EFFECTS OF OCEAN ACIDIFICATION ON EARLY DEVELOPMENTAL STAGES OF THE PACIFIC OYSTER (*CRASSOSTREA GIGAS*) IN AN AQUACULTURE SETTING

by

Manon Picard

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Abstract

Ocean acidification is a great concern worldwide. It has important impacts on the shellfish industry. However, more information is needed to assess the impacts of ocean acidification (OA) on economically important shellfish in a realistic industry setting. The goal of this thesis was to determine the effects of OA on the early developmental stages of the Pacific oyster, *Crassostrea gigas*. To better represent industrial-scale oyster production than is possible in small-volume, short-term laboratory studies, experiments were conducted at the Island Scallops hatchery facility using large volume containers and appropriate time scales. In Chapter 2, I investigated the effects of acidification on fertilization and larval development to explore the potential links between vulnerability at these life stages and documented mass mortality events in hatcheries in the past. Elevated pCO_2 contributed to increased egg fertilization. However, not all fertilized eggs were viable, and elevated pCO_2 reduced the likelihood of further development to later embryonic stages, resulting in decreased overall fertilization success in *C. gigas*. Increased pCO_2 levels also negatively affected oyster larvae by reducing growth and reducing the production sufficiently large to be suitable for aquaculture rearing practices. Larval deformity and mortality also showed a trend towards increases in acidified conditions whereas feeding was reduced. In Chapter 3, I assessed early development in benthic juveniles to test for potential for carry-over and/or acclimation effects of prior larval exposure to elevated pCO_2. Settlement did not depend on pCO_2 conditions during the larval phase. However, metamorphosis was reduced when larvae had been exposed to elevated pCO_2 levels. Juvenile growth and condition were both reduced after juvenile's exposure at higher pCO_2 levels, but shell strength was unaffected.

Overall, these findings suggest that OA had negative impacts on multiple life history stages in *C. gigas*. However, the lack of larval exposure effects on juvenile growth or condition suggests an absence of carry-over effects or acclimation for juveniles. These results have important implications for the aquaculture industry and for the development of best practices for combating the effects of ocean acidification.

Preface

This work was undertaken in collaboration with The Department of Fisheries and Oceans Canada, Pacific Biological Station, based in Nanaimo, BC as well as Island Scallops Ltd in Qualicum Bay, BC. I conducted all of the manipulations and the analyses described in this thesis, but the following people assisted me with various steps. Christopher Beacham supplied manual and troubleshooting assistance for the fertilization, larval and settlement experiments. Michael Van de Pol helped me set up the post-settlement experiment, and Lise Lachance assisted me with the juvenile experiment. Robert Saunders and Erin K. McClelland supplied ideas, technical support, and guidance in every step of this thesis. Kristy M. Miller contributed to many of the research ideas. Christopher D. G. Harley supplied ideas and guidance throughout all the steps of my thesis. John Gosline and Ken Savage designed an apparatus for the crushing experiment. Garth Covernton helped with the conduction of the crushing experiment. Kyle Demes, Christopher D. G. Harley, Megan Vaughan, Katie Marshall and Jennifer Sunday helped with the statistical analyses.

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Ω	saturation state
μm	micrometer
°C	degree Celsius
~	approximately
$[CO_2]$	concentration of carbon dioxide

List of Abbreviations

$CaCO_3$	calcium carbonate
CO_2	carbon dioxide
$CO_{2[aq]}$	aqueous carbon dioxide
CO_3^{2-}	carbonate ion
DIC	dissolved inorganic carbon
HCO_3^-	bicarbonate ion
K^*_{sp}	stoichiometric solubility product
L	Liter
m	meter
OA	ocean acidification
pCO_2	partial pressure of carbon dioxide
ppm	parts per million
ppt	parts per thousand
SE	standard error
µatm	micro atmosphere

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Dedication

À ma famille qui m'a donné tout plein de bonheur et de motivation pour y arriver.

Chapter 1: Introduction

Human activities during the industrial era have led to a substantial increase in carbon dioxide (CO_2) in the atmosphere. Oceans have already absorbed approximately 40% of the CO_2 emissions created since the beginning of the industrial era (GACGC, 2006; IPCC, 2007; Royal Society, 2005; Sabine *et al*., 2004) which is driving a phenomenon known as ocean acidification (OA). Although ocean acidification occurred in the past and has been observed as part of a glacial-interglacial cycle, the rate at which ocean acidification has been happening in the recent years is unprecedented (Pelejero *et al*., 2010). The extent and novelty of the current episode of anthropogenic OA raise the interest in determining the effects that OA has on organisms.

1.1 Chemical Aspects of Ocean acidification (OA)

Ocean acidification occurs when dissolved carbon dioxide ($CO_{2[aq]}$) reacts with water to produce carbonic acid that further dissociates to hydrogen ions, bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) ions. This series of reversible reactions results in a decrease in pH and in calcium carbonate ($CaCO_3$) saturation state. Since the beginning of the industrial era, a decrease of 0.1 pH units in the surface ocean has been observed (Orr *et al*., 2005) corresponding to a 26% increase in acidity as measured by hydrogen ion concentration (IPCC, 2013). A recent assessment of ocean acidification (OA) predicted a pH decline (relative to 1950) of approximately 0.35 - 0.4 units and a pCO_2 of approximately 936 μatm by 2100 under the Representative Concentration Pathway 8.5 (RCP8.5) (IPCC, 2013), which is expected to apply unless significant efforts are made to reduce CO_2 emissions in the coming decades.

The partial pressure of CO_2 (pCO_2), and consequently the pH of sea water, can vary at several spatial and temporal scales. Globally, the pCO_2 is increasing and, because CO_2 is more soluble at lower temperatures, this trend is occurring faster at higher latitudes (Feely *et al*., 2004). Spatially, certain ocean basins, such as the NE Pacific, are already lower in pH (Feely *et al.*, 2008). Deeper water is also more corrosive than surface water (Feely *et al.*, 2008); sea water is considered corrosive when it has the potential to damage organisms upon exposure (i.e. by dissolving shells) (Feely *et al.*, 2008). Deeper water is usually more corrosive due to the accumulation of dissolved inorganic carbon (DIC) from respiration (Feely *et al*., 2004). Additionally, the colder temperatures and increased pressures found at greater depths increase the solubility of $CO_{2[aq]}$. More locally, patterns of upwelling can determine local water chemistry spatially over the scale of kilometers and temporally on a daily basis (Wootton *et al*., 2008; Matson *et al*., 2011). In the Strait of Georgia, British Columbia, upwelling-favourable winds can lead to dramatic changes in pH over the course of a few hours (R. Saunders, pers. comm.).

Changes in pH have important implications for calcium carbonate, which constitute an important structural component of many organisms such as corals, molluscs, coralline algae, etc. Calcium carbonate ($CaCO_3$) can be found in various forms, including calcite, aragonite, and amorphous calcium carbonate. Amorphous calcium carbonate can be found in the earliest life stages of some molluscs, including snails (Hasse *et al.*, 2000), oysters (Weiss *et al*., 2002; Lee *et al*., 2006 but see *Kudo et al*., 2010), and some sea urchins (Beniash *et al*., 1997). Aragonite and calcite can occur individually, simultaneously, or sequentially depending on the age of the organism. Indeed, in certain molluscs such as oysters, aragonite occurs earlier and will be replaced almost exclusively by calcite at later life stages (Stenzel, 1964; Medakovic *et al*., 1997; Lee *et al*.,

2006). Each calcium carbonate form has a specific saturation state that dictates when the material is more likely to be formed or dissolved; the less soluble it is, the less likely it is to dissolve. The saturation state (Ω) of calcium carbonate can be determined from the following equation where each form has its own stoichiometric solubility product (K^*_{sp}).

$$\Omega = [Ca^{2+}] [CO_3^{2-}] / K^*_{sp}$$

Calcite is the least soluble form of $CaCO_3$ with the smallest K^*_{sp} value followed by aragonite, and then amorphous calcium carbonate, which is the most soluble form of $CaCO_3$.

Calcification is a process that has been widely studied with regards to OA, especially in terms of saturation states of $CaCO_3$. Increased pCO_2 not only increases the acidity levels, but also shifts the equilibrium of carbonate, resulting in a decline in available carbonate ions required for calcification. When the saturation state value is lower than 1, $CaCO_3$ dissociates (corresponding to dissolution) or does not form (corresponding to a lack of calcification capacity). However, saturation states above and below 1 do not guarantee or preclude calcification, respectively. For example, some calcifying organisms such as corals, calcifying algae, foraminifera, and crabs have evolved mechanisms to manipulate internal pH at the site of calcification (Ries *et al*., 2009). The pH is maintained at higher levels where calcification occurs than the surroundings, facilitating the conversion of HCO_3^- to CO_3^{2-} which can then be used for $CaCO_3$ precipitation (Ries *et al*., 2009). In addition, organisms such as some coccolithophores can utilize HCO_3^- directly instead of CO_3^{2-} to calcify (Ries *et al*., 2009). Although calcification is generally favored over dissolution at saturation states > 1, recent studies have found that decreases in saturation

state can lead to reduced calcification even when the saturation state remains above 1 (Feely *et al*., 2004).

1.2 Effects of OA on Organisms

Responses to high pCO_2 exposure, although variable, are often detrimental for invertebrates such as sea urchins (Brennand *et al.* 2010; Kurihara, Shimode, & Shirayama, 2004; Kurihara & Shirayama, 2004), brittle stars (Dupont *et al*., 2008), barnacles (Findlay *et al.*, 2010), crabs (Walther *et al.*, 2010), and oysters (Parker *et al.*, 2011). However, experimentally elevated pCO_2 had no effect on some echinoderms such as the sea star *Patiriella regularis* (Byrne *et al*., 2010a) and the sea urchin *Heliocidaris erythrogramma* (Byrne et al., 2009, Byrne *et al*., 2010a; Byrne *et al*., 2010b). Furthermore, for some sea stars, CO_2-induced pH reductions were beneficial and resulted in increased growth (*Pisaster ochraceus* Gooding *et al*., 2009; *Crossaster papposus* Dupont *et al*., 2010).

However, the presence of variation in the effects of OA on organisms blurs predictions and highlights the need for expanding research on inter- and intra-species levels. For instance, previous studies on larval calcification have shown negative effects on the oyster, *Crassostrea virginica,* but neutral effects on the congeneric *C. ariakensis* when exposed to higher pCO_2 (Miller *et al.*, 2009). Moreover, the egg production rates in *Acartia steuri* (Kurihara *et al*., 2004) are reduced but not in *A. tsuensis* (Kurihara & Ishimatsu, 2008). Also, variations are known to occur intraspecifically such as between populations of *S. glomerata* (Parker *et al*., 2011) and of hard clam *Mercenaria* spp. (Waldbusser *et al*., 2010). Variations also occur in the extent of the effects observed between closely related species. For instance, a study on the oysters *Crassostrea*

gigas and *Saccostrea glomerata* found that although these two species were negatively affected by OA, overall the former species was more sensitive to OA than *C. gigas* (Parker *et al*., 2010). In addition, to further add to the complexity of the determination of the effects of OA, variations in the effects exist also between life-stages of organisms (Dupont *et al*., 2013), highlighting the need to study the entire life cycle in depth to predict more accurately the vulnerability of a species.

1.2.1 Effects of OA on Fertilization

Fertilization decreases with exposure to acidification in some organisms such as sea urchins (Kurihara *et al.* 2004; Kurihara and Shirayama 2004), brittlestar (Dupont *et al*., 2008) and oysters (Parker *et al*., 2009; Watson *et al*., 2009). In general, broadcast spawners who release their gametes in the water where fertilization occurs are expected to be more negatively affected by OA than brooders, who carry their eggs in an internal chamber for a certain time during and after fertilization (Noisette *et al*., 2014) based on protective properties of the chamber (Przeslawski, 2004; Chaparro *et al*., 2009). However, recent evidence is challenging this perception by showing that brooding and encapsulating gastropods have reduced shell size in acidified conditions showing the potential limited protective properties towards acidification (Noisette *et al*., 2014).

1.2.2 Effects of OA on Larvae

The larval phase of many marine organisms gains importance especially in terms of dispersal of sessile organisms producing planktonic larvae such as barnacles, oysters, tube-dwelling worms and bryozoans (Tamburri *et al*., 1992). The larval stage is often considered to be the most

vulnerable stage in an animal's life cycle (Kurihara, 2008) and a meta-analysis of empirical data supports this conclusion for molluscs (Kroeker *et al*., 2013). This could be due to restricted physiological capabilities in embryonic and larval stages (Pörtner *et al*., 1998) and the commencement of the shell formation at the same stages (Kurihara *et al*., 2007).

1.2.3 Effects of OA on Settlement

Settlement is the process by which an organism comes in contact with and then attaches to a substrate. For sessile invertebrates, it is an intermediate reversible behavior occurring between the prior exploration of substrates by the planktonic larvae and the metamorphosing into the stationary juvenile stage (Tamburri *et al*., 1992). Most studies that have investigated the effects of OA on settlement have focused on corals (e.g. Albright *et al*., 2008; Albright *et al*., 2010; Albright & Langdon, 2011; Webster *et al*., 2013). These studies generally revealed no effect of OA on physical settlement, although indirect effects were possible when OA altered the characteristics of the biofilm on settlement surfaces (Albright & Langdon, 2011; Webster *et al*., 2013). A study in volcanic CO_2 vents looking at settlement changes over a gradient of pCO_2 found a decline in recruitment of calcareous foraminiferans, serpulids polychaetes, gastropods and bivalves with increased pCO_2 (Cigliano *et al*., 2010). However, it is unclear if the decline is caused by a reduction in larval supply or by early post-settlement mortality as opposed to settlement being affected directly (physiological impairment) or indirectly (mediated by the biofilm).

1.2.4 Effects of OA on Juveniles

The juvenile life stage, which is also considered an early developmental stage, directly precedes the adult stage and has been found to naturally have a very high mortality rate which can reach levels > 90% in benthic marine invertebrates (Gosselin & Qian, 1997). However, mortality at this stage is greatest at the onset of juvenile life (Gosselin & Qian, 1997). This mortality can be exacerbated with OA since juvenile organisms have been found in some instances to be susceptible to OA. For instance, juvenile bivalves clams (*Mercenaria mercenaria*) have been found to be more susceptible than adults to elevated pCO_2 levels and exhibit increased mortality rates arising from the dissolution of $CaCO_3$ (Green *et al*., 2004). They also found that the smaller individuals suffered a higher mortality rate than the larger juveniles.

1.2.5 Potential for Acclimation and Carry-Over Effects

The aforementioned life-stages do not stand alone and, along with the adult stage, they are tightly linked together and performance at one stage can depend upon conditions experienced by earlier stages in the cycle. Acclimation, which occurs when organisms adjust to their environment within their lifetime, is an important concept to consider as it can change the outcomes in a study. For instance, if individual larvae are able to acclimate to high pCO_2 exposure during development, then individuals that had experienced high pCO_2 as larvae may be better able to tolerate high pCO_2 as juveniles. Previous studies have demonstrated that adults oysters, *Saccostrea glomerata*, when exposed to higher levels of pCO_2, produced larvae that were more resilient to higher pCO_2 than adults exposed at lower pCO_2 (Parker *et al*., 2012). Findings from Parker *et al*. (2012) suggest that acclimation to higher pCO_2 levels may occur in certain species of oysters. In contrast, Dupont *et al*. (2013) found that juvenile urchins born to

adults that were acclimated for 16 months at high pCO_2 had a 9-fold increase in mortality, suggesting that acclimation does not occur in all organisms. Such negative carry-over effects can also affect experimental results and should be considered in studies in order to understand how a species respond to OA. A carry-over effect occurs when exposure to a stressor at an earlier stage positively or negatively affects the organism at a later stage. However, since very few studies explored more than one life-stage, there is a need for further studies to understand how these effects either mitigate or worsen the effects of OA on species of interest.

1.3 Effects of OA on *Crassostrea gigas*

The Pacific oyster (*Crassostrea gigas*) has been widely investigated due to its international economic importance in the food industry. For instance, oysters were the most cultivated mollusc world-wide until 2008 with *C. gigas* as the main species, although oysters are now second behind clam and cockle production combined (FAO, 2012). *C. gigas* not only has a tremendous economic value, it also has an ecological importance due to its role in providing habitat and shelter for other many benthic organisms (Kurihara *et al*., 2007) such as barnacles, limpets, and sea anemones amongst others. Moreover, *C. gigas* is the most commonly used species for assessing water quality and the toxicity of pollutants in some regions (His *et al*., 1997).

Additional incentive to study *C. gigas* stems from the fact that it is a mollusc and since molluscs are consistently negatively impacted by OA (Kurihara, 2008; Parker *et al.*, 2009; Gazeau *et al*., 2010; Talmage & Gobler, 2010; Kroeker *et al*., 2010; 2013), they require a greater deal of attention due to their greater susceptibility to OA. Michaelidis *et al*. (2005) have proposed that

oysters and other bivalves' vulnerability to pH are best explained by their poor ability to regulate acid-base ions.

C. gigas are protandric hermaphrodites, meaning that they are separate sexes at any one time but change with time and conditions. Females are usually older individuals that may become the more prominent sex with ample food resources (Cheney & Mumford, 1986). *C. gigas* naturally reproduce by releasing their gametes by broadcast spawning when the temperature is increased above 19° C (Quayle, 1988) and food is abundant in the water where external fertilization takes place. Fertilized embryos develop into planktonic trocophore larvae. The trocophore larva, within approximately 24 hours, develop into a prodissoconch I larva (veliger larva) which further develop into prodissoconch II larva (umbonate larva) within approximately 24 hours. The prodissoconch II larval stage lasts the longest of all the larval stages and is later referred to as pediveliger larvae when the larvae become ready to settle. Pediveliger larvae have an eyespot, a visible and highly mobile foot, and are approximately 300 μm at maximal length. Reaching this stage usually takes around 18 days from fertilization in favorable conditions, although the time of development is highly dependent on temperature (Quayle, 1988). After the larvae settle and metamorphose, they become dissoconch juveniles (Fig. 1-1).

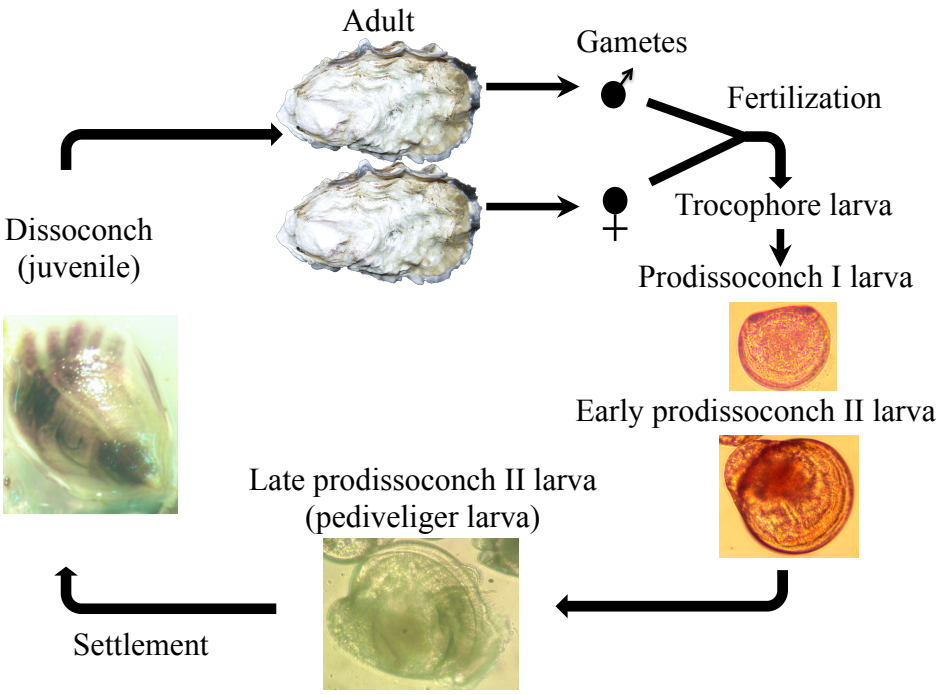

Figure 1-1. Diagram of the life cycle of *Crassostrea gigas*.

In Portugal, Barros *et al*. (2013) observed reduced sperm motility and fertilization and hatching success in *C. gigas*. Moreover, in sea urchins, reduced sperm speed and percent sperm motility were observed after exposure to OA (Havenhand *et al*., 2008, but not in Byrne *et al*., 2009) as well as increased sperm limitation and increased polyspermy (Reuter *et al*., 2011; Bögner *et al*., 2013). The latter result was also found in scallops (Desrosiers *et al*., 1996); however, no study to date has looked upon polyspermy in regard to OA in *C. gigas*. Polyspermy occurs when more than one sperm fertilizes an egg, resulting in the egg's death. Normally, mechanisms relating to post-fertilization block further sperm from entering the egg, but these mechanisms, may be altered by acidification (Reuter *et al*., 2011; Bögner *et al*., 2013). Nonetheless, the impairment of sperm due to OA is not steady everywhere for *C. gigas*. For instance, in Sweden, Havenhand & Schlegel (2009) did not find any significant change in sperm motility and fertilization success

after subjecting *C. gigas* to elevated pCO_2, which is consistent with findings in Japan (Kurihara *et al*., 2007).

The onset of shell mineralization (calcification) in *C. gigas* starts at the late trocophore stage, 6-10 h post fertilization (Waldbusser *et al*., 2013) and coincides with the beginning of observations of negative effects to acidified water as seen in Kurihara *et al*. (2007) who observed no effect on fertilization itself. Waldbusser *et al*. (2013) examined stable carbon isotope ratios to uncover the potential causes of vulnerability to OA at the shell formation stage in *C. gigas*. They found that reduced ability to isolate calcifying fluid from the surrounding seawater, a limited energy budget associated with stress, and a strong kinetic demand for $CaCO_3$ precipitation all play a role in *C. gigas*' sensitivity at the first shell formation stage.

Oysters are thought to be particularly susceptible to increased acidification as larvae as they initially form their larval shell with aragonite until settlement, at which point they begin producing their shell with calcite (Barton *et al*., 2012). Therefore, due to this suspected susceptibility and the economical and ecological importance of *C.* gigas, the number of studies on the effects of OA on *C. gigas* have steadily increased in recent years (Parker *et al*., 2010; Barton *et al*., 2012; Barros *et al*., 2013; Ginger *et al*., 2013, etc.). These studies cover a wide range of pCO_2 levels as well as various responses to those exposures and life stages, resulting in a variety of findings. For instance, Parker *et al*. (2010) found that OA negatively affects *C. gigas* by reducing its fertilization success whereas no effect of OA on fertilization was found by Havenhand & Schlegel (2009). Kurihara *et al*. (2007) did not observe any effect on the embryo until the prodissoconch I stage where they found that the development was reduced and smaller

larvae at the same stage were produced (Kurihara *et al*., 2007; Parker *et al*., 2010). Smaller prodissoconch II and pediveliger larvae as well as dissoconchs were also obtained at higher pCO_2 (Parker *et al*., 2010). Moreover, *C. gigas* has been found to reduce its calcification rate by 10% when dissoconch and adults were exposed at elevated pCO_2 (740 µatm) for only 2 hours (Gazeau *et al*., 2007).

1.4 Implications of OA for the Aquaculture of *C. gigas*

Crassostrea gigas is not only widely studied due to its aforementioned economical importance, but also in light of recent mass mortality events occurring worldwide. *C. gigas* was introduced to British Columbia from Japan, where it naturally resides near Sendai on northern Honshu Island (Quayle, 1988). Since *C. gigas* requires higher temperatures than what can be found naturally in most North American coastal regions, the industry in North America is solely dependent on hatcheries for production (Barton *et al*., 2012). In hatcheries, instead of waiting for the gametes to be naturally released, a technique called strip spawning is widely used to speed up the process of reproduction. This technique involves the direct extraction of the gametes from the gonads, which sacrifices the brooding stock. Hatcheries rear larval *C. gigas* until they are competent to settle, at which point they are referred to as "seed". The seed is then settled on substrate, cultch, which is often composed of empty shells of adult *C. gigas*. Once they settle, they metamorphose and become juveniles, or as they are more commonly known in the industry, spat. The juveniles can then be moved to the ocean and left to grow to the desired size.

From 2005 to 2009, *C. gigas* production declined by 22% on the West coast of the United States, and since most of the industry is situated in Oregon, Washington and California, these areas

naturally exhibited the sharpest population decline (Pacific Coast Shellfish Growers Association 2010). In 2008 alone, the Whiskey Creek Hatchery situated in Oregon, which is one of the three major seed hatcheries of this region, experienced a decline of 80% in *C. gigas* production, representing a loss of approximately $100 million USD (Gouldman *et al*., 2011). Originally, the declines were attributed to a virus *Vibrio tubiashii* (Barton *et al*., 2012); however, since the declines were persistent after preventive measures were taken, other causes such as OA were considered. Although the causes have not been identified with certainty, the declines in the production coincided with acidified coastal upwellings at Whiskey Creek Hatchery (Barton *et al.* 2012). In British Columbia, mass mortality events have also been documented and the few aquaculture companies that remain able to produce *C. gigas* seed still suffer from occasional mass mortality events (R. Saunders, pers. comm.). These mass mortality events in the Strait of Georgia have been suggested to be linked to the upwelling of corrosive seawater (R. Saunders, pers. comm.), although the exact role of low pH has remains unclear.

Armed with increased knowledge regarding the potentially detrimental effects of OA, hatcheries have been implementing various measures to limit the exposure of larvae to low pH. For instance, many facilities are now restricting water intake to periods with acceptable pCO_2 levels in the source water. Some companies have also begun degasing CO_2 when pH levels become low. Unfortunately, these methods do not always prevent massive stock mortalities and monetary loss. Thus, pinpointing the vulnerable stage(s) in the lifecycle of *C. gigas* could allow a better concentration of efforts during the most critical period.

Despite many studies, a consensus has not been reached regarding the early developmental stages at which *C. gigas* are most susceptible to exposure at higher pCO_2 levels. This information is key for aquaculture practices to potentially mitigate the negative effects and make wiser decisions in the harvesting process. Additionally, most of the studies were conducted on a much smaller scale (*i.e.* 20 L used by Barros *et al.*, 2013) than that typical of aquaculture production (*i.e.* 40,000 L). The scale used, if too small, has been found to impede the proper simulation of important processes (Frost *et al*., 2001) such as prey movement that was found to have less effect on prey densities in larger enclosures than in smaller enclosures (Englund, 1997). In addition, most studies to date have been conducted using much higher pCO_2 levels than the predictions on the worst case scenario of the next century (i.e. 2268 µatm used by Kurihara *et al.*, 2007) and thus do not necessarily address the problem faced by growers very realistically. For these reasons, we undertook the present study in order to evaluate the effects of pCO_2 level on the fertilization, larval development as well as on the early juvenile growth and performance of *C. gigas*.

1.5 Thesis Objectives

The goal of this thesis is to answer the following questions about the responses of *Crassostrea gigas* to exposure to near-future pCO_2 levels under realistic hatchery conditions.

1. How does OA affect the fertilization of *C. gigas*?

2. How does OA affect larval *C. gigas* in terms of growth, mortality, deformity, and feeding rate?

3. How does prior larval exposure to OA affect larval settlement in *C. gigas*?

4 a. How does OA affect juvenile *C. gigas*?

4 b. Can individuals acclimate to acidified conditions in the larval stage such that they become more resistant to exposure to high pCO_2 as juveniles?

By answering these questions, I aim to provide a better understanding of the ocean acidification effects on the culture of *Crassostrea gigas*, a locally economically important species, in the hopes of reducing the monetary losses associated with upwelled corrosive water and longer-term trends in ocean pH.

Chapter 2: Effects of Ocean Acidification on Fertilization and Larval Stages Performance of *Crassostrea gigas*

2.1 Introduction

Ocean acidification (OA) has generated a great deal of concern around the world, and its effects are predicted to worsen in the near future. Natural processes are affected and therefore ecological and monetary impacts have already been witnessed in various parts of the planet. Since molluscs have been found to be among the most affected by OA (Kroeker *et al*., 2013), they are one of the most immediate concerns for researchers. Moreover, an increasing amount of research has focused on the effects of OA at early developmental stages of mollucs, given that this stage has been shown to be especially vulnerable to fluctuations in pH (Kroeker *et al*., 2013). The fluctuations in pH experienced recently in the Strait of Georgia, British Columbia, Canada (Marliave *et al*., 2011) suggests that the local oysters' hatchery failures, similar to failures in Oregon and Washington, could be caused by intrusion of low pH water, perhaps associated with periods of upwelling. However, with the lack of industry-related relevance in most of the studies conducted on the effects of OA on *C. gigas* to this date, there is an urgent need for more appropriate studies for the aquaculture industry to better predict and mitigate the effects of OA on this ecologically and economically important species.

The early larval stages is a great concern for the hatcheries locally as there have been prior mass mortality events in the larval stock coinciding with periods of low pH in the intake water suggesting a likely correlation between the two events (R. Saunders, pers. comm.). This likely correlation has been found nearby by growers in Oregon (Barton *et al*., 2012) increasing the

possibility for the correlation to be plausible here as well. Moreover, the larval stage has been found to be vulnerable to OA such as the development to prodissoconch I of *C. gigas* larvae is delayed with elevated pCO_2 (Kurihara *et al*., 2007; Parker *et al*., 2010). Even earlier developmental stages are likely to be the susceptible to OA as well. This is especially true in *C. gigas,* which are broadcast spawners, meaning that fertilization events and early developmental stages occur in the external environment. The early developmental stages of *C. gigas*, which feature key transitions such as the onset of calcification, are thought to be highly susceptible to OA due to the presence of highly soluble amorphous calcium carbonate (Weiss *et al.*, 2002; but see *Kudo et al*., 2010). Later-stage larvae are also highly susceptible to OA because their shells are composed of aragonite, which is quite soluble (Stenzel, 1964).

The goal of larval culture in the hatchery is to produce pediveliger stage larvae, referred to as seed. These larvae are usually gathered in a screen with a minimal mesh size of 253 μm, although larvae screened at 263 μm are preferred as they are believed to be optimal for successful settlement. The seed can be sold to growers at this point, and larger seed, i.e. those screened at 263 μm, are more desirable and command higher prices. Thus, the economic considerations of larval culture include survivorship, development time, and final size. All three of these factors are potentially affected by OA.

Larval experiments have also shown a great variety of responses. For instance, in China, (Ginger *et al*., 2013) OA had no effect on larval size and survival of *C. gigas*, whereas in Portugal (Barros *et al*., 2013) OA reduced size and survival of *C. gigas*. Parker *et al*. (2010) found a reduced larval growth in an Australian population while in Oregon, USA, Barton *et al*. (2012)

observed delayed growth effect on larvae and a correlation of size with aragonite saturation state while experimenting under natural fluctuations. The inconsistency of results among studies, coupled with methodologies (container volumes, feeding methods, pCO_2 levels) that do not correspond to conditions in actual hatcheries, have meant that the degree to which OA will affect hatchery operations is not well understood.

Given the industry's concern about the effects of OA throughout early development, I undertook a series of studies to examine the effects of OA on fertilization and larval development. The fertilization experiment of this study was designed following a fertilization event failure of *C. gigas* at Island Scallops Ltd., found in Qualicum Bay, BC, which was suspected to have been caused by OA. The sperm had been inadvertently rinsed with high pCO_2 water before being mixed with eggs that had been rinsed at ambient pCO_2. The low fertilization rates following this procedure suggested that the sperm of *C. gigas* may be highly susceptible to OA. Therefore, I tested if the prior washing of the sperm with elevated pCO_2 had an effect on the proportion of eggs fertilized as well as the relative development speed 48 hours post-fertilization. Furthermore, I tested if these responses were affected by elevated pCO_2 exposure during fertilization and early development of the embryos. The prior events and literature aforementioned on the subject suggest that the proportion of eggs fertilized will be reduced due to the acidification of the sperm whereas the development speed of the embryos will be reduced due to the acidification during fertilization. Also, to investigate the effects of OA on larval *C. gigas*, I tested how the larvae in the Strait of Georgia respond to OA in terms of size, mortality, deformity, and feeding rate in order to respond to local issues more appropriately. Again based on prior knowledge, I predicted

that all these aspects would be negatively affected, i.e., larval size and feeding rate will be reduced while mortality and deformity will be enhanced when exposed to OA.

2.2 Methods

2.2.1 Fertilization

Four male-female pairs of *Crassostrea gigas* (obtained from Fanny Bay Oysters, Fanny Bay, British Columbia) were strip spawned on October 10th 2012. The gametes for each individual were filtered through a 90 μm screen with the treatment water, half were rinsed with high [CO_2] water (710 ppm) and half at low [CO_2] (510 ppm). (Unless otherwise noted, when measured carbon dioxide levels are being described quantitatively, concentrations (in ppm) are presented as that is what was measured. However, experimental treatments are generally referred to as either high pCO_2 or low pCO_2 throughout the thesis. Although CO_2 concentration is not equivalent to pCO_2 (the partial pressure of CO_2), CO_2 concentration in ppm closely approximates pCO_2 in μatm at the temperatures and pressures used in this study, and they are interchangeable as qualitative descriptors). For this experiment only, naturally high and low pCO_2 water was pumped directly from the ocean bypassing degasing treatments of the hatchery on the same day. The high pCO_2 treatment was taken from the hatchery intake, mixing deeper and shallower water whereas the low pCO_2 treatment was taken directly from a nearby beach to collect surface water. The water was topped up to 1 L then stirred and left for 15 minutes before starting fertilization.

The gametes of a male-female pair were mixed in order to get the following 4 treatments for each couple.

High CO_2 sperm wash High CO_2 egg wash + fertilization	High CO_2 sperm wash Low CO_2 egg wash + fertilization
Low CO_2 sperm wash High CO_2 egg wash + fertilization	Low CO_2 sperm wash Low CO_2 egg wash + fertilization

The density was then homogenized using a plunger. The eggs were counted after filling a 1 mL plastic pipette and placing it under a dissecting microscope. The sperm was counted using a haemocytometer placed under a microscope. Approximately 150,000 (± 2,000) eggs of each female per treatment were combined with 1,000,000 (± 20,000) sperm cells from the corresponding male in each pair to obtain a ratio of 6 sperm cells per egg. The water was topped up to 1 L with the same treatment water as the egg previous treatment. After stirring the water, fertilization was allowed to proceed for 30 minutes, following common hatchery practice and recommendations under Lavens & Sorgeloos (1996). Later, the contents of each container were divided equally into 2 Pyrex flasks and topped to 5L with the same treatment water as the fertilization. Finally, all the flasks were put in a common water bath at 21°C.

After letting fertilization and development proceed for 48 hours, the contents of the flasks were filtered through a 35 μm screen. The filtrate was recovered in a bucket and pH, temperature, pCO_2 and salinity were quantified. The residue was placed in plastic containers for examination under the microscope. A total of 100 randomly sampled individuals from each flask were characterized by their morphology as either eggs (fertilized or not), blastulae, trocophore larvae, or prodissoconch I larvae. Eggs that had been fertilized but had suffered from polyspermy could

not be differentiated from the ones that had not. The experiment was conducted similarly to other studies conducted in various parts of the world looking at the effects of OA on *C. gigas* fertilization (Table 2-1).

Table 2-1. Summary comparing various aspects of this fertilization experiment to similar studies on *Crassostrea gigas*.

Experiment	Volume (L)	pCO_2 level (µatm) Low	High	Duration (h)	Sampling time (h)	Country
Current study	5	518*	721*	48	48	Canada
Kurihara *et al*. 2007	0.05	348	2268	48	2, 3, 8, 24, and 48	Japan
Havenhand & Schlegel 2009	0.005	8.15 (pH)	7.82 (pH)	1	1	Sweden
Parker *et al*. 2010	0.11	375	600, 750, 1000	0-48	2 and 48	Australia
Gazeau *et al*. 2011	4.5	449	494, 1020, 2170, 3730	embryo-72	72	The Netherlands
Barton *et al*. 2012	22,000	Depended on intake		48	24	USA
Barros *et al*. 2013	20	580	1386, 3573	48	2, 3, 4, and 48	Portugal
Timmins-Schiffman *et al*. 2013	3	400	700, 1000	embryo-72	24 and 72	USA

* Concentration of carbon dioxide was measured in ppm and converted to pCO_2 in µatm to facilitate comparisons with the literature

2.2.1.1 Water Chemistry

Water chemistry was taken at the beginning and at the end of the experiment following the methods of Dickson *et al*. (2007) (Table 2-2). The concentration of carbon dioxide [CO_2] was measured using CO_2/H_2O Gas Analyzer (LI-840A) calibrated with known concentrations of CO_2 using pure nitrogen (i.e., 0 ppm CO_2), 500 ppm CO_2 and 750 ppm CO_2. Temperature was measured using a pH meter (JENCO pH 6810) and salinity with a portable refractometer (A366 ATC VISTA series instruments). Dissolved inorganic carbon (DIC) was measured only at the beginning of the experiment using a Dissolved Inorganic Carbon analyzer (model AS-C3

manufactured by Apollo Scitech Inc.) after poisoning the 300 mL sample (Wheaton 13) with 100 µL (Mandel Scientific P200) of Mercuric Chloride (HgCl).

Table 2-2. Seawater carbonate chemistry variables (mean ± SE) at the beginning (t=0) and at the end (t=48 hrs) of the fertilization experiment. Concentration of CO_2 ($[CO_2]$), temperature (Temp), and dissolved inorganic carbon (DIC) were measured at the beginning of the experiment once per treatment as the water mass was distributed to all the flasks; thus, no variance is associated with those measurements. $[CO_2]$, Temp, and pH were measured at the end of the experiment for each flask. Alkalinity (A_T), and saturation state for calcite (Ω_{cal}) and aragonite (Ω_{ara}) were estimated using CO_2calc (Robbins *et al.*, 2010).

pCO_2 Treatment	Time (hrs)	$[CO_2]$ ppm	DIC mmol/ml	Temp °C	A_T mmol/kg	pH	Ω_{cal}	Ω_{ara}
Low	0	510	2655	18.0	2950	8.18	5.59	3.61
Low	48	610 (31)	1942	14.9 (0.09)	2063	7.97 (0.03)	2.32	1.48
High	0	710	1461	18.3	1608	7.80	1.34	0.87
High	48	687 (24)	2036	14.9 (0.08)	2149	7.94 (0.01)	2.27	1.45

2.2.1.2 Statistical Analysis

The arcsin square root proportion of egg fertilized and the arcsin square root proportion of embryos that have developed to the trocophore stage and beyond were analyzed to meet the assumption of homogeneity of the residuals on mixed effects models by nesting within couple (RStudio 0.94.110). The ANOVA values were reported from the models. The data were later backtransformed for graphical presentation. One replicate of the sperm and egg washed and fertilized at high *p*CO_2 was excluded as the contents were in poor condition and the stages could not be easily identified.

2.2.2 Larval Experiment

The larval experiment was conducted with prodissoconch I larvae from a single spawning event using multiple parental origins. Prior to manipulations, larvae were reared to an age of 2 days in a common tank. The experiment was performed in large (4,200 L) tanks in order to mimic aquaculture practices by using a large volume; these large volumes were also better at maintaining constant pCO_2 (personal observation). The volume used in this study was within the range of previous studies (e.g. Parker *et al*., 2010; Barton *et al*., 2012) conducted on larval *C. gigas* in aquaculture facilities (Table 2-3).

Table 2-3. Summary comparing the design of this larval experiment with similar studies on *Crassostrea gigas.*

Larval Experiment	Water volume (L)	pCO_2 level (µatm) High	Low	Experiment duration (Days)	Sampling days	Density (larvae /mL)	Country
Current study	4,200	695*	406*	D-stage-18	5,6,8,10,12, 14,16,18,20	3	Canada
Parker *et al*. 2010	1,000	600, 750, 1000	375	90 µm screened-4 days	4	2.5	Australia
Gazeau *et al*. 2011	4.5	494, 1020, 2170, 3730	449	embryo-72	72	15	The Netherlands
Barton *et al*. 2012	22,000	Depended on intake		0-9 or 0-16	2,4,6,8,10, 12,14,16	Cohort dependent	USA
Ko *et al*. 2013	40	1497, 2386	622	D-stage-settlement	11, 16	20	China
Barros *et al*. 2013	20	1386, 3573	580	0-6 days	4,6	Not provided	Portugal

* Concentration of carbon dioxide was measured in ppm and converted to pCO_2 in µatm to facilitate comparisons with the literature

Four 4200 L tanks were filled with seawater from the Strait of Georgia mixing deep water (intake at 24 m) with shallow water (intake at 9 m) that was later sand filtered and protein skimmed at a pCO_2 of 401 ± 31 ppm. Of these 4 tanks, 2 were bubbled with supplemental CO_2 (using a tank of pure CO_2 and an air stone) to raise the pCO_2 to 685 ± 32 ppm. Approximately

12.6 million larvae were put in each of the four tanks to obtain a density of 3 larvae/mL of seawater.

Larval growth of *C. gigas* is optimal at 27° C (Rico-Villa *et al*., 2009) and the larval development lasts 16-18 days post-fertilization depending on the conditions. However, in hatcheries, the economic benefits of shorter development times (reduced labor costs, etc.) are partially offset by the cost of heating the water as high as 27 °C. Therefore, hatcheries along the west coast of North America rear oyster larvae at approximately 25 °C (Barton *et al*., 2012) to best balance costs and benefits.

2.2.2.1 Water Change

Water exchanges were conducted to mimic hatchery practices using still water. The water in the tanks was partially (50%) changed every 2 days. The older water was poured down from a pvc apparatus screwed in the tank at 2/3 of the tank volume and to keep the larval content in the tank, the apparatus was covered by a 50 µm screen. The tank was being filled from the top in the meantime. A tank with the same size (4,200 L) was used to top the two tanks of the same treatment to ensure that the water had the same chemistry. The water was fully changed every 4 days by opening valves on drainage pipes at the bottom of the tanks and screening the content using a basket lined in the bottom with a 52 µm screen. Saltwater was then used to rinse the tanks in an attempt to collect any remaining larvae. The tanks were next cleaned using bleach then rinsed thoroughly with tap freshwater until all the bleach was removed. The treatment water was then used to rinse the tanks before filling them again with treatment water.

2.2.2.2 Water Chemistry

Water chemistry was characterized following the same methods as described in the water chemistry section of the fertilization experiment. Samples were taken before and after each partial and full water exchange (Table 2-4).

Table 2-4. Means for seawater carbonate chemistry variables with SE in parentheses. Mean values for rearing *Crassostrea gigas* larvae from prodissoconch I stage (2 days old) until they were ready to settle (20 days old). Concentration of CO_2 ($[CO_2]$) and temperature (Temp) were measured every 2 days. Dissolved inorganic carbon (DIC) was measured on days 6 (before and after water changes), 10, 13, 14, 17, and 18 along with salinity, $[CO_2]$, and Temp, this represents a complete set of measurements of all parameters once every full water change. On those days, alkalinity (A_T), pH, saturation state for calcite (Ω_{cal}) and aragonite (Ω_{ara}) were estimated using CO_2calc (Robbins *et al.,* 2010).

pCO_2 Treatment	Replicate	$[CO_2]$ ppm	DIC mmol/ml	Temp °C	A_T mmol/kg	pH	Ω_{cal}	Ω_{ara}
Low	1	397	1447	21.0	1589	8.04	2.39	1.54
		(21)	(16)	(0.4)	(16)	(0.02)	(0.09)	(0.06)
Low	2	404	1442	20.8	1576	8.03	2.29	1.48
		(23)	(17)	(0.4)	(20)	(0.02)	(0.10)	(0.06)
High	1	685	1526	20.8	1608	7.86	1.64	1.06
		(23)	(15)	(0.3)	(14)	(0.03)	(0.10)	(0.07)
High	2	685	1521	20.7	1605	7.86	1.64	1.05
		(22)	(17)	(0.3)	(15)	(0.03)	(0.10)	(0.07)

2.2.2.3 Feeding Methodology and Determination of Feeding Rates

Larvae were fed the same algal composition and concentration as the hatchery stock (Appendix A). Buckets of suspended algal food were poured directly in the tanks. As a control for the larval consumption of algae, a 5 Liter Pyrex flask was also filled with the filtered water (with a 50 µm mesh) to contain the same composition as the tank with the exception of the larval oysters. The flask was then partially submerged in the tank to maintain it at tank temperature.

The algal concentration readings were taken after letting the water and algae mixture mix for an hour after the daily feeding and each morning before the next feeding, which was 18-22 hours later. The samples were taken from four equidistant locations around the perimeter of each tank and by filtering a water sample through a 30 μm screen, which allowed algae to pass but prevented the larvae from entering the water sample. The filtered sample was put in a 20 mL clear plastic container. The algal concentration was measured in a Coulter counter analyzer (Beckman Ztm) after setting up the reading between 4-6 μm. Each sample was measured once for a total of 4 readings per tank twice a day. The larval feeding was measured by subtracting the control final concentration in the flask to the tank final concentration.

2.2.2.4 Sampling

Approximately every other day from day 2 until day 20, photos were taken of a subset of the larvae using a microscope camera (UCMOS 10 000 KPA) and camera control software (Toup view 3.2). I used all the larvae in each photo, approximately 70 individuals per sampling day per tank, to assess morphological deformity (defined as any irregularities in the ventral margin line) and mortality (measured as the number of individuals that had empty shells). Using the first 25 larvae in each image, larval length was quantified using ImageJ (Rasband 1997-2012). Length was measured as diameter for prodissoconch I larvae and maximal length from the umbo for prodissoconch II larvae. Samples for size and shape evaluations were collected near the surface using a 60 μm screen when the larvae were 5, 8, 12, and 16 days old. Mortality was only measured on the days of full water changes in order to get a sample of the whole tank content and avoid the bias associated with dead larvae sinking. Size and deformity were also evaluated

from the whole tank content at the larval age of 6, 10, 14, 18, and 20 days old corresponding to the days of complete water changes.

At the end of the experiment, the remaining larvae were filtered through screens of 263 μm, 253 μm and 90 μm. The mesh sizes were chosen in order to depict the final density at those size ranges. 253 μm is the minimum size to sell seed (i.e., larvae ready to settle) in the industry and 263 μm is the ideal size to sell seed, as they are most likely to settle at that size. The larvae at each size class were placed in a bucket which was then topped up to 20 L with seawater at low pCO_2. The contents were homogenized using a plunger and a 10 mL pipette was then immersed in the bucket, and the number of larvae was counted in six 1 mL subsamples that were averaged and then extrapolated to the number of larvae in the 20 L sample.

2.2.2.5 Statistical Analyses

The larval sizes were analyzed using repeated measures ANOVA nested within tanks. Because the assumption of sphericity was not met, we used the Greenhouse-Geisser corrected F and p values when calculating significance. The size at two days old was excluded from the analysis as larvae were not separated into treatments prior to this day. The sizes at 18 and 20 days old were excluded from the analysis as the larvae had started reaching settlement size. Morphological deformity was analyzed using a Pearson Chi-squared test combining all the measurements over time and by pooling across replicate tanks as well. Mortality in the tanks was analyzed using a Pearson Chi-squared test by analyzing all the larvae photographed from which mortality was defined as empty shell. Concentration of the remaining larvae and the proportion that were smaller than 253 μm were tested using t-Test analyses. The larval feeding rate data were

analyzed using repeated measures nested within tanks including only the days when at least 3 algal replicates were measured per tank per sampling (both initial and final) and when the control was also measured. All statistical analyses were conducted using JMP 9.02.

2.3 Results

2.3.1 Fertilization and Early Development

Fertilization was higher at higher pCO_2 (2-Way ANOVA; $F_{1,24} = 346.827$, p=0.0002) (Fig. 2-1). However, as previously mentioned, the eggs that were fertilized, but had suffered from polyspermy, could not be differentiated from the ones that had not. There was no effect of prior sperm treatment (2-Way ANOVA; $F_{1,24} = 0.541$ p=0.469), nor was there an interaction between the prior sperm treatment and the fertilization treatment (2-Way ANOVA; $F_{1,24} = 0.4911$ p=0.490).

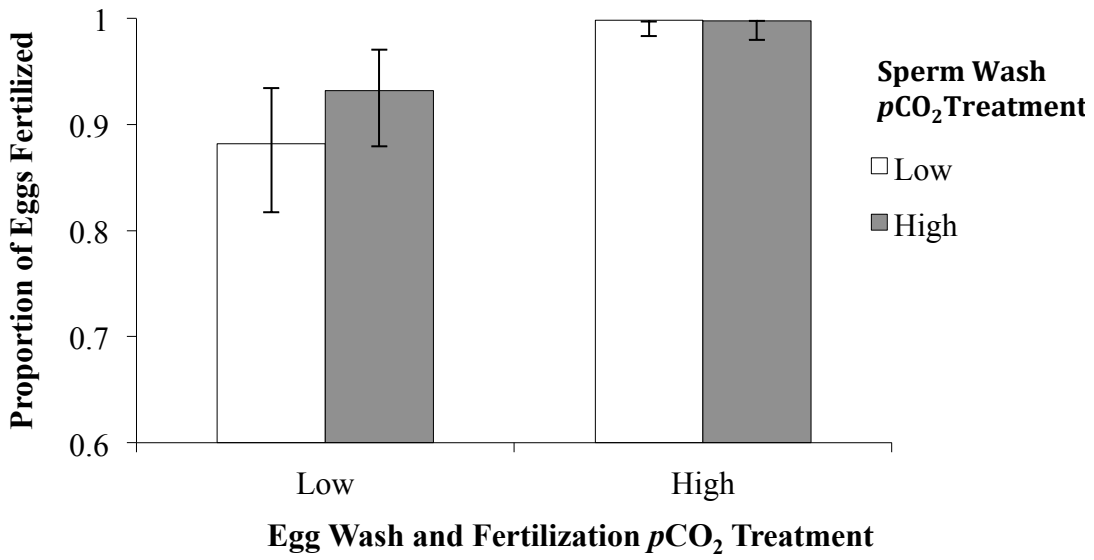

Figure 2-1. Proportion of fertilized eggs of *Crassostrea gigas* after 48 hours (n=4 male-female pairs). Each pair was subjected to all treatments and each treatment was replicated twice per pair except for one replicate of high pCO_2 sperm mixed with high pCO_2 eggs for one of the four families. Data are backtransformed means and pooled standard error.

I found a significant effect of pCO_2 on the development of embryos 48 hours after fertilization (Fig. 2-2). At low pCO_2, of all the fertilized eggs and developing larvae, I found more trocophore and prodissoconch I larvae than at higher pCO_2 (2-Way ANOVA; $F_{1,24} = 11.901$, p=0.002). The prior washing of the sperm however had no effect on the development of the embryo (2-Way ANOVA; $F_{1,24} = 0.001$, p=0.979). Furthermore, there was no interaction between the treatment of the sperm and the one of the fertilization (2-Way ANOVA; $F_{1,24} = 0.438$, p=0.514).

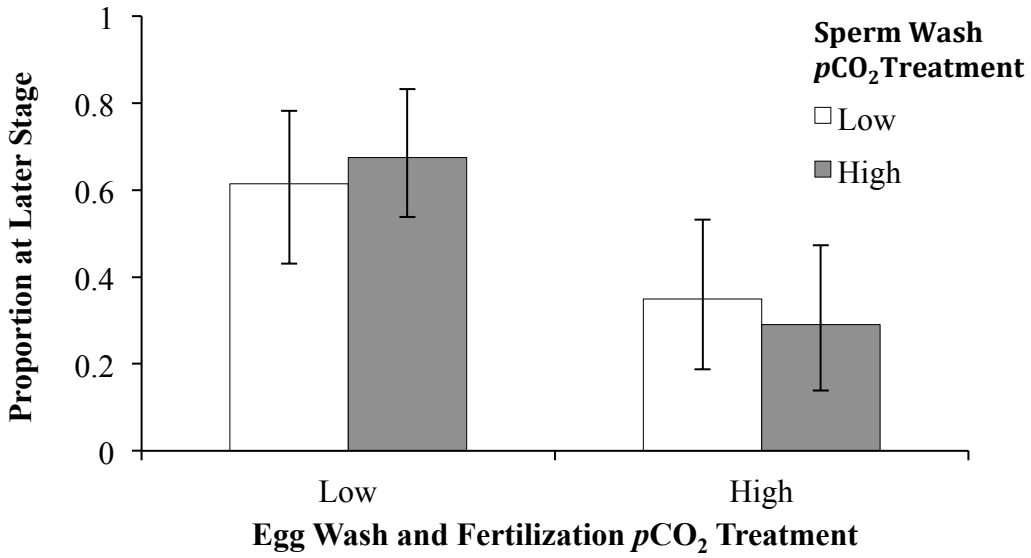

Figure 2-2. Proportion of fertilized eggs of *Crassostrea gigas* at later developmental stage (trocophore and prodissoconch I) after 48 hours (n=4 male-female pairs). Each pair was subjected to all treatments and each treatment was replicated twice per pair except for one replicate of high pCO_2 sperm mixed with high pCO_2 eggs for one of the four families. Data are backtransformed means and pooled standard error.

Overall, I found a significant negative effect of pCO_2 on the development of embryos 48 hours after fertilization (Fig. 2-3). At low pCO_2, of all the eggs that were available for fertilization, I found more trocophore and prodissoconch I larvae than at higher pCO_2 (2-Way ANOVA; $F_{1,24} = 7.304$, $p=0.012$). The prior washing of the sperm however had no effect on the development of the embryo (2-Way ANOVA; $F_{1,24} = 0.042$, $p=0.840$). Furthermore, there was no interaction between the treatment of the sperm and the one of the fertilization (2-Way ANOVA; $F_{1,24} = 0.731$, $p=0.401$).

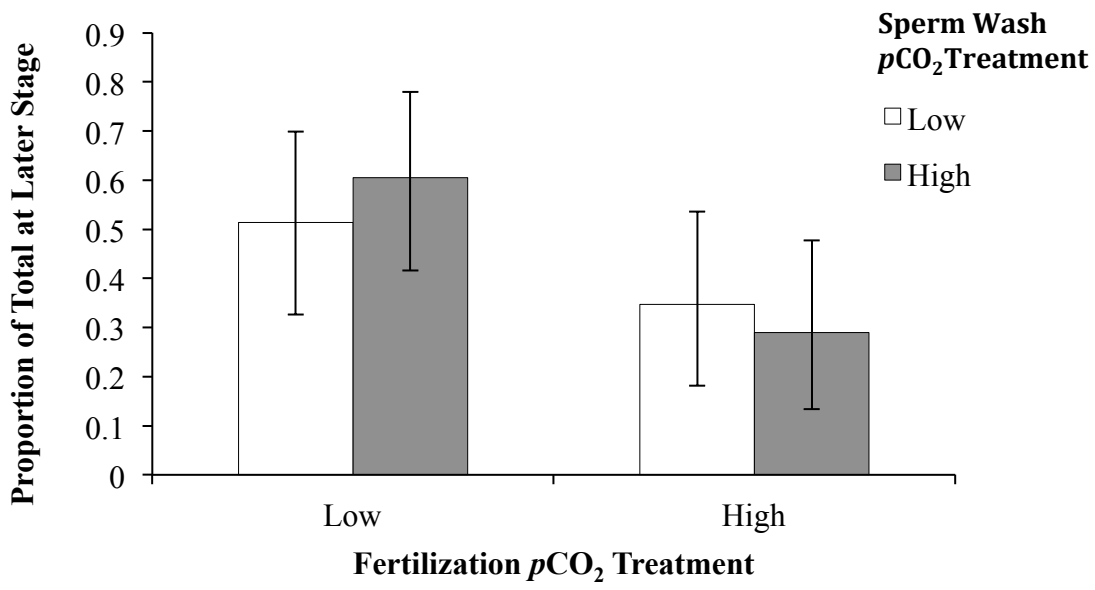

Figure 2-3. Proportion of total eggs of *Crassostrea gigas* at later developmental stage (trocophore and prodissoconch I) after 48 hours (n=4 male-female pairs). Each pair was subjected to all treatments and each treatment was replicated twice per pair except for one replicate of high pCO_2 sperm mixed with high pCO_2 eggs for one of the four families. Data are backtransformed means and pooled standard error.

2.3.2 Larval Development

2.3.2.1 Sizes

There was a significant interaction between time and pCO_2 treatments (RMANOVA; $F_{6.91}$=3.525 p=0.011) (Fig. 2-4). The larvae were growing to similar sizes in both treatments until they were 12 days old. At that point, the larvae at low pCO_2 (207 μm) had become 10% larger than the larvae at high pCO_2 (189 μm). At 16 days old, the same trend was observed but the larvae at low pCO_2 (291 μm) were 7% bigger than the larvae at high pCO_2 (272 μm). The sizes of the larvae at high pCO_2 reached the sizes of the larvae at low pCO_2 by day 18, and by day 20 the larval sizes were even more convergent with both treatment having an average of 311 μm. Time had a

greater effect on the larval sizes than did the pCO_2 treatments (MANOVA; $F_{6,91}$=893.348, p<0.0001).

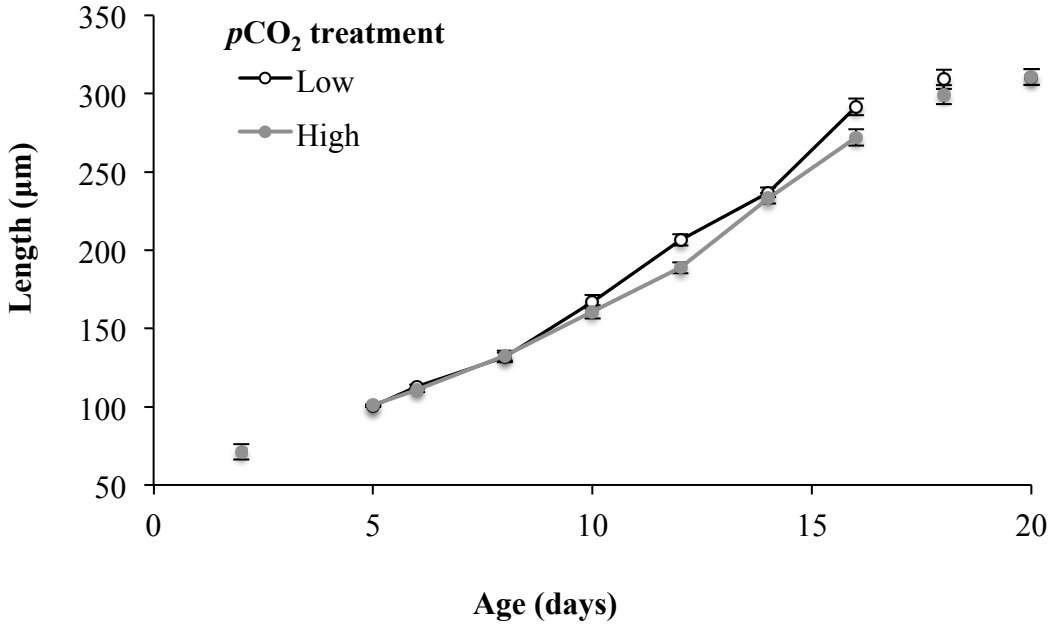

Figure 2-4. Mean length of larval *Crassostrea gigas* in the tank experiment over time. This experiment was run for 18 days on larvae that were 2 days old until they were 20 days old (n=2). Day 2 data were collected prior to experimental manipulation, and days 18 and 20 may have included larvae that had already settled, biasing population-level larval size estimates. The line is drawn only from days 5-16 to illustrate the range used for the repeated measures analysis. Error bars represent standard errors.

The convergence of mean larval size between treatments on days 18 and 20 suggests that the high pCO_2 larvae may have caught up with their low pCO_2 counterparts. The size distribution at day 20 show a greater spread in the size and that more larvae are bigger than 320 µm but also more larvae are smaller than 280 µm in the low than in the high pCO_2 treatment (Fig. 2-4). The lack of further rightwards shift from the 16 days old larvae on the size distribution suggests that

larvae were likely starting to settle out of the low pCO_2 tanks. Despite the convergence of the means of both treatments from day 18 onwards (see Fig. 2-3), the low pCO_2 tanks contained more larvae that were > 320 μm on these later sampling dates.

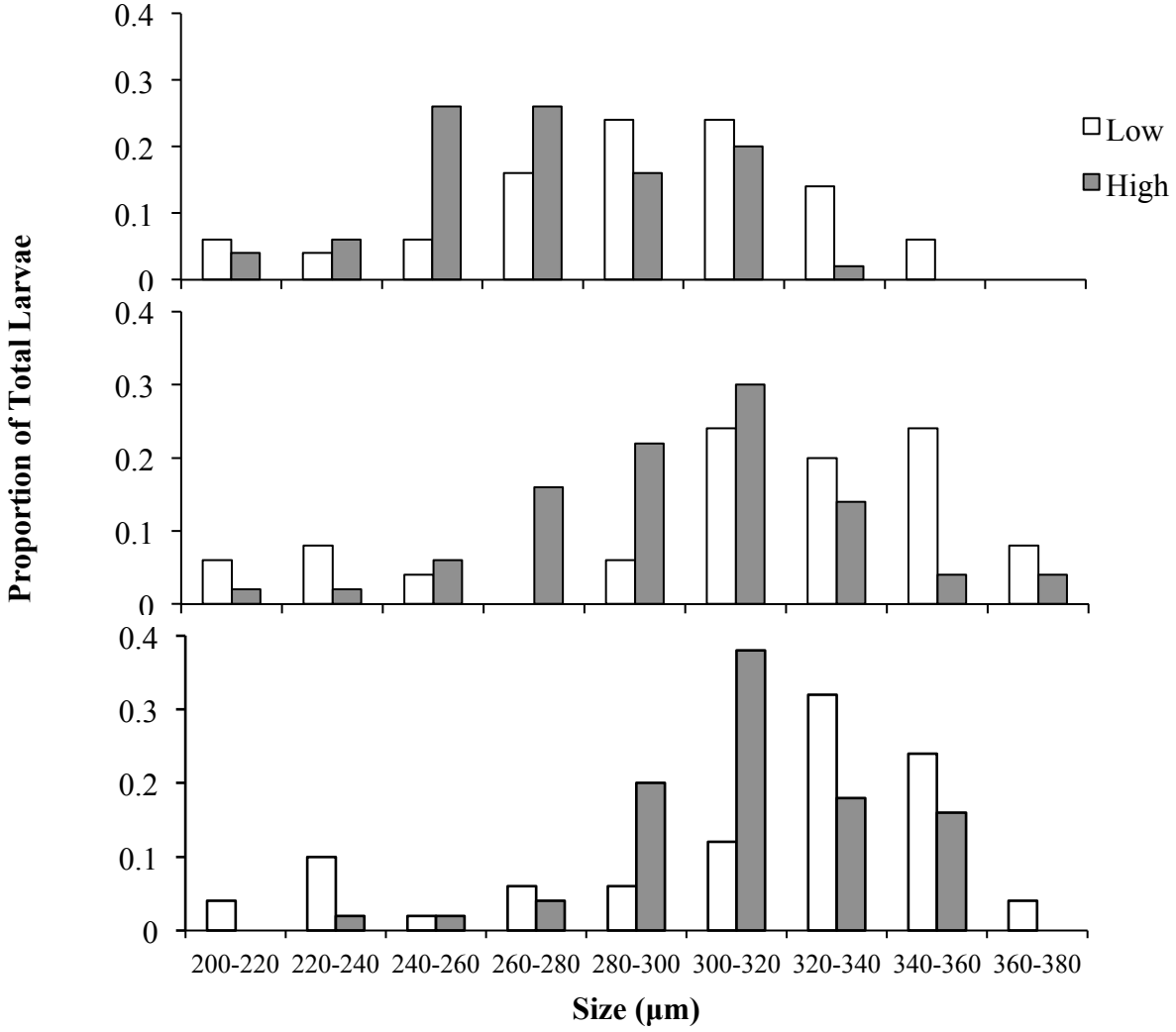

Figure 2-5. Size distribution of 16 (top), 18 (middle), 20 (bottom) days old larval *Crassostrea gigas* at low and high pCO_2 treatment. The means at day 16 are 291 µm (low) and 272 µm (high), at day 18 299 µm (low), and 309 µm (high), and at day 20 are 311 µm (low and high). The 2 tanks within each treatment were combined (n=50 larvae per treatment).

2.3.2.2 Quality and Quantity

At the end of the 18-day experiment, approximately 2.5 times more of the surviving larvae passed through a 253 µm sieve in the high pCO_2 treatment than in the low pCO_2 treatment (2-tailed t=5.008, p=0.043) (Fig. 2-6).

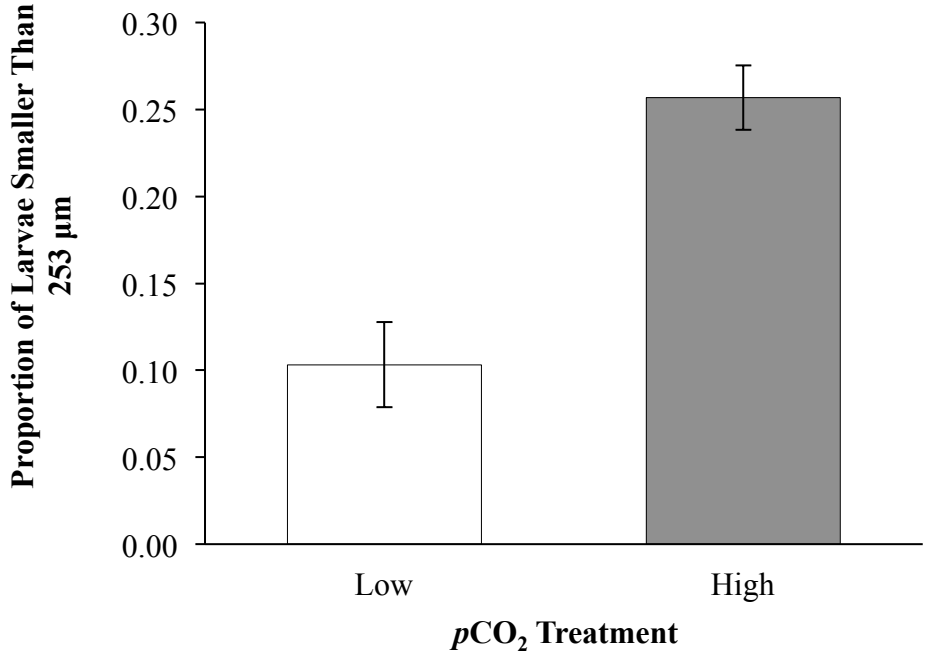

Figure 2-6. Proportion of larval *Crassostrea gigas* that passed through a 253 µm mesh at the end of the 18 day experiment in the tanks (n=2). Note that this size threshold corresponds to larvae that were approximately 320 µm in length (see Figure 2-4). Error bars represent standard errors.

Although there were more larvae that were larger in the low pCO_2 treatment at the end of the experiment, when the larvae were 20 days old, the same concentration of larvae was recovered in both treatments (2-tailed t =0.096, p=0.932) (Fig. 2-7).

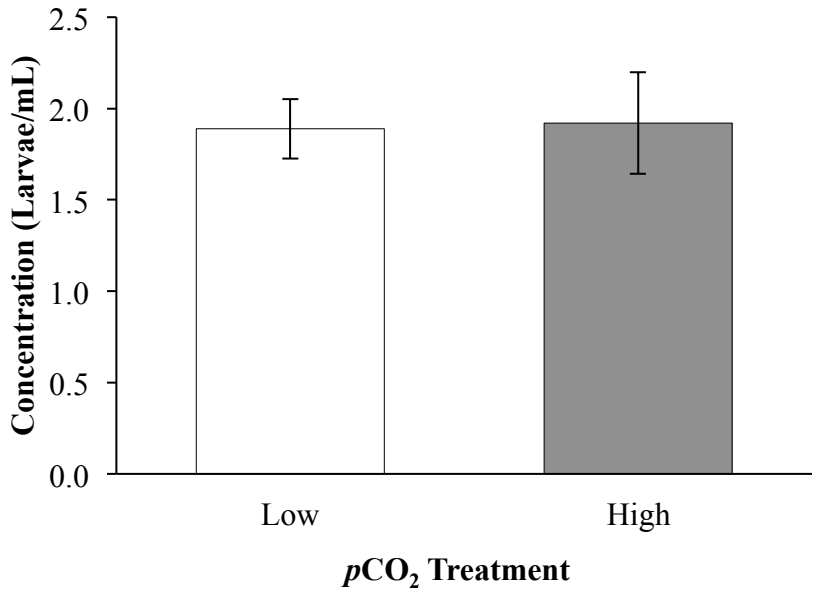

Figure 2-7. Concentration of larval *Crassostrea gigas* remaining at the end of the 18 day experiment in the tanks per mL of seawater (n=2). Error bars represent standard errors.

2.3.2.3 Mortality

Summing across all sampling dates, 8 out of 691 larvae were dead in the low pCO_2 treatment (1.2%) and 20 out of 861 (2.3%) were dead in the higher pCO_2 treatment (DF=1, N=2 χ^2 =3.149; p=0.076). This trend, while not significant, suggests that almost twice as many larvae died in the high pCO_2 treatment than in the low pCO_2 treatment. Regardless, any differences in mortality were small and did not appear to affect the final numbers (see Fig. 2-6).

2.3.2.4 Deformity

Deformity was very minimal in both pCO_2 treatments; 0.8% (7 out of 841 larvae) in the high pCO_2 treatment and 0.1% (1 individual out of 691 larvae) in the low pCO_2 treatment. Although

not statistically significant, there was a trend towards greater deformity at high than at low pCO_2 treatment (DF=1, N=2, χ^2=3.453 p=0.063).

2.3.2.5 Feeding

There was a significant interaction of time and *p*CO2 treatment (RMANOVA; $F_{7,2}$= 7964.582, p=0.0004) on algal consumption rates. The larvae in the low pCO_2 treatment tended to consume more algae than at the high pCO_2 treatment later in the experiment but this tendency was only significant on day 20 (MANOVA; $F_{1, 8}$= 55.025, p<0.0001) (Fig. 2-8). The larvae in both treatments fed more over time (RMANOVA; $F_{7,2}$=28,561.102, p=0.0001).

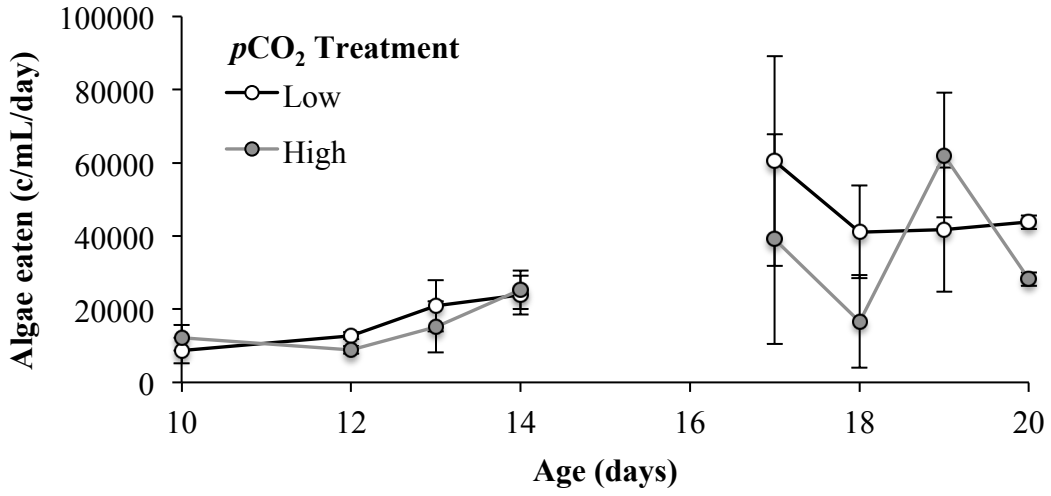

Figure 2-8. Algal consumption, measured in algae cells/mL/day, by *Crassostrea gigas* larvae over time (n=2). The algal consumption is represented only the days when at least 3 reading were taken and when the control was also measured. Error bars represent standard errors.

2.4 Discussion

Ocean acidification is negatively impacting a wide variety of marine species at a number of points in their life cycles. Fertilization through larval development are key processes and are particularly vulnerable to OA. The potential for the impacts of OA to affect an aquaculture company's bottom line via impairment of early development make successful management of these processes a priority for the aquaculture industry.

2.4.1 Fertilization

Fertilization happens when there is a fusion between a spermatozoa and an egg to produce an embryo, which may then develop into a planktonic larva (trocophore larva then prodissoconch I larva). However, instances where multiple spermatozoa fertilize a single egg, polyspermy results in the death of the fertilized egg. Mechanisms to block further sperm from fertilizing the egg do exist to limit the occurrence of this detrimental event in organisms. Fertilization success, therefore, is not only measured as the number of eggs that have been fertilized but also by the number of these fertilized eggs that progress to become a viable larva as both processes have to happen in order for the species to proliferate.

Sperm activity is related to fertilization success in marine invertebrates (e.g., Havenhand *et al*., 2008), and several studies have investigated the effects of OA on sperm activity. They found that reduced motility and speed of sperm could be used to predict the decline of fertilization success in terms of egg cleavage and development to swimming larvae by testing the model with their observations. For *C. gigas*, Havenhand & Schlegel (2009) found no effect of acidification on the activity of *C. gigas* sperm nor on fertilization success, but Barros *et al*. (2013) found the sperm to

have reduced activity with increased acidification as well as the predicted reduced fertilization success. The experiment in the present study did not provide any evidence that pre-fertilization washing of the sperm at high pCO_2 levels had an effect on either the proportion of eggs fertilized or on their development. Based on our results, we cannot conclude that sperm washing was the cause for the fertilization failure experienced at Island Scallops Ltd. However, the high pCO_2 treatment in my experiment was only ~700 μatm and not much higher than the low pCO_2 treatment. By comparison, the intake water in the hatchery can reach CO_2 concentrations as high as 1400 μatm. Further studies will be required to completely rule out OA as a factor in the recent failure.

A greater proportion of *C. gigas* eggs were fertilized at high than at low pCO_2 48 hours post-fertilization. In contrast to our findings, Barros *et al*. (2013) observed a non-significant reduction in proportion of eggs fertilized after 4 hours at a similar level of acidification and a significant reduction at a higher level of acidification, and Parker *et al*. (2010) found a significant reduction at similar level after 2 hours. Conversely, other studies did not find an effect of acidification on the fertilization of *C. gigas* eggs when more acidified water was used, as seen in Kurihara *et al*. (2007) and Havenhand & Schlegel (2009). Differences in acidification levels used and time allotted post-fertilization could have played a role in the discrepancies among studies. If the acidified sperm of *C. gigas* is slower such as was observed in Barros *et al*. (2013), it could explain the difference in the result with this study, as well as Parker *et al*. (2010), and Havenhand & Schlegel (2009) who all monitored eggs no more than 4 hours post-fertilization. Perhaps the sperm had not had enough time to fertilize all of the available eggs due to its reduced activity level. This duration discrepancy, however, cannot explain the difference between the findings of

the present study and Kurihara *et al*. (2007)'s study in China, which used more highly-acidified water.

Polyspermy could potentially explain the simultaneously increased proportion of eggs fertilized and decreased further embryonic development. Reuter *et al*. (2011) found that the time for the egg to block polyspermy significantly increased in another broadcast spawner, the red sea urchin, by more than 10-fold at 1800 µatm compared to 800 µatm or 400 µatm. A comparable study has yet to be conducted on *C. gigas* but if this species is more sensitive to acidification, then the pCO_2 threshold to increase the time for the egg to block polyspermy may be reduced and the onset of increased polyspermy may occur at lower pCO_2 levels than was observed for sea urchins.

Our results could also be potentially explained by a rightward shift in the peak of fertilization success vs sperm density, as was found with sea urchins (Reuter *et al*., 2011). It could mean that when sperm is not limiting, as in this study, fertilization is not affected and can even be increased. This result may apply well to a hatchery setting, where sperm is rarely limiting. However, in natural settings where sperm density is lower, fertilization could potentially be reduced. Further studies are required to further elucidate these patterns.

Although more eggs were fertilized at the elevated pCO_2 level, a smaller proportion of them had advanced to later development stage by 48 hours. More analyses are needed to determine if the development is delayed, as opposed to ceased, which could have serious implications for the industry. The latter result would require greater water chemistry monitoring during fertilization

procedures. However, since 48 hours is much longer than the normal time 6-10 h to reach the trocophore larval stage (Waldbusser *et al*., 2013), we can probably assume that the lack of embryonic development was probably more than a delay. The alternative hypothesis of a developmental delay, however, agrees with most findings with *C. gigas* to date such as Kurihara *et al*. (2007) that found developmental delay, but that delay only set in as of prodissoconch I stage which was attributed to the vulnerability of the onset of calcification. However, Parker *et al*. (2010) dispute this idea as they found negative effects earlier on, which is corroborated by the findings of Barros *et al*. (2013). This reduction in development success could be an important factor in larval stock declines.

Finally, it is also possible that the methodology influenced the results. The technique that was used to fertilize the eggs, strip spawning, was beneficial in terms of time efficiency and prevention from using gametes of adults that showed overall weak or unfit gametes while allowing sex determination. This study and all the fertilization studies mentioned in Table 2-1 used strip spawning with the possible exception of Gazeau *et al*. (2011), who do not report their spawning methodology. However, His *et al*. (1997) mention that this technique can also be detrimental as the gametes that would not normally be released are non-selectively used. Therefore, this could potentially explain the variability that we observed in our results and could have reduced the detection of the extent of the effects of acidification on fertilization success.

2.4.2 Larvae

Reduction in larval sizes of *C. gigas* have been found at the prodissoconch II larval stage (Parker *et al*., 2010; Barton *et al*., 2012) but other studies observed either no effect on larval size (Ginger

et al., 2013) or a larval size reduction at an earlier stage, the prodissoconch I stage (Kurihara *et al*., 2007; Barros *et al*., 2013). The onset of effects of OA on larval size at the prodissoconch II could be explained by the mixotrophic diet of *C. gigas* at prior stages (Rico-Villa *et al*., 2009; Ben Kheder *et al*., 2010 a,b). This could have reduced the dependence of larval growth on the external environment due to the availability of energy reserves in the egg (Barton *et al*., 2012). Since larvae of approximately 120 μm have begun to rely mostly on exogenous resources (Rico-Villa *et al*., 2009; Ben Kheder *et al*., 2010 a,b), this energetic switch could explain the onset of effects of acidification at or near this size (Barton *et al*., 2012). The study of Ginger *et al*. (2013) only monitored growth twice, on days 11 and 16 post-fertilization, which, according to our study, could mean that the range of size divergence was missed. Ginger *et al*. (2013) used warmer temperature, ~25 °C as opposed to my study at ~21 °C, the time of development was likely reduced in their study. The warming, therefore, could have reduced the likelihood of detection of effects of OA on size by reducing the range of size divergence over time. In my study, larval growth was reduced with increased pCO_2, particularly over the second half of larval development, and the larvae took approximately two more days to reach settlement size. The divergence in sizes between treatments began from approximately 160 μm which is slightly larger than the 120 μm that Barton *et al*. (2012) observed. They, however, started to expose *C. gigas* to acidification from fertilization rather than from the prodissoconch I stage (as was done in the present study), which could explain why we observed delayed negative effects on size.

At the end of the experiment, even though the average sizes were the same, the size distribution was different with a greater spread at low than at high pCO_2. Although there was no difference in the quantity of larvae recovered in the two treatments, the relative proportion of marketable

larvae (>253 μm) was greater at low pCO_2. The similar sizes that were found at the end of our study after a period over which sizes differed between treatments may be due to faster growing larvae reaching their maximal size and settlement competency earlier. These larvae may have simply remained at a given size while waiting for an appropriate substrate upon which to settle, or they may have settled on the tank walls and been lost to the larval population. Either possibility could explain the stabilization of the upper end of the larval size frequency distribution on days 18 and 20 at low pCO_2, and allowed the larvae at high pCO_2 to catch up in terms of size. Another possibility would be that the smaller larvae at higher pCO_2 died off during the experiment, resulting in a contraction of the size range of the larvae at high pCO_2. This was possible because mortality, although very low in both treatments, was greater at higher pCO_2. The larger range of size observed at low pCO_2 could also be caused by self-thinning happening in good conditions where a few opportunistic individuals could outcompete the others (Guiñez, 2005). This may result in multilayered sizes including few larger individuals and few smaller ones as well; this effect could also explain the fact that mean size did not differ among treatments despite the presence of more large larvae in the low pCO_2 treatment.

Increased larval mortality with increased acidification was previously reported in Barros *et al*. (2013). However, this was not the case for all studies as others found no effect on mortality (Ginger *et al*., 2013; Timmins-Schiffman *et al*., 2013), although Ginger *et al*. (2013) only monitored twice during the experiment and Timmins-Schiffman *et al*. (2013)'s experiment only lasted 3 days. Moreover, since dead larvae can only be detected within a few days post-mortem due to deterioration of the shell, there could have been an underestimation of mortality in these

studies especially at higher pCO_2 that could increase dissolution rates since the aragonite saturation state was <1 in Ginger *et al*. (2013).

Although the number of larvae recovered at the end of my experiment was similar across all treatments, I observed a slightly but non-significantly greater mortality rate at higher pCO_2. There are three possible explanations for this discrepancy. First, it is possible that the trend towards higher mortality in the high pCO_2 treatment arose by chance. Second, it is possible that the very low rates of mortality, even if the acidification effect was real, were insufficient to generate measurable differences in final population size. Third, since the larvae had begun to reach settlement size earlier in the low pCO_2 treatment, any increased mortality due to elevated pCO_2 could have been counterbalanced by more larvae settling within the tanks in the low pCO_2 treatment. Since settlement within the tanks could not be quantified, we are limited to speculation on this topic. Further research could involve an earlier termination of the experiment, as soon as settlement size is reached by the larvae of any of the treatment, or the addition of settlement surfaces that can be easily removed, on which larvae could easily be quantified.

There is a consistent trend of increased deformity in *C. gigas* after exposure to increased pCO_2 (Kurihara *et al*., 2007; Gazeau *et al*., 2011; Parker *et al*., 2010; Barros *et al*., 2013). Although these studies were all shorter than mine, I also found that deformity increased in *C. gigas* after exposure to increased pCO_2. However the levels of deformity in my study were extremely low compared to other studies such as on abalone finding very high rates (~ 40%) under similar pCO_2 levels (800 μatm) (Crim *et al*., 2011).

In this study, based on the feeding results obtained over time, we don't have consistent evidence that feeding was reduced with OA as only on the last sampling day was a significant reduction in algal consumption observed.

2.4.3 Potential Explanation for Variations in Results

The variations in the results obtained between studies on *C. gigas* such as effects on fertilization and larval size could be due to differences in populations geographically separated at various extents and introduced from Japan at various time periods. Also, some populations could have developed adaptations from distinctive conditions in their introduced environment. The introductions of *C. gigas* worldwide started in the early 1900s from Japan and although at first, in most places, aquaculture was continually supplied by Japan, now most places grow their own oyster stock. For instance, in Puget Sound, in the late 1970s, local hatcheries were established to reduce cost from importation (Cheney *et al*., 1986). In BC, local hatcheries were established even earlier, in 1961 (Quayle, 1988). Geographic differences with varying OA responses have also been observed in the sea urchin, *Heliocidaris erythrogramma* (Havenhand *et al*., 2008; Byrne *et al*., 2009; Parker *et al*., 2010). However, the results obtained here were similar to the findings of Oregon (Barton *et al*., 2012) which, in addition to being the closest study geographically, also used larger containers than most studies, thus better mimicking aquaculture practices.

2.4.4 Implications

In order to understand the overall impacts of OA on *C. gigas* during its early developmental stages, it is important to measure various parameters since these can go in a similar direction,

either showing increased or decreased fitness or they can diverge and hide the overall impacts by cancelling each other out. Also, although mortality is one obvious measure of a loss of fitness, there are other more subtle measurements that can indicate the overall health of an organism. For instance, size metrics are important as indicators of fitness as healthy organisms can afford to allocate more energy into growth than stressed organisms. Larval size can also serve as predictor of larval (Parker *et al*., 2010) or juvenile survival (Barros *et al*., 2013). Anger (1987), Strathmann (1987), and Kurihara *et al*. (2007) suggest that smaller larvae have higher chances of starvation due to reduced frequency of encountering and clearing food. Moreover, juvenile size is affected by prior larval size; when larval size is smaller, juvenile fitness is compromised due to reduced competitive ability and increased mortality post-settlement (Anil *et al*., 2001; Gazeau *et al*., 2010; Hettinger *et al*., 2012). This suggests that smaller larval sizes could ultimately lead to decreased production in hatcheries. Furthermore, deformity can have a serious impact on the larval fitness as it can impede swimming capabilities (Beiras & His, 1994; Kurihara *et al*., 2008). Therefore, the larvae can be more vulnerable to starvation or predation due to inefficient or impaired locomotion, feeding, or predator avoidance. However, care should be taken in making these assumptions (Barros *et al*., 2013) as there exists some evidence of acclimation or adaption to acidification (Parker *et al*., 2012).

In this study, the various parameters measured showed overall negative impacts of OA on fertilization and larval development. Hence, economic losses for the local hatcheries should be expected if measures are not taken to mitigate these effects. However, since the financial investment by the hatchery at these developmental stages is less than it will become at later developmental stages, investigation of acclimation and or carry-over effects have to be

undertaken before deciding how best to conduct fertilization and grow larvae to maximize the cost effectiveness of harvesting oysters at later stages.

Chapter 3: Effects of Ocean Acidification on Settlement and Postsettlement Performance of *Crassostrea gigas*

3.1 Introduction

Ocean acidification (OA) has generated a great deal of interest in the last decades. This interest in the scientific community is mostly due to the magnitude of ongoing and predicted chemical changes and the uncertainties regarding biological responses. The effects of OA can vary among species and among individuals, and even within an organism between its life stages. The increasing interest in OA is shared by the aquaculture industry, especially when its profits are directly dependent upon the marine environment and since declines associated with OA have already been reported. This chapter focuses on both of these aspects by increasing the scientific knowledge on an oyster species with the goal of specifically answering questions that are relevant to the aquaculture industry now or in the near future.

This chapter builds on the work presented in Chapter 2 by extending manipulations to later life stages. Knowledge about the entire life cycle is crucial knowledge, because for species to persist, individuals will need to pass through all stages from fertilization through growth and development and on to reproduction. Furthermore, since carry-over effects can potentially alter the responses of later life stages to a stressor like ocean acidification, studies that span more than one life-stage are essential. A carry-over effect occurs when exposure to a stressor at an earlier stage positively or negatively affects the organism at a later stage. Although few studies have been conducted so far on more than one life-stages (see Dupont & Pörtner, 2013) recent studies

have started to look at carry-over effects on oysters species (*e.g.* Hettinger *et al*., 2012; Parker *et al*., 2012).

Settlement in oysters occurs when a larva permanently attaches to the substrate with its foot. This process has been found to be dependent on waterborne substances released by both conspecific adult and biofilms (Tamburri *et al*., 1992). However, invertebrate larvae have been found to delay settlement when a physical or chemical factor present in their environment has been causing them physiological stress (Pechenik, 1999), and Miller *et al*. (2009) suggested that OA could potentially cause such delay in oysters. *C. gigas* larvae are however limited in their capacity to delay settlement and will perish if they cannot find a suitable site in a timely fashion (Quayle, 1988). Settlement success is known to have a great impact where even small changes in the number of settled larvae have large consequences on the adult population of bivalves (Gosselin & Qian, 1997; Hunt & Scheibling, 1997; Parker *et al*., 2010). Very few studies to date have looked at the effects of prior exposure to acidification on the settlement on oysters. To date, no effect of OA manipulation during the settlement period has been found on settling oysters (Ginger *et al*., 2013) or urchins (Dupont *et al*., 2012). However, if the adults urchins had been subjected to high pCO_2 for 4 months, the settlement was reduced regardless of the pCO_2 treatment at which the settlement was conducted (Dupont *et al*., 2012).

Metamorphosis is a process that immediately follows settlement and is energetically costly to the organism since it relies in its entirety on the energy storage accumulated by the larval stage (Pechenik, 2006; Burke *et al*., 2008). This process in *C. gigas* can be summarized as morphological and physiological changes such as loss of the velum, foot, anterior adductor

muscle, and eyespot (Quayle, 1988) that are no longer needed in a sedentary lifestyle. The formation of the juvenile shell follows immediately metamorphosis (Quayle, 1988). OA does not seem to affect this process in *C. gigas* (Ginger *et al*., 2013).

Once settled and metamorphosed, juvenile *C. gigas* form new shells made primarily of calcite (Stenzel, 1964), although the larval shell made of aragonite remains at the umbo and can persist for some time (Quayle, 1988). The new shell grows quite rapidly and is of the same composition as the adult shell (Quayle, 1988). Studies on various species of oysters have found decreased juvenile growth at high pCO_2 (Parker *et al*., 2010; Hettinger *et al*., 2012). Moreover, the larval prior exposure to acidification resulted in negative carry-over effects on juvenile growth seven days post-settlement that persisted for a least 1.5 months (Hettinger *et al*., 2012). However, there are discrepancies in the outcomes of OA experiments on juvenile survival with one study finding no effect (Hettinger *et al*., 2012) while another found reduced survival (Beniash *et al*., 2010).

Reduced growth is not the only potential effect of OA on juvenile oysters; OA can also reduce the thickness and impair the integrity of the shell. Although very few studies investigated shell strength in acidification conditions, it was found that overall biomineralization process is negatively affected by OA which translates as decreased strength in the acidified shell of *C. virginica* (Beniash *et al*., 2010). However, this might not apply to all oysters species as the thickness of the shells of *Ostrea lurida* was unaffected by OA (Sanford *et al*., 2014). Interestingly, prior acclimation of the adults *S. glomerata* results in stronger and quicker growing shells of larvae at elevated pCO_2 conditions (Parker *et al*., 2012).

Cultured oysters in BC experience varying degrees of pCO_2 fluctuation during their life span which highlights the need for research to consider hatchery practices as they do not reflect the consistency of treatment which most studies to date have focused on. Fertilization, larval growth, and settlement experience constant pCO_2 conditions in hatcheries, whereas juveniles and adults experience fluctuating pCO_2 conditions in the ocean. The juveniles are outplanted to the ocean to grow for approximately 1-2 years until they are sexually mature adults, during which time they experience variable conditions. The adults are then brought to the hatchery to be warmed up to 18 °C and over-fed to trigger gamete production for a minimum of 2 weeks prior to fertilization (Barton *et al*., 2012) while preventing the release of gametes that happens at 19 °C. During this time, they can experience more constant pCO_2 conditions. Since it is difficult or impossible to adjust the pCO_2 conditions in the ocean directly at relevant scales, the knowledge about carry-over effects and acclimation in *C. gigas* from prior exposure to pCO_2 can help the industry in making decisions regarding the ideal pCO_2 levels for growing larvae in order to maximize later developmental success.

In this chapter, my objective is to determine whether it is better to raise larvae in optimal pH conditions (lower pCO_2 level) or at suboptimal pH conditions (higher pCO_2 level) to "harden" them before sending the juveniles to the ocean where they are likely to experience higher pCO_2 levels. In this study, we did not subject *C. gigas* to acidification stress during settlement, as this can easily be conducted in ideal conditions in hatcheries. Rather, we tested the best larvae, in terms of size, from both high and low pCO_2 larval treatments to see if the prior pCO_2 treatment had an effect on the settlement density of the pediveliger larvae. Due to the low energy requirement of this process and the prior studies failing to find effects of OA, I predicted that

there would be no difference in settlement density in previously acidified larvae. However, I expected that if the larvae had previously been stressed by increased pCO_2 exposure as larvae, even though they were able to reach the same size, they might not be able to survive through metamorphosis if their energy stores had been compromised. This would result in fewer settled larvae with extended shells in elevated pCO_2 conditions. Growth, condition, and shell strength of juvenile *C. gigas* were all expected to be negatively impacted. Furthermore, I expected that negative carry-over effects from larval exposure to acidification would exacerbate these effects.

3.2 Methods

3.2.1 Settlement of Pediveliger Larvae

Larvae for the settlement and juvenile growth experiments described in this chapter were collected at the end of the larval experiment described in Chapter 2 using a 263 μm screen. Approximately 130,000 larvae were collected from each tank and were equally divided into 3 plastic bins. Thus, a total of 12 bins – 6 per treatment – were used (Fig. 3-1). The bins contained 8 L of the same source of seawater at 400 ± 50 ppm at 21.3 ± 1 °C and lined with empty adult *C. gigas* halved shells with inner layer up to cover the surface of the bin. After 1, 24, and 48 hours, the shells were shaken to recover only the settled larvae. Then an area of 4 cm^2 was haphazardly selected on the inner layer of two shells per bin and the number of settled oysters was counted under a dissecting scope.

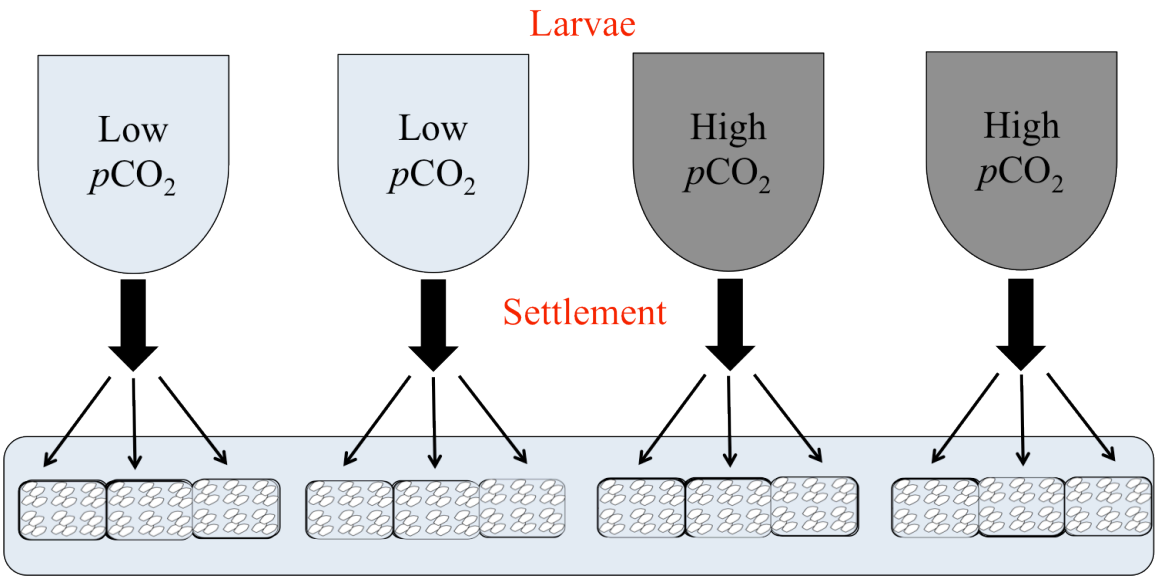

Figure 3-1. Diagram showing how 130,000 larvae from each tank were divided in 3 settlement bins containing adult *Crassostrea gigas* shells in low pCO_2 water. All 12 settlement bins were held in a common water bath for 2 days. Although not depicted in this schematic, bins were randomly placed in the water bath.

3.2.2 Post-Settlement Experiment

The settled oysters from the settlement experiment (see above) were used for this experiment. I divided the shells from each bin into 6 bunches and each bunch was put in a mesh bag (Ittinet Blue). Each bag was then hung in 1 of 6 tanks containing approximately 2,800 L of seawater, following the same intake method as described in section 2.2.2, where 3 tanks were at low (440 ± 35 ppm) and 3 at high (665 ± 50 ppm) [CO_2]. The high [CO_2] corresponding to high pCO_2 treatments were raised to the desired level by being bubbled with supplemental CO_2 (using a tank of pure CO_2 and an air stone). Each bag was completely submerged in the tank and there were a total of 12 bags – one from each of the settlement bins described above – per tank (Fig. 3-2). The experiment was run for 15 days.

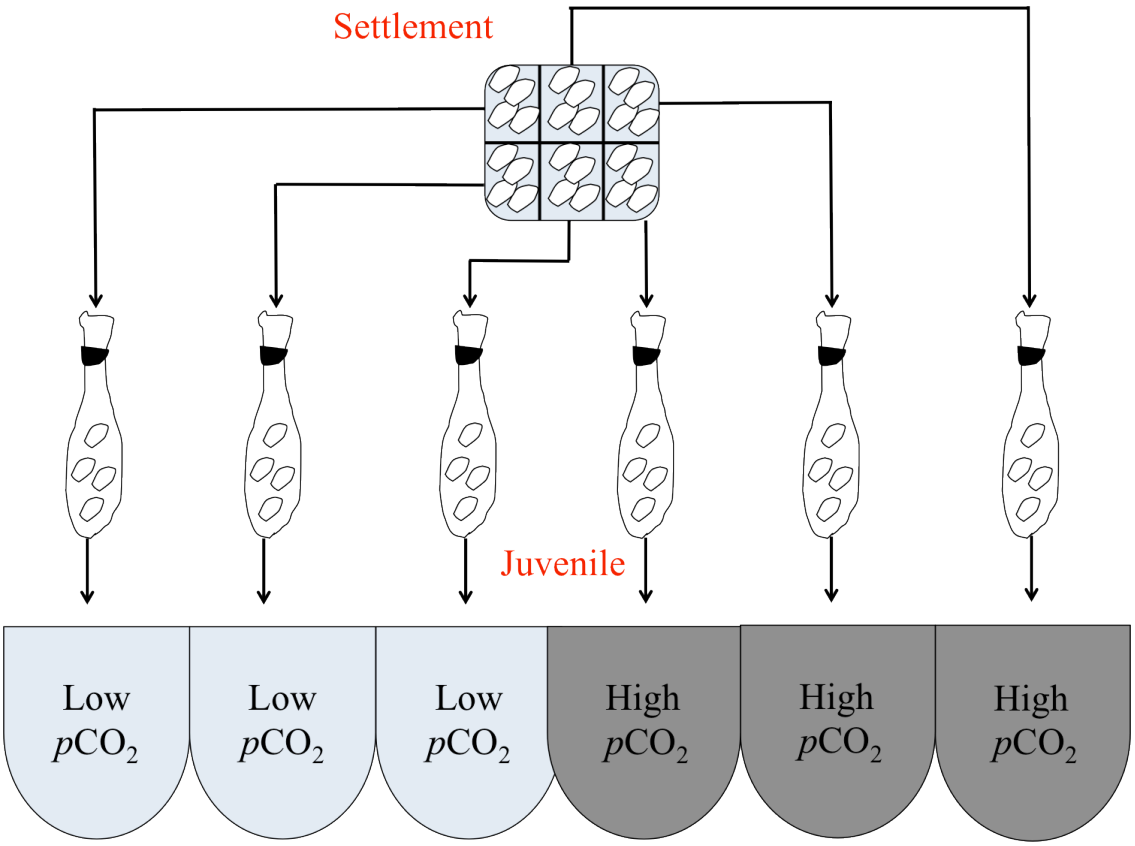

Figure 3-2. Diagram showing the division of each settlement bin in 6 bunches. Each bunch was put in a mesh bag that was hung in a tank.

3.2.2.1 Water Chemistry

Water chemistry was characterized following the same methods as described in the water chemistry section of the fertilization experiment (see Chapter 2). Salinity, pCO_2, and temperature were measured after each full water exchange on days 4, 8, and 12 in the experiment. On day 12, DIC samples were also taken before and after water exchange (Table 3-1). Temperature and pH were also measured on days 5, 7, 11 and, 13 days in the experiment.

Table 3-1. Seawater carbonate chemistry variables (mean ± SE) for rearing settled *Crassostrea gigas*. Mean values (Avg.) for rearing *Crassostrea gigas* from spat (22 days old) for 15 days. Alkalinity (A_T), pH, saturation state for calcite (Ω_{cal}) and aragonite (Ω_{ara}) were estimated using CO_2calc (Robbins *et al.*, 2010) at day 12 of the experiment (34 days old *C. gigas*) using the measured chemistry parameters before and after water change.

Treatment	Replicate	Avg. $[CO_2]$ ppm	Avg. DIC mmol/ml	Avg. temp °C	Avg. A_T mmol/kg	Avg. pH	Avg. Ω_{Ca}	Avg. Ω_{Ar}
Ambient	1	433 (37)	1505 (14)	20.0 (0.7)	1639 (2)	8.01 (0.04)	2.28 (0.22)	1.47 (0.15)
Ambient	2	450 (36)	1507 (25)	20.0 (0.7)	1641 (10)	8.01 (0.04)	2.28 (0.20)	1.47 (0.13)
Ambient	3	438 (31)	1505 (26)	20.0 (0.6)	1642 (17)	8.01 (0.03)	2.30 (0.13)	1.49 (0.08)
Elevated	1	683 (74)	1591 (13)	20.6 0.3)	1673 (15)	7.84 (0.00)	1.64 (0.04)	1.06 (0.03)
Elevated	2	693 (40)	1616 (7)	20.5 (0.2)	1705 (17)	7.86 (0.04)	1.74 (0.13)	1.12 (0.09)
Elevated	3	620 (36)	1587 (5)	20.5 (0.2)	1692 (6)	7.91 (0.04)	1.93 (0.14)	1.25 (0.09)

3.2.2.2 Water Change

The water was fully exchanged every four days until the end of the experiment by opening the bottom of the tanks to remove all the water and the bags were left hanging during the water exchange. The tanks were cleaned following the same methods described in section 2.2.2.1 during each water exchange event.

3.2.2.3 Feeding

The juvenile *C. gigas* were fed various concentrations of mixture of algae (Appendix B) on days 1, 4, 6, 7, 8, 11, 12, and 13. The feeding was done by pouring buckets of concentrated algae directly into the tanks.

3.2.2.4 Sampling

After 5 and 15 days of exposure to the experimental treatments, photographs of at least 25 juveniles were taken using a camera (UCMOS 10 000 KPA) and image software (Toup view 3.2) under a dissecting scope. The sizes of the first 25 juveniles photographed were later measured from the photos using ImageJ (Rasband 1997-2012). Length was measured as maximal length from the umbo. Additional measurements of the gut area as well as overall shell area after 15 days in the experiment were also measured on the first 25 photographed individuals using ImageJ (Rasband 1997-2012). The proportion of settled juveniles that had laid shells was measured on day 5 of the experiment to represent successful metamorphosis from all the photographed individuals (approximately 60 individuals per mesh bag). At day 15 of the experiment, all the shells were put in Ziploc bags filled with the treatment water they originated from for the post-settlement experiment and put in a freezer at -20 °C.

3.2.3 Crushing Experiment

Here, we investigated shell strength by measuring parameters including; instantaneous force at first crack, maximum force of resistance, elasticity, and total force withstood by the shell as a way to compare shell strength among treatments in which elevated pCO_2 was applied to oysters as larvae, juveniles, both, or neither. In order to measure the force required to break juvenile oyster shells, the juvenile oysters were detached from their settlement site on adult shells using forceps, and the juvenile soft tissues were removed to isolate the right valve (outer shell) intact. Before crushing, the shells were photographed to allow further measurements of shell area using ImageJ (Rasband 1997-2012) and then submerged in seawater to prevent them from drying out. If a shell broke during any of the preparation manipulation, the shell was discarded and was

replaced. The shells were compressed using a load cell of 5 N (Instron®, Massachusetts, USA) and the maximum compressive force was set at 2.000 N to stay within the safe range for the instrument. The extension was set at 0.5 mm per minute and the force was recorded instantaneously. In this way, we were able to measure compressive force per mm of extension. Ten juvenile shells were crushed per mesh bag for a total of 720 shells. Afterwards, the shells were photographed again and put individually into separate vials (microcentrifuge snap cap of 1 mL or 1.5 mL) to measure their dry mass with an automated S-microbalance (MX5 Mettler Toledo).

The instantaneous force at first crack, maximum force of resistance, shell elasticity, and the area under the force curve (which represents the total force required to fully crush the shell to the point where the resistance was being provided by the microscope slide rather than the shell itself) were measured from the data obtained. The instantaneous force at first crack was obtained by looking at the first change (relaxation) in compressive force that was more negative than -0.005 N. This value was used because it was higher than noise in the readings and was unlikely to reflect measurement error. The shell maximum force of resistance was measured by using the maximum value of force. The elasticity was measured using the initial slope from the eighth to the forty-eighth measurement. The eight was chosen to ignore the initial noise of the first contact of the load with the shell and the forty-eighth was chosen as it gave enough measurements for the slope and did not encompass the first crack in any cases. The area under the curve, represented the total force withstood by the shell, was estimated using the trapezoidal rule using the equation:

Equation 3-1

$$\int_a^b f(x)dx \approx b - a\left[\frac{f(a) + f(b)}{2}\right]$$

3.2.4 Statistical Analyses

Patterns of settlement density were analyzed using repeated measures ANOVA on the average density count of each bin nested within the prior larval tank used using JMP 9.02. The post-settlement sizes on days 27 and 37, the proportion of the area of the shell occupied by the gut, and the four metrics from the crushing tests were measured using mixed effects models using the nlme package (Pinheiro *et al*., 2014) in R (RStudio 0.94.110) and I reported the results of ANOVAs on the models. The mixed effects models were built with the larval and juvenile pCO_2 as fixed factors and using a hierarchical nesting structure to reflect the experimental design. Individuals were grouped within mesh bags as juvenile, that had been settled in a specific settlement bin while considering the larval tank in which they were grown in the prior experiment (i.e. mesh bag/juvenile tank/settlement bin/larval tank). The proportion of juveniles that showed post-settlement extension of juvenile shell was analysed using a generalized linear mixed effects model with a binomial error distribution using the lme4 package (Bates *et al*., 2014) in R (RStudio 0.94.110). In order to account for the non-independence of the larval and juvenile experiments, the larval tanks were crossed with juvenile tanks in the previously described model. The proportion of the shell occupied by the gut as well as all the data from the crushing section were square root transformed to meet the assumption of normality except for the elasticity data, which were log-transformed. All data were later backtransformed for graphical presentation. For the crushing data, size was included in the model as a covariate.

3.3 Results

3.3.1 Settlement

An increasing number of larvae settled on oyster shells over the course of the 48 hour experiment (MANOVA, time effect; $F_{2,7}$=22.825, p=0.0009) (Fig. 3-3). However, there was no interaction of time and larval prior treatment regarding settlement density (MANOVA; $F_{2,7}$=2.671, p=0.137). The larval treatment prior to settling did not influence the settlement density (MANOVA; $F_{1,8}$=0.147, p=0.711).

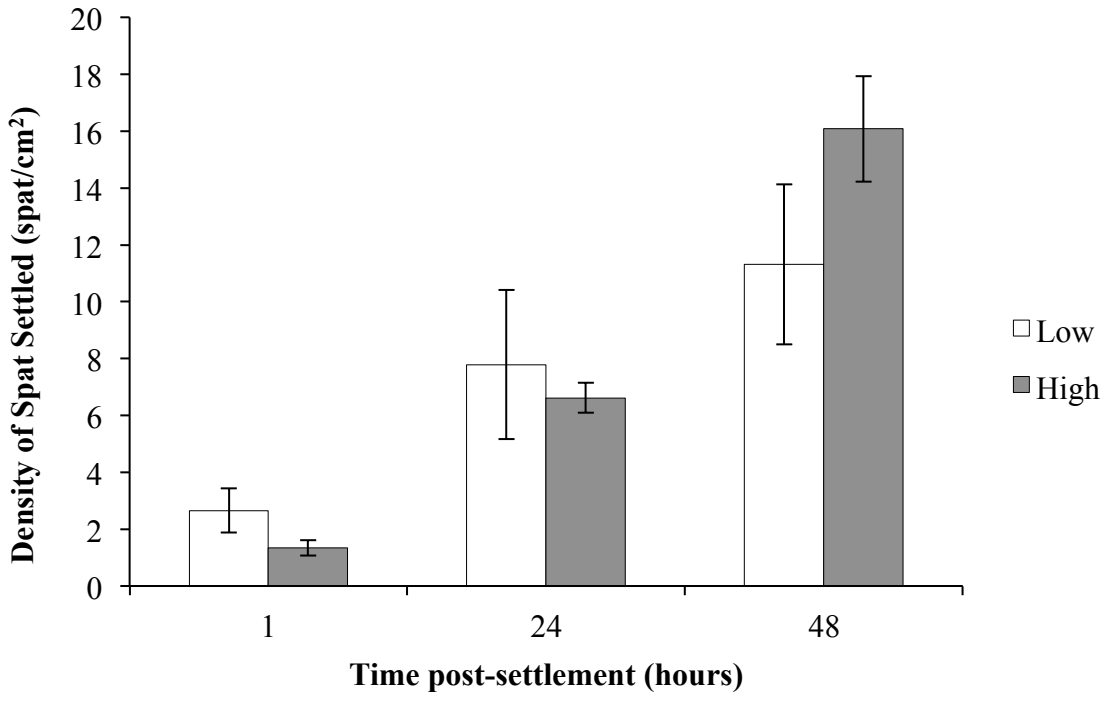

Figure 3-3. Density of spat settled after 1, 24, and 48 hours at low pCO_2 after being reared at low (white bars) or at high pCO_2 (grey bars) as larvae (n=3). Error bars represent standard errors.

3.3.2 Postsettlement Juveniles

3.3.2.1 27 Day-Old Oysters

A smaller proportion of individual *C. gigas* have successfully undergone metamorphosis as indicated by juvenile shell extension when subjected to acidification during their larval development (Table 3-2) (Fig. 3-4). Although not significant, there was a trend towards a reduced proportion of individuals extending their shells when exposed to elevated pCO_2 as juveniles (Table 3-2). The trends are consistent between treatments with no significant interaction between earlier larval treatment and later juvenile treatment.

Table 3-2. Results of a generalized linear mixed effect model of acidification at the larval stage and at the juvenile stage on the proportion of individual *Crassostrea gigas* extending their shell. Contrasts show negative effects of acidification at the larval stage on the proportion of individuals with shell extension. Asterisks (*) denote significance.

	Coefficient	Std. error	z value	P-value
Intercept (L)	2.2239	0.2736	8.127	4.39e-16 *
Larval treatment (H)	-0.9969	0.3272	-3.047	0.00231 *
Juvenile treatment (H)	-0.4302	0.2358	-1.824	0.06811
Larval treatment (H) × Juvenile treatment (H)	0.2319	0.1671	1.388	0.16524

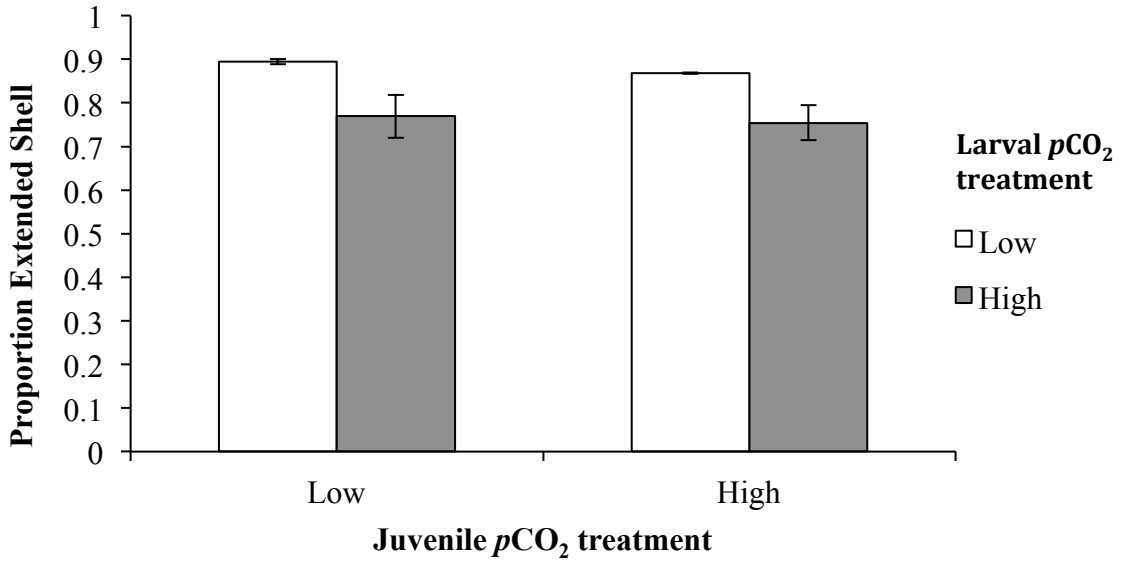

Figure 3-4. Mean proportion of juvenile *Crassostrea gigas* that had extended their shell by 5-7 days after settlement, when the oysters were 27 days old (n=3). Duration of larval and juvenile exposure to carbon dioxide treatments was 18 days and 5 days, respectively. Error bars represent standard errors.

With regards to juvenile size, the settled and metamorphosed oysters held at lower pCO_2 as juveniles were larger (2-Way ANOVA; $F_{1,58}$= 19.839, p<0.0001) (Fig. 3-5). However, their size was similar in regard to larval prior exposure (2-Way ANOVA; $F_{1,2}$= 0.063 p=0.825) and there was no interactive effect between the prior larval treatment and the juvenile treatment on size 5-7 days post-settlement (2-Way ANOVA; $F_{1,58}$= 0.270, p=0.605).

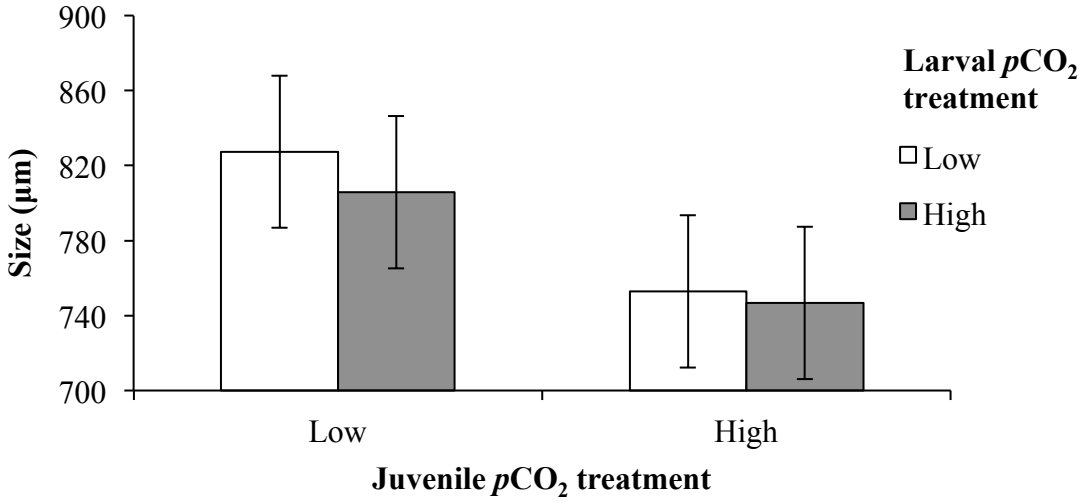

Figure 3-5. Mean length of 27 day-old juvenile *Crassostrea gigas* following exposure to elevated pCO_2 as larvae and/or juveniles. Duration of larval and juvenile exposure to carbon dioxide treatments was 18 days and 5 days, respectively (n=3). Error bars represent pooled standard errors.

3.3.2.2 37 Day-Old Oysters

Although not significant, the juvenile oysters held at lower pCO_2 as juveniles tended to be larger (2-Way ANOVA; $F_{1,58}$= 3.562, p=0.064) but their size was not affected by their earlier larval treatment (2-Way ANOVA; $F_{1,2}$=0.241, p=0.672) (Fig. 3-6). There was no interaction between the prior larval treatment and the juvenile treatment on juvenile size 15-17 days post-settlement (2-Way ANOVA; $F_{1,58}$=0.721, p=0.399).

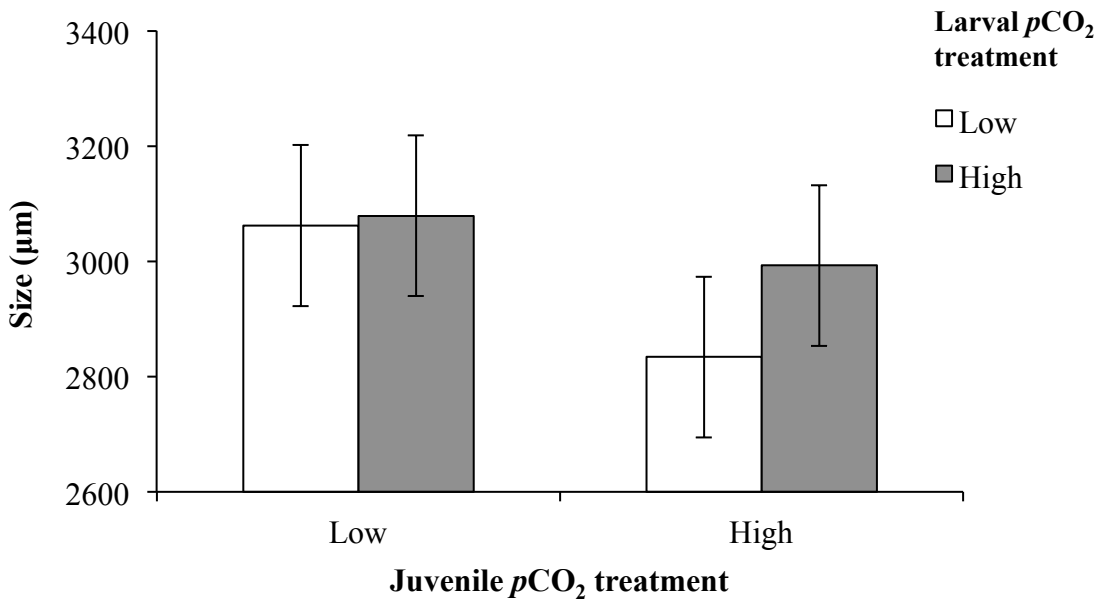

Figure 3-6. Mean length of 37 day-old juvenile *Crassostrea gigas*. Duration of larval and juvenile exposure to carbon dioxide treatments was 18 days and 15 days, respectively (n=3). Error bars represent pooled standard errors.

Juvenile oyster condition, as measured by size of the gut relative to the total size of the animal, was significantly lower when the juveniles were exposed to elevated pCO_2 15-17 days post-settlement (2-Way ANOVA; $F_{1,58}$=13.256, p=0.0006) (Fig. 3-7). However, the larval prior pCO_2 treatment did not have an effect on the juvenile oyster condition (2-Way ANOVA; $F_{1,2}$=0.029, p=0.881) and there was no interaction between the prior larval treatment and the juvenile treatment on the proportion of the juvenile area occupied by the gut (2-Way ANOVA; $F_{1,58}$=2.097, p=0.153).

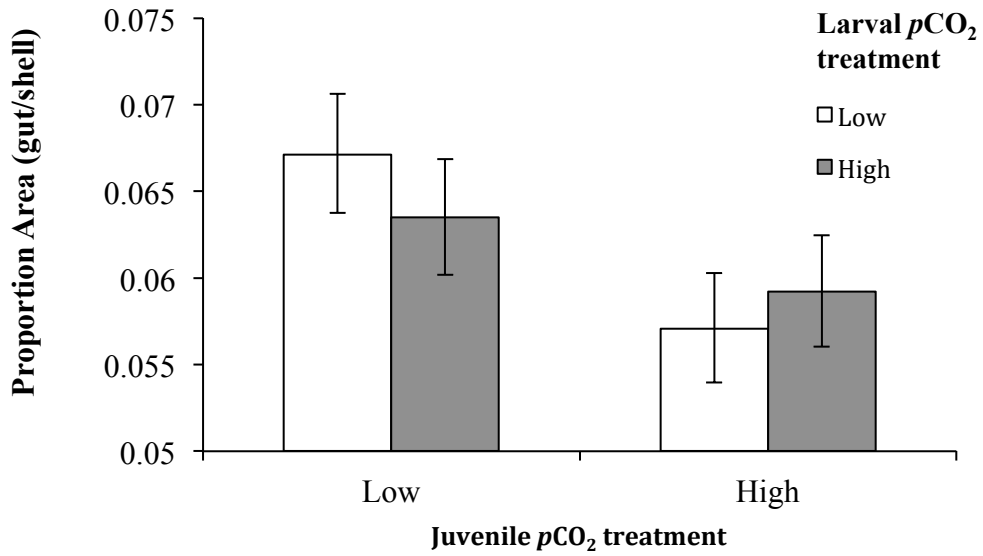

Figure 3-7. Proportion of the whole juvenile area occupied by the gut. Duration of larval and juvenile exposure to carbon dioxide treatments was 18 days and 15 days, respectively (n=3). Error bars represent pooled standard errors.

Experimental pCO_2 exposure had no measurable effects on the biomechanical characteristics of the shells (Fig. 3-8).

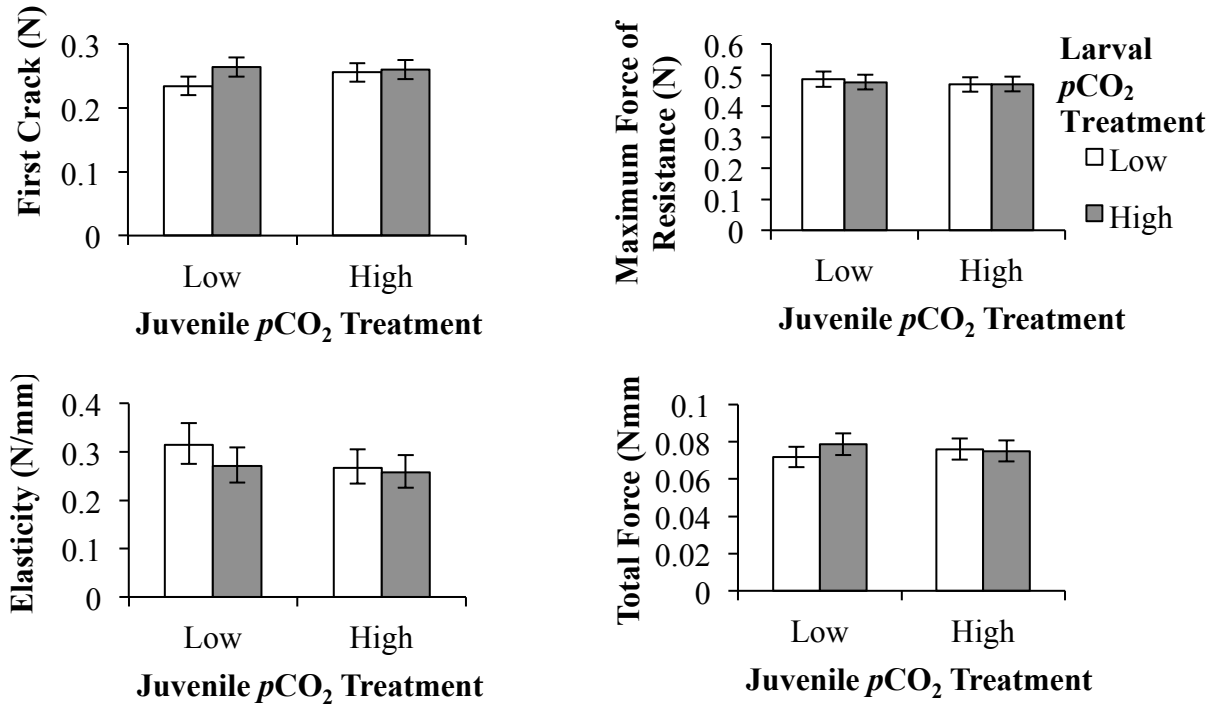

Figure 3-8. Biomechanical characteristics of 37-day old oysters that were previously treated at either low or high pCO_2 as larvae for 18 days and then either at low or high pCO_2 as juveniles for 15 days (n=3). These mean measurements – instantaneous force at first crack (top left), maximum force of resistance (top right), elasticity (bottom left) and total force (bottom right) - were taken by recording the force over extension data using an instron. Error bars represent pooled standard errors.

There was no interaction between the prior larval pCO_2 treatment and the juvenile pCO_2 treatment in any of the measured parameters (Table 3-3). Moreover, in none of the measured parameters was the individual effect of larval pCO_2 treatment or the juvenile pCO_2 treatment having a significant effect on the crushing force of the shells. However, the size of the shell had a significant effect on the maximum force of resistance and total force measurements with a positive relationship in both instances. Therefore, a greater instantaneous force was required to

overcome maximum shell resistance for larger shells, and a shell with a greater shell area resisted crushing with a greater amount of total force during the crushing process.

Table 3-3. Summary of results of ANOVA run on biomechanical measures looking for the effects of prior larval treatment (larval pCO_2 trt) and of juvenile treatment (juvenile pCO_2 trt) and their interaction as well as shell size as a covariate using the surface area of the shell. Asterisks (*) denote significance.

Measure	Effect	DF	F-value	p-value
Instantaneous force at first crack	Larval pCO_2 trt	2	1.253	0.379
	Juvenile pCO_2 trt	57	0.364	0.549
	Larval × juvenile pCO_2 trt	57	1.032	0.314
	Size	628	2.641	0.105
Maximum force of resistance	Larval pCO_2 trt	2	0.034	0.871
	Juvenile pCO_2 trt	57	0.213	0.647
	Larval × juvenile pCO_2 trt	57	0.113	0.738
	Size	628	43.341	<0.0001*
Elasticity	Larval pCO_2 trt	2	0.492	0.556
	Juvenile pCO_2 trt	57	0.643	0.426
	Larval × juvenile pCO_2 trt	57	0.127	0.723
	Size	628	0.476	0.490
Total force	Larval pCO_2 trt	2	0.339	0.619
	Juvenile pCO_2 trt	57	0.006	0.938
	Larval × juvenile pCO_2 trt	57	2.746	0.103
	Size	628	144.074	<0.0001*

3.4 Discussion

The investigation of the effects of OA on marine organisms generally suggests negative outcomes for the large majority of the species studied to date (Kroeker *et al*., 2010, 2013). However, important gaps remain in this area of research and in order to draw firm conclusions, more knowledge is required on multiple life history stages for species of interest. Successful

passage through all the stages is required for the continuation of a species, and conditions experienced at one stage can often affect subsequent stages.

In this study, there was no effect of prior larval exposure to acidification on the larval settlement of *C. gigas*. This lack of effect of OA on the settlement of *C. gigas* is in line with the findings on *C. gigas* in China (Ginger *et al*., 2013), and for the green sea urchins in Sweden (Dupont *et al*., 2013). Conversely, at the volcanic CO_2 vents of Castello Aragonese, a significant decline in settlement of bivalves had been found in acidified water (Cigliano *et al*., 2010). However, their experimental set-up used a natural pCO_2 gradient, which could have tested preference by the larvae more than potential for settlement as the larvae were potentially able to select alternative settlement sites in a nearby, less acidified environment.

In the present study, the adults from which the gametes were obtained had been exposed to fluctuating pCO_2 in the Strait of Georgia for a duration varying between 12 and 24 months. Therefore, acclimation by the parents, or even local adaptation they could have had, may have influenced my results relative to those of other studies as my study population had prior experience with acidification exposure. As mentioned above, there is little information to date about settlement of oysters in regards to OA and this study can only tell that prior larval exposure at approximately 700 μatm, which is not really high considering the levels that can be reached during local upwelling events, does not affect the settlement of oysters. Further studies could include exposing adults to chronic elevated pCO_2 either for the duration of gametogenesis or longer to see if settlement would be increased due to acclimation or reduced due to negative carry-over effects. Settlement may, however, simply be unaffected by OA. A possible

explanation for the lack of an OA effect could be that the process of settlement itself is rapid and does not require an extensive investment of stored energy, unlike the process of metamorphosis that follows.

Metamorphosis appears to have been reduced in the settled oysters that had been exposed to elevated pCO_2 as larvae, with fewer individuals from this treatment demonstrating shell extension as an indicator of successful metamorphosis. Shell extension was not significantly affected by juvenile pCO_2 treatment. A study looking at metamorphosis rates of *C. gigas* reared under experimental pCO_2 conditions as larvae through to settled juveniles found no significant effect of pCO_2 on metamorphosis rates 24 hours post-settlement (Ginger *et al*., 2013). After such a short juvenile exposure, Ginger *et al*. (2013) may have only been testing for the effects of larval prior treatment. My results are not in accordance with Ginger *et al*. (2013), but the longer exposure period in my study could explain this discrepancy. Since we investigated shell extension 5 days post-settlement, it is most likely that the settled larvae that did not show shell extension would end up dying if they had not done so already as metamorphosis happens shortly after settlement. For many settled individuals, it is also likely that metamorphosis had begun during the settlement experiment, in which all individuals were exposed only to low pCO_2. Moreover, the fact that metamorphosis is largely dependent on larval energetic storage (Pechenik, 2006; Burke *et al*., 2008) is consistent with my observation that larval prior treatment had an effect but the post-settlement pCO_2 treatment did not.

At day 27, *C. gigas* were smaller when exposed to increased pCO_2 conditions as juveniles regardless of their larval pCO_2 exposure, although this difference in size was only marginally

significant when the oysters reached 37 days old. Juvenile exposure to increased acidification has also been reported to have negative effects on *S. glomerata* (Parker *et al*., 2010)*, C. gigas* (Parker *et al*., 2010) and on *O. lurida* (Hettinger *et al*., 2012). However, Hettinger *et al*. (2012) found that the exposure to acidification at the larval stage also had a negative impact on growth persisting for at least 1.5 months, which I failed to find in this study. In our findings, the lack of carry-over effects past metamorphosis could be potentially explained with the acidified individuals in poor condition not making it through metamorphosis and therefore leaving the surviving population more fit by filtering the weakest individuals out of the population. Perhaps the individuals *O. lurida* in Hettinger *et al*. (2012)'s study were in better condition than my *C. gigas* and made it past metamorphosis but remained weaker than the non-acidified individuals, resulting in negative carry-over effects. In my study, either metamorphosis acted as a selection pressure for the most fit individuals or once they make it through the metamorphose growth in juvenile start from the same baseline having depleted their larval energetic reserve regardless of their prior treatment.

The proportion of the overall juvenile area occupied by the gut followed the same trend as the size but the trend was still significant when they reached 37 days old. This finding suggests that the 37 day old juveniles exposed to elevated pCO_2 levels were not as healthy as the ones exposed to ambient pCO_2 levels, even though the former are still able to grow about as quickly as the latter, at least over 15 days. This highlights the need for longer terms studies as to determine if this trend persists over time, and if long-term survival and fitness are compromised.

Shell strength was seemingly not affected by OA considering all the measured parameters in this study, showing no differences between any of the treatments applied to larvae or juveniles. Studies on shell strength or its proxy, shell thickness, are rather limited in the OA literature, but our findings of no effect of OA on any of the strength parameters measured by crushing shells align well with the lack of effect of OA on shell thickness found by Ginger *et al*. (2013). However, the lack of effect of OA on shell strength in *C. gigas* does not agree with the negative effects found in Beniash *et al*. (2010) in *C. virginica*. Though, in the present study, size had a significant positive correlation with maximum force of resistance and total force withstood by the shell. Although OA did not affect the strength of shells once size had been accounted for, smaller oysters had weaker shells. Therefore, if OA slows growth, oysters will remain smaller and weaker for longer.

Another consideration noteworthy in this study is that the parents of the *C. gigas* used had been living in the ocean for many months at highly fluctuating pCO_2 levels prior to these experiments. Therefore, the opportunity for acclimation to acidification which could have mitigated or exacerbated the effects seen in this research should be kept in mind. However, this is what *C. gigas* is experiencing now in the aquaculture industry. Hence, in order to understand the present mass mortality events and gain knowledge on the best practices to use in order to maximize harvest, we kept the procedures and conditions as close as we could to understand the effects of OA on the early developmental stages *C. gigas* in the Strait of Georgia.

Overall, the results in this chapter primarily suggest negative effects of acidification on recruitment of *C. gigas*. Even though the settlement process was not affected by prior

acidification treatment, a smaller proportion of the settled oysters went through metamorphosis when grown as larvae in acidified conditions. The juvenile pCO_2 treatment had a significant negative effect on growth and condition as measured by the proportion of the juvenile area occupied by the shell. However, there was no evidence that the strength or material properties of the shell were affected by acidification. Decreased growth, although not seemingly dramatic for the juvenile itself, can have serious implications at the population level since reduced size in early benthic stages has been found to decrease competitive ability, which in turn can increase post-settlement mortality and ultimately lead to decreased recruitment into the adult population (Connell, 1961; Anil *et al*., 2001; Parker *et al*., 2010). Moreover, Waldbusser *et al*. (2010) found that in post-larval clams, capacity to overcome shell dissolution increased with size, making growth an important factor for survival in face of OA. Another important implication of reduced growth for the shellfish industry is the increasing time required for oysters to reach market size, which can have serious impacts for the economic viability of aquaculture operations (Parker *et al*., 2010) by reducing production and increasing investments in labor and expenses.

Chapter 4: Conclusion

4.1 Thesis Objectives

This thesis attempted to determine the overall effects of ocean acidification on an economically valuable species, the Pacific oyster (*Crassostrea gigas*), at early developmental stages under realistic hatchery conditions. There is a growing body of research on this species, but gaps and inconsistencies in our understanding remain, and this thesis provides several novel insights. Moreover, the importance of this study is further evidenced by the urgency to find appropriate answers following mass mortality events in the Strait of Georgia. Chapter 2 aimed at understanding the effects of acidification on the fertilization and larval development in order to determine if the current mass mortality in hatchery could possibly be caused by OA. Chapter 3 was geared towards understanding the implications of an oyster's history of carbon dioxide exposure on the later stages of early development. Although we could not conclude that the mass mortality events of larvae experienced locally are directly connected solely to acidified events, we can infer from these findings that both the fertilization and larval development are negatively affected by ocean acidification. Also, we can conclude that the prior larval treatment matters in terms of recruitment initially with the metamorphosis being reduced with acidification. Yet, once the metamorphosis happens, the larval prior treatment does not seem to have further effects on juveniles. From this study, we conclude that the industry should aim at raising their larvae at ambient rather than elevated pCO_2 conditions to maximize recruitment, since we did not find evidence to suggest that hardening was occurring when juveniles grew at elevated pCO_2 concentrations.

4.2 Fertilization and Larval Development

This experiment looked at prior sperm washing as well as fertilization at elevated pCO_2 levels in 5 L flasks. Positive effects of OA on the fertilization were found with increased proportion of eggs being fertilized but negative effects of OA followed with the decreased proportion of fertilized eggs developing to reach later developmental stages (trocophore and prodissoconch I larvae). The sperm washing treatment had no effect on fertilization success. These findings can be summarized as a decline fertilization success when *C. gigas* is exposed to OA, which agrees with Barros *et al*. (2013) and these conclusions could be explained either from a right shift in the fertilization success over sperm density curve or by increased polyspermy (Reuter *et al*., 2011). However, this finding is not in accordance with the prior finding of no effect of OA on the activity of *C. gigas* sperm nor on the fertilization success of another study (Havenhand & Schlegel 2009).

The larval experiment was conducted on hatchery produced prodissoconch I larvae (beginning at 2 days old) for 18 days at two levels of pCO_2; ambient (400 µatm) and elevated (685 µatm). Growth, production of high quality larvae and feeding were negatively affected by acidification. There were also non-significant trends towards increased mortality and deformity at increased pCO_2 conditions. In terms of growth, divergence between treatments began on day 12 (at approximately 160 µm), whereas Barton *et al*. (2012) observed it earlier at 120 µm. The lack of growth differences in younger larvae could be caused by the mixotrophic diet of *C. gigas* at earlier stages (Rico-Villa *et al*., 2009; Ben Kheder *et al*., 2010a,b). Smaller larval sizes of acidified *C. gigas* were also found in Barros *et al*. (2013), Kurihara *et al*. (2007) and Parker *et al*. (2010), but they did not see the delay in size divergence between treatments that our and Barton

et al. (2012) studies found. However, Ginger *et al*. (2013) found no difference in *C. gigas* larval sizes. Increased mortality with increased acidification was also previously reported in Barros *et al*. (2013). However, Ginger *et al*. (2013) and Timmins-Schiffman *et al*. (2013) did not find any effects of OA on mortality. These variations in results raised the potential effect of population location on the results suggesting that some populations could be better adapted than others to withstand elevated pCO_2 exposure. Deformity, an indicator of the difficulties encountered with the process of calcification, was also found to have increased in *C. gigas* larvae after exposure to increased pCO_2 in Kurihara *et al*. (2007), Gazeau *et al*. (2011), Parker *et al*. (2010), and Barros *et al*. (2013).

Overall, our results agree with most of the prior literature: fertilization and larval development of *C. gigas* are negatively impacted by ocean acidification in general. However, we did not record severe negative impacts in our experiments. Therefore, based on these findings, we cannot conclude that the mass mortality events experienced in the local hatcheries are directly caused by OA, at least not at levels up to ~710 μatm as were used in these experiments.

4.3 Settlement and Post-Settlement Performance

The best quality larvae (those retained by a 263 μm screen) from the larval experiment (see chapter 2) were used to investigate if the prior larval treatment had an effect on the settlement of prodissoconch II larvae. The larvae were allowed to settle at ambient pCO_2 for 48 hours, over which time the density of settled larvae was quantified. We found no effect of larval exposure to acidification on the settlement of *C. gigas*, which is in line with the findings from Ginger *et al*. (2013) on *C. gigas* and Dupont *et al*. (2012) on green sea urchins. Conversely, Cigliano *et al*.

(2010) found a significant decrease settlement of bivalves in acidified water. The absence of an effect in my experiments could be due to the lack of carbon dioxide differences during the settlement period, a potentially small energy requirement for a larva to settle (in which case larval condition may not translate to settlement success), or the parental prior exposure as the broodstock was in the ocean for a long duration (12-24 months) prior to fertilization of their gametes.

The settled larvae were placed in either the original larval treatment (high or low pCO_2) or in a novel treatment (high or low pCO_2) to determine if there were carry-over effects or acclimation from the larval stage to the juvenile stage. Successful metamorphosis, measured as the proportion of juveniles that extended their shell after settlement, was reduced in oysters that had been exposed to elevated pCO_2 as larvae but did not depend on the juvenile pCO_2 treatment. In contrast, Ginger *et al*. (2013) found no difference in metamorphosis success based on acidification. The fact that metamorphosis is largely dependent on larval energetic storage (Pechenik, 2006; Burke *et al*., 2008) may explain why larval prior treatment had an effect, but not the post-settlement pCO_2 treatment. From this, we may infer that recruitment is reduced by increased pCO_2 since, by 5 days after settlement, the individuals that did not metamorphose at this point were likely dead or moribund.

Juvenile exposure to increased acidification had a negative impact on juvenile oysters growth, which also had been reported in *S. glomerata* (Parker *et al*., 2010)*, C. gigas* (Parker *et al*., 2010) and in *O. lurida* (Hettinger *et al*., 2012). However, Hettinger *et al*. (2012) found that exposure to

acidification at the larval stage also had a negative impact on juvenile growth which we failed to find in this study.

The proportion of the overall juvenile area occupied by the gut followed the same trend as the size by being affected negatively by acidification at the juvenile stage only. This suggests poor health for the acidified 37 day-old juveniles.

Shell strength parameters were not affected by juvenile pCO_2 treatment, consistent with the findings of no thickness differences (Ginger *et al*. 2013). Larval pCO_2 treatment also had no effect on shell material properties. However, these findings do not agree with Beniash *et al*. (2010), who found negative effects of ocean acidification on shell strength in *C. virginica*.

Overall, my results all point towards negative effects of acidification on recruitment and overall condition of juvenile *C. gigas*. Based on these findings, I recommend that the aquaculture industry raise larvae at relatively low pCO_2 concentrations (such as those currently found in well-mixed surface waters) in order to maximize recruitment, as my results provide no evidence of acclimation of juveniles following exposure as larvae.

4.4 Recommendations for Future Research

Much research remains to be conducted to fully understand the effects of OA on *C. gigas* and the potential for this species to tolerate, acclimate, and to adapt to exposure to elevated pCO_2. The main avenues that require attention include addressing the effects of fluctuating pCO_2 levels, which would be more realistic for the aquacultured juveniles after being outplanted than what

they experienced in the present study. This is important because if they respond better to fluctuations in pCO_2, exposing the cultured early stages of oysters to variations at earlier stages might be beneficial despite the stock lost in the process.

Moreover, a longer-term study could better inform the industry about the effects of pCO_2 on later-stage juveniles as the size difference started to converge after 15 post-settlement but the gut proportion is significantly smaller, suggesting that delayed effects may be felt as the oysters mature. The longer-term knowledge could change the outcomes on the observed effects and consequently the recommendations for maximizing harvest could be revised accordingly. The longer term could be in relation to the organisms' life of over generations as well. With relatively long exposure to a stressor, species can also acclimate, which can then mitigate the effects of the exposure. If the exposure persists for generations, functional adaptations may also be observed. Adaptation requires that the traits that are beneficial to coping with the stressor be passed on to the next generations.

Furthermore, the levels of pCO_2 used in this study were derived from future projections found in the 2007 IPCC report, which suggested that 700 µatm were likely to be attained by the end of this century. The more recent 2013 IPCC report now predicts a higher likelihood for greater pCO_2 values – approximately 1000 µatm – by the end of the century (IPCC, 2013). Furthermore, these predictions are global means, and local conditions may exceed these levels much sooner. For example, pCO_2 values in excess of 1400 µatm were observed during the summer 2013 in the Strait of Georgia (R. Saunders, pers. comm.). Therefore, in light of this recent information, further research should focus on these higher pCO_2 values to better reflect the actual problem

encountered by hatcheries in British Columbia as well as coastal ecosystems in the Salish Sea and elsewhere.

OA may have already started to have negative effects on the economy and food security and these effects are likely to be exacerbated considering the continual input of CO_2 in the atmosphere. This study is one step towards helping industry adapt to a changing world.

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Appendices

Appendix A

Table A-1. Daily feeding regimen, per tank, for *Crassostrea gigas* larvae for the duration of the larval experiment. TISO (*Isochrysis* sp.), SK (*Skeletonema costatum*), 3H (*Thalassiosira pseudonana*), and CG (*Chaetoceros gracilis*) were given at the following concentrations.

Day	Algal composition	Algal concentration (c/mL)	Total algal concentration (c/mL)
2	TISO	10,000	10,000
3	TISO	10,000	20,000
	SK	10,000	
4	TISO	7,500	15,000
	SK	4,500	
	3H	3,000	
5	TISO	12,000	15,000
	SK	3,000	
6	TISO	17,500	25,000
	SK	3,750	
	3H	3,750	
7	TISO	13,000	20,000
	SK	3,500	
	3H	3,500	
8	TISO	5,000	30,000
	SK	12,500	
	3H	12,500	
9	TISO	4,000	10,000
	SK	3,000	
	3H	3,000	
10	TISO	5,000	30,000
	SK	12,500	
	3H	12,500	
11	TISO	10,000	40,000
	SK	15,000	
	3H	15,000	
12	TISO	14,000	40,000
	SK	10,000	
	3H	10,000	
	CG	6,000	
13	TISO	15,500	50,000
	SK	17,250	
	3H	17,250	
14	TISO	25,000	55,000
	SK	30,000	
15	TISO	12,000	30,000
	SK	12,000	
	3H	6,000	
16	TISO	5,000	40,000
	SK	15,000	
	3H	15,000	

Day	Algal composition	Algal concentration (c/mL)	Total algal concentration (c/mL)
17	CG	5,000	55,080
	TISO	3,000	
	SK	23,540	
	3H	23,540	
18	CG	5,000	65,000
	TISO	10,000	
	SK	27,500	
	3H	27,500	
19	TISO	10,000	65,000
	SK	30,000	
	3H	25,000	

Appendix B

Table B-1. Daily feeding regimen, per tank, for *Crassostrea gigas* juveniles for the duration of the post-settlement experiment (15 days). TISO (*Isochrysis* sp.), SK (*Skeletonema costatum*), 3H (*Thalassiosira pseudonana*), CG (*Chaetoceros gracilis*), and mix (mixture of wild unknown algae composed of short chain diatoms) were given at the following concentrations.

Day	Algal composition	Algal concentration (c/mL)	Total algal concentration (c/mL)
1	TISO	10,000	45,000
	SK	35,000	
4	TISO	19,500	65,000
	SK	26,000	
	3H	19,500	
6	TISO	2,500	10,000
	SK	2,500	
	3H	2,500	
	CG	2,500	
7	TISO	5,000	15,000
	Mix	10,000	
8	TISO	15,000	40,000
	Mix	25,000	
11	TISO	10,000	20,000
	Mix	10,000	
12	TISO	10,000	40,000
	Mix	30,000	
13	TISO	10,000	30,000
	Mix	20,000	