Variable effects of a Sub Lethal 'Heat Wave' on juvenile Olympia Oysters (*Ostrea lurida)* previously exposed to Low pH conditions

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Abstract:

With more anthropogenic CO_2 equilibrating with the oceans and decreasing the concentration of carbonate ions, ocean acidification is a current concern for all marine organisms, especially those that build calcium carbonate shells like the Olympia Oyster. This study looks at the effect of elevated pCO_2 levels with a subsequent heat shock on the mortality rates of juvenile Olympia Oysters. The oysters were bred from adults at Totten Inlet. The juveniles were exposed to pH levels of 7.27, 7.64 and 8.02 and then put through a sub lethal and lethal heat shock. Unexpectedly, the oysters from the lowest pH level seemed to have higher survival rates than the oysters from the highest pH level. This could be attributed to the release of the chaperone protein that can help the organism with a secondary stress.

Introduction:

The input of anthropogenic CO_2 into the atmosphere has and will continue to cause relatively rapid temperature increases and changes in other climatic factors (IPCC 2014, NCA 2014). Climate change acts on the ocean by elevating water temperatures at the surface and eventually at depth, by changing local current regimes and heat transfer, and by increasing the acidity of seawater (Catia et al. 2008, IPCC 2014, Waldbusser and Salisbury 2013). Marine calcifiers are organisms that build a calcium carbonate shell from the surrounding sea water. This final process of increased acidity affects marine calcifiers by creating an undersaturated CO_3^{2-} ion seawater solution, which causes the creation of calcium carbonate for shell formation and homeostasis to require more energy (Byrne et al. 2011). For the deposition of calcium carbonate, there is a certain amount of energy that the primary ions have to acquire before they can form the parent molecule. The increase of H^+ ions and the decrease in CO_3^{2-} makes the energy barrier much higher for the calcifying organism to overcome. Along with ocean acidification, the warming of the ocean and terrestrial environment is also expected to have consequences for marine calcifiers, particularly for those located in the near shore and intertidal environment because they experience greater heat extremes and exposure to solar radiation (Byrne et al. 2011, Waldbusser and Salisbury 2013).

On the West Coast and notably in estuaries like the Puget Sound, upwelling causes input of variable and sometimes extremely low pH water into coastal and near-shore environments (Byrne et al. 2010, Feely et al. 2010). Just off the coast of Washington, surprisingly low pH waters of around 7.6 were found, which is three times lower than the global average (Feely et al. 2008). Other contributing factors such as high respiration of oxygen with the release of

carbon dioxide, low circulation, and anoxia can further exacerbate the stresses of low pH in an estuarine environment, making these areas especially vulnerable to ocean acidification (Feely et al. 2010, Waldbusser and Salisbury 2013).

These stressors have broad implications for bivalves in the Northwest and Puget Sound, which will face a twofold challenge of warming and low pH directly influenced by climate change. An organism of concern is the native Olympia Oyster, a historically overfished oyster coping with diminished populations and low recruitment. For the Northwest, this oyster provides critical ecosystem services such as filtering the seawater of phytoplankton, creating habitat sustaining oyster reefs, and contributing to a local shellfish industry worth billions of dollars (Coen and Luckenback 2000, FAO 2010). Previous experiments have confirmed the oyster's larval survival rate, juvenile growth and survival rate were reduced in low pH conditions (Timmins-Schiffman et al. 2012). Heat stress has also been found to cause decreased disease resistance or fitness (Encomio 2005). Despite the importance of the oyster as a fixture of the Northwest and a potential sustainable economic resource, little is known about the combined effects of low pH conditions and heat stress over variable timescales or intensities. There remains only a relatively small body of studies on the Olympia Oysters in response to climate change compared to other marine calcifiers like the Pacific Oyster or the California Mussel (White et al. 2009).

We investigated the effects of low pH and warming on the Olympia Oyster, first exposing the oysters to low pH conditions, followed by subjecting them to high sub lethal and lethal temperatures over several days. In their natural environment, it is common during summer months (when upwelling and temperatures are increased) for oysters to experience weeks of low pH conditions followed by a heat wave which puts two different stressors on the organisms (IPCC 2014).

We simulated such stressful conditions to test their effects on survival of oysters that had been previously exposed to low pH waters compared to oysters from high pH waters. We posited that the effects of low pH would produce an energetic stress on the oysters thereby weakening their tolerance to cope with a secondary stress. A previous experiment on Pacific Oysters, however, studied the release of heat stress protein 70 and discovered that Pacific Oysters previously exposed to sub lethal air temperatures had better survival rates in subsequent lethal temperatures than oysters without that pre exposure (Encomio 2005). A similar response in our oysters may give an alternative hypothesis: Juvenile Olympia Oysters that have experienced low pH conditions may prove more resistant to additional stressors than oysters without that previous exposure. Though far from exact in mimicking real conditions in a typical Puget Sound near-shore environment, we hope the degree of control with this experiment will shed new light on oyster response to elevated extremes in pH and temperature driven by climate change. In conclusion, this study will look at the effects of heat shock on juvenile Olympia Oysters raised in variable pH conditions.

Methods:

Oysters:

The juvenile Olympia Oysters (*Ostrea lurida*) were collected from Totten Inlet Floating Upwelling System from the Taylor Shellfish Farm during the summer and brought to Friday Harbor Labs. The oysters were kept in a flow though system of filtered seawater from Puget Sound with temperatures of ~12°C and 7.8-7.9pH. The organisms averaged about 27 mm in length and about 19 mm in width and 6 mm in depth. The oysters in the pretreatment of the different pH levels for 9 weeks are from an unpublished experiment studying the effects of ocean acidification on eelgrass growth and oysters (Gover 2014). The pH and temperature levels had been kept the same as our conditions except for a UV light which the eelgrass needed for growth. This caused algae to grow on the oysters, but the plant material quickly died away when the oysters were moved to our reservoirs.

Pre Experiment with Temperature Tolerances:

The pre experiment was developed to discover the lethal and sub-lethal temperatures for juvenile Olympia Oysters. Ideally, the lethal temperature would have 90% mortality and the sub-lethal would have 0% mortality. On October 15, 2014, 100 juvenile Olympia Oysters were collected from ambient sea water and separated into 5 groups of 20 for the first of two trials. Each organism was marked with nail polish with the symbol of their group. Then, each group was placed in different temperature as follows; 15°C, 21°C, 26°C, 30°C and 34°C. The three warmer temperatures were made with heat baths and the heat bath temperatures varied by about half a degree through the experiment. The 21°C water bath was left at room temperature and the 15°C was reached with a RM20 Lauda Brinkman chiller. By using the heat baths, the temperature was ramped to target point in about one hour. For three days, the oysters were put in their respective water baths in sterile sea water in the morning. The pH of the sea water was raised from 7.7 to about 8.0 and the oysters were fed 7 milliliters of algae with a dilution factor of about 100,000 cells per milliliter. The sea water was also kept aerated by air stones and the light exposure matched current day and night conditions. After 10 hours, the oysters were analyzed for mortality and the dead individuals removed. At night, the oysters were placed in the 15°C water bath in new sterile sea water. After the heat shock treatment, the oysters were observed in 15°C sea water at the ambient pH level of about 7.8 for four days to see any delayed mortality effect.

By the end of this heat treatment, there was no mortality, so the temperature had to be raised for the second trial. The same water baths and chiller were used during the second trial and started the night of October 20, 2014. Another 100 juvenile Olympia Oysters were collected

from ambient sea water and separated and marked with nail polish. This time the temperatures were as follows; 15°C, 36°C, 38°C, 41°C and 43°C. The pH was kept the same as ambient sea water for this trial to make sure a pH shock would not be a confounding variable. Otherwise, the routine was the same as the first trial. During the first day, all 20 of the oysters in 43°C died and during the second day all of the oysters in 41°C died. Finally, after the third day, four of the oysters in 38°C died. All of the juvenile Olympia Oysters in 36°C survived. It appeared that the lethal temperature was 41°C and the sub-lethal was 36°C.

Main Experiment:

Seawater Chemistry Manipulation:

The experimental system for pre-treating the juvenile oysters is maintained with a flow-through set-up, where seawater enters the system and is filtered down to 0.2 microns and passed through UV light for complete sterilization. The CO_2 is drawn out of the water with CO_2 depleted membrane contactors under partial vacuum. For our experiment testing, three treatments were chosen at the pH levels of 7.27, 7.64 and 8.02 which is equivalent to CO_2 levels of about 1600, 1000 and 400 micro-atmospheres of CO_2. The set-point pH levels were determined by average CO_2 concentrations in Puget Sound waters during the summer season (Moore 2012). The larval Olympia Oysters were held in 3-L microcosms in a temperature controlled water bath or cooler. Ambient air is stripped of CO2, compressed and used to aerate the seawater in the reservoir. The reservoir pH is monitored with a Durafet pH probe. When the pH level strays from the set point, a solenoid allows more or less CO2 to be injected with the ambient air. The sterile seawater at set pH and temperature was pumped from the reservoir into the oyster microcosms through irrigation drippers. An outflow pipe was fitted at the 3-L mark on the side of the microcosm and drained straight through the system.

Olympia Oyster Manipulation:

By measuring mortality rates of the juvenile oysters during and at the end of the experiment, we learned how juvenile Olympia Oysters are affected by a three day heat shock when they have been exposed to variable pH levels for different temporal intervals. In order to maintain proper conditions for the pre-treatment, the seawater was tested with a spectrophotometric technique to calibrate the Durafet pH probe and then once a week to test if the pH reading was accurate. The total alkalinity was also tested once a week with open cell titrations. Two modified coolers, A and B, were used as reservoirs for the water baths. The coolers A and B have precise control of partial pressure of carbon dioxide and temperature and were set at pH levels of 7.27 and 7.64 respectively. The coolers had 6 microcosm chambers with the juvenile Olympia Oysters set on the bottom. Each 3-L container or microcosm has 45 juvenile Olympia

Oysters. The experimental flow chart is shown in Figure 1. There were two microcosms (A1 and A2) that were kept at a pH of 7.27 for nine weeks and another two microcosms (B1 and B2) that were kept at a pH of 7.64 for nine weeks. There were four microcosms (A3 through A6) that were kept at a pH of 7.27 for three weeks and another four microcosms (B3 through B6) that were kept at a pH of 7.64 for three weeks. Finally, there were two heat shock control groups (HC1 and HC2) kept in a different cooler C, which was kept at a pH of 8.02 and then put through the heat shock treatment. There was also a microcosm (C) that was kept at a constant pH of 8.02 in cooler C and a water temperature of 11°C throughout the entirety of the experiment. These coolers were kept at their respective pH levels and ambient sea water temperature for three weeks.

After the three weeks in these pre-treatment conditions, the heat shock treatment began and the juvenile Olympia Oysters were marked in two groups of 20 from each microcosm and then put in beakers of the same volume. Beakers were chosen so that the oysters could all fit on the bottom in a single layer and be exposed to the heat in the same way. From each microcosm of 45 oysters, 20 were put in a lethal temperature bath and 20 were put in a sub-lethal temperature bath. The extra five oysters from the 45 original oysters in each microcosm were kept aside and measured for morphological differences of total weight (wet and dry), total length, total width and total depth. In total, there were four water baths with two of them set at the lethal temperature of 41°C and the other two set at the sub-lethal temperature of 36°C. Each morning of the heat shock treatment, the juvenile Olympia Oysters were put in their separate beakers and into their specific water baths. After 10 hours of being in the water baths, the beakers were removed and the oysters will be returned to their microcosm in their specific pH reservoirs (A, B and C) and kept at a temperature of 15°C. The oysters were monitored four times a day and pH and temperature levels were recorded at each interval. Then the sea water in each beaker was replaced with new room temperature sea water at the target pH level. The heat shock treatment continued for three days and then oysters were kept in the stable pH coolers for another four days at a temperature of 15°C for the observation period to look for any delayed mortality response.

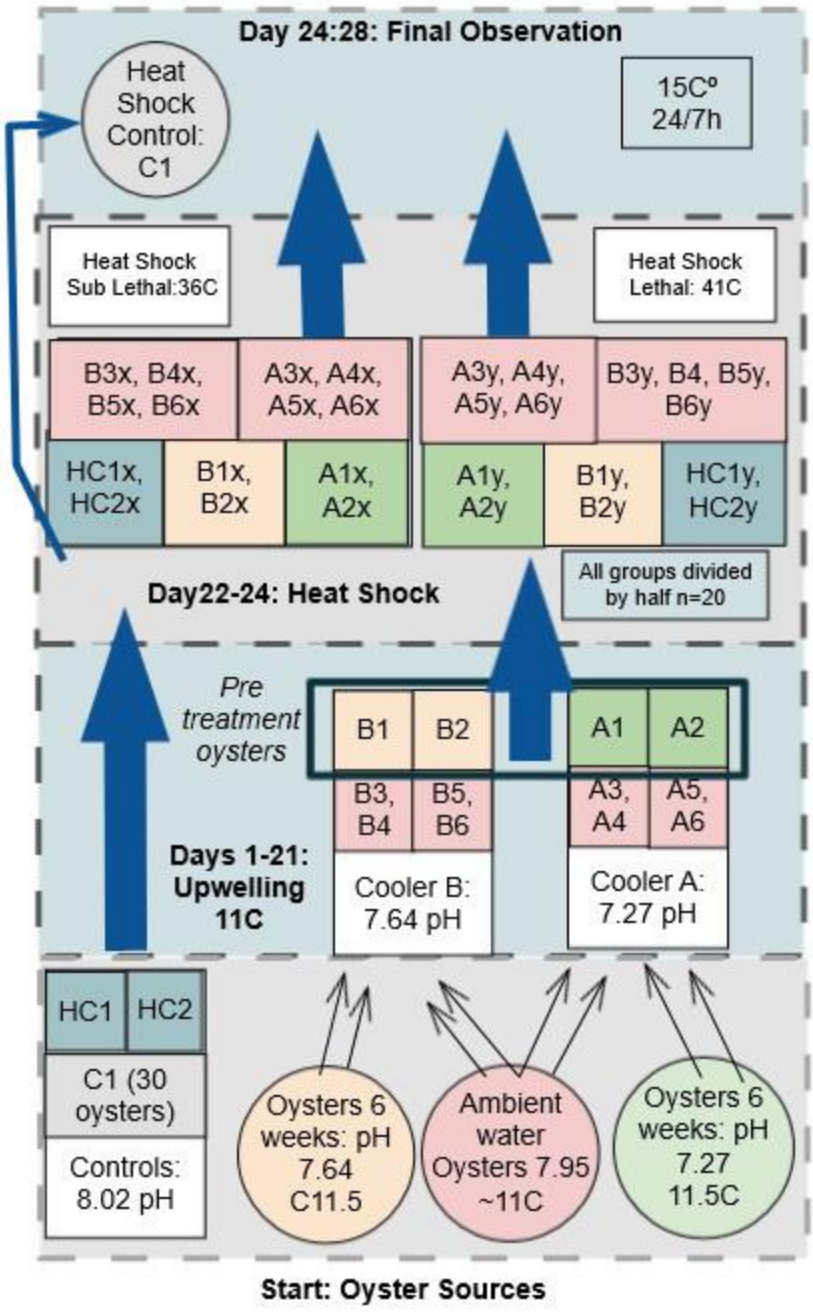

Figure 1: A flow chart that lays out the experimental design. The chart runs on a temporal scale from the bottom to the top.

Results:

Determining Sub lethal and Lethal Temperatures:

In the first trial of the pre-experiment, with temperatures between 24°C and 34°C, there was no mortality in the 20 oysters per group. In the second trial (conditions illustrated in Figure 2), all of the oysters in the 43°C water bath died in the first day. The second day, 16 of the 20 oysters in the 41°C water bath died. On the final day of the heat shock, there was 20% mortality for the oysters in the 38°C water bath and the rest of the oysters died from the 41°C water bath. From these results, the sub lethal temperature limit was chosen to be 36°C (where there was 0% mortality) and the lethal temperature limit was chosen to be 41°C, which had 100% mortality over the course of three days.

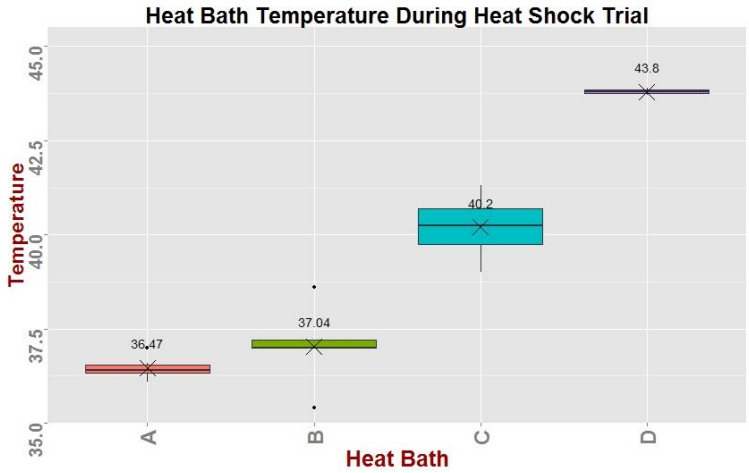

Figure 2: A box and whiskers plot of the temperatures of the heat baths during the second trial of the pre-experiment. Temperature is in Celsius on the y-axis and the specific heat bath is on the x-axis.

Maintenance of Main Experimental Conditions:

The conditions of the coolers were maintained effectively throughout the pre-treatment. Figure 3 shows that both the temperature and pH stayed very close to the target settings for each cooler throughout the study. The mean pH level was consistently near target throughout the experiment (Figure 3a). The temperature was consistently around 11 degrees Celsius and stayed within a degree for all treatments (Figure 3b). The pH was also checked with a spectrophotometer to clarify if the measured pH was close to the real pH. There was a strong correlation between the measured pH levels from the Durafet probes to the actual pH level from the spectrophotometer ($r^2 = 0.97$), showing that the measured pH from the Durafet probes is very accurate. The microcosms, however, had an average of a positive 0.158 offset from the target pH level, determined by spectrophotometry. Incoming salinity of seawater from

the pumping station stayed between a range of 30.27 and 31.04 with average salinity at about 30.75 as measured in PSU.

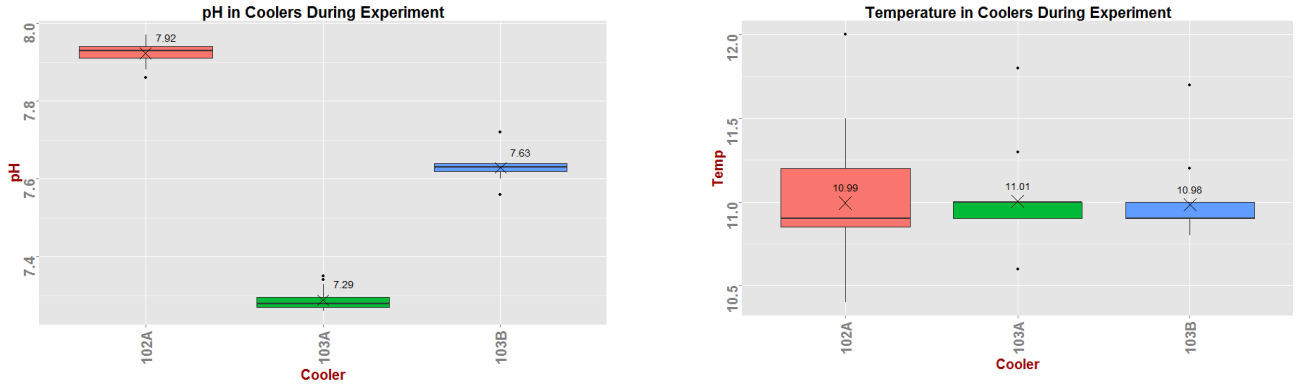

Figure 3: Mean pH levels (left graph, a) and mean temperatures (right graph, b) for the three treatments over the three weeks with measurements taken daily. The X's represent the mean and the middle lines represent the median. The range of the box holds the standard deviation.

Heat Shock Experimental Conditions:

Figure 4 shows that the temperature and pH of the heat baths during the heat treatment were maintained and kept distinct between treatments. The temperatures for the heat baths were precise and accurate with less than one degree of variance (Figure 4a). The pH of the different groups in the heat baths had larger ranges, but still were different (Figure 4b).

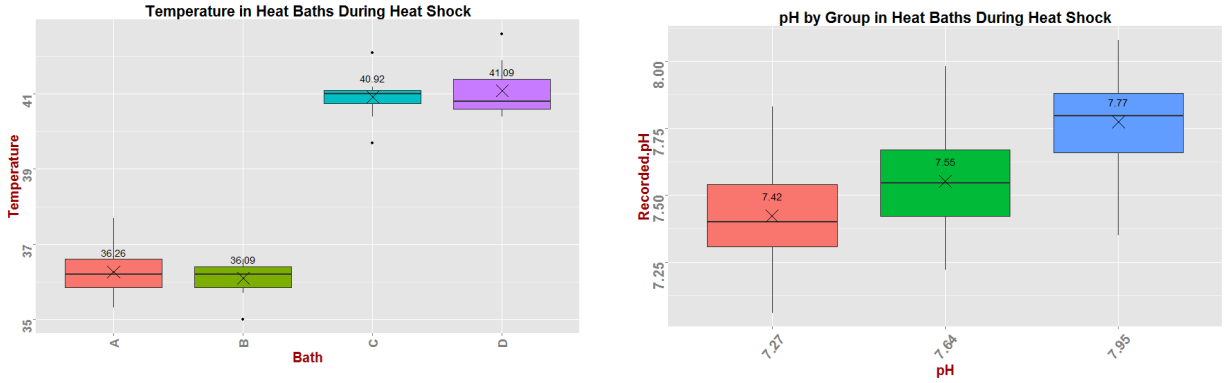

Figure 4: Mean temperature levels of the heat baths during the heat shock treatment (left graph, a) and the pH range for each group during the heat shock (right graph, b). The X's represent the mean and the middle lines represent the median. The range of the box holds the standard deviation.

Figure 5 shows the ranges of pH for each of the beakers in the heat shock treatment. These pH measurements were recorded after the room temperature sterilized sea water at target pH had

been in the microcosms for three hours, which meant that the oysters had been respiring and lowering the pH. The air pumps were used to try and counteract this effect and bubble out carbon dioxide. The input of new water caused a slight temperature drop, but the organisms reached target temperature in 18 minutes and the new water was at target pH.

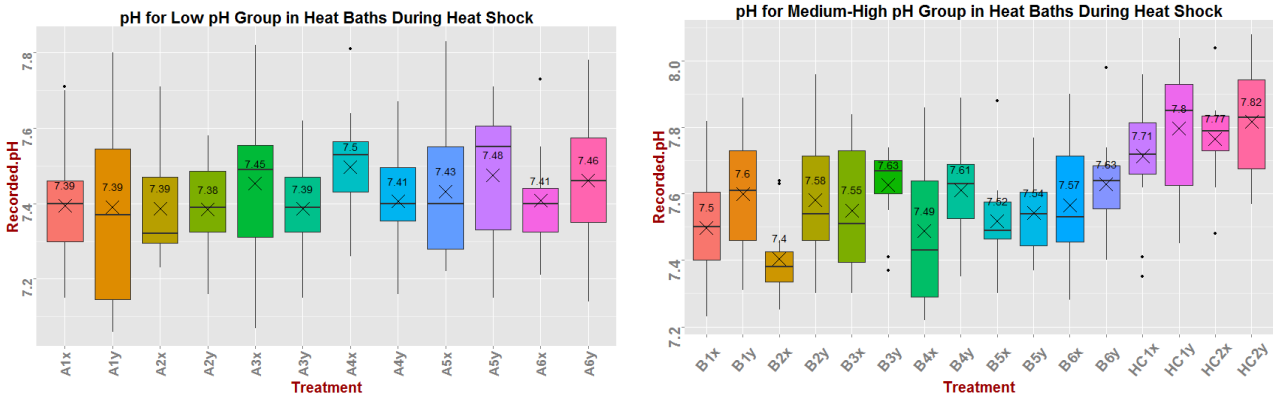

Figure 5 (above): Ranges in pH for each beaker in the treatments. The A's are the lowest pH, the B's are the middle pH and the HC's are the highest pH. The x's on the axis were the in the sub lethal temperature treatment and the y's were in the lethal temperature treatment. The X's represent the mean and the middle lines represent the median. The range of the box holds the standard deviation.

Heat Shock Experiment Mortality:

Mortality over time of the juvenile oysters during the heat shock can be seen in Figure 6. There was a sudden jump in mortality on the evening of the second day, when almost 50% of the oysters in the lethal treatment died. There were very few early deaths in the sub lethal group. Each time period after that, there were fewer deaths in the lethal treatment group. In the sub lethal group, there was never more than one death per beaker per time period.

Figure 7 shows the total proportional mortality of oysters from both pre-treatment groups and at both temperatures. Since the mortality did not seem to be affected by the time length of the pre-treatment, the 3-week and 9-week oysters were pooled together for analysis. Contrary to expectations there was a trend, for the oysters pre-treated with low pH to survive better than the oysters pre-treated in high pH. The proportional mortality of the two extreme pre-treatments was nearly significantly different (one-way ANOVA, $p=0.07$), with the interesting trend of mortality decreasing when the pH of the pre-treatment was low (Figure 7). The sub lethal group had one statistically significant difference ($p=0.03$) between the mid and high pH, but we cannot interpret this trend at this time. Finally, even though there was variability in pH within and among beakers (Figure 8), the mortality in individual beakers was not correlated with the size of the pH range (one-way ANOVA $p = 0.572$, $R^2 = 0.06$) for the lethal bath, or for

the sub lethal bath (p = 0.605, R^2 =0.028). For further information on the mortality in each beaker, see Appendix A.

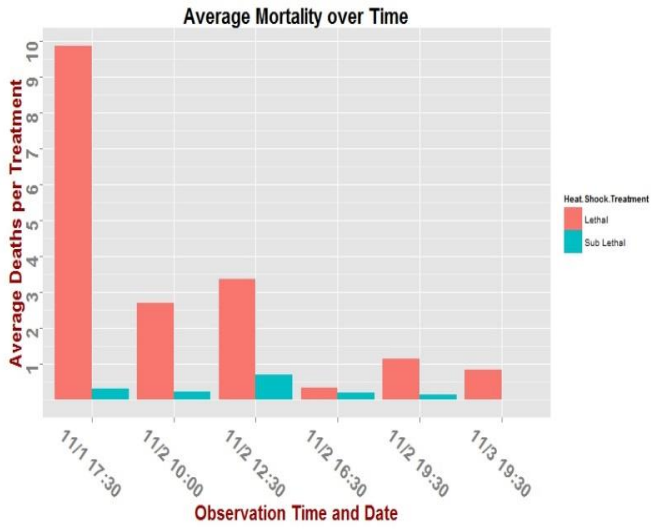

Figure 6 (left): The amount of mortality changing over time with lethal (red) and sub-lethal (black) temperatures. Beakers started with 20 oysters. There were no deaths on the first day and a large percent of the lethal group died on the second evening. Mortality was checked before each water change every three hours.

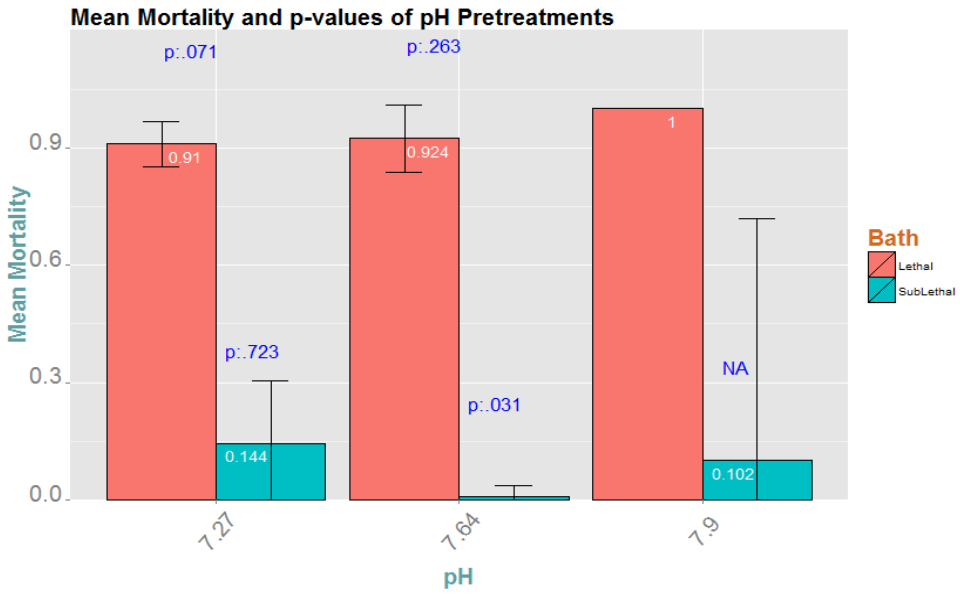

Figure 7: Mortality between the different treatment groups in relation to the three variables of time in treatment, pH of the treatment and the temperature of the heat shock. The p-values are the results of an ANOVA test between each group and the control group.

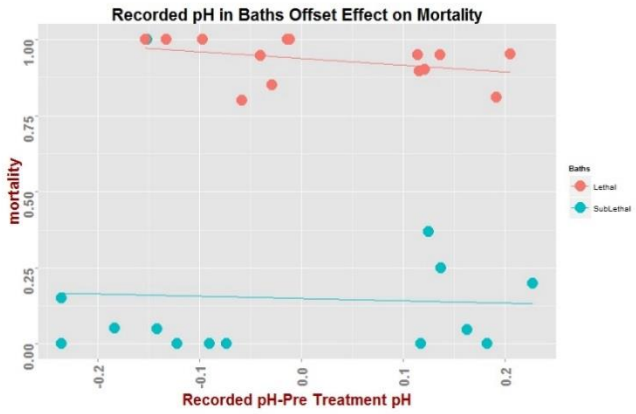

Figure 8: Relationship between the pH and mortality within individual beakers.

Discussion:

The juvenile Olympia Oysters reacted to the lethal heat shock differently than expected. The oysters pre-treated in low pH levels survived slightly better than the oysters pre-treated with high pH levels. We expected the high pH pre-treatment groups to have lower mortality because the organisms had experienced less stress before the heat shock, and therefore should have been more equipped physically to handle a lethal heat shock. Instead, we hypothesize that the oysters from the low pH pre-treatment survived better because they created stress proteins to better prepare their body for a secondary stress in the form of the heat shock. A similar effect was seen in a study by Encomio (2005), where oysters were challenged with lethal and sub lethal heat shocks and then faced with a disease. Those oysters that went through the heat shocks had higher survival rates than those that did not go through a heat shock. Greater tolerance also could result from proteins called chaperones, which include the hsp70 proteins and many other stress proteins (Boutet et al. 2003). The chaperone proteins are produced when an organism is put through a sub lethal stress, and they then help the organism survive another (possibly bigger) stress. It appears that a primary stress such as ocean acidification or upwelling might prepare the organism for a secondary stress like a heat shock. This could be the case for a variety of different factors that affect these Olympia Oysters, both biotic and abiotic.

The sub lethal heat shock experiment produced some curious results. Oysters from the mid-pH pre-treatment group had low mortality compared with both the low and high pH pre-treatment groups; we cannot explain this result.
Our data suggests that length of time in the pre-treatment pH conditions did not affect the mortality rates of the juvenile Olympia Oysters. When put through the heat shock, the 9 week pre-treated oysters had about the same mortality as the 3 week pre-treated oysters. Our power to detect a difference is small, however, because of the small sample size for the 9-week group. It would be interesting to run another experiment with a larger difference in pre-treatment period, and a larger sample size, to see if there is a significant temporal effect.

Conclusion:

In this study, we observed that juvenile Olympia Oysters pre-treated in forecasted low pH levels seemed to survive better under conditions of a lethal heat shock than those pre-treated in a current pH level. In addition, we found that the oysters pre-treated for different time lengths did not have noticeably different mortality rates in lethal and sub lethal heat shocks. It appears that a primary stress such as ocean acidification or upwelling may be able to prepare the organism for a secondary stress like a heat shock. This preparation is most likely associated with different stress proteins released to help the organism retain homeostasis. To evaluate this possibility further, future research should test the mortality in juvenile Olympia Oysters with larger sample sizes and larger differences in pre-treatment pH levels and times.

Appendix A: Raw data from the heat shock experiment. The first column shows the number of beakers in the experiment. The second column displays the pH level of pre-treatment of that group. The third column is what each beaker of oysters was labeled. The fourth column displays the amount of oysters in each beaker. The fifth column shows the mortality percentages from each beaker after the heat shock. The sixth column shows the numerical pH value of the pre-treatment the oysters were in and the last column displays the temporal length of the pre-treatment.

Treatments	Groups	Oysters	Amount	Mortality	pH	weeks
1	Low pH	A1y	20	0.9	7.27	9
2	Low pH	A2y	20	0.95	7.27	9
3	Low pH	A3y	19	0.895	7.27	3
4	Low pH	A4y	20	0.95	7.27	3
5	Low pH	A5y	21	0.952	7.27	3
6	Low pH	A6y	21	0.81	7.27	3
7	HI pH	HC2y	20	1	8	9
8	Med pH	B1y	19	0.947	7.64	9
9	Med pH	B2y	20	0.8	7.64	9
10	Med pH	B3y	20	1	7.64	3
11	Med pH	B4y	20	0.85	7.64	3
12	Med pH	B5y	20	1	7.64	3
13	Med pH	B6y	20	0.95	7.64	3
14	HI pH	HC1y	20	1	8	9
15	Low pH	A1x	19	0.368	7.27	9
16	Low pH	A2x	20	0	7.27	9
17	Low pH	A3x	20	0	7.27	3
18	Low pH	A4x	20	0.2	7.27	3
19	Low pH	A5x	21	0.048	7.27	3
20	Low pH	A6x	20	0.25	7.27	3
21	HI pH	HC1x	20	0.15	8	9
22	Med pH	B1x	20	0.05	7.64	9
23	Med pH	B2x	20	0	7.64	9
24	Med pH	B3x	20	0	7.64	3
25	Med pH	B4x	20	1	7.64	3
26	Med pH	B5x	20	0	7.64	3
27	Med pH	B6x	20	0	7.64	3
28	HI pH	HC2x	19	0.053	8	9

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