INTRODUCTION

 Global climate change is expected to affect all ecosystems at an accelerating pace over the next century (IPCC 2007; Caldeira and Wickett 2003). Many of the projected changes will have significant consequences for natural populations, from molecular physiological changes to broadscale range shifts (Talmage and Gobler 2011; O’Donnell et al. 2009; Wong et al. 2011; Tomanek et al. 2011; need range shift citations). Ocean acidification has been gaining increasing attention as one of the more insidious changes to accompany global climate change. As atmospheric anthropogenic CO2 reaches equilibrium with ocean surface waters it dissolves and more H+ ions are released into the water. The increase in H+ ion concentration in seawater is the direct cause for decreased pH (acidification); however, other changes resulting from this same chemical reaction may also have significant effects on aquatic organisms. Increased CO2 concentration in seawater can change the gradient between an organism’s internal fluids and its environment, resulting in a greater diffusion of CO2 into animal tissues (Fabry et al. 2008). Increases in internal CO2 concentrations can result in acidosis of metabolic fluids, as seen in cuttlefish (Gutowska et al. 2010), the sea urchin *Psammechinus miliaris* (Miles et al. 2007), and the mussel *Mytulis edulis* (Michaelidis et al. 2005). The dissolution of CO2 in seawater not only produces more H+, it also produces greater concentrations of bicarbonate (HCO3-) and makes carbonate ion (CO32-) less available. The latter effect is of great importance to calcifying organisms since they rely on the availability of CO32- to make carbonate-based structures. All of these changes have the potential to increase the environmentally induced stress that an aquatic organism experiences.

 Aquatic invertebrates, as environmental conformers, may be especially susceptible to changes such as ocean acidification since they do not have extensive physiological mechanisms to counteract environmental shifts. In particular, acidification-induced changes can lead to inhibited calcification or shell dissolution and alterations in ion balance. The most apparent effect of ocean acidification on calcification is that it makes CO32- less available to form and maintain calcified structures and it also is more corrosive to already formed CaCO3. After 20 weeks at a pH of 7.5 compared to a control of 8.2, *C. virginica* adults had reduced shell mass and thicker calcitic laths in their shells (Beniash et al. 2010). Similarly, *M. edulis* raised at elevated CO2 for 8 weeks had decreased shell length and mass (Thomsen and Melzner 2010). Additionally, many aquatic invertebrates dissolve their calcified structures to make HCO3- more available as a buffer against internal acidosis caused by ocean acidification. This phenomenon has been observed across a range of invertebrate species: *C. gigas* (Lannig et al. 2010), the limpet *Patella vulgata* after just 5 days at high pCO2 (Marchant et al. 2010), and *P. miliaris* (Miles et al. 2007).

 Ocean acidification also changes ion balances across membranes, leading to an increased energy demand in organisms as they correct the imbalance or as they launch a stress response in reaction to it. Changes in ionic concentration due to hypercapnia and carbonate structure dissolution can result in overall shifts in cellular ion gradients, which could significantly alter cellular processes (Dissanayake et al. 2010). Overall, changes in environmental and internal organismal carbonate chemistry create a more stressful and energetically demanding environment for aquatic invertebrates.

 The stresses induced by ocean acidification can have secondary effects on organism growth, physiology, behavior, and life history. Typically, invertebrate larvae that are reared at higher pCO2 are smaller and have more developmental abnormalities than those at ambient pCO2 (Chan et al. 2011; Parker et al. 2010). A host of physiological changes are also associated with changes in pCO2. Invertebrate larvae raised at elevated pCO2 show an inhibited ability to launch a heat shock response (O’Donnell et al. 2009) and alterations in both transciptomic and proteomic profiles (Todgham and Hofmann 2009; Wong et al. 2011). Increasing attention, however, is being put on the importance of changes in carbonate saturation state (Ω) and how it affects larval growth and development. *C. gigas* larval survival in the hatchery is impacted by decreased Ω (Barton et al. in review). Growth is also inhibited under lower Ω, although not affected by lower pH (Gazeau et al. 2011). Ocean acidification is a current and future challenge and will continue to negatively impact aquatic organisms. These impacts are of both ecological and economic concern since the effects of ocean acidification are seen across taxonomic groups (i.e. on an ecosystem-level scale) and have the potential to decrease the viability of commercially important populations.

 This study aims to characterize the effects of two elevated levels of pCO2 on growth and physiology during early larval stages of the Pacific oyster, *Crassostrea gigas*. Oysters are ecologically important because they filter water and form oyster reefs that create habitat for a myriad of other estuarine invertebrates. They are also economically important, forming the basis of multi-million dollar industries (CITE). Recently, in the Pacific Northwest of the U.S., there has been increasing concern over the already apparent effects of corrosive, acidified water on both natural and hatchery production of *C. gigas* larvae (B. Eudeline pers. comm.; Feely et al. 2010). Larval *C. gigas* were reared in 2 elevated levels of pCO2 (700 and 1000 µatm) and ambient (400 µatm) from fertilization through 4 days post-fertilization. During these four days, the larvae were assessed for growth, calcification, and their physiological response to ocean acidification.

METHODS

*Ocean acidification system*

 The ocean acidification scenarios were simulated using a flow-through system that draws in seawater from Friday Harbor, WA. Thus, the total alkalinity mirrors natural fluctuations in the local water. Once the water enters the system, it is filtered to 0.2 µm and stripped of CO2 by heating it, etc. Ambient air is also stripped of CO2 by this process and added back to the low-CO2 seawater. As the seawater enters the experimental system, CO2 (Praxair) is added back in with a Venturi injector (manufacturer) at the correct levels to simulate the desired atmospheric level of pCO2 – 400, 700, or 1000 ppm. The water enters large Igloo coolers that hold the replicate 3 L microcosms where the larval *C. gigas* are kept. The water is pumped by a DuraFET pH probe (manufacturer), which is attached to the cooler. The pH probe communicates with a Honeywell controller (manufacturer), which records the pH every minute and adjusts the amount of CO2 injected by the Venturi injector if the pH deviates from the desired level. As the water re-enters the cooler from being pumped by the DuraFET, it drips into the bottom of separate microcosms at xx L per minute. An outflow tube at the top of the microcosms fitted with 35 µm mesh allows for turn-over of the water. The water flows out of the microcosms through outflow pipes. The systems were equilibrated to the correct treatment levels for 48 hours prior to the start of the experiment.

*Oysters*

 Fourteen adult *C. gigas*, 10 females and 4 males, were collected from Argyle Creek in Friday Harbor, WA at low tide. The oysters were strip spawned and eggs and sperm were pooled separately (day 0). The pooled eggs were divided equally between 18 small fertilization dishes containing water pre-equilibrated to the correct pCO2 (6 replicates per 3 pCO2 treatments). Remaining pooled eggs were divided between 6 50-mL Falcon tubes containing pre-equilibrated seawater. The pooled sperm was diluted in seawater and a small amount was added to the eggs to avoid polyspermy. After sperm addition, the eggs were gently agitated and left to incubate for 15 minutes to allow for fertilization. After 15 minutes, the fertilized eggs in the fertilization dishes were gently poured into 3 L microcosms containing pre-equilibrated treatment water held in the coolers filled with water set at 21°C. The microcosms were left static for 24 hours. The Falcon tubes containing fertilized eggs were also placed in the coolers.

 At 1 hour post-fertilization, the Falcon tubes were inverted gently 3 times and 500 µL of fertilized eggs were removed. This was repeated for a total of 1 mL from each tube. The 1 mL of fertilized eggs and seawater were allowed to settle to the bottom of a 2 mL microcentrifuge tube for 5 minutes, after which the seawater was drawn off and they were fixed in 4% paraformaldehyde (PFA) buffered in filtered seawater. At 6 hpf, the remaining material in the Falcon tubes was filtered on a 35 µm screen and rinsed using 2 mL of seawater into 2 mL tubes. The trocophore larvae were relaxed with 7.5% MgCl2 before being fixed in 4% PFA.

 On days 1 and 3 post-fertilization, larvae were sampled and fixed in 4% PFA from 2 replicate microcosms in each treatment. Briefly, all larvae were filtered out of their microcosms onto 35 µm mesh screens and washed into 50 mL Falcon tubes of seawater at the same treatment pCO2. The tubes were inverted gently 3 times between each of 3 aliquots, the volume of which was estimated to get a total of 100 larvae. The microcosms were cleaned and refilled and the remaining larvae were poured back. All larval microcosms were similarly cleaned at this time even if the larvae were not sampled.

Larvae were analyzed microscopically for size and calcification. Using a Nikon and some kind of software, larval hinge length and shell height were measured at 10x magnification. Using an inverted microscope, larval calcification was measured using double polarized light at 20x magnification. Larvae were scored as calcified on day 1 post-fertilization if they had obvious calcification at their hinge; larvae were uncalcified if there was no apparent calcification. On day 3 post-fertilization, larvae were fully calcified if the polarized light reflected a “maltese cross” in the larval shell (LaBarbera 1974). If the cross was not apparent, then the larvae were counted as partially calcified or uncalcified (these two counts were grouped for analysis).

 On day 4, 2 replicate microcosms from each treatment were sampled for transcriptomics. All larvae were filtered onto a screen from each microcosm and rinsed into a 2 mL tube. The larvae were spun down for 1 minute at 2,500 rpm. The seawater was drawn off with a pipette and the larvae were immediately flash frozen in liquid nitrogen.

*Carbonate chemistry*

 Throughout the experiment, salinity and temperature were measured using probes whenever samples were taken for carbonate chemistry. Daily spectrophotometric pH using *m-*cresol purple was measured of the water flowing into the microcosms following SOP 6b in Dickson et al. (2007). pH was measured within 2 replicate microcosms per treatment on days 0, 1, and 3. Final pH values reported here have been corrected for dye addition and for temperature at which the samples were taken. Total alkalinity (AT) was also measured following SOP 3b (Dickson et al. 2007). Samples for AT were taken of the incoming water in each treatment and of 2 replicate microcosms on days 0, 2, and 3. Dissolved inorganic carbon (DIC) was measured of the incoming water for each treatment on days 0, 2, and 3 following SOP 2 (Dickson et al. 2007). AT and pH were used to calculate calcium carbonate saturation state (Ω) of aragonite and calcite using the following constants (A. Dickson, pers. comm.): Lueker et al. 2000 for CO2 Constants, Dickson 1990b for KHSO4, Total scale (mol/kgSW) for pH scale, and Wanninkhof 1992 for Air-Sea Flux.

*RNA extraction and qPCR*

 Total RNA was extracted from the larvae collected on day 4 using Tri-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer’s protocol. RNA was reverse transcribed to complementary DNA (cDNA) using M-MLV reverse transcriptase according to the manufacturer’s protocol (Promega, Madison, WI). Primers were made to amplify only cDNA by designing them to span introns of genes with known genomic structure using Primer3 in Geneious Pro v. 5.3.6 (Drummond et al. 2010). Primer sequences are in Table 1. qPCR was performed using 1 µL of cDNA in a 20 µL reaction containing 10 µL of 2x SsoFast Eva Green Master Mix (Bio-Rad, Hercules, CA), 8 µL of nuclease-free water, and 0.5 µL each of 10 µM forward and reverse primers (Integrated DNA Technologies, Coralville, IA). Thermal cycling and fluorescence detection were performed using a CFX96 Real-Time Detection System (Bio-Rad). Cycling parameters were as follows: 98°C for 2 minutes; 40 cycles of 98°C for 2 seconds, 55°C for 5 seconds plus a plate read; melt curve from 75-95°C at 0.2°C for 10 seconds, followed by a plate read.

 Average Ct (fluorescence-based cycle threshold) values across replicates and average gene efficiencies were calculated with PCR Miner (Zhao and Fernald 2005, <http://www.miner.ewindup.info/version2>). Gene expression (R0) was calculated based on the equation R0 = 1/(1+E)Ct, where E is the average gene efficiency and Ct is the cycle threshold for fluorescence.  All expression values were normalized to expression of elongation factor 1α (GenBank Accession Number AB122066). All qPCRs were run in duplicate.

*Statistics*

Daily growth rate was calculated by subtracting mean larval size (shell hinge length or height) for day 1 from the same mean measurement for day 3 and dividing by 2 within each treatment. Larval size and daily growth rate were analyzed using an ANOVA with fixed effects of treatment and day, followed by Tukey’s Honestly Significant Difference test (Tukey’s HSD) when the ANOVA showed significant differences. Larval calcification was compared between treatments using a generalized linear model (GLM). Binomial error distributions were used for all analyses. GLMs assess the difference between groups while maintaining robustness to variable sample sizes. Differences in gene expression between treatments were calculated using ANOVA with treatment as a fixed effect. All analyses were performed in R (R Development Core Team 2011).

RESULTS

*Carbonate chemistry*

Throughout the experiment, DIC and pH were different between the three treatments, while AT varied, but was the same across treatments (Table 2). The pH profiles presented in Figure 1 are from the controller logs of the DuraFET pH probes. The pH values from the probes are corroborated by the spectrophotometric pH (Table 2). For the 400 ppm control treatment, the average pH was 7.99 from day 0 to day 4 as measured by the DuraFET pH probes (Figure 1). The average pH in the 700 ppm treatment was 7.75 pH units and in the 1000 ppm treatment it was 7.66 pH units. DIC was highest in the 1000 ppm treatment and lowest in the 400 ppm control (Table 2). Total alkalinity varied between 1965.59 and 2031.41 µmol kg-1 (Table 2). Aragonite and calcite saturation states were greater than 1 for the duration of the experiment, except at 1000 µatm on days 0 and 3 of the experiment (Table 2). Temperature was consistently around 21°C across treatments, with a minimum temperature of about 19.4°C, a maximum of 21.1°C and average of about 20.4°C (Table 2).

*Size, growth, and calcification*

 Shell size, measured by both height and hinge length, was significantly different between days 1 and 3 and between treatments (Figures 2 and 3). The ANOVA was significant for a difference in shell hinge length across treatments (p=1.302e-6) and between days (p=0.000903). Based on Tukey’s HSD, hinge length was significantly different in every pairwise comparison between treatments (Table 3). Results were similar for shell height, except the pairwise comparison of 400 and 700 µatm was not significant (p>0.05, Table 3). Daily growth rate for both shell hinge length and height were not significantly different between treatments (data not shown).

 Larval calcification was significantly different between treatments for days 1 and 3 post-fertilization. On day 1 post-fertilization, calcification was greater at the highest pCO2 of 1000 µatm (z-value=2.084, p=0.0372, Figure 4). On day 3, the trend had switched and the larvae at 1000 µatm were significantly less calcified than the other two treatments, which had similar numbers of fully calcified larvae (z-value=-3.203, p=0.00136, Figure 5).

*Gene expression*

 Genes were expressed…somehow….stay tuned!

DISCUSSION

*C. gigas* larvae are affected by OA in early development – they are smaller and are less calcified at elevated pCO2 (however, 700 µatm is not as bad as 1000). A number of other people have seen similar results in their studies of oyster larval development and here is what they found…

Our gene expression assays also showed these differences which are really really cool

What does it mean?

Stress affects energy budget, less able to grow, calcify (see Stumpp et al. 2011)

Decreased energy budget from OA can lead to decreased ability to feed and grow, which may mean that there are intrinsic negative effects of OA that the larvae are unable to overcome

OA-mediated selection at this stage of development could have significant effects on later stages and then at the population and ecosystem levels

Broadcast spawners have an advantage, but their offspring need to have the plasticity necessary to grow and survive in OA conditions in order for natural selection to work on future generations

Most importantly, OA is not just a problem of the future. It is already happening, especially in nearshore ecosystems