The Effects of Ocean Acidification on Multiple Life History Stages of the Pacific Oyster, *Crassostrea gigas*: Implications for Physiological Trade-offs

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Abstract

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# Chapter I: Elevated pCO2 causes developmental delay in early larval Pacific oysters, *Crassostrea gigas*

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## Abstract

Increasing atmospheric CO2 equilibrates with surface seawater, elevating the concentration of aqueous hydrogen ions. This process, ocean acidification, is a future and contemporary concern for aquatic organisms, causing failures in Pacific oyster (*Crassostrea gigas*) aquaculture. This experiment determines the effect of elevated *p*CO2 on the early development of *C. gigas* larvae from a wild Pacific Northwest population. Adults were collected from Friday Harbor, Washington, USA (48°31’70N, 12°1’10W) and spawned in July 2011. Larvae were exposed to Ambient (400 μatm CO2), MidCO2 (700 μatm), or HighCO2 (1,000 μatm). After 24 h, a greater proportion of larvae in the HighCO2 treatment were calcified as compared to Ambient. This unexpected observation is attributed to increased metabolic rate coupled with sufficient energy resources. Oyster larvae raised at HighCO2 showed evidence of a developmental delay by 3 days post-fertilization, which resulted in smaller larvae that were less calcified.

## Introduction

Ocean acidification is expected to affect ecosystems at an accelerating pace over the next century (Caldeira and Wickett 2003; IPCC 2007). Seawater pH declines (acidifies) in association with the uptake of anthropogenic CO2 and resultant increased H+ ion concentration. Projected changes in atmospheric *p*CO2 may have significant consequences for natural populations ranging from physiological changes to broad-scale range shifts (Talmage and Gobler 2011; O’Donnell et al. 2009; Wong et al. 2011; Tomanek et al. 2011; Banks et al. 2010; Perry et al. 2005). Acidification of nearshore waters can occur via a variety of processes, including equilibration with elevated *p*CO2 in the atmosphere, upwelling events, terrestrial run-off, and respiration. The upper ocean acidification in the North Pacific is proportional to the anthropogenic increase in atmospheric CO2, enforcing that the present-day pH changes are outside the range of natural variability (Byrne et al. 2010a, b). In addition to atmospheric sources of CO2, oceanic upwelling and nearshore respiration further reduce the pH of water in which larvae develop (as low as pH 7.4 along the west coast of North America) and increasingly result in waters undersaturated with respect to aragonite (Feely et al. 2008, 2010). During the spring and summer off the US west coast, upwelling of waters rich in CO2 and respiration from nearshore biological activity can cause under saturation of nearshore waters (Feely et al. 2008, 2010; Fassbender et al. 2011). These contemporary processes occur in the same area where planktonic invertebrate larvae congregate. As CO2 emissions continue to equilibrate with ocean surface water, these habitats that already experience low pH could see further and more sustained increases in *p*CO2.

Numerous studies have examined developmental consequences of ocean acidification on marine bivalve larvae. Exposure to low-pH water early in development caused decreased mid-stage growth and survival in *C. gigas* (Barton et al. 2012). *C. gigas’s* congener, *Crassostrea virginica*, grew more slowly and incorporated less CaCO3 into their shells at elevated *p*CO2 when compared to controls (Miller et al. 2009). Similarly, ocean acidification conditions decreased both shell integrity and tissue mass in larval mussels, *Mytilus californianus* (Gaylord et al. 2011). Larval Sydney rock oysters (*Saccostrea glomerata*) demonstrated reduced survival and slower growth and development when reared under conditions simulating future oceanic *p*CO2 (Watson et al. 2009). Both clam (*Mercenaria mercenaria*) and scallop larvae (*Argopectens irrandians*) were impacted by elevated *p*CO2 in their metamorphosis, growth, and lipid synthesis (Talmage and Gobler 2011). The effects of ocean acidification have been studied on populations of *C. gigas* from Japan (Kurihara et al. 2007), Australia (Parker et al. 2010, 2012), and Europe (Gazeau et al. 2011), but few studies to date look at these effects on populations of *C. gigas* from the United States. Due to differences in experimental design, it is difficult to directly compare the three aforementioned studies, but overall *C. gigas* larvae are smaller when raised at elevated *p*CO2 (Kurihara et al. 2007; Parker et al. 2010; Gazeau et al. 2011), demonstrate a developmental delay (Kurihara et al. 2007), and have morphological and shell deformities (Kurihara et al. 2007; Parker et al. 2010; Gazeau et al. 2011).

Pacific oyster larvae are planktotrophs, spending an extended period of one to three weeks in the plankton, where they undergo a variety of important morphological and physiological changes (Strathmann 1985). These developmental changes are frequently associated with environmental cues (Bonar et al. 1990), and their successful completion is necessary for larval metamorphosis into a settled juvenile oyster. Organismal responses to ocean acidification vary among and within taxa suggesting that ecological and evolutionary history may influence responses to ocean acidification. Thus, empirical studies are needed to understand the mechanistic responses of species to a specific environmental stress and how the stress corresponds to the species’ or population’s original ecological niche.

One of the primary means by which marine organisms are directly influenced by ocean acidification is through relative concentrations of H+ and associated decreased availability of CO3 2-. These changes in water chemistry impact calcifying organisms as they rely on CO32- to form and maintain carbonate-based structures (Beniash et al. 2010; Thomsen and Melzner 2010), while greater H+ concentration can cause acidosis of body fluids. Acidosis can result in dissolution of calcium carbonate structures, reducing shell thickness and releasing ions into the hemolymph. Many adult aquatic invertebrates can make use of dissolved calcified structures or possibly actively dissolve their shell, to make HCO3- more available as a buffer against internal acidosis. Excess HCO3- for buffering can also be acquired from the aquatic environment. This phenomenon has been observed in Dungeness crabs, *Cancer magister* (Pane and Barry 2007); blue crabs, *Callinectes sapidus* (Henry et al. 1981); limpets, *Patella vulgata* (Marchant et al. 2010); and urchins, *Psammechinus miliaris* (Miles et al. 2007); however, internal acidosis was not successfully avoided in oysters, *C. gigas* (Lannig et al. 2010). It is not clear to what degree larvae can utilize this mechanism to maintain homeostasis under elevated *p*CO2 conditions, but some invertebrates that inhabit naturally CO2-rich environments are able to reproduce and the larvae settle without apparent adverse effects (Thomsen and Melzner 2010).

Sustained environmental change, such as ocean acidification, can negatively affect both the ecosystem and economy. Shellfish, including oysters, provide important ecosystem services such as improved water quality and benthic-pelagic coupling through the filtration of large volumes of water, release of feces to the benthos, and creation of habitat via reef formation (Coen and Luckenbach 2000). In addition to their ecological roles, mollusks are economically important to many coastal communities worldwide. In 2008, mollusks comprised 64.1 % (or 13.1 million tons) of worldwide aquaculture production, with oysters accounting for 31.8 % of the total production (FAO 2010). The global economic cost of ocean acidification to the mollusk fishery is unclear but has been estimated to increase with rising atmospheric CO2 levels and terrestrial sources of acidification (Narita et al. 2012). Recently, in the Pacific Northwest of the US, concern has heightened over the already apparent effects of corrosive, acidified water on both natural and hatchery production of *C. gigas* larvae (Elston et al. 2008; Feely et al. 2010; Barton et al. 2012). Hatchery water supply comes from adjacent natural bays, and when upwelling events occur, the water that enters the hatchery can reach *p*CO2 near 1,000 μatm (S. Alin, unpublished data; B. Eudeline, pers. comm.). These upwelling events have been linked to mortality episodes in the hatchery, perhaps due to a combination of acidic water and pathogens associated with the water masses (Elston et al. 2008). Acidification events are projected to become more frequent and sustained as atmospheric *p*CO2 continues to rise.

This study characterized the effects of two elevated levels of *p*CO2 on size, calcification, and development during early larval stages of the Pacific oyster, *Crassostrea gigas*. Oyster larvae were raised in two elevated levels of *p*CO2 (700 and 1,000 μatm) and ambient (400 μatm) seawater through 72 h following fertilization. The chemistry scenarios simulated in this study are based on projections for the coming century, but these values of low pH and Ω are already occurring with increasing frequency in nearshore upwelling systems off the US West coast (Feely et al. 2010; Hauri et al. 2009).

## Materials and methods

### Seawater chemistry manipulation

Experimental conditions were maintained using a flowthrough seawater system in Friday Harbor, Washington, USA. Water entering the system was filtered (to 0.2-μm), UV-sterilized, and CO2-depleted using membrane contactors (Membrana, Charlotte, North Carolina, USA) under partial vacuum. Three experimental treatments were chosen to correspond with dissolved CO2 levels of 400, 700, or 1,000 ppm in the atmosphere. These three treatments will be referred to throughout the manuscript as Ambient, MidCO2, and HighCO2. Set-point pH levels were determined with the program CO2SYS (Robbins et al. 2010) using an average total alkalinity of 2,060 μmol kg-1 based on total alkalinity measurements taken the week prior to the experimental trial.

Larval *C. gigas* were held in 3-L microcosms within a large reservoir filled with the respective treatment water. Ambient air stripped of CO2 by a CO2 adsorption unit (Twin Tower Engineering, Broomfield, Colorado, USA) was used to aerate the seawater within the reservoirs through a Venturi injector into the larger reservoir of treatment water. This replaced oxygen lost through the degassing process. Reservoir pH was continuously monitored by a Durafet III pH probe (Honeywell, Morristown, New Jersey, USA). When the probe registered that the treatment’s pH strayed from its set point, a solenoid would open or close to allow more or less pure CO2 (Praxair, Danbury, Connecticut, USA) to be injected via the Venturi. The Durafet probe information was fed into a Honeywell UDA2182 pH controller, which also controlled the solenoids.

Seawater was pumped from the reservoir into larval microcosms through irrigation drippers (DIG Industries, Sun Valley, California, USA) at a rate of 1.9-L h-1. An outflow tube at the top of the microcosms fitted with 35-μm mesh allowed water to exit the microcosms while retaining larvae. All systems were equilibrated to the correct treatment level 48 h prior to the start of the experiment. Water temperature was held at 20.4 ± 0.4°C.

### Oysters

Ten female and four male adult *C. gigas* were collected from Argyle Creek in Friday Harbor, Washington, in July 2011. Oysters were strip-spawned into Ambient seawater with eggs and sperm pooled separately (day 0). Pooled eggs (approximately 2 million) were divided equally into 18 7.5-cm diameter containers. Sperm was diluted (so as to approximate a 1:1 sperm/egg ratio) in Ambient seawater and added to each container of eggs. After the addition of sperm, the eggs were gently agitated and incubated for 15 min to allow for fertilization.

Six containers of fertilized eggs were transferred to microcosms containing one of three treatment conditions. Initial densities post-hatching were approximately 1 larva mL-1. On days 1 and 3 post-fertilization, larvae were randomly sampled to determine survival, size, developmental stage, and presence or absence of calcification. For each microcosm sampled, larvae were filtered onto 35-μm mesh screens and washed with the appropriate seawater. Approximately 100 larvae were removed for each sample, relaxed with 7.5 % MgCl2, and fixed in 4% paraformaldehyde buffered in filtered seawater. The remaining larvae were returned to cleaned microcosms filled with new seawater. Larvae were fed *Dunaliellia* sp. and *Isochrysis* sp. at concentrations of 30,000 cells mL-1 each (concentrations for optimal larval growth) on day 2. During feeding, water flow was turned off in microcosms for 2 h. All microcosms were cleaned at each sampling event.

Larvae were examined using light microscopy to determine survival, size, developmental stage, and shell presence/absence. Survival was determined at 20–40x: larvae were counted as dead if there was a complete absence of ciliary movement. Larval hinge length and shell height were measured at 10x magnification with a Nikon Eclipse E600 and NIS Elements Basic Research software (Nikon, Tokyo, Japan). Larval developmental stage and shell presence were determined at 20x magnification using an inverted microscope and double polarized light. Larvae were scored as calcified on day 1 post-fertilization if calcified shell was observed at the hinge (Fig. I.1a). On day 3 post-fertilization, larvae were classified as fully calcified if polarized light produced a ‘‘Maltese cross’’ in the larval shell (Fig. I.1b; LaBarbera 1974).

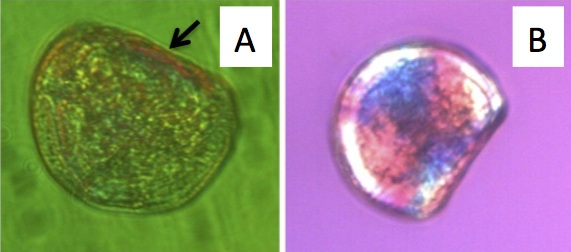


Figure I.1. D-hinge larvae under polarized light portraying calcification at the hinge without a Maltese cross in the shell (a) and full calcification as evidenced by the Maltese cross (b).

### Carbonate chemistry

Salinity was measured with a conductivity meter (Hach sensION5; Loveland, Colorado, USA), and temperature was measured using a Fluke 1523 thermometer (Fluke, Everett, Washington, USA). Seawater pH entering the microcosms was measured daily using the spectrophotometric technique outlined in SOP 6b by Dickson et al. (2007) to confirm pH measurements from the Durafet probe. When any discrepancies were observed, the Durafet probe was recalibrated. Seawater pH measurements were taken from two microcosms per treatment on days 0, 1, and 3. Final pH values reported here have been corrected for dye addition and temperature. Total alkalinity (AT) was measured following the open cell titration of SOP 3b (Dickson et al. 2007). Samples for AT were taken from incoming water and from two microcosms in each treatment on days 0, 2, and 3. CO2SYS (Robbins et al. 2010) was used to calculate calcium carbonate saturation state (Ω) of aragonite and calcite, carbonate ion concentration, and *p*CO2 with AT and pH as inputs using the following constants: Lueker et al. (2000) for CO2 constants; Dickson (1990) for KHSO4, total scale (mol kg-1 SW) for pH scale; and Wanninkhof (1992) for air–sea flux.

### Statistics

Differences in larval size and mortality across treatments were examined using a two-way ANOVA with fixed effects of treatment and day followed by Tukey’s Honestly Significant Difference test (Tukey’s HSD). A one-way ANOVA was also used to test for differences in larval size among treatments using the combined fixed factor of daytreatment. Larval calcification and developmental stage were compared among treatments using a generalized linear model (GLM). Binomial error distributions were used for GLM analyses. The occurrence of a developmental delay was assessed by fitting the regression of shell height on hinge length to a linear model and testing for differences

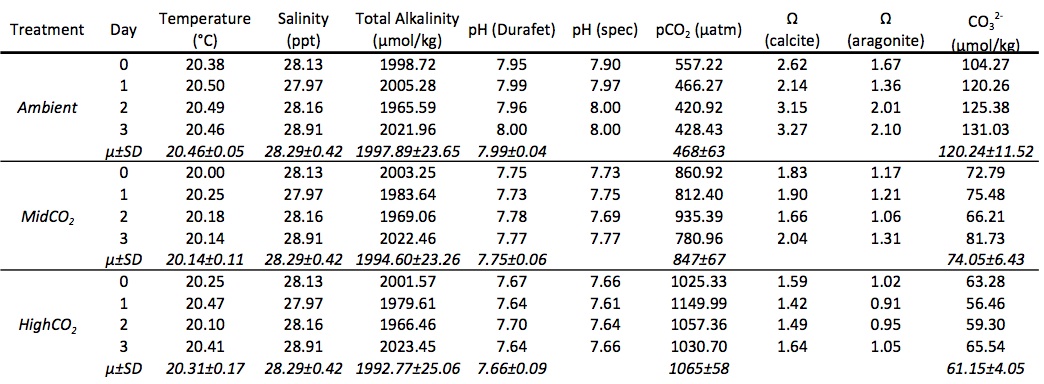
in the slopes of these lines across treatments. Developmental delay would be demonstrated if the larvae maintained the same allometry across treatments (the slopes of the lines were the same) but were different in size. At least two replicates within treatments and time points were used for all statistical analyses. All analyses were performed in R (R Development Core Team 2011).

## Results

### Carbonate chemistry

Throughout the experiment, seawater pH differed across treatments and AT varied slightly but to the same degree across treatments (Table I.1). Mean seawater pH was consistent within but varied among treatments (Fig. I.2). Mean pH (±standard deviation), as measured by the Durafet pH probes (Fig. I.2), was 7.99 ± 0.04 in the Ambient treatment, 7.75 ± 0.06 in the MidCO2 treatment, and 7.66 ± 0.09 in the HighCO2 treatment. Aragonite and calcite saturation states were >1.0 for the duration of the experiment, except in the HighCO2 treatment on days 1 and 2 (Table I.1). Carbonate ion concentration was lowest in the HighCO2 treatment (average ± SD of 61.15 ± 4.05 μmol kg-1 seawater, N = 4, Table I.1), intermediate in MidCO2 (74.05 ± 6.43 μmol kg-1, N = 4), and highest in the Ambient treatment (120.24 ± 11.52 μmol kg-1, N = 4). Partial pressure of CO2 in the seawater averaged 468 ± 63 μatm in the Ambient treatment, 847 ± 67 μatm in the MidCO2 treatment, and 1,065 ± 58 μatm in the HighCO2 treatment.

Table I.1. Salinity, total alkalinity (AT), and spectrophotometric pH are point measurements taken each day. Partial pressure CO2, Ω, and CO32- were calculated from spectrophotometric pH and AT. Mean and standard deviation (μ±SD) for the following parameters are given for all 3 days: temperature, salinity, AT, pH, *p*CO2, and CO32-.



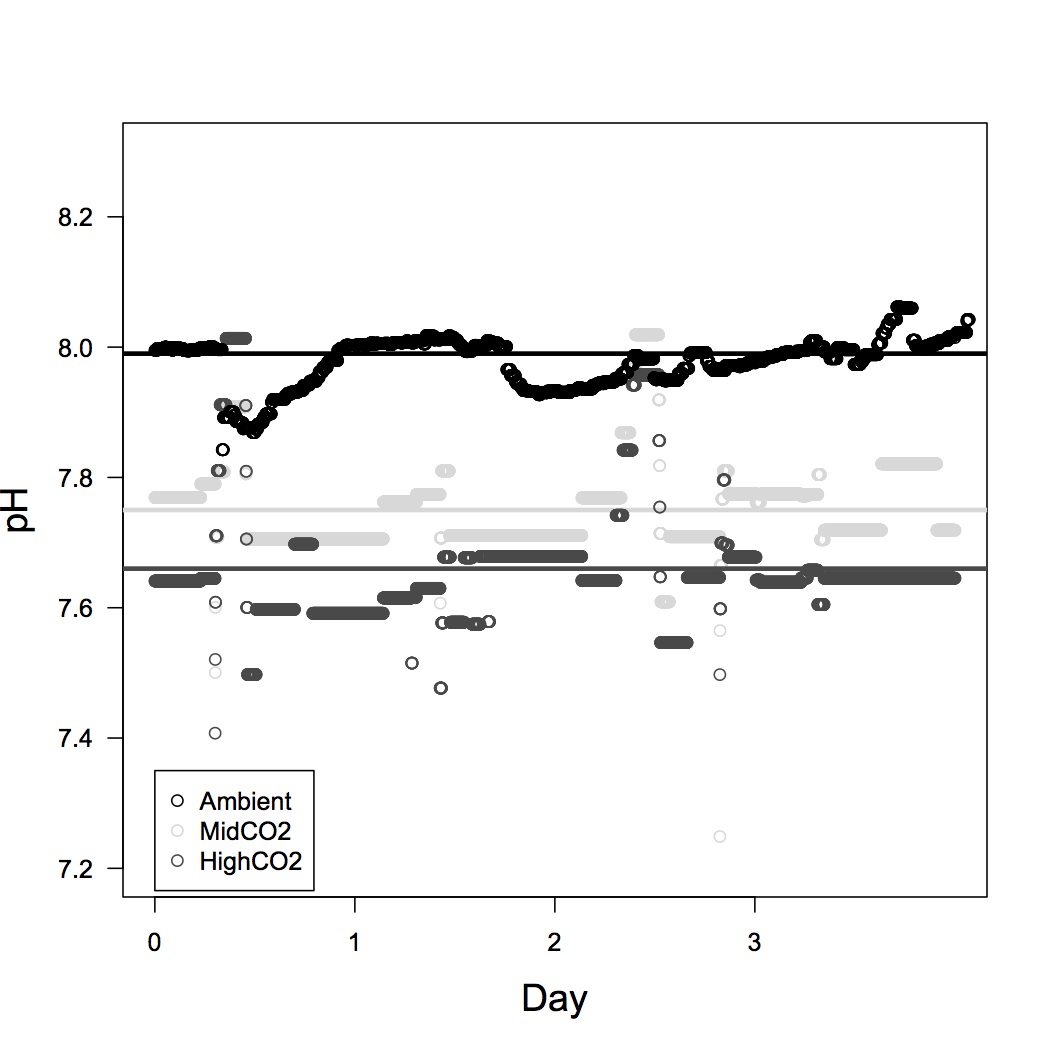


Figure I.2. Profiles of pH measurements in the three different treatments – Ambient (black), MidCO2 (light gray), and HighCO2 (dark gray). Average pH for the experiment for each treatment is represented by solid lines. The DuraFET probes recorded pH measurements every minute.

### Size, development, and calcification

Survival was near 100 % in all treatments on day 1 (Ambient = 99.0 %, MidCO2 and HighCO2 = 99.7 %). On day 3, survival was 92.9 % in the Ambient treatment

and was approximately 88.6 % in the MidCO2 and 85.6 % in the HighCO2 treatment. Mortality was similar across treatments (F = 0.59, P > 0.05) but different across days (F = 17.7, P < 0.05).

On day 1, a slightly greater proportion (0.977) of larvae at HighCO2 were at the D-hinge stage (compared with those that were still trocophores), but this difference was not significant (z value = 1.016, P = 0.310; data not shown). The proportion of larvae at the D-hinge stage on day 1 in the Ambient treatment was 0.875 and in MidCO2 was 0.833. Amount of larvae with shell was significantly different among treatments for days 1 and 3 post-fertilization. Following 24 h of treatment (day 1), the proportion of larvae with shell present was inversely proportional to *p*CO2 level with the greatest number of larvae with shell in the HighCO2 treatment (z value = 2.084, P = 0.0372, Fig. I.3). On day 3, fewer larvae at HighCO2 conditions had full shell compared with the other two treatments (z value = -3.203, P = 0.00136).

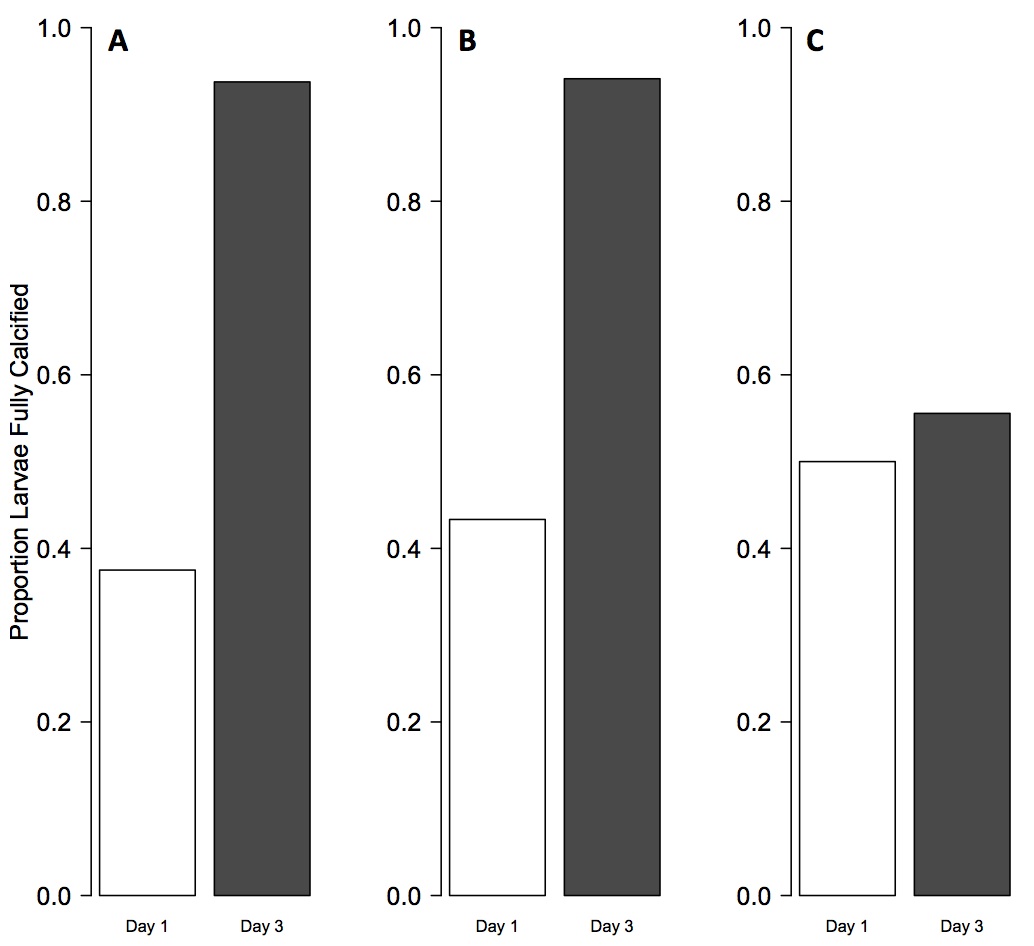
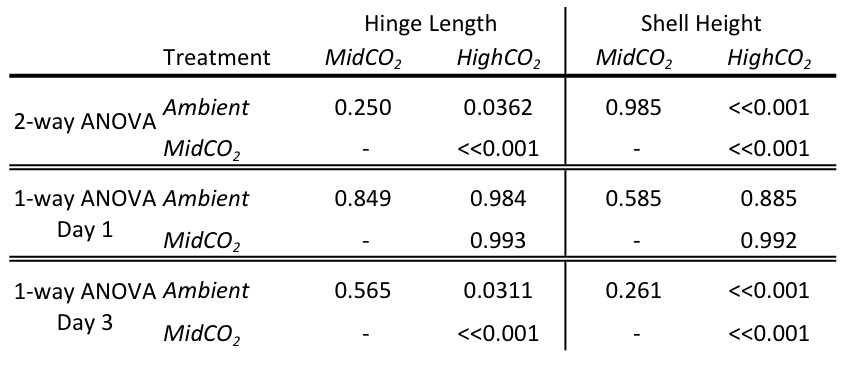


Figure I.3. Proportion of calcified larvae at different *p*CO2 treatments. Bars represent calcification on day 1 (white) and day 3 (gray). Proportion of larvae calcified are provided from the Ambient treatment (panel A), MidCO2 treatment (panel B), and HighCO2 treatment (panel C). There is a significant difference in calcification among treatments, with the highest proportion of larvae calcified at HighCO2 on day 1 and the fewest larvae calcified in HighCO2 on day 3.

Larval size (shell height and hinge length) was similar across experimental treatments after 24 h; however, by day 3, larvae grew significantly larger (height and length) in the Ambient and MidCO2 as compared to the HighCO2 treatment (Table I.2, Figs. I.4, I.5). Between days 1 and 3, larvae increased in size under Ambient conditions (shell height, P < 1e-7) and MidCO2 conditions (shell height and hinge length, P < 1e-7 and P = 7.4e-6, respectively; Figs. I.4, I.5) but did not significantly increase in size under HighCO2 conditions. By day 3, all larvae observed across treatments were at the D-hinge stage. The slope of the linear regression through shell height versus hinge length for the larvae raised at Ambient *p*CO2 was 0.6459 (Fig. I.6), which was not significantly different from the slope of the regression line through the MidCO2 data (0.8583, P > 0.05) or from the line through the HighCO2 size data (0.3625, P > 0.10).

Table I.2. Results from post hoc Tukey’s HSD following ANOVA for comparisons of hinge length and shell height among treatments.



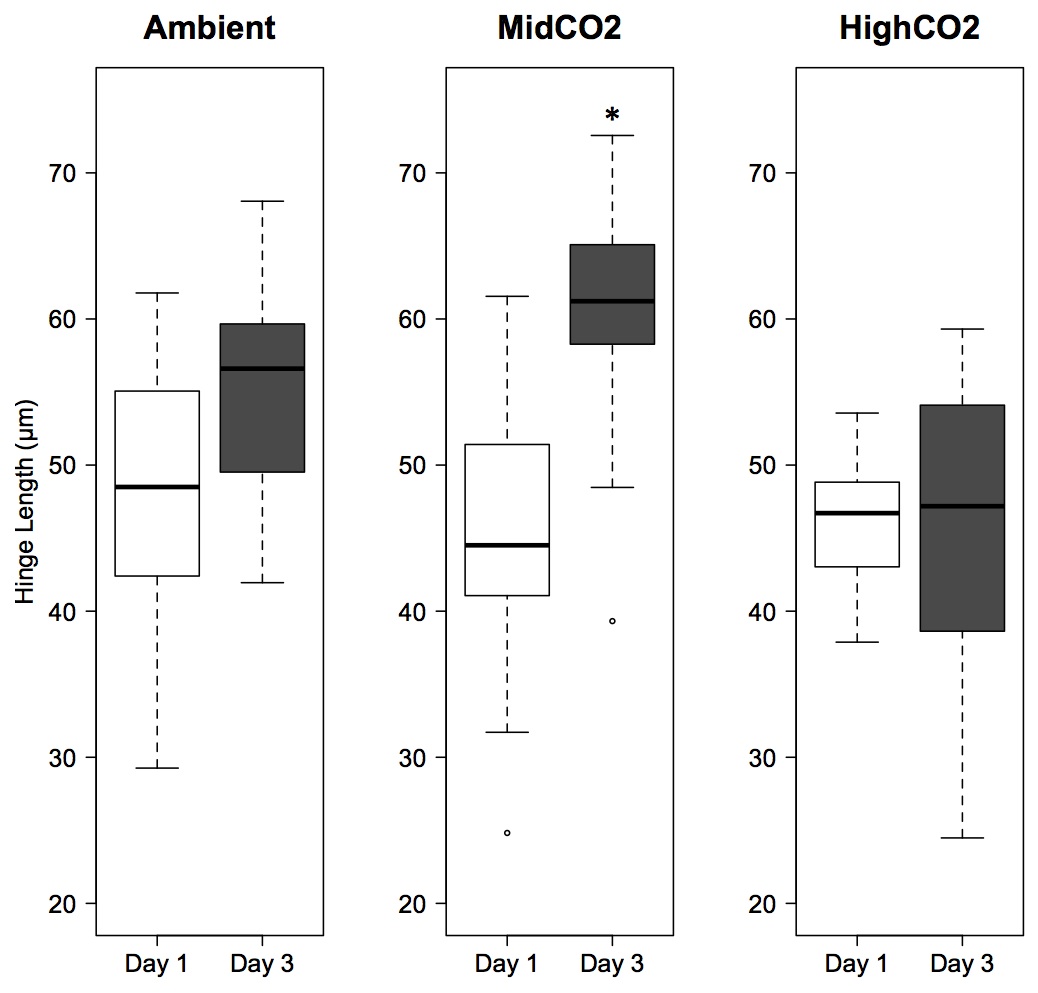


Figure I.4. Larval hinge length on day 1 (white boxplots) and day 3 (gray boxplots). Results are shown for the Ambient treatment, MidCO2 treatment, and HighCO2 treatment. Boxplots contain the middle 50% of the data and dashed lines encompass the data with 1.5x the spread of the middle 50%. Open circles represent outliers. Horizontal black bars indicate median values. An asterisk indicates significant differences within a treatment. On day 3, larvae in the HighCO2 treatment were significantly smaller than those in the other two treatments (P < 0.05).

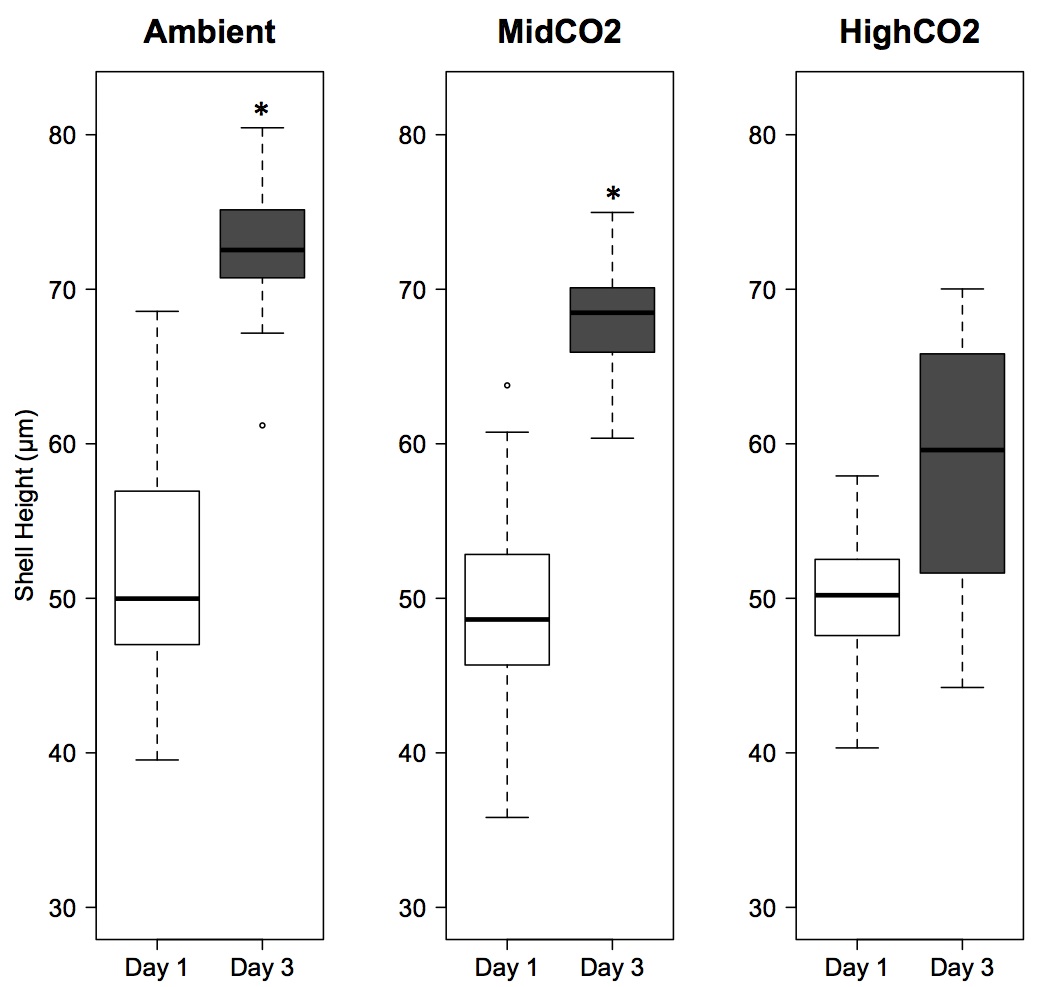


Figure I.5. Larval shell height on day 1 (white boxplots) and day 3 (gray boxplots). Results are shown for the Ambient treatment, MidCO2 treatment, and HighCO2 treatment. Boxplots contain the middle 50% of the data and dashed lines encompass the data with 1.5x the spread of the middle 50%. Open circles represent outliers. Horizontal black bars indicate median values. An asterisk indicates significant differences within a treatment. On day 3, shell height was reduced in larvae at HighCO2 relative to those raised at Ambient and at MidCO2 (P < 0.01),

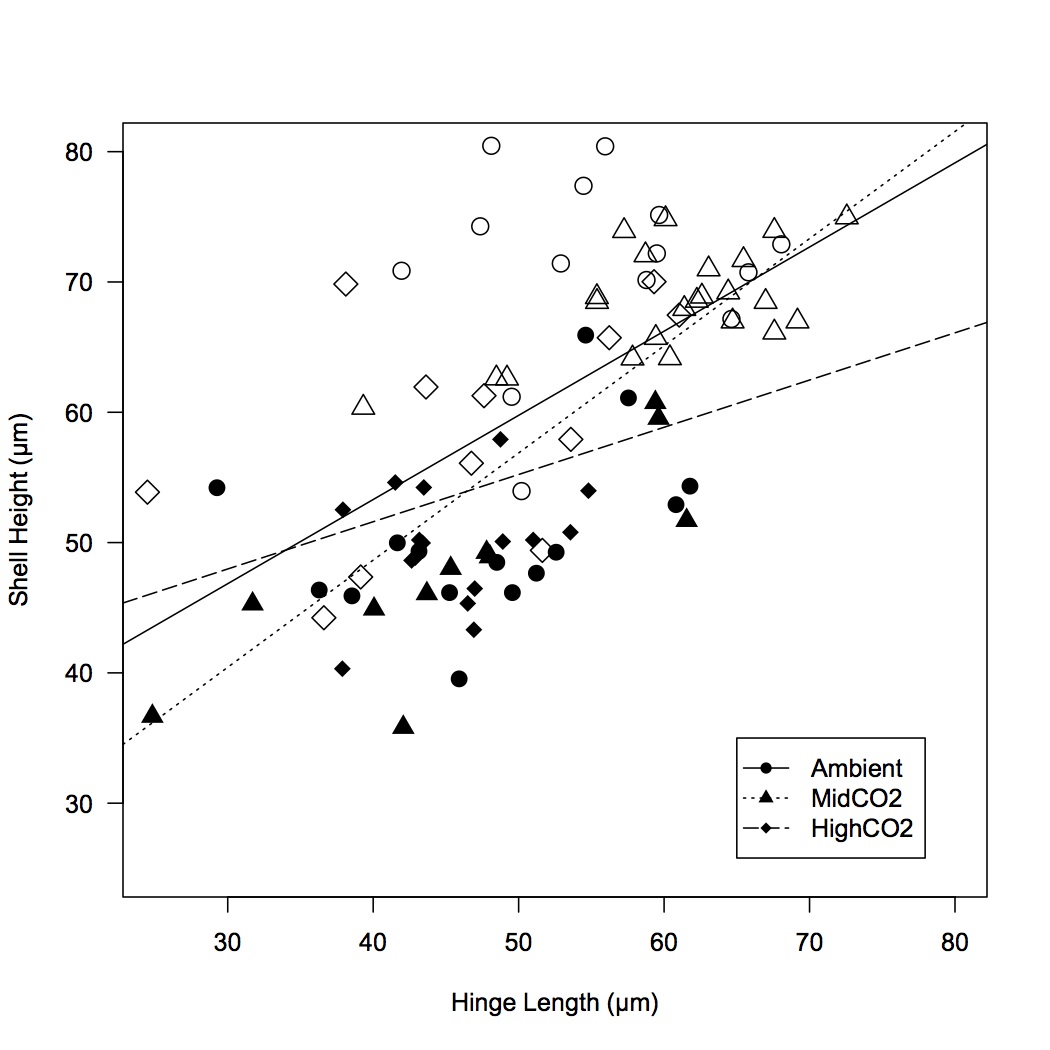


Figure I.6. Regression of larval shell height on hinge length by treatment and day. Data from larvae raised under Ambient *p*CO2 conditions are represented by circles, MidCO2 are triangles, and HighCO2 are diamonds. Size data from day 1 are in black and day 3 are in white. The solid line is the regression line for Ambient data (intercept = 27.47, slope = 0.65), dotted for MidCO2 (intercept = 15.76, slope = 0.82), and dashed for HighCO2 (intercept = 37.10, slope = 0.36). The slopes of all lines are statistically the same (P > 0.05).

## Discussion

Oyster larvae raised at HighCO2 showed evidence of a developmental delay by 3 days post-fertilization, which caused them to be smaller and have less calcified material than controls. These results are consistent with other studies of *Crassostrea* spp. larvae in which elevated *p*CO2 resulted in decreased growth and shell mineralization (Kurihara et al. 2007; Miller et al. 2009). Kurihara et al. (2007) raised *C. gigas* to 48 h post-fertilization at an elevated *p*CO2 of about 2,268 μatm, much higher than *p*CO2 projected for the coming century, and observed a negative effect on calcification as early as 24 h post-fertilization. The authors also observed a developmental delay in reaching the D-hinge stage at 48 h post-fertilization (Kurihara et al. 2007). Since we did not measure growth or calcification in our larvae at 48 h post-fertilization, we are not able to draw direct comparisons with this time point, but we did observe a developmental delay by 72 h post-fertilization. Similarly, *C. virginica* larvae raised from 72 h post-fertilization through competency at different *p*CO2 grew more slowly at elevated *p*CO2 (560 and 800 latm) and biomineralized less CaCO3 than controls; however, *Crassostrea ariakensis* showed no effect of *p*CO2 treatment (Miller et al. 2009). It is likely the observed differences between the studies are related to the much higher *p*CO2 level used by Kurihara et al. (2007) and species- and population-specific differences in acclimation to ocean acidification.

The developmental delay is evidenced by similar growth trajectories across treatments (Figs. I.3, I.6) coupled with the smaller size of larvae in the HighCO2 treatment. This suggests that change in size is not a direct effect of ocean acidification on shell growth and maintenance. In a study comparing faster growing hybrid *C. gigas* larvae to slower growing inbred larvae, slower growth was attributed to reduced feeding rate and differing allocation of internal energy reserves for metabolic processes (Pace et al. 2006). The stress of elevated *p*CO2 can induce similar physiological changes via effects on metabolic demands, resulting in a developmentally delayed phenotype (Stumpp et al. 2011a). It is difficult to detect developmental delay with complete confidence in studies that do not follow larvae through to settlement. In one such study, larval *Strongylocentrotus purpuratus* were exposed to elevated *p*CO2 throughout their larval period, and from this perspective, it was apparent that ocean acidification caused a delay in development, although at discrete time points, this delay could be interpreted as overall smaller size (Stumpp et al. 2011a). Developmental delay may give these species the energetic resources they need to survive stress and reach the later developmental stages of metamorphosis and settlement. However, a delay in development opens the possibility for a host of other complications for pelagic larvae, such as greater potential to be advected to unsuitable habitat (Strathmann 1985), greater chance of being exposed to predators (Underwood and Fairweather 1989), and an overall longer time in the water column where environmental conditions are variable and risky for a free-floating larva.

A greater percentage of the larvae in the HighCO2 treatment had shell present by 24 h post-fertilization compared with both Ambient and MidCO2. The impact of ocean acidification on larval invertebrates can change in direction and magnitude as the larvae switch from a non-feeding to a feeding stage. The larvae at HighCO2 were most likely able to maintain a normal developmental rate and calcified structures early in development because an increased metabolic rate would have been supported by sufficient maternal energy resources. In early development, *C. gigas* depend on maternal lipid reserves, but after 24 h in the plankton, the larvae become dependent upon external resources (Gallager et al. 1986). Environmental stress frequently instigates an elevated metabolic rate (Lannig et al. 2010; Stumpp et al. 2011a). During the non-feeding stage, larvae may have enough maternal resources to support their increased metabolic rate and sustain normal or even accelerated growth and development. In the non-feeding lecithotrophic larvae of the common sun star (*Crossaster papposus*), larvae at low pH developed and grew faster than those in ambient conditions (Dupont et al. 2010). Once the metabolic switch to external resources occurs, the larvae may not be able to get enough resources to sustain the increased metabolic rate as well as normal development. A similar trend is seen in larval purple sea urchins, *S. purpuratus*. Ocean acidification had a larger impact on the feeding larval stage of *S. purpuratus* than it did on the non-feeding stage (Stumpp et al. 2011a). Similarly to *C. gigas*, *S. purpuratus* demonstrated a developmental delay starting with the onset of feeding (Stumpp et al. 2011a). At the same time, routine metabolic rate increased in both elevated *p*CO2 and ambient treatment, but increased more at low pH (Stumpp et al. 2011a). The results from these studies suggest that the maintenance of homeostasis becomes more difficult under the energetic demands of ocean acidification stress; however, the physiological stress is realized as developmental delay, with associated phenotypes of less shell and smaller size, only when larvae are in a feeding stage. Reallocation of resources associated with invertebrate responses to ocean acidification has been shown to affect several processes, including as soft tissue growth (Gaylord et al. 2011; Beniash et al. 2010), scope for growth (Stumpp et al. 2011a), and shell integrity (Gaylord et al. 2011; Melzner et al. 2011).

Larval shell formation is closely linked to development and begins by 24 h post-fertilization. Numerous species experience decreased calcification when water is undersaturated with respect to aragonite (Kurihara et al. 2007; Miller et al. 2009; Crim et al. 2011; Gazeau et al. 2011; Byrne et al. 2010a, b), although some species are still able to form apparently normal calcified structures in undersaturated conditions (Dupont et al. 2010; Catarino et al. 2011; Yu et al. 2011). Early *C. gigas* larval shells are made of amorphous calcium carbonate and aragonite (Weiss et al. 2002), two of the more soluble forms of CaCO3 at low pH. Invertebrates are able to control calcification through amorphous mineral precursors and metabolites (Weiss 2011), thus decreasing the potential effects of a corrosive environment. On days 1 and 2, ΩAr was below 1.0, causing the seawater to be undersaturated with respect to aragonite. Calcification can become energetically costly due to scarcity of CO32- ions in the environment and disruption of ionic gradients of the calcifying compartment from changes in H+. If oyster larvae remove a fixed number of H+ from their calcifying fluid versus maintaining a fixed ratio of extracellular/intracellular H+, then their energy budget would be more taxed during environmental hypercapnia (Ries 2011). This added stress on the process of calcification could have contributed to the energy budget shifts that led to a developmental delay.

In this study, *C. gigas* tolerated the MidCO2 treatment through 3 days post-fertilization. The lack of negative effects on shell formation and maintenance in the larvae from the MidCO2 treatment suggests that a cut-off of ΩAr < 1.0 is significant in terms of the ability of this population to biomineralize at this time point in development. It is also possible that the high level of food available to the larvae modulated the impact of ocean acidification and could have led to an underestimation its effect in this treatment (Melzner et al. 2011). An elevated *p*CO2 of 750 μatm (ΩAr of about 1.0) had significant negative effects on hard clam (Mercenaria mercenaria) and bay scallop (Argopecten irradians) larvae after about 3 weeks of exposure as evidenced by decreased survival, development, growth, and lipid synthesis (Talmage and Gobler 2011). The comparable exposure conditions in our study (MidCO2) did not have a negative impact over the time period observed. Due to the similarities of carbonate chemistry parameters with Talmage and Gobler (2011), the differential responses observed across species are likely indicative of variability in species, developmental stage tolerances, or length of exposure. Longer experiments in larvae have demonstrated that the negative effects of ocean acidification persist and sometimes worsen in mussels *M. californianus* (Gaylord et al. 2011), urchins *S. purpuratus* (Stumpp et al. 2011a, b), abalone *Haliotis kamtschatkana* (Crim et al. 2011), and oysters, *Crassostrea ariakensis* and *C. virginica* (Miller et al. 2009). The compounding negative effects of ocean acidification during an experiment may be due to a species’ decreasing ability to tolerate a specific environmental stress as their metabolic needs change throughout development.

## Conclusions

In this study, we observed that an acute, 72-h exposure to the end-of-century projections of ocean acidification (HighCO2) has a negative impact on development in oyster larvae. Additionally, this study revealed that moderate changes in seawater chemistry (MidCO2, about 800 μatm, mean ΩAr > 1.19 ± 0.10) did not have an observed significant impact on larvae through 3 days post-fertilization. It appears the effects of an environmental stress, such as ocean acidification, vary depending on developmental and metabolic stage of *C. gigas* larvae. This is most likely directly associated with a switch in larval energy metabolism as the oysters develop from a non-feeding stage to a feeding stage. In order to effectively evaluate the possibility of acclimation or adaptation, future research should focus on characterizing larvae from diverse genotypes and locations as well as assessing any influences that might be experienced later in development.

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# Chapter II: Shotgun proteomics as a viable approach for biological discovery in the Pacific oyster

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## Abstract

Shotgun proteomics offers an efficient means to characterize proteins in a complex mixture, particularly when sufficient genomic resources are available. In order to assess the practical application of shotgun proteomics in the Pacific oyster (*Crassostrea gigas*), liquid chromatography coupled with tandem mass spectrometry was used to characterize the gill pro­teome. Using information from the recently published Pacific oyster genome, 1043 proteins were identified. Biological samples (*n* = 4) and corresponding technical replicates (three) were similar in both specific proteins identified and expression, as deter­mined by normalized spectral abundance factor. A majority of the proteins identified (703) were present in all biological sam­ples. Functional analysis of the protein repertoire illustrates that these proteins represent a wide range of biological processes, supporting the dynamic function of the gill. These insights are important for understanding environmental influences on the oyster, because the gill tissue acts as the interface between the oyster and its environment. *In silico* analysis indicated that this sequencing effort identified a large proportion of the complete gill proteome. Together, these data demonstrate that shotgun sequencing is a viable approach for biological discovery and will play an important role in future studies of oyster physiology.

## Introduction

Fluctuations in gene and protein expression can be sensitive and specific indicators of biological processes. At the tran­script level, several methodologies can be used to characterize expression from the gene-centric to systems level, including quantitative PCR (e.g. Griffitt *et al.*, 2006; Stumpp *et al.*, 2011), microarrays (e.g. Todgham and Hofmann, 2009; Lockwood *et al.*, 2010), and high-throughput sequencing (e.g. Polato *et al.*, 2011; Philipp *et al.*, 2012). The use of high-throughput sequencing technology has exponentially increased available genome and transcript information for taxa of ecological interest in recent years. While these results provide an accurate portrayal of changes at the molecular level, it is common that proteins have a more direct role in regulating physiological processes and responding to environ­mental change.

Historically, there have been several technical and analyti­cal challenges in characterizing global protein expression. One challenge is the need to have sufficient genomic resources available to describe proteins of interest. Specifically, protein sequencing generally produces short amino acid fragments that require a known corresponding gene for identification and annotation purposes. However, lack of genomic resources has not completely hampered proteomic studies. For example, researchers characterized the physiological response of *Gillichthys mirabilis* gill tissue exposed to osmotic and tem­perature stress using two-dimensional gel electrophoresis without sequencing proteins (Kültz and Somero, 1996). In another study, researchers used surface enhanced laser desorp­tion/ionization and identified 11 differentially expressed pro­teins in the gill tissue of *Oncorhynchus mykiss* exposed to zinc stress (Hogstrand *et al.*, 2002). Four proteins were identi­fied based on a combination of their physical properties (i.e. mass and binding) coupled with sequence similarity compari­sons with the limited number of teleost protein sequences in the SwissProt database (Hogstrand *et al.*, 2002).

The use of predicted protein sequences in closely related species can assist in annotation, but species-specific informa­tion will provide more accurate results. This is evident in a study on protein expression in pea (*Pisum sativum*) chloro­plasts, where concurrent complementary DNA sequencing facilitated the identification of a greater number of proteins compared with identification through homology searches with closely related model species (Bräutigam *et al.*, 2008). The reason that species-specific information provides such an advantage is due to how modern-day protein sequence iden­tification is executed. The vast majority of high-throughput mass spectrometry (MS) proteomics is accomplished by matching observed peptide fragmentation patterns (tandem mass spectra) to theoretical spectra. This is possible because peptides fragment in a predictable manner, allowing for theo­retical tandem mass spectra to be created *in silico* from a given protein sequence, stressing the importance of the data­base used. These correlation-based algorithms require the peptide mass (precursor mass) and peptide fragmentation (tandem mass spectrum). Even when employing databases of closely related species, a large number of viable tandem mass spectra of peptides might not be assigned accurately to a pro­tein, because a single amino acid mutation could significantly alter the peptide mass and resulting fragmentation pattern.

As technological advances have continued to increase the accessibility of whole transcriptomes and genomes to researchers, there is increasing interest in leveraging these data to carry out proteomic studies for both biological dis­covery and for better characterizing physiological responses to environmental change. Recently, the Pacific oyster (*Crassostrea gigas*) genome was sequenced (Zhang *et al.*, 2012). Given the availability of this resource, our objective was to quantify the level of information (and respective vari­ability) attainable in proteomic studies in oysters. There have been several prior studies examining protein expression in oysters using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) with samples separated by two-dimensional gel electrophoresis (2-DE) beforehand. In larval oysters, these proteomic techniques have identified spe­cific proteins that are responsible for early developmental changes in *C. gigas* (Huan *et al.*, 2012) and the larval *C. gigas* response to elevated partial pressures of CO2 (ocean acidifi­cation; Dineshram *et al.*, 2012). These methods have also been used to identify and sequence proteins that are differen­tially regulated in a range of physiological situations in adult oyster species. The discoveries include the following: the up-regulation of antioxidant proteins in response to ocean acidi­fication (Tomanek *et al.*, 2011); expression profiles denoting high-quality oocytes (Corporeau *et al.*, 2012); differing pro­teomic profiles between disease-resistant and disease-suscep­tible oysters (Simonian *et al.*, 2009); and specific responses to metal exposure (Thompson *et al.*, 2011, 2012a, 2012b; Liu and Wang, 2012) and acid sulfate run-off (Amaral *et al.*, 2012). These seminal studies in marine invertebrate pro­teomics demonstrate that analysis of global protein expres­sion is a powerful tool to facilitate our understanding of the molecular physiological response to environmental stressors.

An alternative to 2-DE approaches is to perform shotgun proteomics. Shotgun proteomics is the sequencing of a com­plex mixture of peptides using LC-MS/MS without prior separation (i.e. 2-DE). One of the main advantages of using 2-DE methods is that information on the physical properties of the proteins (mass and isoelectric point) can be used in the protein identification, whereas these empirical data are lost in the strictly tandem MS approaches. However, tandem MS has significantly greater data efficiency than gel-based approaches. The use of shotgun proteomics allows for a greater number of proteins to be identified rapidly from a single sample, providing a more complete metabolic picture of cellular function and physiology. This method has been demonstrated by Muralidharan *et al.* (2012), who used shot­gun proteomics to uncover *Saccostrea glomerata* haemocyte proteomic responses to metal contamination, and by Dheilly *et al.* (2012, 2013), who explored the proteomic response of coelomocytes to immune challenge in two urchin species.

In this study, we used shotgun proteomics to sequence the gill proteome of the Pacific oyster, *Crassostrea gigas*. The gill is the interface between bivalves and their environment, necessi­tating that the tissue performs a variety of physiological func­tions in response to the environment (e.g. David *et al.*, 2007; Wang *et al.*, 2010). The identification of proteins that are expressed in gill tissue supports the development of tools that can help to guide future research on the molecular physiology of molluscs faced with stresses such as climate change and dis­ease. The goal of this study was to determine the effectiveness of using a shotgun proteomics approach and to carry out func­tional characterization of proteins expressed in gill tissue.

## Methods

### Oysters

Pacific oysters (*C. gigas*, 11 months old) were collected in Shelton, WA, USA. Oysters were transferred to Friday Harbor Laboratories (Friday Harbor, WA, USA) into a flow-through system at 13°C for 6 weeks. Eight 4 L vessels contain­ing six oysters each were kept in a water bath with seawater flowing through at 57.5 ml min-1. Vessels were cleaned every other day with fresh-water and salt-water rinses. Oysters were fed Shellfish Diet 1800 (Reed Mariculture, Campbell, CA, USA). At the end of six weeks, gill tissue was removed from four oysters and immediately flash frozen in liquid nitrogen for proteomic analysis.

### Protein digestion and desalting

Gill tissue samples (50–100 mg) were homogenized in 50 mM NH4HCO3 (100 μl) using RNAse-free plastic pestles. Each homogenized gill sample was sonicated four times with a probe sonicator and stored on dry ice between sonications. After sonication, protein concentrations were measured using the Bradford assay, following the manufacturer’s protocol (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Urea (36 mg) was added to each sample (for a total concentration of 6 M) to denature and solubilize peptides. Next, 1.5 M Tris (pH 8.8; 6.6 μl) was added, followed by 200 mM (*tris*(2-car­boxyethyl)phosphine) (2.5 μl). Samples were incubated for 1 h at 37°C on a shaker. To alkylate the proteins, 200 mM iodoacetamide (20 μl) was added. Samples were then vor­texed, and incubated for 1 h at room temperature in the dark. To absorb excess iodoacetamide, 200 mM dithiolthreitol (20 μl) was added, followed by vortexing and incubation at room temperature for 1 h. A volume equal to approximately 100 μg was removed, and the remainder was discarded. Ammonium bicarbonate (200 μl of 25 mM) was added to dilute the urea, and then high-pressure liquid chromagogra­phy (HPLC) grade MeOH (50 μl) was added to each tube. Trypsin was solubilized in a trypsin dilution buffer (20 μl) to a concentration of 1 μg/μl (Promega, Madison, WI, USA), and 3 μl of this solution was added to each sample to digest the proteins enzymatically. The samples were incubated over­night at 37°C. The next day, dilute formic acid was added, and the samples were evaporated on the speed vac to near dryness. Samples were reconstituted in 200 μl of 5% acetoni­trile and 0.1% trifluoroacetic acid.

Samples were desalted by passage through a pre-prepared MacroSpin column, following the manufacturer’s specifica­tions (The Nest Group, Southborough, MA, USA). After desalting, the remaining solvent was evaporated using a speed vac.

### Liquid chromatography and tandem mass spectrometry

Mass spectrometry was performed at the University of Washington Proteomics Resource (Seattle, WA, USA). Samples were resuspended in 2% acetonitrile and 0.1% for­mic acid in water (100 μl). Samples were then vortexed to mix and spun down at 21130 × g for 10 min. The superna­tant was aliquoted to autosampler vials. Nano LC separation was performed with a nanoACUITY system (Waters, Milford, MA, USA) interfaced to an LTQ Orbitrap XL mass spectrom­eter (Thermo Scientific, San Jose, CA, USA). Peptides were trapped on a 100 μm i.d. × 20 mm long pre-column packed with 200 Å (5 μm) Magic C18 particles (C18AQ; Michrom, Auburn, CA, USA). For separation, a 75 μm i.d. × 250 mm long analytical column with a laser pulled emitter tip packed with 100 Å (5 μm) Magic C18 particles (C18Q; Michrom) was used and analysed in positive ion mode. For each LC-MS/ MS analysis, an estimated amount of 0.5 μg of peptides was loaded onto the pre-column at 2 μl min-1 in water/acetonitrile (98%/2%), with 0.1% (v/v) formic acid. Peptides were eluted using an acetonitrile gradient flowing at 240 nl min-1, using a mobile phase consisting of the following components: Solvent C (water, 0.1% formic acid) and Solvent D (acetoni­trile, 0.1% formic acid). The gradient programme was as fol­lows: 0–1 min, Solvent C (98%) and Solvent D (2%); 1 min, Solvent C (90%) and Solvent D (10%); 90 min, Solvent C (65%) and Solvent D (35%); 91–101 min, Solvent C (20%) and Solvent D (80%); and 102–120 min, Solvent C (98%) and Solvent D (2%). Peptide spectra were acquired by scans in the Orbitrap followed by the ion trap.

### Data acquisition

High-resolution full precursor ion scans were acquired at 60 000 resolution in the Orbitrap over 400–2000 *m*/*z* while six consecutive tandem mass spectra were acquired by colli­sion-induced dissociation in the linear ion trap (LTQ). The data-dependent ion threshold was set at 5000 counts for MS/ MS, and the maximum allowed ion accumulation times were 400 ms for full scans and 100 ms for MS/MS measurements. The number of ions accumulated was set to 1E6 for Orbitrap scans and 1E4 for linear ion trap MS/MS scans. An angioten­sin and neurotensin standard was run after every eight injec­tions. Each sample was injected in triplicate in a novel randomized order.

### Protein identification and data analysis

Peptide sequence and corresponding protein identification for all mass spectra was carried out using SEQUEST (Eng *et al.*, 1994) and the *C. gigas* proteome version 9 (Zhang *et al.*, 2012, http://dx.doi.org/10.5524/100030). A DECOY database was created by reversing the *C. gigas* proteome and adding it to the forward database. This was completed in order to determine false positive matches of peptide spectra matching, and yielded a false discovery rate of ~0.6%. Search parameters included trypsin as the assigned enzyme and a precursor mass accuracy of ±3 Da. SEQUEST results were analysed using PeptideProphet and ProteinProphet in order to evaluate peptide matches statistically and assign protein probabilities (Nesvizhskii *et al.*, 2003). Only pro­teins with a probability of ≥0.9 (estimated false discovery rate of 0.6%), a minimum of two unique peptide hits within a single replicate, and a minimum of four total tandem mass spectral assignments in the combined technical and biologi­cal replicates were used in further characterizations described below.

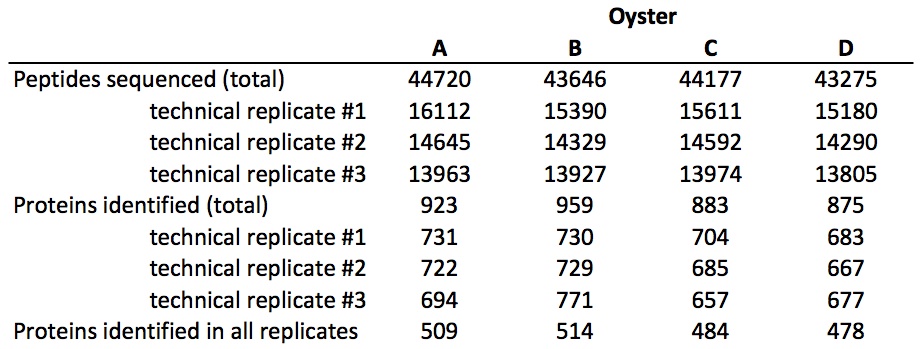
In order to annotate corresponding proteins, the *C. gigas* proteome (version 9) was compared with the UniProtKB/ Swiss-Prot database (www.uniprot.org) using Blastp with an e-value limit of 1E-10. Associated gene ontology (GO) terms were used to classify sequences based on biological process, as well as to categorize genes into parent categories (GO Slim). Enrichment analysis was used to identify over-represented biological processes in the gill proteome com­pared with the entire proteome [Database for Annotation, Visualization and Integrated Discovery (DAVID), version 6.7; Huang *et al.*, 2009a, 2009b, http://david.abcc.ncifcrf. gov/]. The results of the enrichment analysis were visualized in REViGO (Reduce and Visualize Gene Ontology; Supek *et al.*, 2011, http://revigo.irb.hr/). Normalized spectral abundance factor (NSAF; Florens *et al.*, 2006) was used to calculate expression for each protein in each oyster. Technical replicates were pooled by taking the sum of total independent tandem mass spectra for each protein (SpC). For each protein, SpC was divided by protein length (*L*). The NSAF is calculated from SpC/*L* divided by the sum of all SpC/*L* values for the proteins for a particular oyster. Comparisons of proteins identified across biological sam­ples were visualized using Venny (Oliveros, 2007).

The minimum number of peptides needed to be sequenced to optimize unique protein identifications was determined using an *in silico* approach. A list was constructed of all sequenced peptides and their matching protein identification. Redundancies were maintained in this list, so that if a certain peptide was sequenced multiple times it was included multiple times in the list. Randomized subsets of this list were generated using the sample function in R (R Development Core Team, 2009). The number of hypothetically sequenced peptides in these lists ranged from 500 to 70 000. A plot was generated to visualize the relationship between each sample size of randomly chosen peptides and the number of unique proteins identified.

## Results

A combined total of 175 818 tandem MS spectra were gener­ated across all four biological and three technical replicates using the Orbitrap mass spectrometer (Table II.1). Expression values were comparable between biological replicates, with *r*2 ranging from 0.800 to 0.889 (Supplementary Data II.1). A total of 54 521 unique peptides contributed to the identification of 2850 proteins, with a probability score threshold of 0.9 (Supplementary Data II.2). Of these proteins, 1043 had at least two unique peptide hits and four tandem mass spectra in the combined replicates. The mean coverage of proteins by sequenced amino acids was 13.3%. Protein identifications for each injection, including protein probability scores, num­ber of total and unique spectra, and peptide sequences, are provided in Supplementary Data II.3. The NSAF values for each protein are provided in Supplementary Data II.4.

Table II.1. Summary of the number of peptides sequenced and proteins identified for each oyster (labeled A-D)



For all biological samples, the number of proteins identi­fied in each technical replicate was consistent with minimal standard deviation (1.2–3.5%). In each biological replicate, the proteins were identified from between 43 275 and 44 720 sequenced peptides (standard deviation as a percentage of the mean ranged from 4.8 to 7.4%). For each oyster, 54–55% of the identified proteins were present in all three technical rep­licates. Using spectral counts as a proxy for relative expres­sion, protein expression levels were consistent across technical replicates (Fig. II.1).

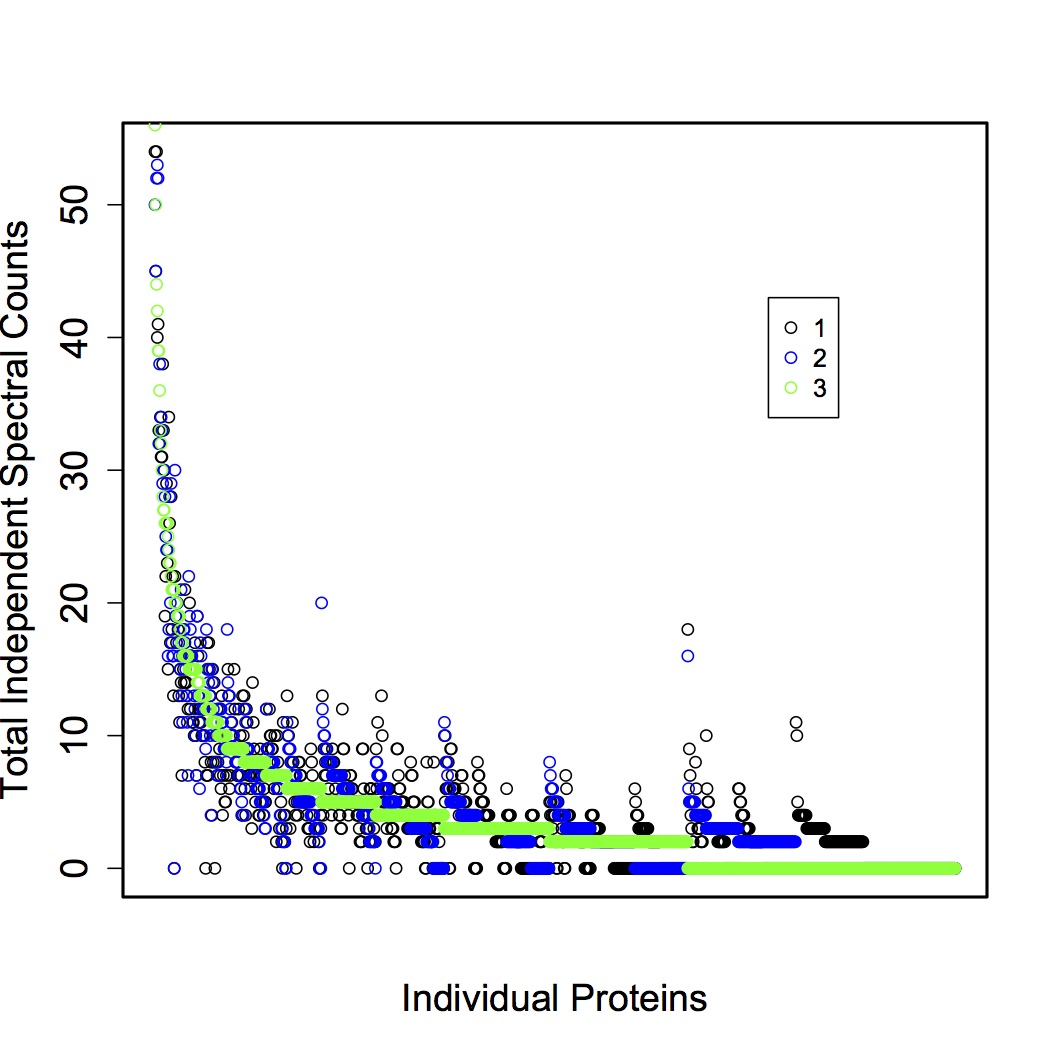


Figure II.1. Total independent spectral counts for three technical replicates for oyster A plotted for each protein (n = 1500). Similar patterns were observed for the other three oysters (data not shown).

The number of proteins identified in each oyster (after pooling technical replicates; see Methods) was 923, 959, 883, and 875 (Table II.1). Most proteins (*n* = 703) were identi­fied across all biological samples (Fig. II.2).

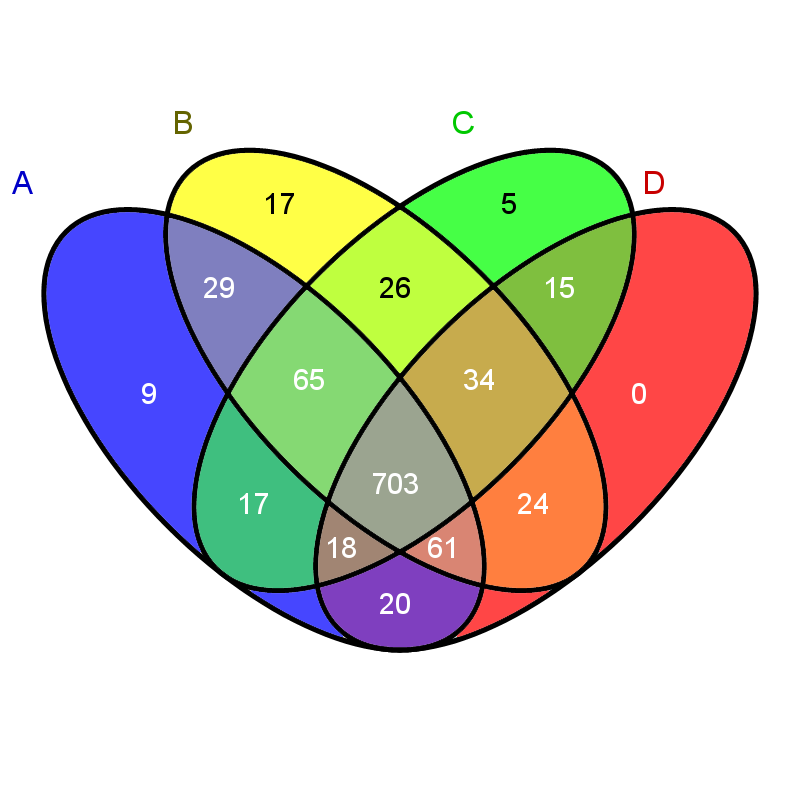
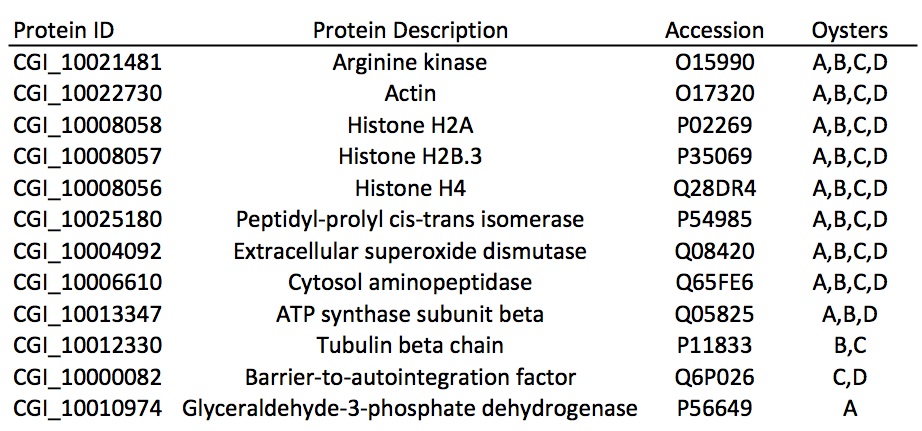


Figure II.2. Venn diagram of proteins identified among biological samples. Proteins identified in oyster A are in the blue ellipse, B in the yellow, C in green, and oyster D proteins are in the red ellipse.

In order to evaluate general protein expression and assess sample variability, the 10 most highly expressed proteins in each oyster were identified. There was not complete overlap in this group of proteins among the four oysters, so that a total of 12 proteins represent the most highly expressed for the entire dataset (Table II.2). The 12 most abundant proteins across the four oysters analysed represent core cell structure and function, such as nucleosome assembly, cytoskeleton structure, muscle components, turnover of intracellular pro­teins, and protection against oxidative stress. Eight of these 12 proteins (arginine kinase, actin, histone H2A, histone H2B.3, histone H4, peptidyl-prolyl *cis-trans* isomerase, extracellular superoxide dismutase, and cytosol aminopepti­dase) were identified in the top 10 most expressed proteins in all four oysters.

Table II.2. The 12 most abundant proteins in the gill proteome as determined by identifying the 10 most abundant proteins in each oyster.



Of the 1043 proteins expressed across all samples, 1033 were annotated using the UnitProt-KB/SwissProt database. Of the annotated proteins, 888 were associated with Gene Ontology classifications. A majority of proteins were associ­ated with the biological process of protein metabolism (*n* = 273), followed by cell organization and biogenesis (*n* = 201), and transport (*n* = 165) (Fig. II.3).

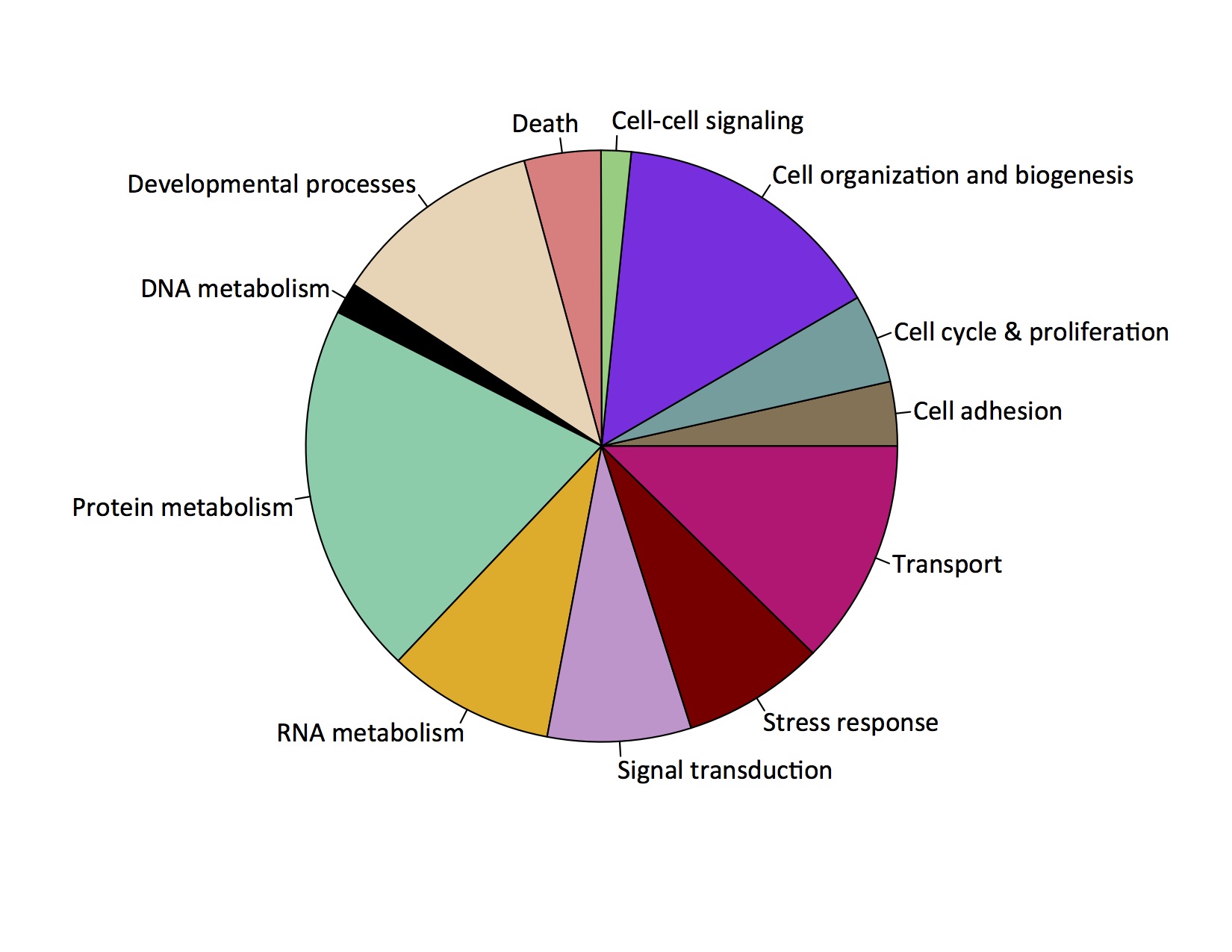


Figure II.3. Representation of biological processes corresponding to the proteins identified from oyster gill tissue.

Enrichment analysis was carried out to determine which biological processes were over-represented in gill tissue in comparison to the entire proteome. Several of the functional groups identified were associated with the abundant proteins involved in metabolism and transport, as well as structural processes (i.e. actin-filament and microtubule) and oxida­tion–reduction. The most significantly enriched biological process was generation of precursor metabolites and energy. Protein IDs (accession numbers starting with “CGI”) corre­sponding to the proteins that contributed to GO term enrich­ment are listed in Supplementary Data II.5.

The number of unique proteins identified with different numbers of sequenced peptides created an exponential curve (Fig. II.4). The plateau began around 30 000–40 000 sequenced peptides, with a total of 2400–2516 unique peptides identi­fied. New unique peptides were still identified in larger sam­ple sizes of peptides, but the return per sequenced peptide diminished.

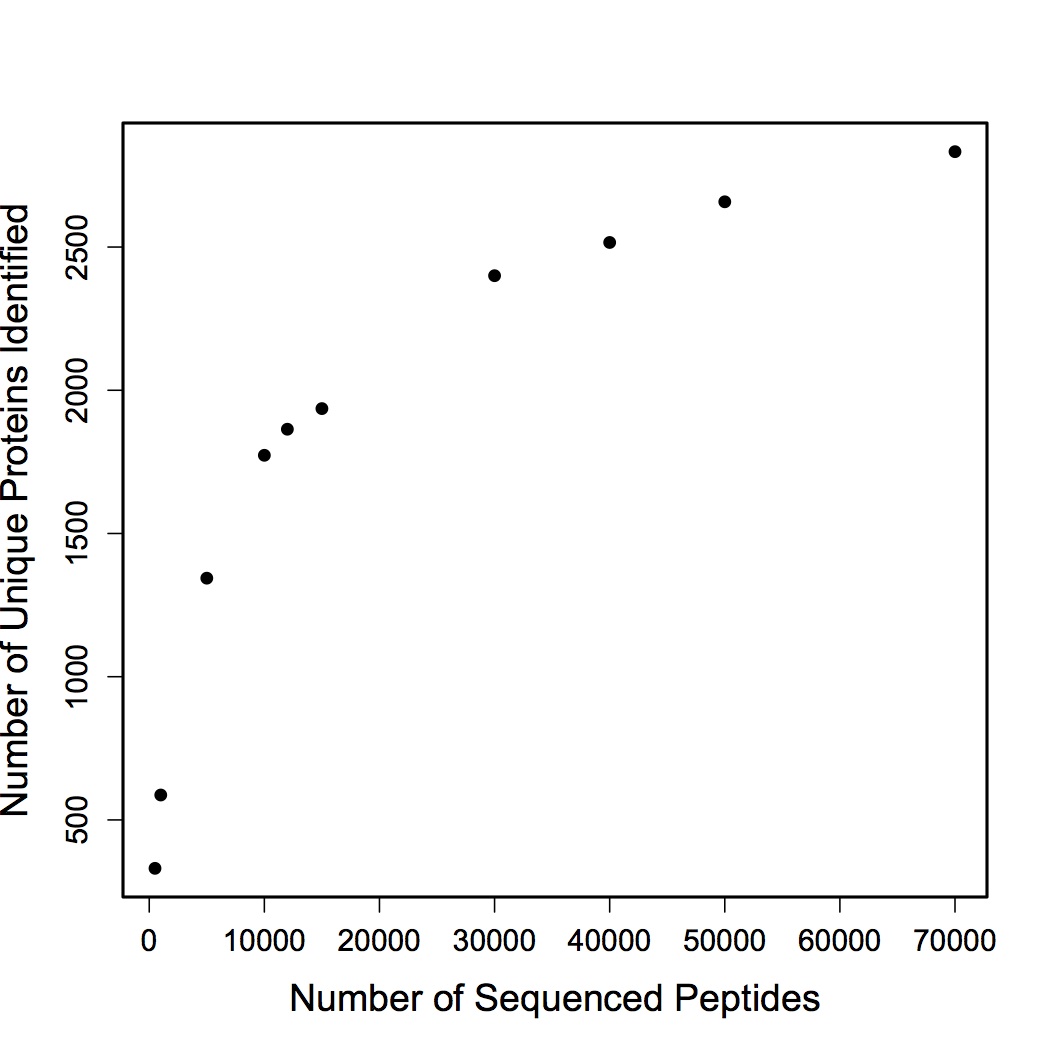


Figure II.4. Predicted number of unique proteins that would be identified based on a sequential increase in peptide sequences.

## Discussion

Technical and analytical challenges have resulted in limited focus on quantitative proteomics approaches in environmen­tal physiology. Given the recent technological advances in the proteomics field (Yates *et al.*, 2009) and release of the Pacific oyster genome (Zhang *et al.*, 2012), we set out to assess the practical use of quantitative proteomics in this model species. For all biological samples, a majority of the proteins identi­fied (54–55%) were present in all respective technical repli­cates. Relative expression across technical and biological replicates was also consistent (Fig. II.1, Supplementary Data II.1). However, there were some proteins not identified in all tech­nical replicates. Thus, proteins with limited expression might not be detected and/or expression levels might not be reflected accurately. It should be noted that the inclusion of proteins in our analysis is highly dependent on threshold selection. In the present study, a protein was included only if it had two unique spectral hits within a replicate and had four total spectra across the combined technical replicate data. If the threshold were adjusted to be more conservative (i.e. a greater total spectral count threshold), variability would be reduced. With a total spectral count threshold of five, 983 proteins are identified and 56–57% of the proteins are in all three techni­cal replicates; with a threshold of 10, 845 proteins are identi­fied and 61–63% of the proteins are in all technical replicates (data not shown).

The number of proteins identified and subsequently anno­tated can vary tremendously based on experimental design, target tissue, match thresholds, and genomic resources avail­able. In the present study, the majority of the proteins (703) were identified in all biological samples. Based on *in silico* analysis (Fig. II.4), we have sequenced a relatively complete proteome for oyster gill tissue. In a study of European white­fish, *Coregonus lavaretus*, proteomics on fish larvae yielded sequencing of peptides corresponding to 1500 proteins (Papakostas *et al.*, 2012). The similar number of protein identifications in whitefish compared with our study (1043) is likely to be associated with the tissue complexity. In the whitefish study, whole body tissue was examined. In a meta­proteomics study of marine microbes, 2273 distinct proteins were identified across 10 samples (Morris *et al.*, 2010). The large number of proteins identified by Morris *et al.* (2010) is evidence of the large number of organisms and ecological niches that were sampled in their study. Previous proteomics studies on Sydney rock oyster haemolymph have found rela­tively few proteins compared with the present study in gill tissue, with the number of identified proteins ranging from 49 to 514 (Simonian *et al.*, 2009; Thompson *et al.*, 2011, 2012a, 2012b; Muralidharan *et al.*, 2012). The identification of fewer proteins in haemolymph is probably because there are fewer cell types present in haemolymph compared with the gill.

In addition to assessing the feasibility of shotgun pro­teomics in the Pacific oyster, we were also able to provide a functional characterization of the gill proteome. Gene ontol­ogy characterization identified a majority of proteins associ­ated with protein metabolism, cell organization and biogenesis, and transport (Fig. II.3). These biological functions would be expected, because gill tissue is the primary interface between the oyster and the environment (water), where the tissue’s major functions include ion regulation, respiration, and sorting of food particles. The high number of proteins involved in these GO categories is not necessarily unique to gill tissue but is likely to reflect the multifunctional nature of a tissue that responds to variable environments.

Enrichment analysis was performed to identify which functional groups of proteins expressed in gill tissue were over-represented in comparison to the complete protein reper­toire. Several of the functional groups identified were associ­ated with the abundant proteins involved in metabolism and transport, as well as cellular structure. These enrichment analysis findings are consistent with a previous transcrip­tomic comparison between *C. gigas* gill tissue and other tis­sues, with genes predominantly expressed in the gill being involved in epithelia morphogenesis, cilia movement, and detoxification and defense (Dheilly *et al.*, 2011). Some of the cytoskeletal proteins identified in gill were tektin-3, microtu­bule-associated protein futsch, and actin. Tektin is part of cilia and flagellar microtubules and has been found to change expression in response to an elevated partial pressure of CO2 (Dineshram *et al.*, 2013), and has also been identified in Sydney rock oyster haemolymph (Thompson *et al.*, 2012b). Transport proteins included ATP synthases and v-type proton ATP synthase. ATP synthase is a good marker of environmen­tal stress in *C. gigas*, because its transcript expression is altered in response to hypoxia (David *et al.*, 2005) and pesti­cide exposure (Tanguy *et al.*, 2005). The most significantly enriched biological process was generation of precursor metabolites and energy. Many of the proteins that contrib­uted to the over-representation of this GO category in the gill tissue are involved in metabolic processes, such as 2-oxoglutarate dehydrogenase, dihydroplipoyllisin-residue acetyltransferase, glycogen phosphorylase, triose phosphate isomerase, and hexokinase. These enzymes are all involved in the breakdown of carbohydrates and other food inputs, and thus underline the important metabolic processes that occur in the gill.

Proteins involved in oxygen metabolism and reactive oxy­gen species defense were also enriched in gill tissue, providing further support for the importance of gill tissue in response to environmental change. Previous transcriptomics-based stud­ies of oysters support that the oxidative stress response plays an important role in the gill tissue (e.g. David *et al.*, 2007; Fleury and Huvet, 2012). Genes and proteins responding to production of reactive oxygen species increase in oysters in many instances of environmental stress, such as exposure to contaminants (e.g. David *et al.*, 2007; Muralidharan et al., 2012), as well as exposure to ocean acidification (Tomanek *et al.*, 2011) and temperature stress (Meistertzheim *et al.*, 2007). Specific proteins that contribute to reactive oxygen species defense are enzymes instrumental in the physiological response to oxidative stress, such as the antioxidants super­oxide dismutase, peroxiredoxin, and catalase.

The success of the shotgun sequencing effort was due in part to the recent publication of the *C. gigas* genome, emphasizing that the dissemination of genomic resources provides invaluable opportunities for advancement for the scientific community. The sharing of these large data sets, such as the genome and the gill proteome, will support further research into the effects of environmental changes on the oys­ter in terms of both acclimatization and adaptation. The char­acterization of the scope of acclimatization and adaptation are instrumental in understanding how the Pacific oyster, an ecologically and economically important species, can respond to climate change at the physiological and population levels. These research results demonstrate that shotgun sequencing of oyster gill tissue is a viable approach for biological discov­ery and that it will be likely to play an important role in future studies on oyster physiology.

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# Chapter III: From shell deposition to protein expression: An integrative assessment of ocean acidification impacts on a marine invertebrate

Submitted to Journal of Experimental Biology

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## Abstract

Ocean acidification as a result of increased anthropogenic CO2 emissions is occurring in marine and estuarine environments worldwide. The coastal ocean experiences additional daily and seasonal fluctuations in pH that attain and exceed projected end of century open ocean pH reductions. In order to assess the impact of ocean acidification on marine invertebrates, Pacific oysters (*Crassostrea gigas*) were exposed to one of four different *p*CO2 levels for four weeks: 400 µatm (pH 8.0), 800 µatm (pH 7.7), 1000 µatm (pH 7.6), or 2800 µatm (pH 7.3). Oysters in all four *p*CO2 environments deposited new shell during the experiment, but growth rate was not different among the treatments. However, micromechanical properties of the new shell were compromised by elevated *p*CO2. Elevated *p*CO2 affected neither whole body fatty acid composition nor mortality rate associated with acute heat shock. Shotgun proteomics revealed that several physiological pathways were significantly affected by ocean acidification, including antioxidant response, carbohydrate metabolism, and cell growth. Additionally, the proteomic response to a second stress differed with *p*CO2, with numerous processes significantly affected by mechanical stimulation at high versus low *p*CO2. These data contribute to an integrative view of the effects of ocean acidification on oysters as well as physiological trade-offs during environmental stress.

## Introduction

Current measurements of surface ocean pH have revealed decreases that are in accordance with modeled predictions of a pH decline of at least 0.3 units corresponding to atmospheric *p*CO2 of 650-970 ppm by the year 2100 (Caldeira and Wickett, 2005; Feely et al., 2010, 2012; Friedrich et al., 2012; Gruber et al., 2012; Orr et al., 2005). The coastal ocean, home to productive fisheries and diverse ecosystems, may see even greater changes in pH due to a plethora of anthropogenic effects (i.e. deforestation, agriculture, mining, increasing population sizes) (reviewed in Duarte et al., 2013). Although some species that live in the coastal ocean show a degree of adaptation to variable pH (see Kelly et al., 2013; Pespeni et al., 2013), sessile invertebrates are sensitive to acute low pH exposures across life stages. In bivalves, low pH results in significant changes to larval development (e.g. Barton et al., 2012), reduced shell deposition in most species (e.g. Gazeau et al., 2007; Melzner et al., 2011), and decreased integrity of the shell (Dickinson et al., 2012; Dickinson et al., 2013) and byssal threads (O’Donnell et al., 2013). In addition to phenotypic impacts, elevated *p*CO2 can result in significant shifts in marine invertebrate metabolism and resource utilization (e.g. Stumpp et al., 2011).

The Pacific oyster, *Crassostrea gigas*, is a marine invertebrate that has been well studied in terms of its response to ocean acidification. Larvae experience developmental delay and shell malformations in response to low pH (Barton et al., 2012; Gazeau et al., 2011; Kurihara et al., 2007; Parker et al., 2010; Timmins-Schiffman et al., 2013a). Adults are also affected negatively by ocean acidification. Fertilization success is reduced at moderately elevated *p*CO2 (600 µatm) (Parker et al., 2010). Calcification rates for adult *C. gigas* decrease linearly with increasing *p*CO2 (Gazeau et al., 2007). Reduced pH also alters response to other environmental variables. The standard metabolic rate of Pacific oysters at low pH was significantly elevated in response to increasing temperature compared to oysters at ambient pH (Lannig et al., 2010). Such studies illustrate that ocean acidification causes profound physiological changes in *C. gigas* that may have long-term consequences on fitness.

To examine the underlying processes associated with the biological impacts of ocean acidification on marine invertebrates, the current study takes an integrative approach in examining the response of adult oysters from alternations in protein expression to shell deposition rates. Oysters were exposed to one of four *p*CO2 levels for one month: 400, 800, 1000, or 2800 µatm. The *p*CO2 values represent approximate current-day surface ocean *p*CO2 (400 µatm) and three elevated values reflecting potential end-of-century scenarios as well as *p*CO2 variation that is currently experienced in the nearshore environment. At the end of one month the impacts of elevated *p*CO2 on shell growth, shell micromechanical properties, lipid metabolism, response to acute heat shock, and response to mechanical stress were assessed.

By taking this integrative approach, these data highlight the complex nature of phenotypic impacts of ocean acidification, while at the same time uncovering the less accessible underlying physiological processes. The latter was made possible by the use of shotgun proteomics, a powerful non-biased approach in the investigation of biological responses, which also offers insight into underlying mechanisms that could lead to phenotypic effects. Together these data demonstrate the scope of effects that ocean acidification can have on a marine invertebrate.

## Methods

### Ocean acidification system

This experiment was conducted at the Friday Harbor Labs Ocean Acidification Environmental Laboratory, Friday Harbor, Washington, USA where oysters were exposed to *p*CO2 values of 400 µatm, 800 µatm, 1000 µatm, or 2800 µatm. The system and control of water chemistry has been previously described in detail (O’Donnell et al., 2013; Timmins-Schiffman et al., 2013a). Briefly, incoming water was filtered (0.2 µm) and stripped of CO2. As the water flowed into the different treatment tanks, CO2-free air and CO2 were added back to reach set points that were continuously monitored by a DuraFET III pH probe (Honeywell, Morristown, NJ, USA). From the treatment tanks, water flow into the eight replicate chambers for each of the four treatment levels was 57.5 mL/min. For this experiment, set points were calculated for 13°C and estimated total alkalinity (AT) of 2100 µmol/kg for *p*CO2 values of 400 µatm (pH 8.03), 800 µatm (pH 7.76), 1000 µatm (pH 7.67), and 2800 µatm (pH 7.24).

*Seawater Chemistry Analysis*

Spectrophotometric pH was measured for all treatments 19 out of the 29 days of the experiment as described in SOP 6b by Dickson et al. (2007). On days 5, 7, 11, 14, 20, 24, and 26 spectrophotometric pH was used to measure the pH of the water inside two of the eight experimental chambers per treatment to ensure consistency with set points. Salinity was recorded with a conductivity meter (Hach sensION5, Loveland, CO, USA) and treatment temperature was verified with a Fluke 1523 thermometer (Fluke, Everett, WA, USA) whenever spectrophotometric pH was measured. Total alkalinity (AT) was measured using an open cell titration as described in SOP 3b (Dickson et al. 2007) for the treatment reservoir water and for two chambers on days 5, 11, 20, and 26. If the AT titration was not done on the day of collection, the water sample was poisoned with mercuric chloride and stored in a sealed borosilicate glass jar. CO2calc (Robbins et al. 2010) was used to calculate calcium carbonate saturation state of aragonite and calcite, carbonate ion concentration, and *p*CO2 with AT and pH as inputs and using the following constants: Lueker et al. (2000) for CO2 constants; Dickson (1990) for KHSO4; total scale (mol/kg SW) for pH scale; and Wanninkhof (1992) for air-sea flux.

### Experimental design

Adult oysters (average shell length ± s.d. = 51± 5 mm, average width = 38 ± 6 mm) collected from Oyster Bay, Washington were maintained in 3.5 L chambers (n = 6 oysters per container) and acclimated for two weeks (T = 13°C, pH = 8). The oysters originated from the same spawning event in March 2011 from approximately 25 broodstock oysters. Oysters were fed 120,000 cells per mL per day of Shellfish Diet 1800 (Reed Mariculture, Campbell, CA, USA). Containers were cleaned every other day with freshwater to prevent fouling. At the beginning and end of the experiment, buoyant weight was taken. Relative growth rate of oyster cohorts within each treatment was calculated for buoyant weight based on Hoffmann and Poorter (2002). For each treatment, the difference in means of natural log-transformed mass data was divided by 29 days. Analysis of variance was used to determine the main effects and interactions of time and *p*CO2 on buoyant weight, using the model:

( 1 )

where *bw* is the measured buoyant weight for an oyster, *t* is time point (either start or end of the experiment) and *pCO2* is the treatment condition. Growth rate analyses were performed in R (R Core Team, 2013).

Oysters were held in one of four treatments for 29 days. At the end of the treatment period oysters were either immediately sampled (n =16), subjected to mechanical stress by centrifugation in a standard salad spinner (5min, ~100rpm) and sampled (n = 8), or subjected heat shock for one hour and sampled (n = 24). Centrifugation has previously been shown to stimulate a stress response in oysters as evidenced by increased circulating noradrenaline and impacts on hemocyte function (Lacoste et al., 2001a; Lacoste et al., 2001b).

For sampling, a section of the posterior gill lamellae was dissected and immediately flash frozen in liquid nitrogen for protein expression analysis. Only samples held at 400 µatm (control) and 2800 µatm, both mechanically stressed and without additional stress, were considered for protein analysis. Remaining viscera from all oysters were put in a separate tube and flash frozen for fatty acid analysis. Both shell valves were gently cleaned of remaining tissue and left to air dry for characterization of shell mechanical properties.

### Shell micromechanical properties

Micromechanical testing was conducted on left shell valves of *C. gigas* that had been exposed to 400, 1000 or 2800 µatm. All reagents, supplies and equipment for sample preparation were purchased from Allied High Tech Products, Inc. (Rancho Dominguez, CA, USA) unless otherwise stated. Micromechanical testing was conducted within the outermost 3 mm of the shell posterior, the region of the shell where growth occurs most rapidly. Although we could not definitively differentiate shell grown during the experimental exposure from pre-existing shell, observations of growth during the course of the experiment were consistent with a 3 mm deposition of new shell.

To prepare samples, shell valves were first cut across their width using a water-cooled diamond tile saw (Skilsaw, #3540), separating the anterior from the posterior portion of the shell. The posterior segment of valves (approximately 35 mm in length) was then cleaned using Micro Organic Soap and a cotton ball to remove oil and debris and mounted on a glass microscope slide using mounting wax. Slides with mounted shells were secured to the cutting arm of a low speed diamond saw (TechCut 4, cooled with proprietary cutting fluid) and the shell segment was cut longitudinally, transecting the most posterior edge. Sectioned shell valves were removed from slides, cleaned again with Micro Organic Soap, dried on a hot plate at 70°C, and mounted in epoxy resin. Mounted samples were then ground and polished on a manual grinding/polishing machine (M-Prep 5) by passing samples through a grinding series of 180, 320, 600 and 800 grit and then polishing with a 1 µm diamond suspension and finally a 0.04 µm colloidal silica suspension. Samples were cleaned with Micro Organic soap and checked under a metallurgical microscope after each step of the grinding/polishing process, and were re-polished if necessary until the surface of each sample was completely even and free of scratches. No etching of shells was observed during grinding or polishing.

Vickers microhardness tests were conducted using a microindentation hardness tester (Clark Instrument MHT-1, SUN-TEC, Novi, MI, USA) on polished shells at 0.245 N load and 5 s dwelling time. Indents were made within the bulk, foliated layer of the shell. Seven to eight indentations were made per sample and each indent was placed at least 45 µm away from other indents and the sample’s edges. Vickers hardness numbers (VHN) were calculated as:

( 2 )

where F is the applied load and d is the mean length of the two diagonals produced by indentation. VHN were averaged for each shell sample. Following microhardness testing, each indent was photographed at 80x magnification on a metallurgical microscope (Jenco MET-233, Portland, OR, USA) equipped with a camera (Leica EC3, Buffalo Grove, IL, USA). Photographs were used to quantify the longest crack produced by each indent, which was measured using image analysis software (Leica LAS EZ, Ver. 3.0) as the radius of a circle radiating from the center of the indent enclosing all visible cracks (Figure S III.1). Hardness and crack radius measurements were used to calculate fracture toughness (Kc) for each sample as described elsewhere (Anstis et al., 1981; Baldassarri et al., 2008):

( 3 )

where 0.0154 is a calibration constant, E is an elastic modulus (empirically determined for *C. gigas* as 73 GPa: Lee et al., 2008), H is hardness in GPa, P is applied load in N and C is crack radius in μm.

Statistical analysis for micromechanical properties was conducted using SPSS (Ver. 19, IBM, Armonk, NY, USA). Outliers were calculated in SPSS as values greater than 1.5 times the interquartile range below or above the first or third quartile respectively, and were removed from the dataset (at most two per treatment group). Data were analyzed using one-way analysis of variance followed by post-hoc testing. Normality and equal variance was tested using a Kolmogorov–Smirnov test with Lilliefor’s correction and a Levene test, respectively. Fracture toughness data met both assumptions and a Tukey HSD post-hoc test was used. As hardness data was normally distributed but did not meet the equal variance assumption, a Welch ANOVA followed by Games-Howell post-hoc testing was applied.

### Fatty acid analysis

Fatty acid analysis was carried out on oysters from three pCO2 treatments (400, 800, and 2800 µatm; n = 8 per treatment). Whole body tissue (minus the dissected gill) was lyophilized overnight and tissues were homogenized with a pestle for use in fatty acid extractions (2.5 mg per extraction). Extractions were performed following the protocol described in Galloway et al. (2012) except two chloroform removals were carried out. Fatty acid methyl esters were identified by running the samples on a HP 6958 gas chromatograph with an auto-sampler and flame-ionization detector using an Agilent DB-23 column (30 m, 0.25 mm diameter, 0.15 µm film) (Supelco, Bellefonte, PA, USA). Peaks were identified based on comparison of retention times with known standards. Individual amounts of fatty acids were normalized within each replicate by dividing the peak area by the sum of all fatty acid peak areas for that sample. Normalized fatty acid data were log-transformed and non-metric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix was used to compare fatty acid profiles among treatments. NMDS was performed in R with the vegan package (R Core Team, 2013; Oksanen et al., 2013).

### Heat shock

Oysters from each of the *p*CO2 treatments of 400, 800, 1000, and 2800 µatm were subjected to acute heat shock. Three temperature shocks were implemented consisting of two sublethal temperatures (42° and 43°C) and one lethal temperature (44°C). The lethal heat shock temperature was previously determined for this group of oysters and is defined as the temperature at which 100% mortality occurs within one week after a one hour exposure (Clegg et al., 2009). The heat shock exposure occurred in 800 mL of seawater equilibrated to the correct temperature in a circulating water bath. Since oysters considerably decrease the temperature of the bath, we added a pre-heating step of 10 minutes in one beaker after which the oysters were transferred into another beaker for the full hour. After heat shock, oysters were returned to the flow through system at pH = 8.03 and 13°C. Mortality was the only parameter assessed for the temperature treatment. Differences in mortality across treatments were assessed using ANOVA in R (R Core Development Team, 2013) with pCO2, day, and temperature as fixed factors.

### Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Protein extraction and desalting were performed on gill tissue from four mechanically stressed and four control oysters held in the present day and the highest treatment level, 400 and 2800 µatm, respectively (n = 16 oysters total) as described in Timmins-Schiffman et al. (2013b). Each of the 16 protein samples was injected into the LC-MS/MS three times, with injections occurring in a randomized order. LC-MS/MS and data acquisition were carried out as previously described (Timmins-Schiffman et al., 2013b).

### Protein informatics analysis

Peptide tandem mass spectra were correlated to *in silico*-generated tandem mass spectra resulting from the Pacific oyster proteome (Fang et al. 2012) using SEQUEST (Eng et al., 1994). Using PeptideProphet from the trans-proteomic pipeline (TPP), peptides were assigned a relative score for best match to the database (Eng et al., 2008; Eng et al., 1994). Only peptides with a PeptideProphet probability score of at least 0.9 were considered for further analysis. Additionally, a protein was considered for analysis only if it had at least 8 spectral counts across all 48 injections (1 spectral count = 1 peptide matched to that protein). Within a biological replicate, a protein was considered to have a non-zero expression value if it had at least 2 unique peptide matches.

NSAF (normalized spectral abundance factor), a metric based on spectral counting (Florens et al., 2006), was used to quantify protein expression. Total spectral counts (SpC) for each oyster were averaged across the three technical replicates. NSAF was calculated by dividing average SpC for each protein by the protein length (L) and then dividing SpC/L by the sum of all SpC/L within a biological replicate (Florens et al., 2006). This workflow was executed in SQLshare (Howe et al., 2011).

Oyster proteins (Fang et al. 2012) were annotated by comparing sequences to the UniProt-KB/SwissProt database (<http://uniprot.org>) using the blastp algorithm (Altschul et al., 1997) with an e-value limit of 1E-10. Based on homology with the SwissProt database, oyster proteins were further annotated with Gene Ontology (GO) and GO parent categories (GO Slim).

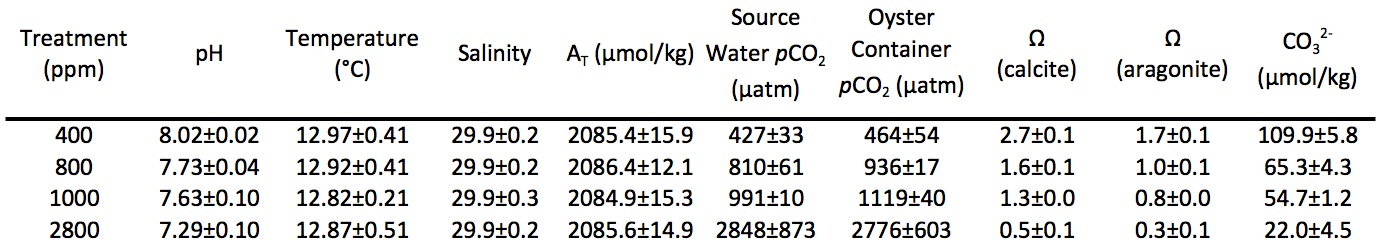
Fold change in protein expression between treatment groups was found by dividing the average NSAF of four biological replicates in one treatment by the average NSAF of the other treatment. In order to statistically test for significant expression differences treatments were compared in a pairwise fashion (400 vs. 2800 µatm, 400 µatm vs. 400 + mechanical stress, and 2800 µatm vs. 2800 + mechanical stress) using the qvalue package in R (R Core Team, 2013; Dabney and Storey) with a q-value cut-off of 0.1. Use of a q-value instead of a p-value from a t-test allows for a multiple comparisons correction using the positive false discovery rate (Storey and Tibshirani, 2003; Storey, 2002). In an effort to determine the biological processes that were influenced by altered environmental conditions, proteins were considered differentially expressed if either there was 1) a 5-fold difference in expression between treatment groups or 2) a q-value < 0.10. The two caveats to these classifications were that only proteins expressed in more than one oyster were considered for fold-based analysis and only proteins expressed by all oysters within a treatment group were considered significant for the q-value cut-off. Proteins expressed in only one treatment were considered having a greater than 5-fold difference and were included in the differentially expressed protein group. Enrichment analysis was performed on these differentially expressed proteins using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v. 6.7 (Huang et al., 2009a, 2009b) (<http://david.abcc.ncifcrf.gov/>). The background protein list was made from the entire sequenced gill proteome. Biological processes were considered significantly enriched if p-value < 0.075. Overlaps in the responses to different stressors were explored using a Venn diagram of the proteins that were differentially expressed between treatments in eulerAPE v. 1.0 (http://www.eulerdiagrams.org/eulerAPE/).

## Results

### Seawater chemistry analysis

The *p*CO2 levels for the four different treatments remained consistent throughout the one month experiment (Table III.1). Average pH (± s.d.) for treatments as measured by the DuraFET probe were 8.02 ± 0.02, 7.73 ± 0.04, 7.63 ± 0.10, and 7.29 ± 0.10 for the 400, 800, 1000, and 2800 µatm treatments, respectively. Spectrophotometric pH corroborated the DuraFET measurements (spectrophotometric pH data not shown). The *p*CO2 in containers with oysters was approximately 40 µatm higher than the source water *p*CO2 , except for the 2800 µatm treatment where it was approximately 75 µatm lower than the source water. Total alkalinity was 9% higher in the chambers (data not shown) compared to the source water for 400 µatm, 10% higher at 800 µatm, 13% higher at 1000 µatm, and 3% lower at 2800 µatm. Calcite was undersaturated (Ωc < 1.0) only at the highest *p*CO2 level and aragonite was undersaturated at the two highest *p*CO2 levels (1000 µatm and 2800 µatm).

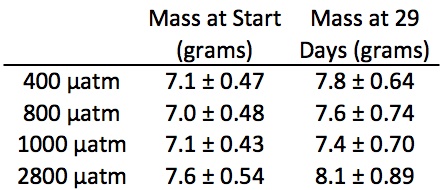
Table III.1. Water chemistry summary data. Mean and ± standard deviation are provided for the 29 day experiment. Salinity is an average of nineteen measurements and AT was measured four times. pH and temperature values are from the continuous monitoring by the DuraFET probe. pH, temperature, salinity, and AT were directly measured and all other parameters were calculated using CO2calc (Robbins et al., 2010).



### Oyster growth

Relative growth rate (RGR) for shell mass exposed to increased *p*CO2, as measured by buoyant weight, was not significantly different among treatments (p > 0.05) (Table III.2). There was a significant effect of time on shell mass across all treatments (F = 6.1190, p = 0.014), indicating shell growth in all treatments.

Table III.2. Average buoyant weight ± 95% confidence intervals at start and end of experiment.



### Micromechanical properties

Micromechanical properties were tested within the outer 3 mm of the growing edge (posterior) of left shell valves for oysters in the treatments of 400, 1000, and 2800 µatm. Both Vickers microhardness and fracture toughness differed significantly among *p*CO2 treatments (microhardness: Welch ANOVA, p = 0.014; fracture toughness: one-way ANOVA, p = 0.003) (Figure III.1). The microhardness of shells grown at 1000 µatm was significantly lower than that of shells grown at 400 µatm (Games-Howell: p < 0.05). Shells grown at 2800 µatm showed a trend toward lower microhardness as compared to the 400 µatm control group, but this comparison was not statistically significant (Games-Howell: p = 0.119). In contrast, fracture toughness was significantly lower in shells grown at 2800 µatm as compared to both the 400 and 1000 µatm treatments, but the 400 and 1000 µatm treatments did not differ (Tukey HSD: p < 0.05). Representative cracks formed by micromechanical testing are shown in Figure S III.1.

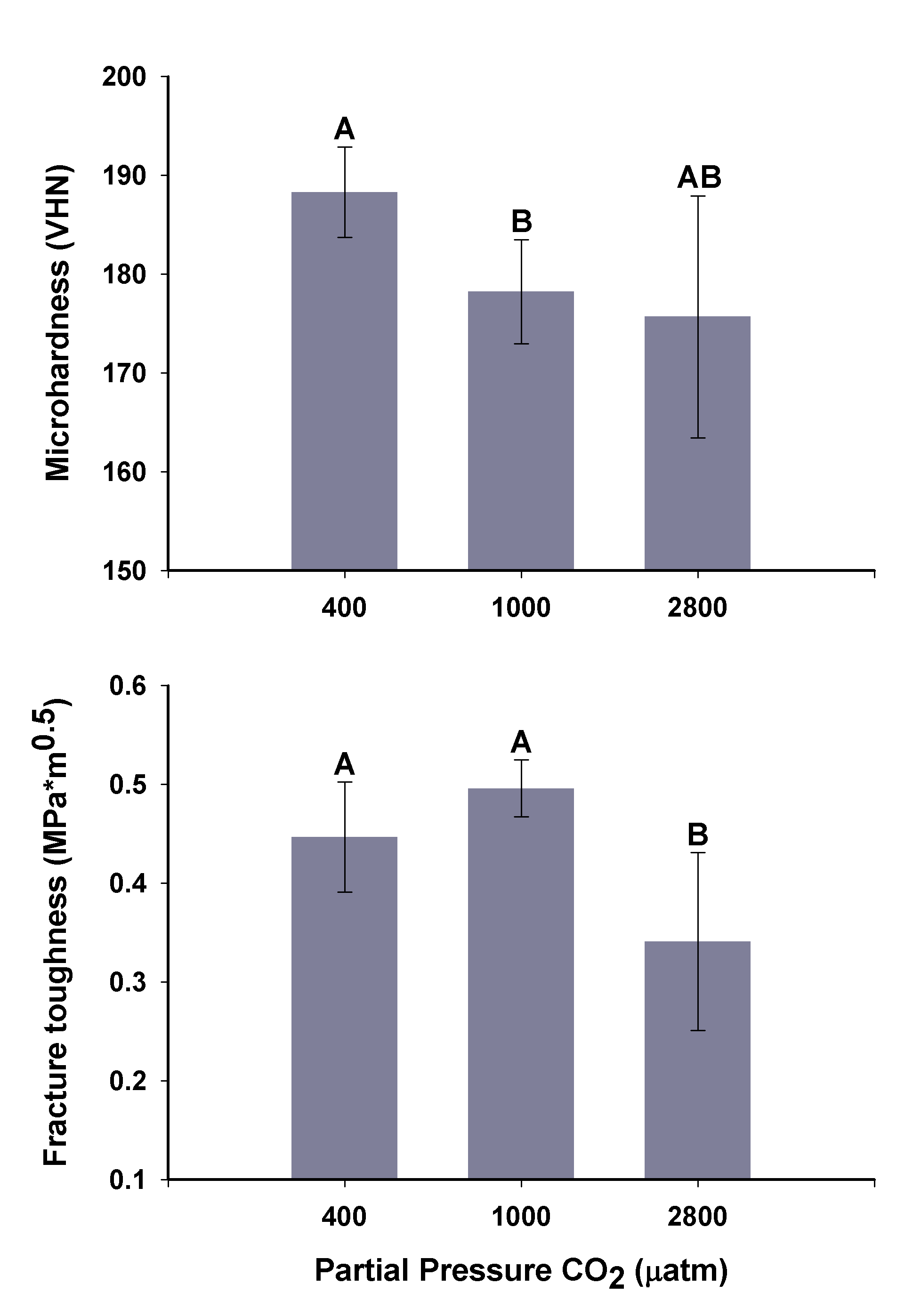


Figure III.1. **Mean Vickers microhardness and fracture toughness of *C. gigas* shells (± 95% C.I.), tested within the outer 3 mm of the shell’s growing edge.** Groups marked with different letters are significantly different (p < 0.05; n = 5-7 shells per treatment).

### Fatty acids

There was no difference in fatty acid profiles among three treatments (400, 800, and 2800 µatm) (Figure III.2). The nonmetric multidimensional scaling (NMDS) approach shows the relative position of each oyster according to its entire fatty acid profile (i.e. oysters that are plotted close together have similar fatty acid profiles). There is also no significant difference in total fatty acid per milligram tissue (data not shown). Twenty-one fatty acid peaks were identified in the 24 samples, which is within the range of 16-35 fatty acids found in other studies of bivalves (Both et al., 2011; Milke et al., 2004; Pettersen et al., 2010; Soudant et al., 1999). Among those fatty acids identified were 16:0; 18:0; 18:1n-9; 18:1n-7; 18:2n-6; ALA (18:3n-3); ARA (20:4n-6); EPA (20:5n-3); DPA (22:5n-6); n-3 DPA (22:5n-3); and DHA (22:6n-3). Raw and normalized fatty acid data are available in Table S III.1.

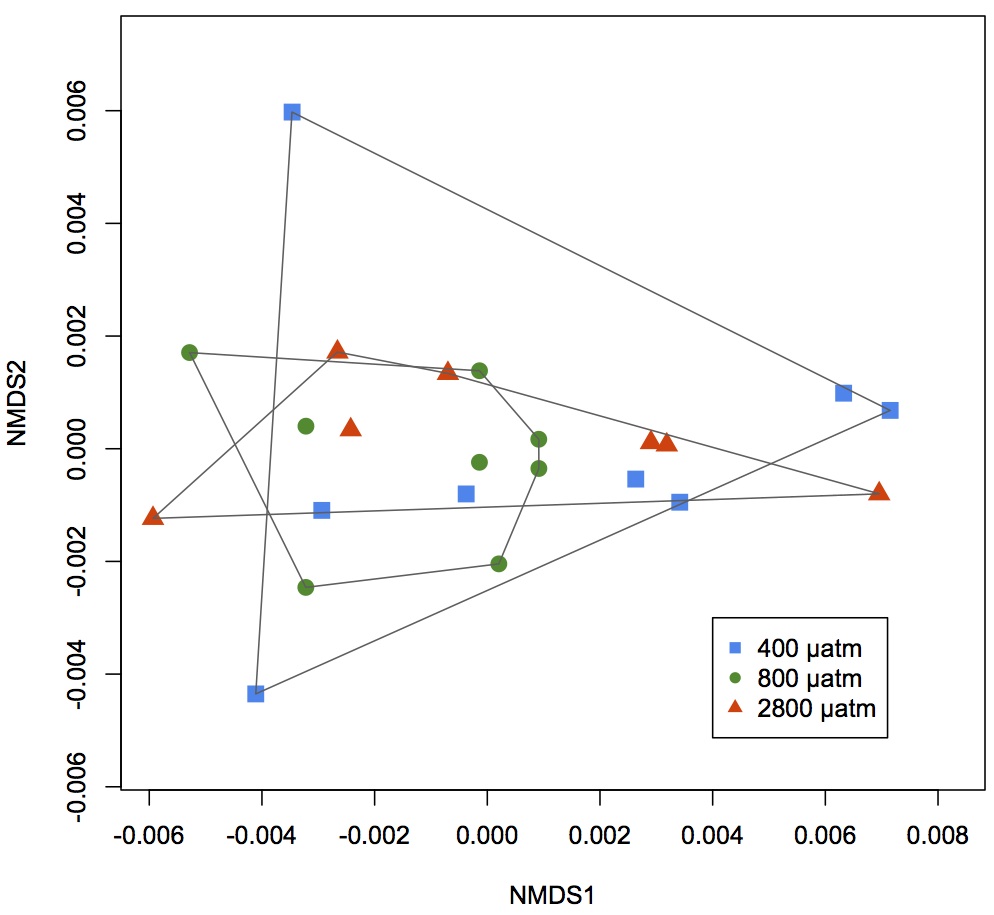


Figure III.2. **Non-metric multidimensional scaling (NMDS) analysis of fatty acid profiles for oysters from 400, 800 and 2800 µatm.** There are no differences in relative amounts of fatty acids among the three treatment groups.

### Heat shock response

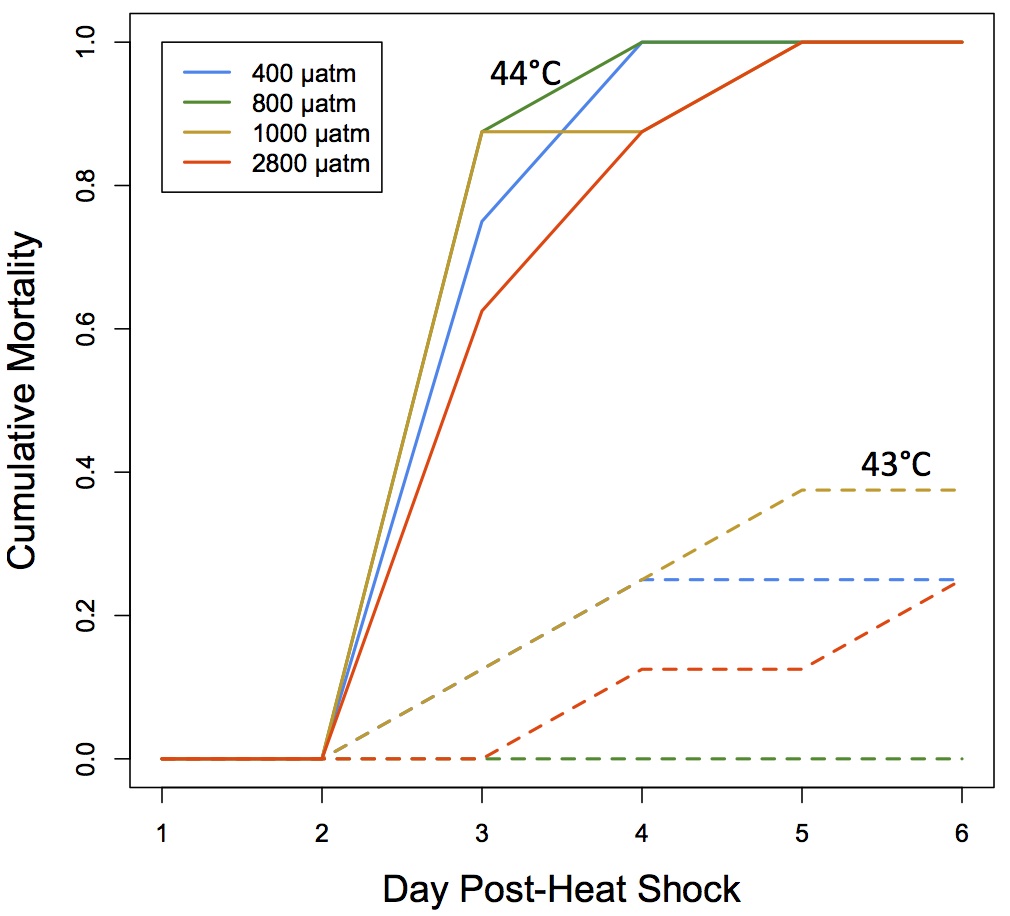
There was no difference in mortality in response to heat shock due to *p*CO2 (p > 0.05) (Figure III.3). There was a significant difference in mortality due to temperature (F = 78.5191, p = 6.0E-13) and day (F = 31.8392, p = 3.5E-7). One hundred percent mortality (n = 8 oysters per temperature per treatment) occurred across all three treatments by day 5 post-heat shock at the lethal temperature (44°C). There was no mortality by day 6 post-heat shock in the 42°C group (data not shown).

Figure III.3. **Oyster mortality after 1 hour heat shock at 43°C (dashed lines) or 44°C (solid lines).** Mortality from heat shock did not differ among groups of oysters exposed to different pCO2 for 1 month - 400 µatm (blue), 800 µatm (green), 1000 µatm (yellow), 2800 µatm (orange).

### Proteomics

After filtering, 700 733 peptides were considered for analysis corresponding to 1 616 proteins (Table S III.2, Table S III.3, Table III.3). Eighty-nine percent (1 449) of proteins were annotated using the UniProt-KB/SwissProt database and 77% (1 250) of those were further categorized with Gene Ontology information (Table S III.4).

Pairwise comparisons were made based on gill protein expression between 1) oysters held at 400 µatm versus oysters held at 2800 µatm, 2) oysters held at 400 µatm versus oysters held at 400 µatm subjected to subsequent mechanical stress, and 3) oysters held at 2800 µatm versus oysters held at 2800 µatm subjected to subsequent mechanical stress. When the proteomic response of oyster gill tissues under high *p*CO2 conditions was examined compared to present day conditions, 64 proteins were identified as elevated under high *p*CO2 conditions whereas 55 proteins were expressed at decreased levels (Figure III.4A, Figure S III.2). Proteins identified as differentially expressed include those involved in carbohydrate metabolism (i.e. α-L-fucosidase, probable β-D-xylosidase 5, succinyl-CoA ligase, and UDP-glucose 4-epimerase), cell growth (i.e. thymidine phosphorylase and tyrosine-protein kinase Yes), transcription and translation (i.e. calcium-regulated heat stable protein 1, prohibitin-2, and translational activator BCN1), and response to reactive oxygen species (i.e. glutathione S-transferase omega-1). Gene enrichment analysis revealed enriched proteins are associated with cell junction organization / assembly (i.e. contactin, talin) and morphogenesis (i.e. cadherin-23, Neurogenic locus notch homolog protein 1, Integrin β -PS) (Table S III.5).

Ninety-nine proteins were differentially expressed upon mechanical stress exposure at 400 µatm. Forty-seven were elevated under mechanical stress and 52 proteins were expressed at a lower level (Figure III.4B, Figure S III.2). The proteins that were differentially expressed included those involved in apoptosis (i.e. programmed cell death protein 5, CDGSH iron-sulfur domain-containing protein 2), carbohydrate metabolism (i.e. α-L-fucosidase, lysosomal α-mannosidase, phosphoacetylglucosamine mutase), and transcription and translation (i.e. histone deacetylase complex subunit SAP18, eukaryotic translation initiation factor 3 subunit A). No significantly enriched biological processes were identifiedin response to mechanical stress at 400 µatm.

Seventy proteins were elevated when oysters held at 2800 µatm were subjected to mechanical stress, and 53 proteins were expressed at decreased levels (Figure III.4C, Figure S III.2). Polysaccharide and monosaccharide metabolism processes were affected by mechanical stress as evidenced by their enrichment in this treatment, resulting from the differential expression of proteins including β -hexosaminidase subunit β, Lysosomal α-glucosidase, putative glycogen [starch] synthase, Glycogenin-1, α-L-fucosidase, 6-phosphogluconolactonase, 60 kDa SS-A/Ro ribonucleoprotein, and N-acetylgalactosamine kinase (Table S III.3). Differentially expressed proteins included those involved in response to reactive oxygen species (i.e. glutathione S-transferase Mu 3 and dual oxidase 2) and apoptosis (i.e. caspase-7 and engulfment cell motility protein 2). Similar biological processes as those identified in the response to ocean acidification alone were also enriched in this comparison, including cell junction organization / assembly (i.e. neurexin-4, talin-1, contactin) and tissue morphogenesis (i.e. lysosomal α-glucosidase, tyrosine-protein kinase Btk29A) (Table S III.5).

Table III.3. Total number of proteins identified for each oyster across all three technical replicates with numbers of proteins for each individual technical replicate in parentheses. Number of proteins shared among all three technical replicates (with percentage of total) are in the third column.

|  |  |  |
| --- | --- | --- |
| Oyster (pCO2 – biological replicate) | Total Proteins (Technical replicate #1, #2, #3) | Proteins Across All 3 Replicates (% of Total) |
| 400-1 | 882 (869, 871, 873) | 853 (96.7%) |
| 400-2 | 878 (861, 863, 861) | 841 (95.8%) |
| 400-3 | 836 (819, 824, 819) | 802 (95.9%) |
| 400-4 | 867 (855, 856, 857) | 839 (96.8%) |
| 400-MechS1 | 861 (850, 850, 846) | 832 (96.6%) |
| 400-MechS2 | 815 (803, 806, 801) | 788 (96.7%) |
| 400-MechS3 | 854 (840, 848, 841) | 826 (96.7%) |
| 400-MechS4 | 862 (842, 850, 848) | 825 (95.9%) |
| 2800-1 | 873 (854, 858, 856) | 837 (95.9%) |
| 2800-2 | 924 (910, 917, 910) | 894 (96.8%) |
| 2800-3 | 901 (888, 884, 893) | 871 (96.7%) |
| 2800-4 | 874 (861, 866, 865) | 849 (97.1%) |
| 2800-MechS1 | 889 (877, 878, 879) | 858 (96.5%) |
| 2800-MechS2 | 891 (886, 876, 880) | 867 (97.3%) |
| 2800-MechS3 | 867 (860, 859, 856) | 844 (97.3%) |
| 2800-MechS4 | 941 (923, 925, 929) | 905 (96.2%) |

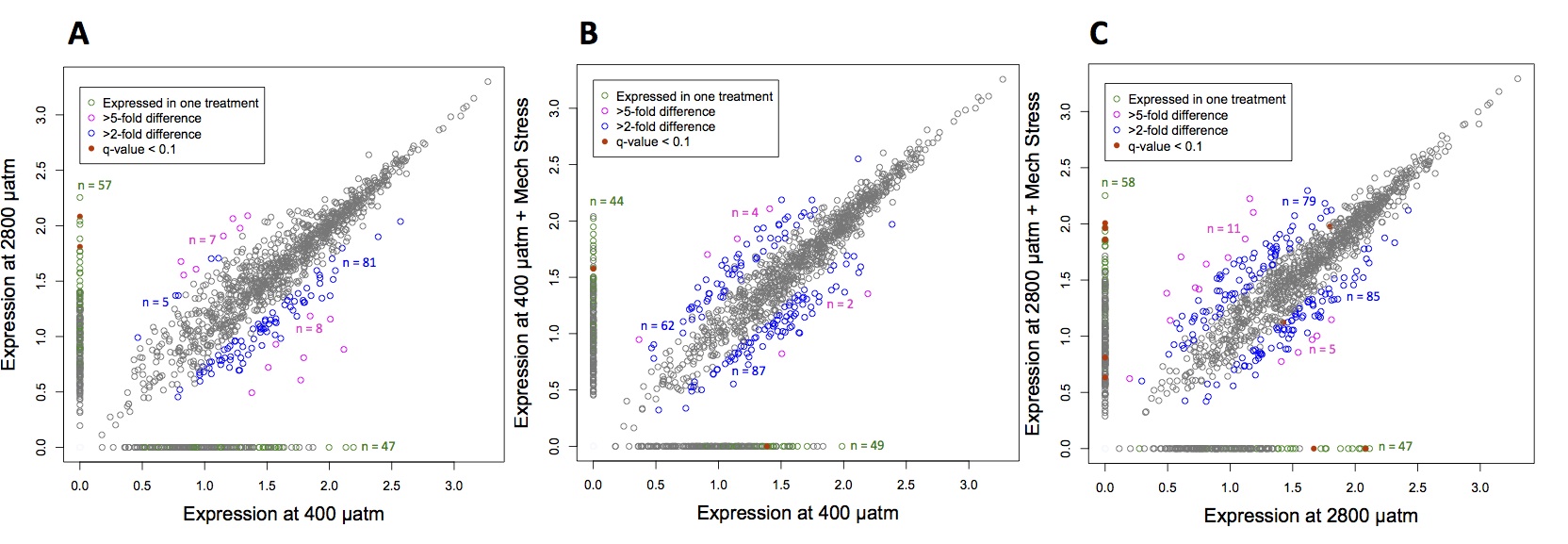


Figure III.4. **Characterization of** **protein expression values (log([normalized spectral abundance factor]\*10000)) for experiments designed to examine the influence of ocean acidification (A), mechanical stress at 400 µatm (B), and mechanical stress at 2800 µatm (C).**

The magnitude of the proteomic responses in the three different between-treatment comparisons was similar (99-123 differentially expressed proteins), though certain proteins were treatment specific (Figure III.5). In addition, for those proteins identified as differentially expressed in more than one comparison, the directional responses of that protein to each treatment often diverged. For example, proteins in the carbohydrate metabolism and cell growth and apoptosis pathways responded differently across the three comparisons (Figures III.6A, B). These proteomic profile discrepancies were the most dramatic in a comparison between the responses to ocean acidification and to mechanical stimulation at high *p*CO2. All 46 differentially expressed proteins shared between these two responses were expressed in opposing directions (Figures III.6A, B, Table S III.3 and Figure S III.2).

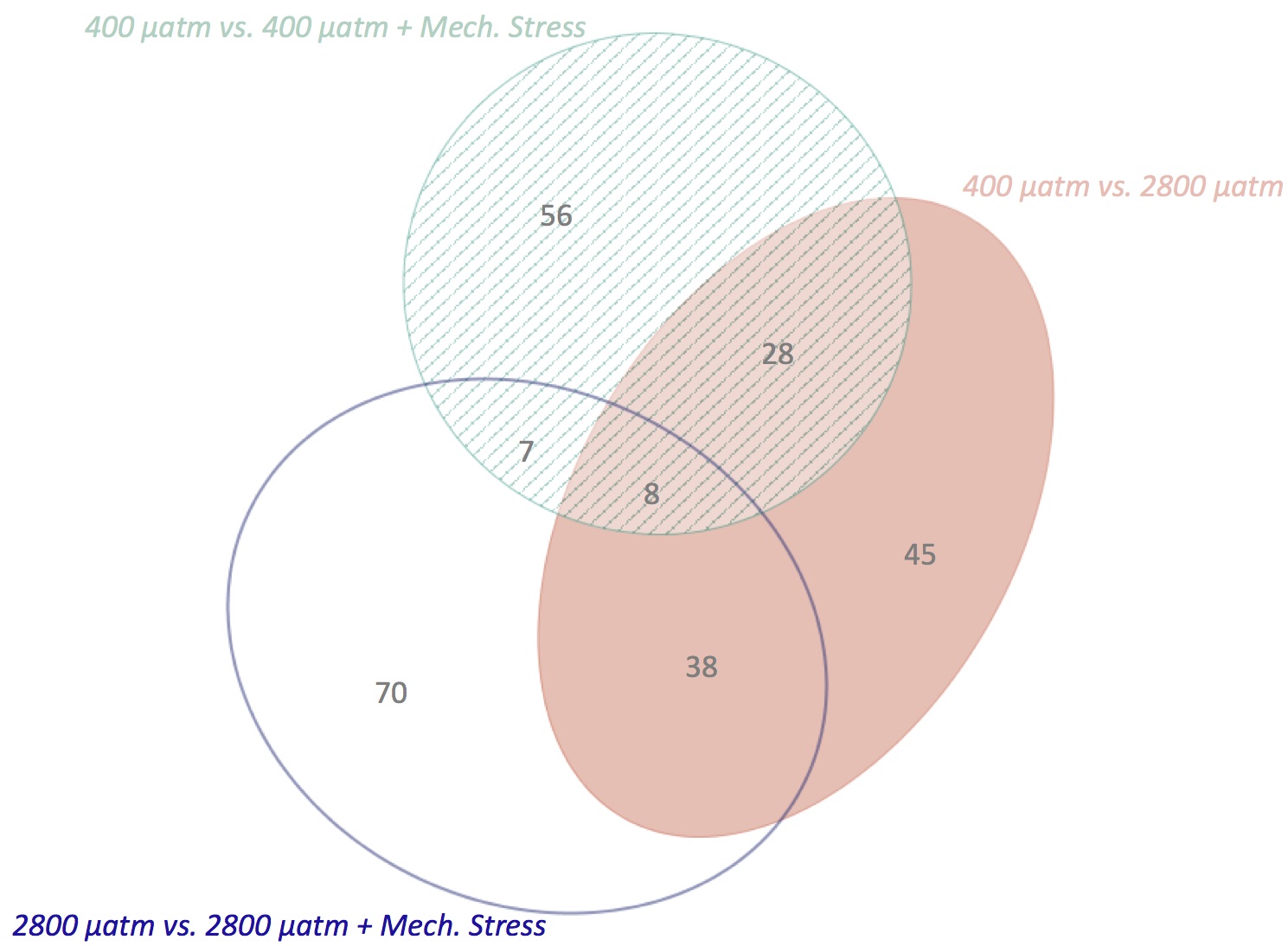
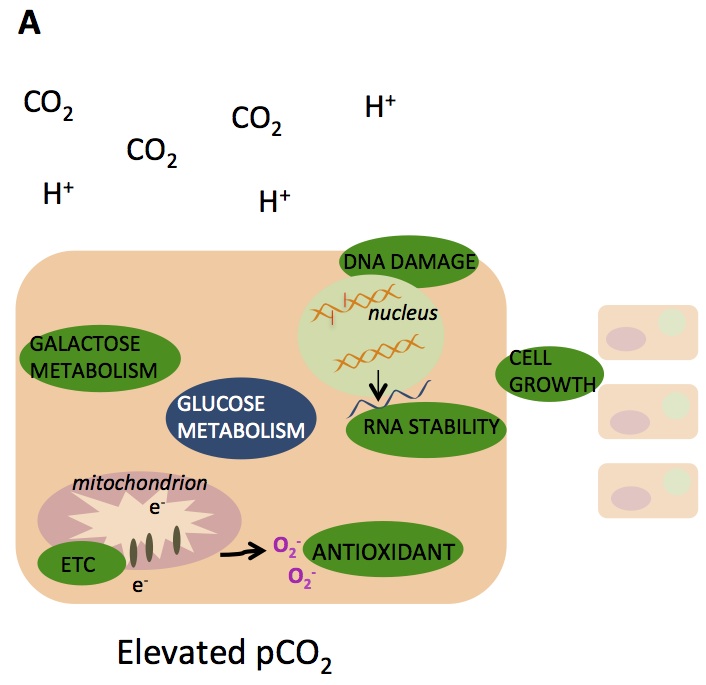


Figure III.5. **Differentially expressed proteins among treatments.** The proteins represented by the solid pink ellipse were those implicated in the response to ocean acidification alone, those in the open blue ellipse are different in response to mechanical stress in the 2800 µatm-exposed oysters, and those in the striped aqua ellipse changed in response to mechanical stress at 400 µatm. Numbers represent the number of proteins in each segment of the ellipses.



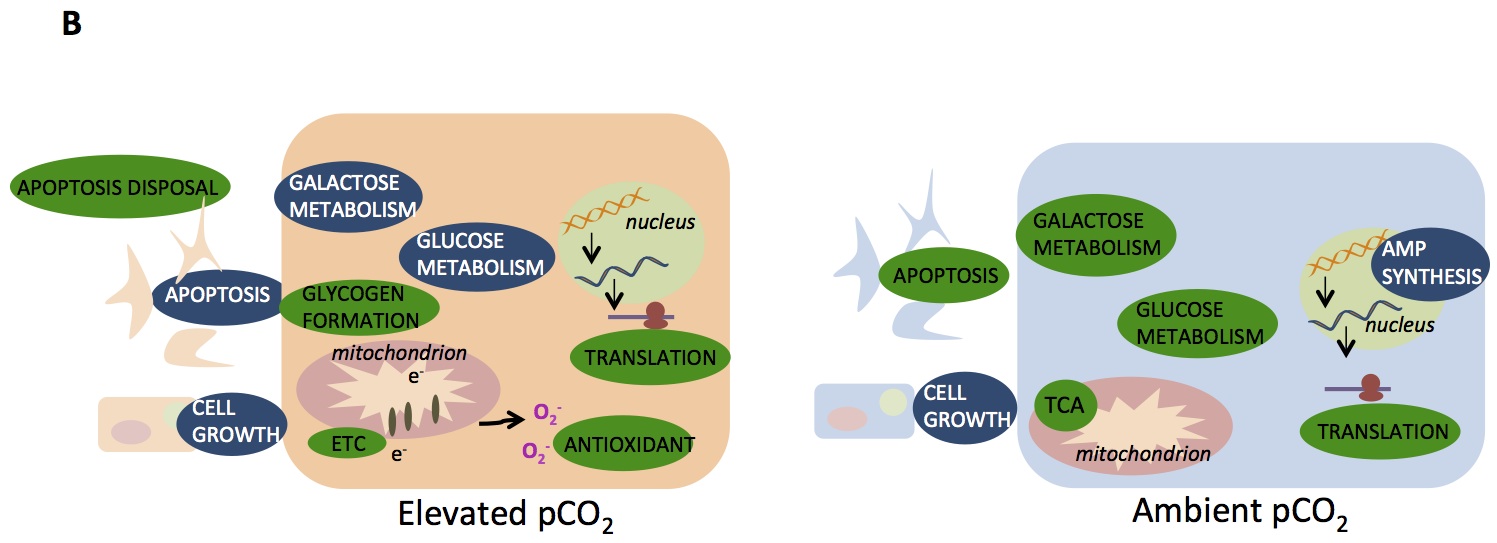


Figure III.6. **Cartoon** **representation of key molecular changes that occur in response to different stressors.** Responses to (A) ocean acidification and to (B) mechanical stress at the two different pCO2 levels are depicted separately. Green or blue ellipses represent greater or decreased expression of protein(s) in the indicated pathway, respectively. Processes are in all capital letters and organelles are in italics. Processes are labeled generally and specific protein information can be found in supplementary material Table S4. “ETC” stands for electron transport chain.

## Discussion

Ocean acidification is an on-going and global scale phenomenon that has been shown to negatively impact most taxonomic groups. A large number of studies have characterized these negative impacts across species and life stages (e.g. Kroeker et al., 2013; Kroeker et al., 2010). With the goal of achieving a better understanding of the effects of ocean acidification and responses to additional acute stressors across multiple physiological processes, we employed an integrative approach, combining analyses of shell micromechanical properties, fatty acids, and proteomics. Our findings illustrate the value of using complementary approaches to explore interactions and trade-offs among different fundamental processes during environmental stress.

Relative shell growth was not impacted by exposure to elevated *p*CO2 (800, 1000 and 2800 µatm) for one month. The lack of significant difference in growth among treatments is somewhat surprising, especially considering that calcite, the main component of adult oyster shells, was undersaturated in the 2800 µatm treatment. Over longer exposures to ocean acidification, other bivalve species have demonstrated decreased shell growth as compared to individuals held at ambient *p*CO2. After six weeks, mussel (*Mytilus edulis*) shell length growth was negatively impacted by elevated *p*CO2 of 2400 and 4000 µatm (Melzner et al., 2011). Juvenile oysters, *Crassostrea virginica*, also showed lower shell mass after 45 days at 1 665 µatm (Talmage and Gobler, 2011) and after a 20 week exposure at 3 523 µatm (Beniash et al., 2010). Based on these data we might expect to see differences in shell growth if exposure was continued for a longer period of time. In addition to the duration of exposure, level of *p*CO2 may also be an important factor in effects observed on shell growth. Elevated *p*CO2 may in fact prompt an over-compensation in terms of shell deposition. For instance, hard shell clams, *Mercenaria mercenaria*, had higher shell mass at moderately elevated *p*CO2 (about 800 µatm) after 16 and 21 weeks of exposure, but no significant difference in shell mass between the control treatment and highly elevated *p*CO2 (about 1500 µatm) was observed (Dickinson et al., 2013). At least some of the variability in the effects of elevated *p*CO2 on shell growth is likely due to intra- and interspecies differences in population history of exposure to low pH events.

Given that shell growth persisted in all elevated *p*CO2 treatments, we were able to perform mechanical testing on new shell that was deposited. Shell microhardness and fracture toughness were both affected by exposure to elevated *p*CO2. Microhardness, a measure of a material’s resistance to deformation, was reduced at elevated *p*CO2 (1000 µatm) as compared to the control (400 µatm), whereas fracture toughness, a measure of the propensity for cracks to propagate within a material, was reduced only at 2800 µatm. Given that both measurements depend on the microstructural arrangement of the shell and the extent and distribution of elastic elements within the shell (i.e. the shell organic matrix) (Lee et al., 2008), exposure to elevated *p*CO2 may lead to dose-dependent differences in the structure and/or composition of newly formed shell. Such changes could result either from alterations in the physiology of shell deposition or the ability to prevent dissolution under varying seawater hydrochemistry. In the current study, changes to micromechanical properties were detected at both Ωcalcite (Ωc) = 0.5 (2800 µatm) and at Ωc = 1.3 (1000 µatm). Alterations in shell growth and structure would be expected at Ωc < 1, however there is increasing evidence that shell modifications occur even at Ωc > 1. Even when seawater is saturated with respect to calcite, bivalves experience both shell dissolution (Dickinson et al., 2013; Lannig et al., 2010) and changes to shell microstructure (Beniash et al., 2010; Dickinson et al., 2013; Welladsen et al., 2010). These results indicate that as Ωdecreases the driving force towards biomineralization is reduced. Since calcification is an energetically intensive process (Paine, 1971; Palmer, 1992; Rosenberg & Hughes, 1991), one explanation for significant effects on CaCO3 structures at Ω> 1 is that resources for energy metabolism are being reallocated to other, non-calcification physiological processes in order to maintain homeostasis.

In order to gain insight into changes in energy metabolism that could be a result of elevated *p*CO2, we also examined fatty acid profiles. There was no difference in relative proportions of fatty acids in whole body tissue after one month of exposure to 800 µatm or 2800 µatm *p*CO2. This was contrary to our hypothesis that the extended stress of ocean acidification would alter fundamental metabolites. Oysters and other bivalves are highly dependent on fatty acids as a main energy source, especially poly-unsaturated fatty acids (Milke et al., 2004; Pettersen et al., 2010; Soudant et al., 1999; Trider and Castell, 1980). Changes in physiological state, such as those induced by reproduction or poor nutrient supply, can alter the relative proportions of fatty acids in oyster tissue (Both et al., 2011; Soudant et al., 1999). It is possible that an environmental stress can trigger a change in bivalve physiology and result in changes in fatty acid metabolism and/or storage, however, exposure time and life stage may also play important roles. In the case of ocean acidification, *C. gigas* is able to maintain homeostasis of both total and relative amounts of important fatty acids. After 11 weeks of exposure to *p*CO2 of 800 µatm, juvenile *C. virginica* had significantly less lipid per gram body weight than control oysters (Dickinson et al., 2012). The extended stress of the 11 week exposure in Dickinson et al. (2012) may have overwhelmed *C. virginica*’s ability to maintain lipid homeostasis, suggesting that oyster energy metabolism may fail under consistent ocean acidification stress. However, there are some instances where ocean acidification did not influence lipid levels in invertebrates. For example, in larval sea urchins, despite the fact that individuals at elevated *p*CO2 were smaller than control larvae,they maintained the same lipid and protein levels (Matson et al., 2012).

Consistent with the hypothesis that elevated *p*CO2 impacts underlying physiology, we predicted that lower pH would depress the temperature threshold for mortality after heat exposure. The lack of influence on acute heat shock response across the four *p*CO2 treatments could be evidence that *p*CO2 has little effect on the oyster’s macro-physiological response. We are not aware of other studies that have investigated ocean acidification and acute heat stress, but there are studies that have explored moderately elevated temperatures and ocean acidification over an extended period. In *C. virginica*, elevated *p*CO2 did not impact the oyster’s response to elevated temperature (Ivanina et al., 2013). In fact, Ivanina et al. (2013) demonstrated that exposure to elevated *p*CO2 limited high temperature-associated mortality in *C. virginica*. The modulating effects of elevated *p*CO2 on temperature-associated mortality, as well as the lack of negative impacts of acute temperature evidenced herein, suggest that the physiological response stimulated by one stress offers tolerance to negative effects of a second stress.

To achieve a comprehensive assessment of the underlying physiological impact of ocean acidification on oysters, we characterized the proteomic response. Ocean acidification significantly affected the underlying molecular physiology of *C. gigas* after one month of exposure. One clear trend identified in gill protein expression patterns in oysters exposed to elevated *p*CO2 was that proteins associated with carbohydrate metabolism were differentially expressed. This included proteins involved in gluconeogenesis (serine-pyruvate aminotransferase) as well as galactose metabolism **(**UDP-N-acetylhexosamine pyrophosphorylase and N-acetylgalactosamine kinase). Both of these pathways can lead to production of glucose. One explanation for these changes is that during ocean acidification there is a need for increased glucose production in order to provide energy resources necessary to maintain homeostasis. Further, the fact thatserine-pyruvate aminotransferaseexpression was decreased and proteins associated with galactose metabolism increased in expression could indicate a shift in glucose production pathways (Figure 6A). In other taxa, the general stress response usually leads to increased carbohydrate metabolism and decreased carbohydrate storage (Mizock, 1995; Parrou et al., 1997). Similarly, larval urchins had decreased expression of carbohydrate, lipid, and protein metabolism-related genes after ocean acidification exposure (Todgham and Hofmann, 2009).

Cellular respiration and the electron transport chain proteins were also affected by exposure to ocean acidification conditions**.**  Increased expression of proteins associated with both of these processes(cysteine-tRNA ligase, cytochrome c oxidase, and NADH dehydrogenase) implies oysters exposed to elevated *p*CO2 experienced a heightened demand for energy (i.e. increased ATP production in mitochondria). Metabolic rate can also be affected by the combination of elevated *p*CO2 and temperature, but in a species-specific manner: *C. gigas*’s metabolic rate was suppressed (Lannig et al. 2010) while *M. mercenaria*’s was elevated (Matoo et al., 2013). Heightened metabolism can occur in response to an ongoing stress (Mizock 1995) and may be an important adaptive strategy to counteract the physiological effects of elevated environmental *p*CO2 (Melzner et al., 2009). Further evidence of increased mitochonodrial metabolism is the elevated expression of an antioxidant response protein (glutathione S-transferase omega-1). The cellular need for an antioxidant response could arise from greater reactive oxygen species generation due to increased metabolism (Mohanty et al., 2000). Additionally, the cellular environment resulting from ocean acidification may directly lead to oxidative stress due to elevated cellular CO2 and H+ (Dean, 2010; Tomanek et al., 2011). Resulting from either direct or indirect (increased metabolism) effects of elevated *p*CO2, invertebrates frequently experience oxidative stress during ocean acidification exposure as evidenced at the molecular level (Todgham and Hofmann, 2009; Tomanek et al., 2011). All of these changes to molecular processes in the mitochondria are evidence of an increase in energy production, and perhaps energy requirements, in oysters exposed to ocean acidification.

The proteomic response to a secondary stress in conjunction with ocean acidification goes beyond the response to elevated *p*CO2 alone to investigate how certain responses are altered by this environmental change. The ultimate goal of investigating a second stress was to attempt to assess whether the “normal” physiological response to a stress event (i.e. mechanical stimulation) would be significantly impacted under ocean acidification conditions. Qualitative differences between the proteomic responses to mechanical stimulation at 400 and 2800 µatm are reflected in the enrichment analysis, which identifies differences between treatment groups at the process level. No biological processes were enriched during the “normal” (400 µatm) response to mechanical stimulation. Numerous processes including neuromuscular processes, cell junction assembly, tissue morphogenesis, and carbohydrate metabolism were significantly impacted by at 2800 µatm upon mechanical stress, clearly demonstrating how ocean acidification impacts the normal physiological response to an exongenous stress.

Ocean acidification appears to significantly impact apoptotic responses of oysters exposed to mechanical stress (Figure III.6B). Changes to cell growth and morphogenesis are reflected in the enrichment of these processes in oysters exposed to elevated *p*CO2 with and without mechanical stimulation. Cell growth pathway proteins (receptor-type tyrosine-protein phosphatase β, thymidine phosphorylase, and tyrosine protein kinase Yes) were expressed at lower levels after mechanical stimulation at both *p*CO2. Proteins associated with apoptosis increased expression with mechanical stimulation at 400 µatm (programmed cell death protein 5 and CDGSH iron-sulfur domain-containing protein 2) and decreased at 2800 µatm (caspase-7). These data suggest that mechanical stress is associated with apoptosis, which would be consistent with cell damage. While based on a limited number of proteins, these results provide evidence that apoptosis decreased under elevated *p*CO2 conditions. However, it should be noted there was an increase in expression of a protein responsible for disposal of apoptotic cells (engulfment cell motility protein 2) with mechanical stress at 2800 µatm. Changes to the normal apoptotic response could be detrimental to oysters, highlighting the importance of considering other environmental conditions when examining biological impacts of ocean acidification

Further evidence of the interaction of multiple stresses on the proteomic response is how proteins that were differentially expressed in the response to ocean acidification alone (compared to 400 µatm) and in the response to mechanical stress at 2800 µatm underwent a change in relative levels. In response to elevated *p*CO2 alone, expression levels of proteins involved in carbohydrate metabolism were increased. Proteins in this pathway were expressed lower at 2800 µatm in conjunction with mechanical stress, compared to 2800 µatm exposure alone. Specifically, in oysters exposed to elevated *p*CO2 alone, there was increased expression of a protein involved in anabolism of carbohydrates (succinyl-CoA ligase subunit B mitochondrial)and decreased expression of a fucose metabolism protein (α-L fucosidase). In contrast, oysters exposed to the additional mechanical stress at 2800 µatm exposure demonstrated a decreased expression of a carbohydrate anabolism protein (6-phosphogluconolactonase) and greater expression of α-L fucosidase compared to 2800 µatm exposure alone. These data suggest that when an oyster is exposed to both environmental stressors used in this experiment a tipping point was reached where there were not sufficient resources for carbohydrate anabolism and carbohydrate stores were needed to meet the energetic demands imposed by the stress. Given the resources needed to sustain a response to an environmental stress, this phenomenon could be detrimental. Another example is with serine-pyruvate aminotransferase, a protein in the tricarboxylic acid cycle. Serine-pyruvate aminotransferase was expressed at lower levels under elevated *p*CO2 alone, but, in comparing oysters exposed to elevated *p*CO2 to those exposed to mechanical stimulation at elevated *p*CO2, expression was increased. Stress from mechanical stimulation impacts expression of proteins involved in many of the processes that were influenced by elevated *p*CO2, further illustrating the potential synergistic impacts of these two stressors on oyster physiology. Together these protein expression patterns indicate the complex nature of how multiple stressors influence physiology and how exposure to additional stressors (i.e. increased temperature or disease exposure) in combination to ocean acidification could have significant implications for survival and potential for adaptation.

### Conclusion

Even though ocean acidification is frequently portrayed as detrimental to marine calcifiers, its effects on invertebrates range well beyond changes to the calcification process. In this study, a wide variety of processes and responses were assessed in the Pacific oyster’s response to elevated *p*CO2 to better understand the physiological trade-offs that occur during this particular stress response. Shell growth was not affected by ocean acidification after one month, but elevated *p*CO2 did affect the integrity of the deposited shell material. Relative amounts of fatty acids, which are necessary for continued survival and execution of other energy-consuming processes, were also unaltered at elevated *p*CO2. Mortality in response to acute heat shock remained unaffected as well. The proteomic profile of *C. gigas* gill tissue was significantly altered by ocean acidification, elucidating the molecular physiological costs of elevated environmental *p*CO2. From a proteomics perspective, ocean acidification also affected *C. gigas*’s response to an additional stress event. Shell integrity and response to a second stress become important in a dynamic environment, or when there are multiple predators, and in this way ocean acidification may decrease *C. gigas* fitness under chronic exposure.

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# Appendix A: Exploration of enzymatic activity of Pacific oyster antioxidant proteins in response to ocean acidification

## Abstract

## Introduction

## Methods

*Carbonate Chemistry*

This experiment was conducted at the Friday Harbor Labs Ocean Acidification Environmental Laboratory, Friday Harbor, Washington, USA where oysters were exposed to pCO2 values of 400 µatm, 1000 µatm, or 2800 µatm.  The system and control of water chemistry has been previously described in detail (O’Donnell et al., 2013; Timmins-Schiffman et al., 2013a). Briefly, incoming water was filtered (0.2 µm) and stripped of CO2. As the water flowed into the different treatment tanks, CO2-free air and CO2 were added back to reach set points that were continuously monitored by a DuraFET III pH probe (Honeywell, Morristown, NJ, USA).  From the treatment tanks, water flow into the eight replicate chambers for each of the three treatment levels was 57.5 mL/min.  For this experiment, set points were calculated for 13°C and estimated total alkalinity (AT) of 2100 µmol/kg for pCO2 values of 400 µatm (pH 8.03), 1000 µatm (pH 7.67), and 2800 µatm (pH 7.24).

*Oysters*

Adult oysters (average shell length ± s.d. = 51± 5 mm, average width = 38 ± 6 mm) collected from Oyster Bay, Washington were maintained in 3.5 L chambers (n = 6 oysters per container) and acclimated for two weeks (T = 13°C, pH = 8).  The oysters originated from the same spawning event in March 2011 from approximately 25 broodstock oysters.  Oysters were fed 120,000 cells per mL per day of Shellfish Diet 1800 (Reed Mariculture, Campbell, CA, USA).  Containers were cleaned every other day with freshwater to prevent fouling.

At the end of 29 days, oysters were shucked and gill tissue was dissected and immediately flash frozen in liquid nitrogen.  Before protein extraction, tissues were ground to a powder in liquid nitrogen using a mortar and pestle.

*Glutathione S-transferase: protein extraction and enzymatic assay*

For the glutathione S-transferase (GST) assay, oysters gill tissue was extracted using Triton® X-100 (Bio Rad, Hercules, CA) extraction buffer for 4 oysters from the 400 µatm treatment, 4 from the 1000 µatm, and 2 from 2800 µatm. Whole tissue samples (18.7-98.4 mg) were homogenized on ice using an Ultra turrax (Kinematica, Luzern, Switzerland) in 300 µl of Triton buffer (10 mM PBS, 1 mM EDTA, and 50 µl of Triton®  X-100).  An additional 100 µl of the Triton buffer was used to rinse remaining tissue homogenate off of the Ultraturrax.  The Ultraturrax was rinsed in two separate baths of deionized water between samples.  The homogenate was incubated for 40 minutes at room temperature and then spun at 3000xg for 1 hour at 4°C.  The upper layer (excluding the pellet) was pipetted into a clean tube and then centrifuged again at 10,000xg for 45 minutes at 4°C.  The final supernatant (excluding the upper lipid layer) was transferred to a clean tube and stored at -80°C until analysis.

GST enzyme activity was measured using the Glutathione S-Transferase Assay Kit (SIgma, St Louis, Missouri, USA) following the manufacturer’s instructions for the 96-well plate.  Twenty microliters of undiluted protein extraction was used for the assay.  All samples were measured in triplicate.  The assay was read on a Synergy HT spectrophotometer at 340 nm (BioTek, Winooski, VT, USA) with Gen5 v. 2.03 software (BioTek Instruments Inc.) with readings every 50 seconds over a 20 minute period.

*Superoxide dismutase: protein extraction and enzymatic assay*

Protein extractions were executed similarly to the GST assay for superoxide dismutase (SOD) activity except a house-made lysis buffer was used instead of Triton buffer.  Total protein was extracted from gill tissue (23.3-96.8 mg) from 4 oysters from the 400 µatm treatment, 4 from 1000 µatm, and 6 from 2800 µatm.  The lysis buffer is described in Guévélou et al. (2013) and the extraction method was carried out exactly as described above for the Triton buffer.

The activity of SOD was measured using the 19160 SOD determination kit (Sigma) following the manufacturer’s instructions.  Samples were diluted 1:2 in the kit dilution buffer before use in the assay.  A standard curve was prepared using superoxide dismutase (SOD bovine Sigma Reference S7446) diluted to 20, 8, 4, 2, 1, and 0.5 U/mL.  All samples were run in triplicate.  The assay was read on a Synergy HT spectrophotometer at 450 nm (BioTek) with Gen5 v. 2.03 software (BioTek Instruments Inc.) over a 20 minute period with readings every 1 min, 10 sec.  A single time point corresponding to the linear portion of the curve for each sample (at 15 minutes, 10 seconds) was chosen to calculate SOD activity.

*Protein Concentration*

For protein concentration, protein samples were diluted 1:10.  Protein concentration was determined using the Bio-Rad DC Protein Assay kit following manufacturer’s instructions, with a standard curve from 0-1500 µg/ml of BSA.  Each sample was run in triplicate and absorbances were read at 750 nm on a Synergy HT spectrophotometer (BioTek) using KC4 v. 3 software (BioTek Instruments Inc.).  If the triplicates had a coefficient of variation greater than 20%, then the outlier was removed from the average of protein concentration.

*Statistical Analysis*

All statistical analyses were executed in R (R Development Team 2013. To calculate GST activity, the first 5 data points were excluded to ensure linearity of the kinetic curve.  The change in absorbance (ΔA) was calculated with the following:

ΔA = (Af-Ai)/(Tf-Ti)

Where A = absorbance at 340 nm, T = time point, the subscript f = final and the subscript i = initial.  GST specific activity is then calculated by the equation:

([ΔA]\*0.2mL)/(5.3 mM-1\*0.02mL)

where 0.2 mL = reaction volume, 5.3 mM-1 is the extinction coefficient for CDNB in a 96-well plate, and 0.02 mL is the enzyme volume.  For each sample, GST activity was divided by sample concentration to achieve an activity expressed per mg of tissue (µmol/mg/min).

SOD activity is measured as an inhibition rate of the assay.  The inhibition rate is:

{([Ablank 1]-[Asample])/(Ablank 1)} \* 100.

Blanks 2 and 3 were also run, as per the manufacturer’s protocol, but since their kinetic slopes = 0 they were excluded from the calculations for not contributing significantly to the background SOD activity.  Replicates were excluded (1 each for 6 samples) if linearity of the reaction curve was not achieved by the time point 15 min, 10s.  The linear relationship between % inhibition and ln[standard concentration] was plotted and the equation for the line of best fit was used to calculate U/ml of SOD (based on % inhibition) for all of the samples (Supplementary Figure AppA.1).

For GST and SOD activity, effect of treatment on enzyme activity was explored with a one-way ANOVA with treatment as a fixed factor and significant differences were further investigated using Tukey’s HSD.

## Results

*Glutathione S-transferase*

Mean GST activity (mean *±* 95% confidence interval) was 0.0198 *±* 0.0098 µmol/mg/min for oysters in the 400 µatm treatment, 0.0156 *±* 0.0078 µmol/mg/min for oysters from 1000 µatm, and 0.0161 *±* 0.093 µmol/mg/min for oysters from 2800 µatm (Figure AppA.1).  There was no significant difference in GST activity among treatment groups (F=0.3517, p=0.7893).

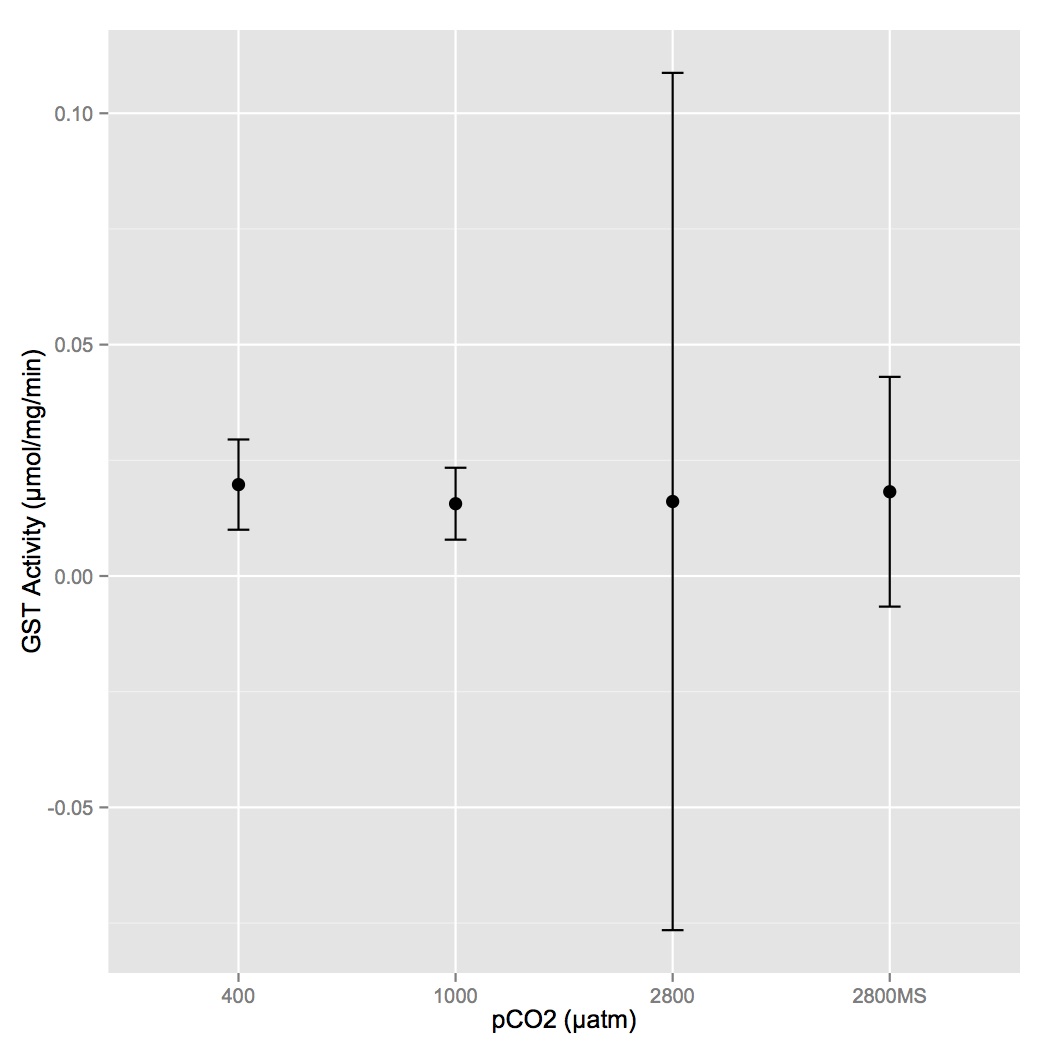


Figure AppA.1*.* Mean GST activity per treatment with 95% confidence intervals.  There is no statistical difference among the treatments.

*Superoxide dismutase*

Mean SOD activity (mean *±* 95% confidence interval) was 0.75 *±* 0.3 U/mg for the 400 µatm oysters, 1.05 *±* 0.7 U/mg for the 1000 µatm oysters, 2.60 *±* 0.4 U/mg for the 2800 µatm oysters (Figure AppA.2).  There was significant effect of treatment on SOD activity (F=17.011, p=3.122E-5).  This difference was found to be due to the elevated SOD activity in oysters exposed to 2800 µatm compared to the other two treatments (p < 0.05), which were not different from each other.

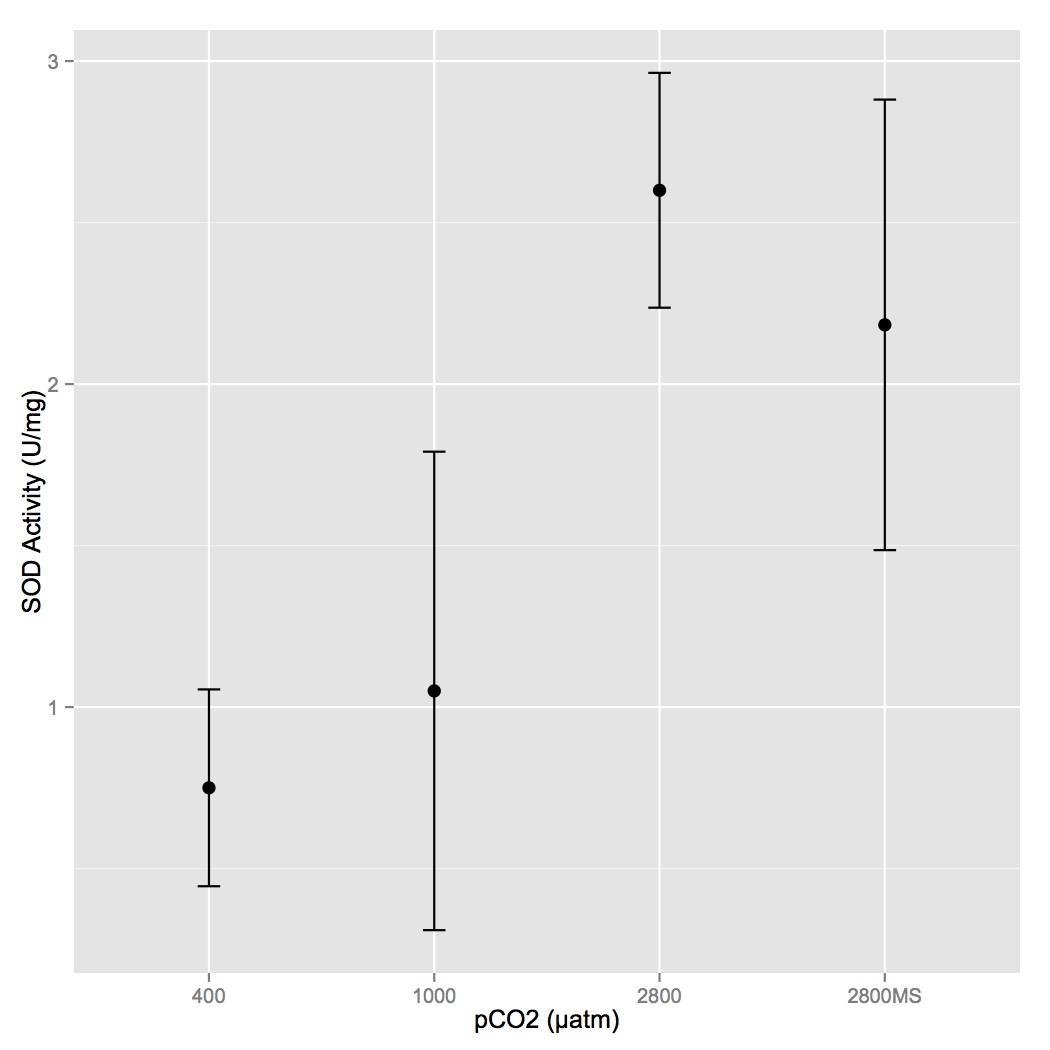


Figure AppA.2. Mean units of SOD per mg of tissue with 95% confidence intervals for each treatment.  SOD activities in 2800 µatm and 2800 µatm + mechanical stress are statistically different from those in 400 and 1000 µatm.

## Discussion

# Synthesis and Conclusion