come from their tolerance to the present day natural fluctuation in pH along the coast. Although,

Evans, Chan, Menge, and Hofmann (2013) suggest that the recovery time between fluctuations may be necessary for tolerance, as they are unable to maintain their coping response through prolonged exposure to acidified pH conditions. Genetic variation may also play a role in their ability to adapt, as Kurihara (2008) has noted that the impacts of increasing pCO_2 on fertilization in echinoderms (*H. pulcherrimus*, *E. mathaei*) varied between females. Furthermore, Pespeni et al. (2013) suggest that current populations of *S. purpuratus* have the genetic variation to adapt to frequently changing ocean conditions.

Adaptation capability of *S. purpuratus* may occur if the genetic diversity of the population is reduced. For example, future ocean acidification may cause significant genetic change to the growth and biomineralization genes. If changes in ocean acidification occur at a faster rate than *S. purpuratus* can adapt, an initial population decline would likely occur, causing a reduction in the overall genetic variation of the population, and could put the future of the population at risk. One way to explore this idea would be to examine the response multiple, successive generations of this species to altered oceanic conditions.

Environmental Management Response

Current ocean acidification monitoring efforts vary along sections of the North American Pacific Coast, with research being conducted in different manners and intensities in Canada and the United States. In the United States, research and monitoring efforts are conducted by the Pacific Marine Environmental Laboratory (PMEL) for the National Oceanic and Atmospheric Administration (NOAA), as well as the Partnership for Interdisciplinary Studies of Coastal Oceans (PISCO) and the Ocean Margin Ecosystem Group for Acidification Studies (OMEGAS) consortiums, which focus on changes in the ocean environment in the California Current Large

Marine Ecosystem (CCLME) (Hofmann et al., 2014). In Canada, there is no organized national program, although the Department of Fisheries and Oceans Canada (DFO) has included some monitoring efforts toward ocean acidification research. Another effort in monitoring has been undertaken by the Ocean Networks Canada, an initiative started by University of Victoria. Some tools are available for sharing research and data collected through monitoring efforts, namely through websites and publications, although, there is a lack of a central database or authority to combine research findings.

In consideration of the cross-border impacts of ocean acidification and the challenges associated with long-term monitoring, environmental management at the governance level needs to be cooperative and span political borders. Environmental management approaches need to consider the negative effects of anthropogenic decision-making on non-target organisms in various trophic levels. The results of detrimental decisions may take years to show, thankfully in part to a healthy ecosystem's ability for adaptive response. However, a decrease in an ecosystem's biodiversity increases the likelihood of eventual ecosystem collapse that could take generations to recover.

Utilizing holistic, sustainable environmental management would benefit both commercial fisheries, and the species in the affected ecosystems. In areas such as the eastern Pacific Coast, collaborative environmental management polices between different countries will ensure that one party's environmental efforts are not diminished by the detrimental effects of another's lack thereof. Considering ocean acidification and populations of sea urchins along the Pacific coast are not mindful of human-derived economic and political borders, oceanic and near shore ecosystem management plans need to respond in kind, breaching economic and

political zones. It is imperative that governing bodies use collaborative approaches, such as ocean zoning (Crowder et al., 2006; Halpern, McLeod, Rosenberg, & Crowder, 2008; Kenchington & Day, 2011). This approach incorporates marine ecosystem and climate change research, and acts to add an important spatial dimension into existing regulations (Crowder et al., 2006). Ocean zoning acts to identify ecosystem services and acts as a tool to enable management decisions according to the varied intensities of multiple stressors on the environment (Halpern et al., 2008). This approach has already been utilized in the Great Barrier Reef, Australia, with success, where zoning has enhanced the health of the areas outside of those zoned for protection (Kenchington & Day, 2011). Cross-border collaboration will likely unearth unique challenges, but allows the opportunity to work together to achieve common goals, realizing a balance between economic and industrial growth, while protecting coastal ecosystems.

Conclusions and Recommendations

Larval development was decreased by 41% to 68% by high pCO_2 conditions representing a pH decrease of ~0.4. Decreased development compared to ambient conditions was greatest at test temperatures of 15°C and 18°C. However, significant decreases in larval development by ~30% were seen with a pH decrease of 0.1 – 0.3 from ambient. In light of the effects on larval development by pH changes less than the projected pH decrease of 0.3 – 0.4 by the year 2100 (Orr et al., 2005; Solomon et al., 2007), *S. purpuratus* may need to adapt rapidly. Fertilization and larval survival were tolerant to ocean acidification and all combinations of stressors; however, these survival results should be taken under precaution, as they only represent the larval survival rate at 96 hours and not the latter stages of growth where confounding factors such as feeding, disease and predation may occur.

Increased understanding of the urchins' ability to adapt is key in the determination of the fate of the populations along the Pacific Coast. Multiple stressor studies will continue to play an important role in this understanding, as there still remains uncertainty and gaps in our knowledge, such as the rate in which *S. purpuratus* will be able to adapt to multiple ocean acidification stressors. More research is necessary to determine if a population shift or migratory effects would occur as an adaptation response. Therefore, it is important to consider the interactive effects of multiple stressors and ocean acidification when making environmental management decisions

Continued monitoring of changing ocean parameters is a necessity, considering the far-reaching implications of ocean acidification. As conditions continue to change, pushing

buffering capacities and species' thresholds, we must be aware of and prepared for any impending tipping points that have the potential to affect entire ecosystems, not just an isolated species. This potential demonstrates the need for a collaborative effort toward proactive, adaptive, and sustainable environmental management that incorporates the regulation of anthropogenically produced CO_2.

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Appendix A. Summary of Tasks for Experiments

Table A1

Summarized Timeline of Events and Tasks for Initiation and Completion of Experiments

Test Day	Brief Summary of Tasks Performed for Each Round of Testing
Day -1	
	Prepared test treatments; adjusted temperature and salinity
	Measured and recorded water quality (salinity, pH, temperature, dissolved oxygen) for each treatment (prior to CO_2 addition)
	Placed treatments in a temperature controlled water bath and allowed to aerate overnight with its respective CO_2 treatment (390, ~900, and 1500 ppm) to achieve equilibrium
Day 0	
	Transported urchins from holding facility to testing lab
	Measured and recorded water quality (salinity, pH, temperature, and dissolved oxygen) of each test treatment
	Divided total volume of each treatment for both experiments: Transferred ~900 ml to larval development test vessels (~300 ml into each of the three replicate 350 ml jars), and five 5 ml aliquots to fertilization test vessels (five replicate test tubes)
	Took subsamples from remaining volume of each treatment for initial confirmatory chemistry (DIC, TP, and TS) and preserved as necessary
	Spawned urchins, checked gamete quality
	Prepared and added gametes (sperm and eggs) to the 20-minute fertilization tests; ended fertilization test by preserving each test tube with 10% buffered formalin
	Prepared and added gametes (pre-fertilized embryos) to the development tests
Days 1 - 3	
	Monitored airlines, flow rates, and CO_2 tank volumes
	Measured and recorded pH of each larval development treatment
	Visually monitored larval development in development tests according to known developmental stages by microscopic examination of 1 ml aliquots from selected test treatments
Day 4	
	Measured & recorded water quality (salinity, pH, temperature, & dissolved oxygen)
	Monitored larval development in development tests according to known developmental stages, by microscopic examination
	Ended larval development test; removed six 10 ml aliquots from each treatment, transferred to holding vials, and preserved with 2 ml 10% formalin
	Took subsamples from remaining volume of each treatment for final confirmatory chemistry (DIC, TP, and TS) and preserved as necessary

Appendix B. Summary of Experimental Test Conditions

Table B1

Summary of Test Conditions Used for Sea Urchin Fertilization and Development Tests

Parameter	**Conditions and Methods**	
Organism Information	**Fertilization Test**	**Development Test**
Species	Pacific Purple Sea Urchin, *Strongylocentrotus purpuratus*	Pacific Purple Sea Urchin, *Strongylocentrotus purpuratus*
Organism Source	Marine Research Educational Products, San Marcos, CA. Field collected from Mission Bay, CA.	Marine Research Educational Products, San Marcos, CA. Field collected from Mission Bay, CA.
Acclimation and Holding Conditions	Urchins held in 35L tanks; in-tank filters to clean and circulate water; frequent removal of waste and renewal with clean holding water	Urchins held in 35L tanks; in-tank filters to clean and circulate water; frequent removal of waste and renewal with clean holding water
Water Used for holding	Natural Seawater (Vancouver Aquarium), 5 µm filtered and UV Sterilized before use and Artificial salt water prepared from Instant Ocean Sea Salts	Natural Seawater (Vancouver Aquarium), 5 µm filtered and UV Sterilized before use and Artificial salt water prepared from Instant Ocean Sea Salts
Feeding Regime	Carrots and seaweed; fed ~3 times/week	Carrots and seaweed; fed ~3 times/week
Laboratory Conditions - Acclimation	12°C with 16 hours light:8 hours dark photoperiod; ambient laboratory lighting	12°C with 16 hours light:8 hours dark photoperiod; ambient laboratory lighting
Mode of Transport	Packed dry between moist paper towel with ice packs in a cooler; same day shipment	Packed dry between moist paper towel with ice packs in a cooler; same day shipment
Test Specific Conditions	**Fertilization Test**	**Development Test**
Methods Used	Environment Canada (2011)	Adapted from ASTM (1998) and USEPA (1995)
Laboratory Conditions	~15°C with 14 hours light:10 hours dark photoperiod; ambient laboratory lighting	~15°C with 14 hours light:10 hours dark photoperiod; ambient laboratory lighting

Water source	SFU Biology Lab B2105; Vancouver Aquarium 1 µm filtered and aerated with oil-free compressed air before use	SFU Biology Lab B2105; Vancouver Aquarium 1 µm filtered and aerated with oil-free compressed air before use
Test Treatments	27 (3 CO_2 concentrations, 3 salinities, 3 temperatures)	27 (3 CO_2 concentrations, 3 salinities, 3 temperatures)
Test Manipulation - Salinity	Treatments adjusted to desired salinity with Deionized water or 0.2 µm filtered 90 ppt hypersaline brine (prepared from Vancouver aquarium water)	Treatments adjusted to desired salinity with Deionized water or 0.2 µm filtered 90 ppt hypersaline brine (prepared from Vancouver aquarium water)
Test Treatment Manipulation - Temperature regulation	Test vessels placed in water baths to achieve and maintain desired test temperature for the duration of the test	Test vessels placed in water baths to achieve and maintain desired test temperature for the duration of the test
Replicates/treatment	5	3 test vessels; total of 6 aliquots preserved for survival and development analyses
Test Vessels	20 ml Borosilicate glass test tubes	350 ml glass jars for duration of development test; 20 ml vials for preserved test aliquots
Test treatment Volume	5 ml	300 ml
Test Duration/Exposure time	20 minutes total duration (10 minutes sperm, 10 minutes sperm+egg)	96 hours
Spawning induction	1.0 ml of 0.5M KCl injected into peristomal membrane beside aristotle's lantern	1.0 ml of 0.5M KCl injected into peristomal membrane beside aristotle's lantern
Number of males and females used	12°C – 3 males, 6 females 15°C – 6 males, 3 females 18°C – 4 males, 4 females	12°C – 3 males, 6 females 15°C – 6 males, 3 females 18°C – 4 males, 4 females
Sperm: egg ratio	75:1 for all tests	Estimated ~7-10:1; as needed to achieve 90% fertilization
Total number of gametes/test vessel	849 – 905 eggs and 63,739 - 67,877 sperm	Estimated: 7,200 – 7,700 Actual embryo counts: 6,570 – 7,200

Gamete density/ test replicate	170 - 181 eggs/mL 12,748 - 13,575 sperm/mL	Estimated: 24 – 26 embryos/mL Actual: 22 – 24 embryos/mL
Water Quality parameters monitored	Salinity, DO, pH and temperature at test initiation	Salinity, DO, pH, and temperature at test initiation and at test completion; pH was measured at each 24 hour time point
Feeding	No feeding during test	No feeding during test

Chemical Analyses	**Fertilization Test**	**Development Test**
Sampling time and technique	Subsamples were taken at test initiation	Subsamples were taken at test initiation and at test completion
Dissolved inorganic carbon (DIC)	Instrument: Apollo SciTech AS C3 DIC Analyser w/ LI-7000 Infrared CO_2 detector. Method: Apollo SciTech Manual. Location: Harley Lab, University of British Columbia	Instrument: Apollo SciTech AS C3 DIC Analyser w/ LI-7000 Infrared CO_2 detector. Method: Apollo SciTech Manual. Location: Harley Lab, University of British Columbia
Total Phosphate (TP)	Methods of SW Analysis, Section 10.2.5 (Grabhoff, 1999). Instument: Hach DR 5000™ UV-Vis Spectrophotometer. Location: COPE lab, Simon Fraser University	Methods of SW Analysis, Section 10.2.5 (Grabhoff, 1999). Instument: Hach DR 5000™ UV-Vis Spectrophotometer. Location: COPE lab, Simon Fraser University
Total Silicate (TS)	Methods of SW Analysis, Section 10.2.11 (Grabhoff, 1999). Instument: Hach DR 5000™ UV-Vis Spectrophotometer. Location: COPE lab, Simon Fraser University	Methods of SW Analysis, Section 10.2.11 (Grabhoff, 1999). Instument: Hach DR 5000™ UV-Vis Spectrophotometer. Location: COPE lab, Simon Fraser University

Appendix C. Laboratory Room Temperature Monitoring Log

Table C1

Minimum/Maximum Room Temperature Log

Date	Thermometer #1			Thermometer #2			Daily Averages			Average Room Temp
	Min	Max	Current	Min	Max	Current	Min	Max	Current	
2013 Feb 20	11.2	16.1	14.6	-	-	-	11.2	16.1	14.6	13.7
2013 Feb 22	14.1	16.9	15.9	13.9	16.4	15.0	14.0	16.7	15.5	15.3
2013 Feb 26	13.6	16.5	14.2	13.5	16.1	14.1	13.6	16.3	14.2	14.9
2013 Feb 27	13.5	16.2	14.1	13.3	16.1	13.7	13.4	16.2	13.9	14.8
2013 Feb 28	13.4	16.1	14.3	13.6	16.1	14.5	13.5	16.1	14.4	14.8
2013 Mar 01	13.4	16.1	13.9	13.3	16.2	13.6	13.4	16.2	13.8	14.8
2013 Mar 02	13.4	15.7	13.7	13.2	15.9	13.5	13.3	15.8	13.6	14.6
2013 Mar 03	13.3	16.1	13.6	13.3	15.9	13.6	13.3	16.0	13.6	14.7
2013 Mar 07	13.2	16.5	14.0	13.2	16.1	14.0	13.2	16.3	14.0	14.8
2013 Mar 10	13.5	16.4	14.0	12.7	16.0	13.0	13.1	16.2	13.5	14.7
2013 Mar 11	13.4	16.4	14.0	12.6	15.3	13.0	13.0	15.9	13.5	14.4
2013 Mar 12	13.4	16.6	13.6	12.7	15.3	13.1	13.1	16.0	13.4	14.5
2013 Mar 13	13.1	16.5	13.6	12.7	15.2	13.3	12.9	15.9	13.5	14.4
2013 Mar 14	13.3	16.6	13.7	12.5	14.5	13.0	12.9	15.6	13.4	14.2
2013 Mar 15	13.4	16.4	13.7	12.7	14.7	13.1	13.1	15.6	13.4	14.3
2013 Mar 17	12.7	16.4	13.1	12.5	15.9	13.6	12.6	16.2	13.4	14.4
2013 Mar 18	13.0	15.1	13.7	13.4	16.0	14.4	13.2	15.6	14.1	14.4
2013 Mar 19	12.9	15.4	13.1	13.6	16.3	13.9	13.3	15.9	13.5	14.6
2013 Mar 20	12.7	15.2	13.1	13.4	16.4	14.0	13.1	15.8	13.6	14.4
2013 Mar 21	12.9	15.1	13.2	13.5	16.3	14.1	13.2	15.7	13.7	14.5
2013 Mar 22	13.5	15.9	13.5	13.5	16.5	14.0	13.5	16.2	13.8	14.9
Overall Average Room Temperature (±SD):										**14.6 (1.5)**

Appendix D. Schematic of Test Treatment Preparation

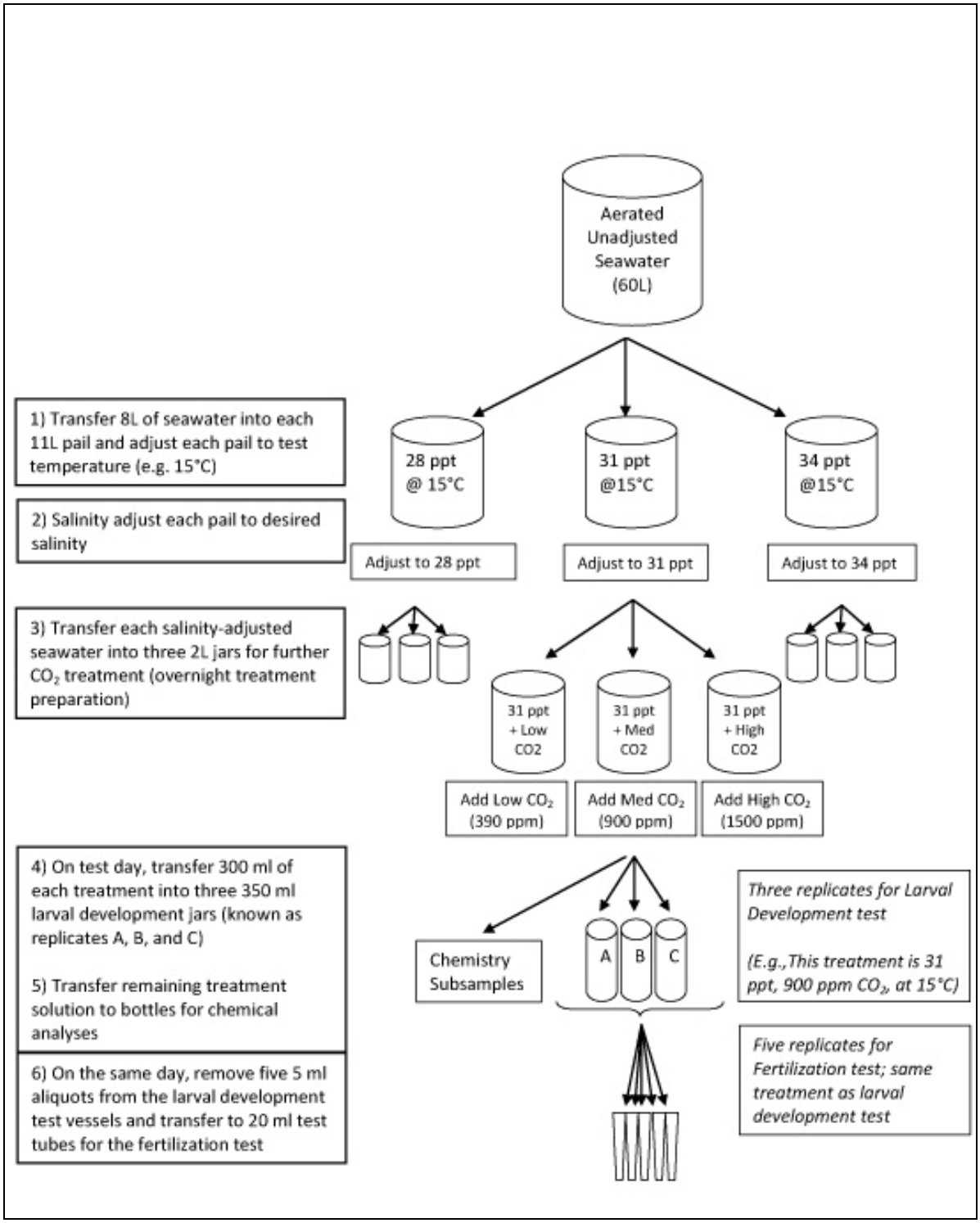

Figure D1. Schematic of test treatment preparation detailing stages of salinity adjustment and addition of pCO_2 prior to transferring to test vessels.

Appendix E. Water Quality Measurements for Larval Development Tests

Table E1

Summary of Water Quality Measurements for Urchin Larval Development Tests

Treatment	Day 0[1]				Day 1	Day 2	Day 3	Day 4			
	Salinity	Temp	pH	DO	pH	pH	pH	Salinity	Temp	pH	DO
12°C											
LS/LC	28.2	13.4	8.28	8.9	8.14	8.12	8.11	27.9	12.4	8.11	9.3
MS/LC	31.0	13.4	8.21	8.9	8.17	8.14	8.14	31.0	12.4	8.13	9.3
HS/LC	34.0	13.4	8.24	8.9	8.19	8.16	8.15	34.0	12.4	8.15	9.3
LS/MC	28.1	13.5	7.91	8.9	7.80	7.76	7.81	28.0	12.6	7.83	9.3
MS/MC	31.0	13.5	7.96	8.9	7.95[2]	7.93	7.87	31.0	12.5	7.84	9.4
HS/MC	34.0	13.5	8.10	8.9	7.76	7.76	7.81	34.0	12.5	7.86	9.2
LS/HC	28.1	13.5	7.68	8.9	7.68	7.67	7.67	28.0	12.4	7.67	9.3
MS/HC	31.0	13.4	7.69	8.9	7.70	7.70	7.69	31.0	12.4	7.69	9.2
HS/HC	34.0	13.4	7.77	8.9	7.75	7.71	7.73	34.0	12.4	7.71	9.2
15°C											
LS/LC	28.4	14.7	8.21	8.5	8.20	8.18	8.12	28.0	14.6	8.09	8.6
MS/LC	31.1	14.8	8.22	8.6	8.23	8.19	8.15	30.9	14.4	8.12	8.5
HS/LC	34.0	14.8	8.18	8.6	8.20	8.15	8.14	33.9	14.3	8.14	8.6
LS/MC	28.4	14.9	8.30	8.6	8.03	7.99	8.01	28.2	15.2	8.02	8.6
MS/MC	31.1	14.9	8.29	8.5	8.00	7.98	8.03	31.0	15.2	8.03	8.6
HS/MC	34.0	14.9	8.25	8.5	8.14[3]	8.03	8.05	34.0	15.3	8.06	8.6
LS/HC	28.3	14.9	8.07	8.5	7.69	7.65	7.67	28.0	14.5	7.69	8.7
MS/HC	31.0	14.9	8.02	8.6	7.67	7.63	7.68	30.9	14.3	7.67	8.7
HS/HC	34.0	14.9	7.67	8.5	7.73[3]	7.67	7.70	33.9	14.2	7.72	8.5
18°C											
LS/LC	27.8	16.6	8.37	8.1	8.27	8.19	8.15	28.1	17.3	8.12	8.3
MS/LC	30.9	16.7	8.31	8.0	8.29	8.20	8.16	31.1	17.3	8.14	8.3
HS/LC	34.1	16.8	8.29	8.1	8.26	8.20	8.15	34.2	17.4	8.14	8.3
LS/MC	28.0	16.8	8.40	8.1	8.16	8.03	8.02	28.2	17.9	7.95	8.1
MS/MC	31.1	16.9	8.27	8.0	8.05	8.01	8.01	31.2	18.1	7.94	8.1
HS/MC	34.1	16.7	8.05	8.1	8.10	8.01	8.01	34.2	18.0	7.94	8.0
LS/HC	28.0	16.6	7.79	8.1	7.73	7.72	7.68	28.2	17.1	7.68	8.3
MS/HC	31.1	16.8	7.99	8.0	7.90	7.75	7.69	31.3	17.2	7.67	8.3
HS/HC	34.0	16.7	8.04	8.0	7.78	7.74	7.72	34.2	17.3	7.68	8.3

[1] *Day 0 water quality measurements represent the parameters for the 20-minute fertilization test*

[2] *Aeration was low; adjusted*

[3] *Vessel was not aerating; adjusted airlines*

Table E2

Summary of Mean pH readings (Test Initiation to Completion) for Urchin Larval Development Tests

Treatment Grouping	Treatment	pH Measurement					Average Initial pH	Average Final pH
		Day 0	Day 1	Day 2	Day 3	Day 4		
12°C								
Low Carbon	LS/LC	8.28	8.14	8.12	8.11	8.11	8.24	8.13
(390 ppm)	MS/LC	8.21	8.17	8.14	8.14	8.13		
	HS/LC	8.24	8.19	8.16	8.15	8.15		
Medium Carbon	LS/MC	7.91	7.80	7.76	7.81	7.83	7.99	7.84
(900 ppm)	MS/MC	7.96	7.95	7.93	7.87	7.84		
	HS/MC	8.10	7.76	7.76	7.81	7.86		
High Carbon	LS/HC	7.68	7.68	7.67	7.67	7.67	7.71	7.69
(1500 ppm)	MS/HC	7.69	7.70	7.70	7.69	7.69		
	HS/HC	7.77	7.75	7.71	7.73	7.71		
15°C								
Low Carbon	LS/LC	8.21	8.20	8.18	8.12	8.09	8.20	8.12
(390 ppm)	MS/LC	8.22	8.23	8.19	8.15	8.12		
	HS/LC	8.18	8.20	8.15	8.14	8.14		
Medium Carbon	LS/MC	8.30	8.03	7.99	8.01	8.02	8.28	8.04
(900 ppm)	MS/MC	8.29	8.00	7.98	8.03	8.03		
	HS/MC	8.25	8.14	8.03	8.05	8.06		
High Carbon	LS/HC	8.07	7.69	7.65	7.67	7.69	7.92	7.69
(1500 ppm)	MS/HC	8.02	7.67	7.63	7.68	7.67		
	HS/HC	7.67	7.73	7.67	7.70	7.72		
18°C								
Low Carbon	LS/LC	8.37	8.27	8.19	8.15	8.12	8.32	8.13
(390 ppm)	MS/LC	8.31	8.29	8.20	8.16	8.14		
	HS/LC	8.29	8.26	8.20	8.15	8.14		
Medium Carbon	LS/MC	8.40	8.16	8.03	8.02	7.95	8.24	7.94
(900 ppm)	MS/MC	8.27	8.05	8.01	8.01	7.94		
	HS/MC	8.05	8.10	8.01	8.01	7.94		
High Carbon	LS/HC	7.79	7.73	7.72	7.68	7.68	7.94	7.68
(1500 ppm)	MS/HC	7.99	7.90	7.75	7.69	7.67		
	HS/HC	8.04	7.78	7.74	7.72	7.68		

Appendix F. Summary of Silicate, Phosphate, and DIC Results

Table F1

Summary of Silicate Results for all Treatments and Timepoints

			12 °C Test		15°C Test		18°C Test	
CO_2 Grouping	SW Treatment	Treatment Code	Day 0	Day 4	Day 0	Day 4	Day 0	Day 4
Low CO_2	Low Salinity	LS/LC	44.76	42.24	39.81	16.21	11.83	10.72
	Medium Salinity	MS/LC	53.60	59.89	46.77	38.76	12.62	14.49
	High Salinity	HS/LC	59.45	60.60	53.29	41.58	18.11	17.99
Medium CO_2	Low Salinity	LS/MC	45.61	44.76	38.68	23.75	11.49	11.48
	Medium Salinity	MS/MC	55.14	53.87	46.44	7.35	14.00	13.35
	High Salinity	HS/MC	61.39	61.26	55.79	31.53	18.88	16.91
High CO_2	Low Salinity	LS/HC	47.34	45.17	39.88	21.19	11.61	3.90
	Medium Salinity	MS/HS	55.35	55.35	46.63	21.05	14.61	5.52
	High Salinity	HS/HC	62.66	63.23	55.52	28.36	18.97	17.49

Table F2

CO_2 Grouping	SW Treatment	Treatment Code	12 °C Test		15°C Test		18°C Test	
			Day 0	Day 4	Day 0	Day 4	Day 0	Day 4
Low CO_2	Low Salinity	LS/LC	0.31	0.32	0.19	0.22	0.27	3.52
	Medium Salinity	MS/LC	0.27	0.41	0.27	1.49	0.41	0.48
	High Salinity	HS/LC	0.36	0.45	0.31	1.06	0.74	0.76
Medium CO_2	Low Salinity	LS/MC	0.18	0.13	0.19	0.94	0.32	0.49
	Medium Salinity	MS/MC	0.22	0.45	0.32	0.85	0.59	0.57
	High Salinity	HS/MC	0.52	0.40	0.31	0.98	0.71	0.85
High CO_2	Low Salinity	LS/HC	0.13	0.18	0.19	0.73	0.31	0.38
	Medium Salinity	MS/HS	0.35	0.22	0.26	0.84	0.46	0.47
	High Salinity	HS/HC	0.40	0.37	0.40	0.74	0.70	0.75

Summary of Phosphate Results for all Treatments and Timepoints

Table F3

CO_2 Grouping	SW Treatment	Treatment Code	12 °C Test		15°C Test		18°C Test	
			Day 0	Day 4	Day 0	Day 4	Day 0	Day 4
Low CO_2	Low Salinity	LS/LC	1950.58	2055.66	2093.68	2196.11	1904.56	2028.35
	Medium Salinity	MS/LC	2157.26	2234.34	2265.71	2359.76	2081.00	2178.20
	High Salinity	HS/LC	2310.20	2396.55	2487.54	2555.11	2226.93	2310.20
Medium CO_2	Low Salinity	LS/MC	2126.63	2148.93	2056.47	2242.11	1888.22	2100.09
	Medium Salinity	MS/MC	2284.64	2347.38	2233.87	2413.87	2109.45	2284.71
	High Salinity	HS/MC	2390.30	2528.31	2439.26	2619.89	2385.49	2398.29
High CO_2	Low Salinity	LS/HC	2186.91	2207.72	2190.74	2359.89	2174.32	2170.99
	Medium Salinity	MS/HS	2384.60	2413.78	2401.75	2532.70	2252.41	2329.99
	High Salinity	HS/HC	2557.42	2608.28	2771.94	2763.96	2365.39	2486.30

Summary of Dissolved Inorganic Carbon (DIC) Results for all Treatments and Timepoints

Appendix G. CO2SYS Output Results

Table G1
CO2SYS Output results – Day 0

Treatment Name	Desired Test temp (°C)	Salinity (ppt)	Temp (°C)	pH	P (dbars)	Total P (µmol/kg SW)	Total Si (µmol/kg SW)	TA (µmol/kg SW)	TCO_2 (µmol/kg SW)	fCO_2 (µatm)	pCO_2 (µatm)	HCO_3 (µmol/kg SW)	CO_3 (µmol/kg SW)	CO_2 (µmol/kg SW)	Revelle	ΩCa	ΩAr	xCO2 (dry at 1 atm) (ppm)
Low CO_2 Treatments																		
LS/LC	**12**	28.2	13.4	8.28	0	0.31	44.76	2160.4	1950.6	293.4	294.5	1786.2	152.387	11.997	11.630	3.78	2.38	299.0
	15	28.4	14.7	8.21	0	0.19	39.81	2289.4	2093.7	377.6	379.0	1931.3	147.565	14.813	12.467	3.66	2.31	385.3
	18	27.8	16.6	8.37	0	0.27	11.83	2177.0	1904.6	236.1	237.0	1699.1	196.686	8.776	10.114	4.92	3.11	241.4
MS/LC	**12**	31.0	13.4	8.21	0	0.27	53.60	2368.2	2157.3	374.0	375.4	1985.7	156.519	15.051	12.134	3.82	2.43	381.1
	15	31.1	14.8	8.22	0	0.27	46.77	2500.0	2265.7	387.8	389.2	2074.7	176.074	14.938	11.841	4.30	2.74	395.7
	18	30.9	16.7	8.31	0	0.41	12.62	2364.8	2081.0	289.9	291.0	1864.1	206.364	10.560	10.313	5.06	3.23	296.4
HS/LC	**12**	34.0	13.4	8.24	0	0.36	59.45	2572.9	2310.2	361.4	362.8	2102.7	193.159	14.301	11.244	4.63	2.96	368.2
	15	34.0	14.8	8.18	0	0.31	53.29	2738.4	2487.5	457.3	458.9	2279.0	191.213	17.330	11.945	4.59	2.94	466.5
	18	34.1	16.8	8.29	0	0.74	18.11	2543.7	2226.9	316.4	317.5	1985.6	230.081	11.289	10.067	5.52	3.56	323.5
Medium CO_2 Treatments																		
LS/MC	**12**	28.1	13.5	7.91	0	0.18	45.61	2201.5	2126.6	780.0	782.9	2021.2	73.594	31.809	17.421	1.83	1.15	794.8
	15	28.4	14.9	8.30	0	0.19	38.68	2296.5	2056.5	297.6	298.6	1868.1	176.811	11.601	11.199	4.39	2.77	303.6
	18	28.0	16.8	8.40	0	0.32	11.49	2180.5	1888.2	216.7	217.5	1670.4	209.843	7.998	9.734	5.24	3.32	221.6
MS/MC	**12**	31.0	13.5	7.96	0	0.22	55.14	2394.0	2284.6	724.1	726.8	2159.5	96.053	29.051	16.031	2.35	1.49	737.9
	15	31.1	14.9	8.29	0	0.32	46.44	2507.2	2233.9	321.7	322.8	2019.5	202.049	12.352	10.919	4.94	3.14	328.2
	18	31.1	16.9	8.27	0	0.59	14.00	2375.5	2109.4	324.8	325.9	1903.2	194.542	11.748	10.727	4.76	3.05	332.1
HS/MC	**12**	34.0	13.5	8.10	0	0.52	61.39	2582.3	2390.3	527.6	529.6	2221.2	148.319	20.813	13.205	3.56	2.27	537.6
	15	34.0	14.9	8.25	0	0.31	55.79	2731.3	2439.3	377.3	378.7	2206.7	218.267	14.256	11.001	5.24	3.36	385.0
	18	34.1	16.7	8.05	0	0.71	18.88	2573.1	2385.5	613.0	615.2	2216.3	147.291	21.936	13.247	3.54	2.28	626.7
High CO_2 Treatments																		
LS/HC	**12**	28.1	13.5	7.68	0	0.13	47.34	2196.1	2186.9	1367.3	1372.4	2086.4	44.733	55.761	19.110	1.11	0.70	1393.3
	15	28.3	14.9	8.07	0	0.19	39.88	2331.0	2190.7	556.4	558.4	2054.9	114.185	21.704	14.700	2.84	1.79	567.7
	18	28.0	16.6	7.79	0	0.31	11.61	2224.5	2174.3	1091.9	1095.8	2070.4	63.414	40.534	18.241	1.58	1.00	1116.2
MS/HC	**12**	31.0	13.4	7.69	0	0.35	55.35	2405.7	2384.6	1417.8	1423.1	2273.4	54.117	57.060	18.805	1.32	0.84	1444.6
	15	31.0	14.9	8.02	0	0.26	46.63	2546.2	2401.7	669.4	671.8	2255.2	120.828	25.721	14.947	2.95	1.88	683.1
	18	31.1	16.8	7.99	0	0.46	14.61	2388.9	2252.4	686.8	689.2	2114.4	113.053	24.915	14.753	2.77	1.77	702.3
HS/HC	**12**	34.0	13.4	7.77	0	0.40	62.66	2617.0	2557.4	1234.1	1238.7	2432.9	75.726	48.832	17.921	1.82	1.16	1257.4
	15	34.0	14.9	7.67	0	0.40	55.52	2803.1	2771.9	1715.2	1721.4	2638.5	68.643	64.806	18.496	1.65	1.06	1750.1
	18	34.0	16.7	8.04	0	0.70	18.97	2546.4	2365.4	623.2	625.4	2200.5	142.534	22.314	13.390	3.42	2.20	637.2

Legend: Data Entered into CO_2SYS | Output data generated by CO_2SYS

Table G2

CO2SYS Output results – Day

Treatment Name	Desired Test temp (°C)	Salinity (ppt)	Temp (°C)	pH	P (dbars)	Total P (µmol/kg SW)	Total Si (µmol/kg SW)	TA (µmol/kg SW)	TCO_2 (µmol/kg SW)	fCO_2 (µatm)	pCO_2 (µatm)	HCO_3 (µmol/kg SW)	CO_3 (µmol/kg SW)	CO_2 (µmol/kg SW)	Revelle	ΩCa	ΩAr	xCO2 (dry at 1 atm) (ppm)
Low CO_2 Treatments																		
LS/LC	**12**	27.9	12.4	8.11	0	0.32	42.24	2190.6	2055.7	464.8	466.5	1929.5	106.506	19.648	14.605	2.65	1.66	473.1
	15	28.0	14.6	8.09	0	0.22	16.21	2340.8	2196.1	531.7	533.7	2057.7	117.447	20.970	14.603	2.92	1.84	542.4
	18	28.1	17.3	8.12	0	3.52	10.72	2196.2	2028.4	468.1	469.8	1884.6	126.741	17.013	13.207	3.17	2.01	478.9
MS/LC	**12**	31.0	12.4	8.13	0	0.41	59.89	2405.3	2234.3	466.2	467.9	2083.1	131.920	19.363	13.605	3.22	2.04	474.6
	15	30.9	14.4	8.12	0	1.49	38.76	2545.1	2359.8	514.9	516.7	2194.6	145.103	20.099	13.512	3.55	2.26	525.1
	18	31.1	17.3	8.14	0	0.48	14.49	2381.5	2178.2	464.3	465.9	2007.4	154.165	16.596	12.344	3.78	2.42	475.0
HS/LC	**12**	34.0	12.4	8.15	0	0.45	60.60	2606.8	2396.5	463.4	465.1	2217.6	160.000	18.921	12.737	3.84	2.45	471.7
	15	33.9	14.3	8.14	0	1.06	41.58	2782.9	2555.1	516.1	518.0	2358.3	176.947	19.873	12.701	4.25	2.72	526.3
	18	34.2	17.4	8.14	0	0.76	17.99	2546.5	2310.2	479.3	481.0	2115.9	177.528	16.794	11.760	4.26	2.75	490.4
Medium CO_2 Treatments																		
LS/MC	**12**	28.0	12.6	7.83	0	0.13	44.76	2196.5	2148.9	941.9	945.5	2049.4	59.967	39.543	18.602	1.49	0.93	959.1
	15	28.2	15.2	8.02	0	0.94	23.75	2366.3	2242.1	644.4	646.7	2111.8	105.357	24.921	15.544	2.62	1.65	657.7
	18	28.2	17.9	7.95	0	0.49	11.48	2206.0	2100.1	732.3	734.9	1981.7	92.210	26.139	15.742	2.30	1.46	749.8
MS/MC	**12**	31.0	12.5	7.84	0	0.45	53.87	2412.2	2347.4	976.1	979.7	2234.2	72.817	40.412	17.951	1.78	1.13	993.7
	15	31.0	15.2	8.03	0	0.85	7.35	2564.6	2413.9	658.8	661.2	2263.4	125.365	25.085	14.791	3.07	1.95	672.5
	18	31.2	18.1	7.94	0	0.57	13.35	2409.5	2284.7	794.5	797.3	2149.7	107.273	27.736	15.207	2.63	1.69	813.7
HS/MC	**12**	34.0	12.5	7.86	0	0.40	61.26	2616.2	2528.3	978.8	982.4	2399.4	89.090	39.839	17.120	2.14	1.36	996.4
	15	34.0	15.3	8.06	0	0.98	31.53	2815.3	2619.9	648.6	651.0	2437.9	157.797	24.213	13.700	3.79	2.43	662.1
	18	34.2	18.0	7.94	0	0.85	16.91	2543.5	2398.3	813.0	815.8	2248.9	121.424	27.998	14.548	2.92	1.88	832.4
High CO_2 Treatments																		
LS/HC	**12**	28.0	12.4	7.67	0	0.18	45.17	2210.2	2207.7	1396.2	1401.4	2106.4	42.342	58.985	19.224	1.05	0.66	1421.3
	15	28.0	14.5	7.69	0	0.73	21.19	2374.6	2359.9	1459.6	1464.9	2251.2	50.977	57.738	19.279	1.27	0.80	1488.8
	18	28.2	17.1	7.68	0	0.38	3.90	2191.8	2171.0	1411.9	1416.0	2069.1	50.331	51.558	18.719	1.26	0.80	1444.3
MS/HC	**12**	31.0	12.4	7.69	0	0.22	55.35	2431.1	2413.8	1418.9	1424.2	2301.9	52.930	58.934	18.959	1.29	0.82	1444.4
	15	30.9	14.3	7.67	0	0.84	21.05	2550.3	2532.7	1594.2	1600.1	2413.8	56.436	62.427	18.946	1.38	0.88	1625.7
	18	31.3	17.2	7.67	0	0.47	5.52	2359.0	2330.0	1509.8	1515.1	2218.1	57.852	54.071	18.278	1.42	0.91	1544.5
HS/HC	**12**	34.0	12.4	7.71	0	0.37	63.23	2643.0	2608.3	1430.0	1435.4	2484.8	65.091	58.392	18.531	1.56	1.00	1455.7
	15	33.9	14.2	7.72	0	0.74	28.36	2810.1	2764.0	1512.6	1518.2	2630.7	74.792	58.421	18.436	1.80	1.15	1542.3
	18	34.2	17.3	7.68	0	0.75	17.49	2530.4	2486.3	1542.3	1547.7	2363.6	68.535	54.199	17.776	1.64	1.06	1577.8

Legend: Data Entered into CO_2SYS; Output data generated by CO_2SYS