Variable Effects of a Sublethal and Lethal 'Heat Wave' on Juvenile Olympia Oysters, *Ostrea lurida,* previously exposed to Low pH Conditions

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Abstract:

Prevalent upwelling in summer months in the Northwest United States can bring anomalously acidified waters to Puget Sound. This, combined with the effects of increasing summer peak water temperatures, poses a joint threat from two separate abiotic factors directly linked to climate change. We investigated the response in mortality of Olympia Oysters, *Ostrea lurida,* to these two factors by pretreating oysters in three different pH conditions for different lengths of time before exposing them to a three-day lethal and sublethal heat shock with different day and night temperatures. We were able to determine the lethal and near-sublethal temperature for Olympia oysters in a three-day heat shock as 41°C and 36°C, respectively. When analyzing mortality, our experiment yielded ambiguous statistical results but did indicate that oysters pretreated in low pH conditions may have less mortality in a lethal heat shock than oysters in high pH conditions. This suggests that Olympia oysters could be able to develop resistance to a primary stress after previous exposure to an unrelated secondary stress. We plan to investigate morphological and structural qualities of these oyster shells to determine what additional effects the various pH pretreatments might have had on the oysters.

Introduction

Anthropogenic CO_2 in the atmosphere has and will continue to cause relatively rapid temperature increases and other changes in climatic and environmental regimes (IPCC 2014, NCA 2014). Climate change acts on the ocean by elevating water temperatures, changing local current patterns and heat transfer in the ocean, and increasing the acidity of seawater (Catia et al. 2008, IPCC2014, Waldbusser et al. 2013). This final process can be expected to affect marine organisms that produce calcium carbonate shells, which depend on readily available carbonate for shell formation (Byrne et al. 2011). Warming of the ocean and coast is also expected to have additional consequences for marine calcifying organisms, especially for those in the intertidal zone such as oysters and mussels, because of greater heat extremes and exposure (Byrne et al. 2011, Waldbusser et al. 2013). On the West Coast of the United States, upwelling causes variable and sometimes extreme low pH deep-water input into coastal and near-shore environments (Byrne et al. 2010, Feely et al. 2010)., Acidified waters with a pH of 7.6 were recently found off the coast of Washington, which is three times lower than the global average (Feely 2008).

Estuaries like Puget Sound can be strongly affected by warming and ocean acidification because of other contributing factors such as high organismal respiration, low water circulation, and anoxia, which can further exacerbate the phenomenon (Grantham et al. 2004, Feely et al. 2010, Waldbusser et al. 2013). These factors make some areas such as Hood Canal especially vulnerable to climate change because of preexisting natural vulnerabilities. These vulnerabilities can be exacerbated in summer months when upwelling and warming are at their greatest and most prolonged (Hoyer 1983).

Climate change has broad implications for bivalves in Puget Sound, which will face a twofold challenge of warming temperatures and decreasing pH (Gibson 2011). An organism of concern is the native Olympia oyster, *Ostrea lurida*, a historically overfished species coping with diminished populations and low recruitment (White et al. 2009). This oyster has historically provided critical ecosystem and economic services in the northwestern United States, such as filtering the seawater of phytoplankton, creating habitat-sustaining oyster reefs, representing cultural value to native peoples, and contributing to a multimillion-dollar shellfish industry (Ramsay J. 2012, Blake 2003, Hudson et al. 2012). Previous experiments with this species have indicated decreased growth and higher energetic demands under low pH conditions (Hettinger et al. 2010, Hettinger et al. 2012, Hettinger et al. 2013). Thermal stress and acidification can affect fitness and homeostasis in other oyster species (Lannig et al. 2010, Gibson 2011). However, there is only a small body of study on the Olympia oyster in response to climate change, and little is known about the effects of acidified ocean conditions combined with heat stress over variable timescales and degrees of intensity on the oyster.

We seek to investigate the effects of ocean acidification and warming on the Olympia oyster by experimentally combining these stressors using two different low pH pretreatments over different durations. We also test the effects of thermal stress and low pH conditions synergistically by concluding with three-day sublethal and lethal heat shocks while the oysters were under low pH conditions. According to one study, increased temperatures and ocean acidification should act synergistically to lower the range of thermal tolerance in marine ectotherms (Partner and Farrell 2008). Therefore we hypothesize that the effects of low pH will produce an energetic stress on their system, thereby weakening their tolerance to cope with a subsequent heat shock of three days. However, Clegg (1998) demonstrated that Pacific Oysters previously exposed to sublethal temperatures had better survival of subsequent lethal temperatures for up to two weeks than oysters without that pre-exposure. Thus an alternative hypothesis is that juvenile Olympia oysters reared in low pH conditions for some time may prove more resistant to additional stressors than oysters without previous exposure. Though we cannot experimentally mimic natural conditions in a typical Puget Sound near-shore environment, completing this experiment while exercising careful control of additional variables should improve our understanding of oyster response to elevated extremes in pH and temperature driven by climate change.

Methods

Oysters:

Juvenile Olympia oysters (*Ostrea lurida*), born in spring of 2014, were taken from Totten Inlet Floating Upwelling System (Taylor Shellfish Farms) during the summer and brought to Friday Harbor Labs. At the end of our experiment the juvenile oysters were about 7 months old. Oysters were kept in an outdoor flow-through tank of filtered seawater with a constant temperature and pH comparable to waters in Friday Harbor of ~11°C and 7.8-7.9pH, referred to hereafter as ambient (Friday Harbor Ocean Acidification Lab, University of Washington). Oysters pretreated with low pH conditions for six weeks prior to our treatment were obtained from another unpublished experiment investigating the relationship between ocean acidification, eelgrass, and oysters (Groner unpubl. data). Treatment of these oysters was entirely the same as our treatment except that Groner's experiment used LED lights (Marineland), which mimicked day and night light conditions, causing some algal growth on the oysters. Upon transferring oysters to our experimental units for the remaining three weeks, the algal growth dramatically reduced.

Seawater Chemistry and Environment:

The juvenile Olympia oysters were held in 3.5-L plastic tubs with transparent lids. Each had a hole with a fitted PVC pipe in the top one-third of the tub, which allowed water to exit the tub when it was full. These tubs ("microcosms") were placed in a temperature-controlled modified cooler filled with the same treated water. The modified cooler was a large insulated plastic cooler with a volume of 100 gallons that contained our treated seawater and had input and output ports near its bottom so treated seawater could constantly move through it. Seawater was drawn from Friday Harbor via the Friday Harbor Labs pumping system, filtered down to 0.2 microns, and passed through UV light for complete sterilization. The CO_2 was degassed from the water with CO_2 depleted membrane contactors under a partial vacuum. Ambient air was stripped of CO_2 by a CO_2 absorption unit (Twin Tower Engineering, Broomfield, CO, USA) compressed, and used to aerate the seawater by a Venturi injector into the cooler. The cooler pH was monitored with a Durafet III pH probe (Honeywell, Morristown, New Jersey, USA). When the probe registered that the treatment's pH deviated from its set point, a solenoid valve would open or close to allow more or less pure CO_2 (Praxair, Danbury, Connecticut, USA) to be injected into the seawater by the Venturi. The Durafet probe information was fed into a Honeywell UDA2182 pH controller. Seawater was pumped from the cooler through irrigation drippers (DIG Industries, Sun Valley, California, USA) into each microcosm at a rate of 1.9-L h-1 and was continually mixed by 5V aquarium pumps, 1 per microcosm. The seawater was tested with a spectrophotometric technique to calibrate the Durafet pH probe about once a week or when the displayed pH deviated greatly from target values. If the probe was significantly off, a new set point was inputted using the value of the actual pH, as measured by the spectrophotometric technique. Daily salinity measurements were taken from the incoming seawater for the lab from Friday Harbor, except when running water samples for which a roaming salinity probe was used. Three treatments were chosen for the experiment at the pH levels of 7.47, 7.84 and 8.22, which is equivalent to CO_2 levels of about 1600ppm, 1000ppm, and 400ppm of CO_2. To obtain these target pH conditions within each microcosm, we offset each pH treatment to be 0.2 units lower than the target pH levels in our coolers. This was done because the effect of offgassing from high flow and mixing within the microcosms elevated pH levels by about 0.2 units.

Thermal Limits Experiment:

In a pre-experiment we determined the Olympia oyster's thermal limits for prolonged, extreme temperature exposure. The temperatures determined in this experiment as lethal or sublethal were then used in the main experiment as our sublethal or lethal conditions. On 10/16/2014, four batches of 20 oysters each, all taken from ambient sweater in the outdoor flow-through tank, were placed in four separate microcosms filled with 3-L of filtered seawater. Respective daytime temperatures in the four microcosms were A: 21°C, B: 26°C, C: 30°C, and D: 34°C and nightly temperatures were 15°C for all microcosms. An additional group of 20 oysters in a fifth microcosm was exposed to a constant temperature of 15°C for the entire duration of the experiment. pH was elevated from about 7.7 to just above 8.0 with sodium bicarbonate and maintained there. pH was monitored when temperature was measured by a Directline DL421 pH probe (Honeywell). Temperatures were maintained during the day by two Thelco (Precision Scientific Co) and one 1230T (VWR Scientific Inc) heat baths with one microcosm in each. The fourth microcosm was kept at room temperature and not placed in any heat bath. At night a RM20 Lauda (Brinkmann) chiller set for 15°C was used. Heat baths and the chiller were calibrated over a period of 24 hours and held at steady temperature within one degree overnight for 12 hours. Afterwards the baths were shut off and allowed to cool before being turned back on to measure the time needed to reach their selected temperature. For their calibration, temperatures were recorded regularly with a DP8891 (Omega) temperature probe for 30 minutes to an hour while adjustments were made and again several hours after to check for stability. During the experiment, temperatures were checked at least three times a day by the DP8891 temperature probe and adjusted as necessary to maintain target temperature. A Quiet Power (fusion) and Silent-AIR X4 (Penn-Plax) air pump were used to oxygenate and circulate the water throughout the day and night in all microcosms. During the day oysters were placed in the heat baths for 10 hours in their respective tubs then transferred back to a single tub at night were they remain for 14 hours. We replaced the tubs with new seawater of 12°C with a pH of about 7.8 twice a day, immediately before any transfer of the oysters. The experiment was run for 7 days, during which daily mortality was measured, with three days of heat shock conditions and four days of observation. Oysters remained in the nighttime chiller at 15°C for the entire four-day observation period.

After three days no oyster deaths were observed in the 21-34°C range so we began a second trial on 10/20/2014. The new respective temperatures were A:36°C, B:38°C, C:41°C, D:44°C, and E:15°C and the nightly temperature remained at 15°C. A third Thelco (Precision Science Co) heat bath and a whisper 600 (Second Nature) air pump were added since we would no longer have a room temperature batch. After temperatures were again fine-tuned and observed for stability, the experiment was repeated as previously with one exception: we did not elevate the pH with sodium bicarbonate to above 8.0. Additionally with greater temperatures, warming from about 15°C to target temperatures took about 2.5 hours on average. At the end of the three days, results were recorded and sublethal and lethal temperatures defined based on mortality in the tubs. The lethal water temperature regime was defined as that which killed 100% of oysters in a group. Sublethal was defined as that temperature which killed 0% of oysters in a tank and is immediately below a temperature with some lethality. The goal was to establish both the sublethal and lethal temperatures given our three-day heat shock regime for the juvenile Olympia oysters. Ample food of 7 milliliters of ~10^8 cells/ml (Shellfish Diet 1800 Instant Algae, Reed Mariculture Campbell CA) was provided daily, and light exposure matched current day and night conditions.

Heat Shock Experiment Methods:

For our primary experiment we tested oysters pretreated with different pH levels and for different lengths of time to lethal and sublethal heat shocks to see if there was a significant difference in mortality between pretreated oyster groups. We used three coolers, 103A, 103B, and 102A, which are represented by the rectangles near the bottom of Figure 2, and were set at pH levels of 7.27 and 7.64, and 7.95 respectively. All pH levels were maintained throughout the experiment at these experimental levels, and all coolers were kept at 11°C during the pH treatment phase. Coolers 103A and 103B held six microcosms each while 102A held three of our microcosm containers for a total of 15 microcosms of oysters with 45 oysters per container. Two microcosms each for coolers A and B (A1:2 and B1:2) were treated in their respective cooler conditions for a total duration of nine weeks while the remaining microcosms (A3:6 and B3:6) underwent this treatment for three weeks. In Cooler C we kept our pH of 8.22 (HC1:2 and Heat Shock Control C1), which was treated for a total of nine weeks. During this time each microcosm of 45 oysters received 15ml of ~10^8 cells/ml (Shellfish Diet 1800 Instant Algae) once daily.

At the end of the three-week pretreatment period we randomly removed five oysters from each microcosm for measurements in a separate study, leaving 40 oysters in each tub. Each microcosm from coolers 103A and 103B plus 2 microcosms from 102A with their remaining 40 oysters were randomly divided into half and placed in beakers, thereby doubling our n value and randomly distributing the oysters in each microcosm to the two treatment groups. For instance, the 40 remaining oysters in microcosm A1 were equally divided into beaker A1x and beaker A1y. We then prepared a lethal and sublethal temperature treatment regime as determined in the thermal limits experiments. We allowed temperatures in the heat baths to stabilize for a period of 12 hours with regular observation of temperatures. All the beakers labeled x went to the sublethal treatment group and all the beakers labeled y went to the lethal group. This totaled to 28 beakers or 14 beakers per treatment. As a summary, our oyster sources are listed at the bottom of Figure 2. In the box above, oysters are shown in the pretreatment phase for three weeks. Following this they transfer to the three-day heat shock before ending in the four-day observation stage. Only the C1 group, which was our heat shock control, did not go through the heat shock, but moved straight to observation.

On the morning of 11/30/14 we began the experiment by removing the oysters from their microcosms, separating them into their treatment beakers, and placing these beakers in the heat baths. We followed the same procedures and equipment used in trial two of the thermal limits experiment, except that 400mL beakers were used during the day instead of the microcosms, and were filled with 250ml of treated seawater matching their original pH levels. To compensate for the smaller volume and keep the seawater fresh, we replenished seawater four times a day, or about every two hours, with seawater taken from the coolers the oysters were in. We allowed the water taken from the coolers to reach room temperature (21°C) to minimize temperature changes when the water was changed. The time for replenished seawater to reach target temperature in the beakers was about 10 minutes. We also used air stones in each beaker, running at very low rates, to ensure that the water remained oxygenated and mixed during the experiment. 10 hours oysters were placed in mesh bags with x or y markers and returned to their respective cooler microcosms. We then fed the oysters, as we had been previously, but reduced the amount of food proportionally depending on the number of oysters left in each microcosm. Throughout the experiment all coolers were kept at 15°C to replicate the pretreatment conditions. pH and temperature were also closely measured in the beakers five times a day and recorded. At the end of the heat shock period on the evening of 11/02/14, we transferred the oysters back into the chillers where they remained under observation for an additional four days after which observed mortalities were compiled into a data set to be analyzed.

Analysis Methods:

Averages and standard deviations were calculated for daily temperature, pH, and salinity in the coolers to determine environmental values for our microcosms. In addition, the pH and temperature average offset between the seawater in the microcosms and the coolers was determined using the same spectrophotometric technique used to confirm pH in the coolers. We plotted target pH and recorded pH to determine an R squared value using a linear regression. Actual temperature averages and standard deviations in heat baths were calculated by the same method from temperature data taken multiple times per day from the heat baths. Recorded pH in the heat baths was also plotted in box and whisker plots. We then subtracted the target pH levels from the recorded values to find the difference. A linear regression was run between this pH offset and percentage of mortality to determine what influence conditions in individual beakers might have had on mortality in the beakers. Length of time exposed to low pH seawater and intensity of exposure were compared in a two-way ANOVA test to determine to what degree each independent variable affected the dependent variable of mortality. All calculations and graphing were performed in R (R Development Core Team 2014).

Results:

Observed Sublethal and Lethal Temperatures for a three day heat shock:

In our first test of lethal temperatures we found no mortality of oysters between 24°C and 34°C. In the second trial, all oysters in the 43°C water bath died in the first day (Table 1). Since 90% of the 20 oysters in the 41°C water bath died by the second day with the remaining dead by the third, we decided to use this as our lethal heat shock as it was proven fatal and did not kill all oysters until the final day (Table 1). It also was only three degrees higher than bath B, which had 20% mortality on the third day, meaning that it couldn't be too lethal. At the end of the observation period no further deaths had been observed. From these results, the sublethal temperature limit was chosen to be 36°C as it had 0% mortality and was immediately below a partially lethal temperature. The lethal temperature limit was chosen to be 41°C with 100% mortality over the course of three days. Our sensitivity range was well within 1°C during our treatment as shown in Figure 3. Because of the temperatures chosen for our thermal limits experiment we were not able to rule out 35°C as the sublethal nor 39-40°C as the lowest lethal temperature.

Maintenance of Pretreatment Experimental Conditions:

The conditions of the coolers were maintained at target levels throughout our experiment. Both the temperature and pH stayed very close to our settings for each cooler with a temperature sensitivity of less than half a degree and a pH sensitivity range of about .05 units (Fig. 4). Mean temperature levels were almost exactly on target with a range of less than a degree for each cooler (Fig. 4a). The pH levels measured with the Durafet probes were highly correlated with the pH levels from the spectrophotometer (R^2 value of 0.97), which shows that the measured pH from the Durafet probes is extremely accurate. The microcosms within the coolers experienced an average positive 0.158 offset from the target pH level as confirmed by spectrophotometry. This was 0.042 units less than we had planned, making the CO_2 microatmosphere values slightly higher than our target, but still acceptably close. During the course of our experiment, incoming salinity of seawater from the pumping station stayed between a range of 30.27-31.04 with average salinity at about 30.75 as measured in PSU.

Heat Shock Experimental Conditions:

The temperature and pH of the heat baths during the heat treatment were kept distinct between treatments (Fig. 5). The temperatures for the heat baths were precise and accurate with less than one degree of variance (Fig. 5a). The pH of the different groups in the heat baths had larger ranges, but still were differentiated as designed (Fig. 5b). Figure 9 in the apendix shows the degree of variability for pH within each beaker. To ensure that the pH variation in the beakers was not affecting mortality, we compared offset from the target pH to the mortality in each beaker using a One-way ANOVA and found no significant effect ($p=0.572$ for the lethal bath, $p=0.605$ for the sublethal). Additionally, because each pH value was recorded immediately before we changed the water, which was 2.5 hours after the previous water change, oysters were not constantly exposed to these conditions. Instead, they represent the lowest pH levels the beakers reached before returning to their target pH levels.

Heat Shock Experiment Mortality:

Average mortality over time for juvenile oysters during the heat shock was closely distributed as we had wanted to see for a lethal and sublethal temperature. Our first day of heat shock is not included in this graph because there was zero mortality in any beakers. The first deaths involved 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 deaths in the sublethal group. At each subsequent sampling period we saw some mortality in our lethal treatment , so that numbers of remaining oysters continued to dwindle until all or almost all oysters had died. In the sublethal group, there was never more than one death per beaker on average for every sampling period. Though some beakers had more mortality in total than we had expected, overall this graph strongly indicated a near sublethal treatment with maximum stress present because mean mortalilty remained below 1 for every sampling period, yet some mortality was observed. Both high pH beakers in the sublethal treatment ended the experiment with a few deaths so this low frequency of mortality observed is not a result of more mortality in low pH treatments. On average, the low pH pretreatment groups exhibited less mortality overall than the high pH groups in the sublethal bath, although this difference was not significant (One-way ANOVA p-value = 0.32) (Fig. 7). Unexpectedly, we also found the mean mortality was slightly lower for the low pH pretreatment group than for the high pH group in the lethal temperature baths (Fig. 7). The p values shown in Figure 7 were generated from One-way ANOVAs, comparing each low pH treatment group to the high pH treatment group. Since we were not able to see any significant differences among groups exposed to the low and medium pH conditions for different lengths of time, we pooled the nine week and three week treatments when analyzing the percentage of mortality in Figure 7. When all three pH groups in the lethal treatment are considered together the model yields no strong differences in mortality among the three groups (p-value = 0.16.), unlike when only the lowest and highest pH is considered (Fig.7). For Figure 8 no clear pattern emerges between groups pretreated for different times. Additionally, because the the pretreatment sample size for the 9 weeks exposure only contained two beakers per heat bath treatment, error bars are large and differences are negligible (Figure 8).

Discussion:

Our experiment demonstrated that there may be a positive effect of pretreatment in low pH conditions on survival of a heat shock, suggesting that oysters acclimated to low pH conditions may be better equipped to cope with additional stresses. While some of our statistical tests were ambiguous, the proper form of analysis is uncertain. pH change is logarithmic and oyster tolerance may have a certain threshold after which the effects of low pH exponentially increase. Thus we cannot assume a linear relationship between pH and survival and do not have enough different pH levels to investigate a nonlinear one. Similarly, although it appears there was no effect of length of pretreatment time on the percentage of mortality from heat shock, our samples sizes may have been too low to pick up any significant differences.

The apparent decline in mortality after low-pH pretreatment was unexpected. One hypothesis is that the decreased mortality could be due to the activation of defensive stress-associated molecules such as chaperonins. If so, it is possible that after a certain amount of exposure to mild stress the degree of beneficial resistance given would not continue to increase. Molecular chaperonins are cellular defensive mechanisms, often in the form of heat shock proteins, which increase the cells' resistance to stress and may help slow premature folding of proteins (Hartl 1996). If defensive stress compounds were increasing resistance to additional stresses then we suspect that even an increased sample size would not show any large degree of difference in mortality between three-week and nine-week groups because the benefits of such defensive molecules could only accrue to a certain extent before their maximum benefit would be attained. More exploration into greater differences in time of exposure to low pH stress would certainly be worthwhile, such as testing several months of low pH exposure compared to several days.

In the thermal limits experiments and subsequent heat shock trial we were able to discover a near sublethal and lethal temperature limit for a three-day heat shock for Olympia oysters. Many thermal limits experiments have only tested an hour or a few hours (Encomio 2005, Clegg 1998, Ivanina et al. 2009). Our experiment looks at extreme water temperatures for a period of three days. For our experiment we were not looking to simulate realistic temperature extremes found or predicted in Puget Sound. We only wanted to create the most stressful conditions possible for three days, giving the oyster a longer time to respond to the intense thermal stress. This could be useful in the future for investigating longer time scales of heat stress and their associated effects on Olympia oysters.

We found we were successful in establishing a lethal and sublethal temperature for the juvenile oysters on our time scale. The time it took for all oysters in the high pH group to die in the lethal treatment lasted till the end of the experiment with some survivorship in other groups. A sudden spike in death with rapid decrease is also what we would expect from a lethal treatment as many in the group are killed, leaving a remaining group of more resistant oysters which experience mortality at a gradual rate. This confirms 41°C as an accurate lethal temperature for a three day heat shock. For our sublethal heat shock we cannot be as sure about the effectiveness of the target temperature. 15% of oysters died in one of the high pH beakers. This was in contrast to the 0% mortality seen in the second thermal limits experiment and was above our goal of 0%. This may be due to the fact that oysters in ambient conditions had been constantly at a lower pH than our high pH oysters. It could also be due to an increase in sample size or other factors we weren't able to measure. For future studies on this species, we would recommend the sublethal temperature used be 35°C to decrease mortality.

We were quite surprised at Olympia oysters' extreme degree of thermal tolerance. We posit that the Olympia oysters must at some point experience temperatures of a similar intensity, or else they would not have developed the ability to so successfully cope with temperatures well above summer ocean temperature conditions. Dethier et al. (2010) found near shore temperatures of up to 16°C and surface temperatures of 18°C where oysters can settle. Temperatures are expected to increase across multiple fronts in the near future due to global warming, so it should be assumed that these temperatures will increase by at least a few °C (NCA 2014). It is important to continue to study the combined effects of low pH and increasing temperatures on the Olympia oyster as they are both a reality in the Puget Sound due to climate change. Because both present environmental stressors to the oysters simultaneously, they must be able interact and affect how oysters cope with such stresses. Our study indicates one such response. A further study of interest for Olympia oysters would be to raise oysters at different pH levels for several months before treating the oysters with elevated temperatures such as 15°C for periods on the scale of weeks in order to see what metabolic effects could be observed.

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Treatment	E(15°C), A(36°C)	B(38°C)	C(41°C°C)	D(43°C)
Mortality	No Deaths	4 Deaths (20%)	20 Deaths (100%)	20 Deaths (100%)

Table 1: Treatment showing the temperature bath, Mortality is percentage and number of deaths per group.

Pre-experiment Flowchart daily cycle

Duration: 3 days (per tub)

N=20 oysters #5N

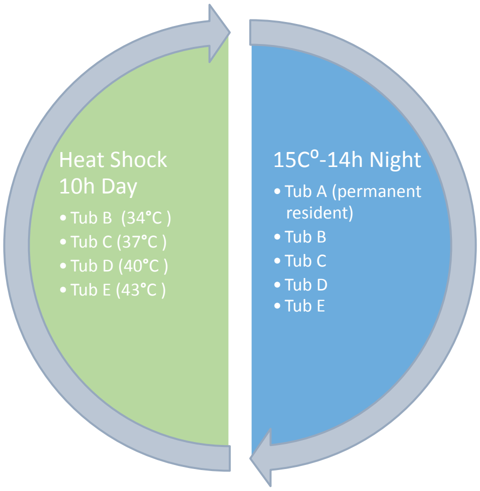


Figure 1: This shows the 24 hour cycle of our heat shock trial experiment with arrows representing the progression. Tubs are the treatment groups with an n of 20.

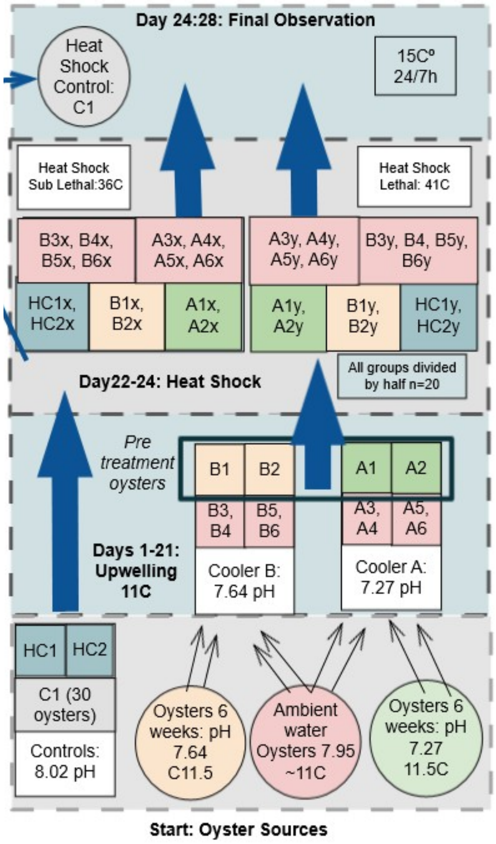


Figure 2: Flowchart marking the progression of our main experiment. Oysters were sourced from 4 different environments in the lowest box. Oysters already being treatment from the Groner experiment are shown in green, tan, and blue. The heat shock control group is in grey. Oysters from the outdoor rearing tanks are shown in pink. Each phase is written bold and arrows show the progression each phase. All pH levels remained as they started and temperature changes are noted when they do occur.

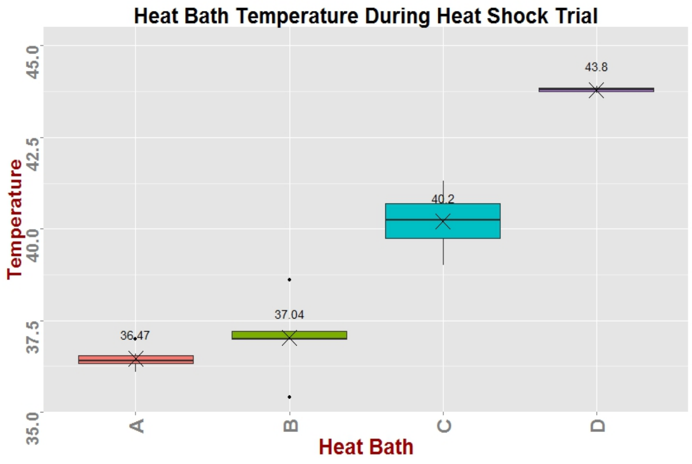


Figure 3 (left): Heat Bath Trial 2 temperatures in box and whisker plots. The x axis is the temperature groups; the y axis is temperature in Celsius. The X's and numbers above the graphs mark the means. Bars in the box are the medians.

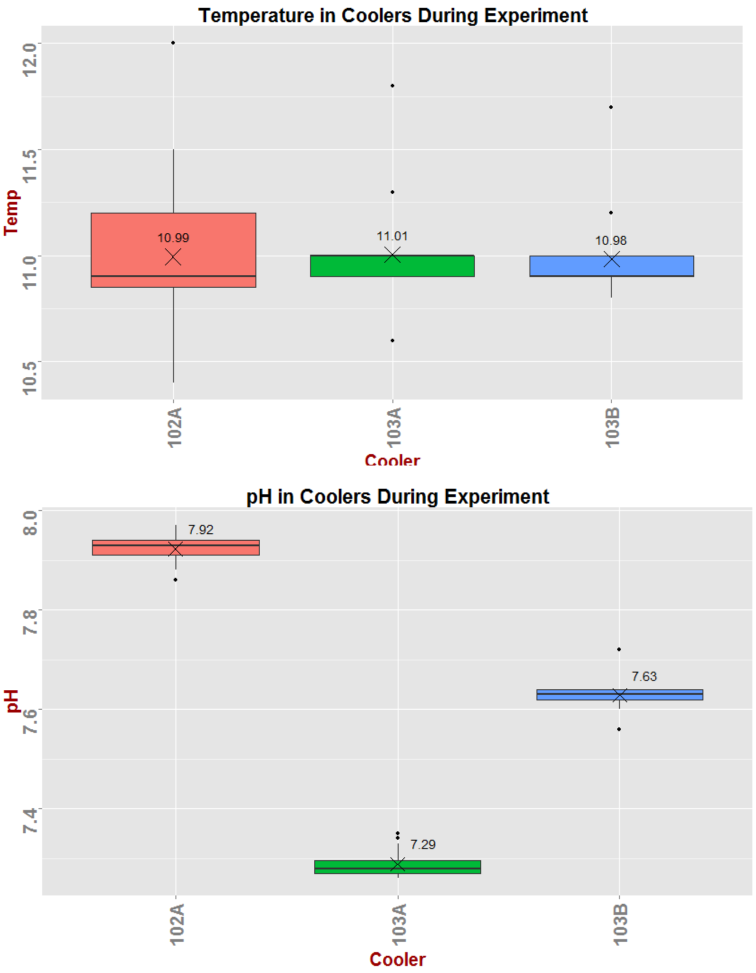


Figure 4: Mean pH levels (top graph, a) and mean temperatures (bottom graph, b) for the three different coolers, each a different treatment over three weeks with measurements taken daily. The X's represent the mean and the middle lines represent the median. The range of the box is the standard deviation.

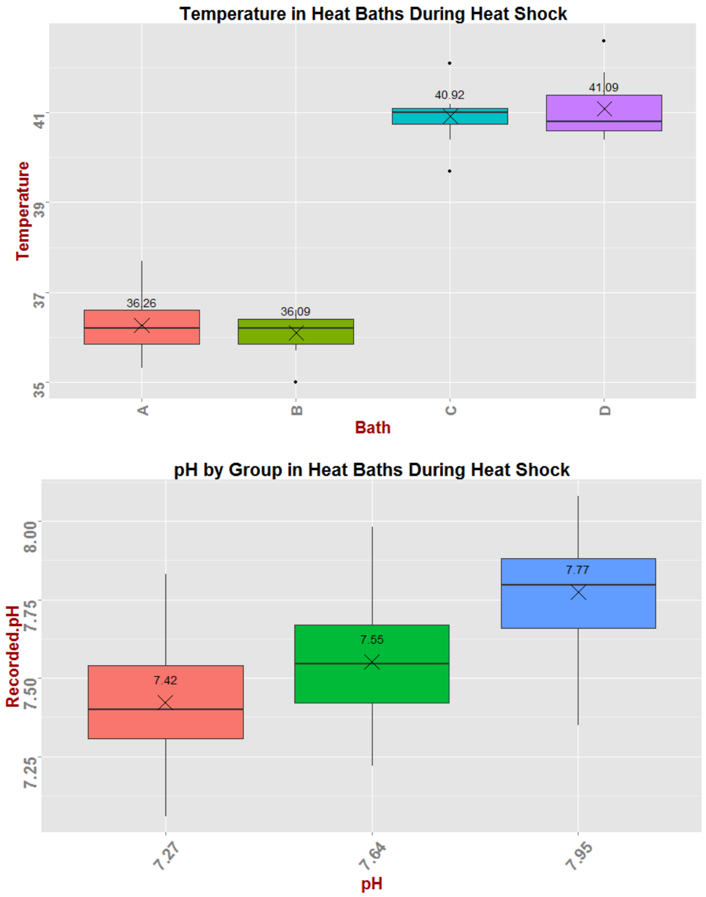


Figure 5: Mean temperature levels of the heat baths during the heat shock treatment (left graph, a) and the pH range for each group during the heat shock (right graph, b). The X's represent the mean and the middle lines represent the median. The range of the box holds the standard deviation.

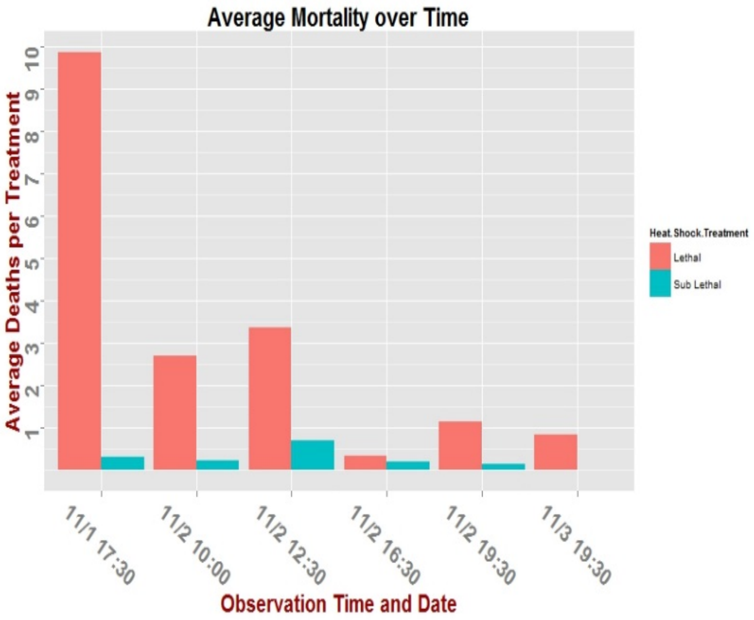


Figure 7: Mean percentage of mortality between the different pretreatment groups, showing both the sublethal and lethal heat shock treatments. Mean mortality is shown in white while the p-values attained from running one-way ANOVA tests between each low pH pretreatment group and the high pH pretreatment group are shown in blue. Only low lethal treatment is considered for significance.

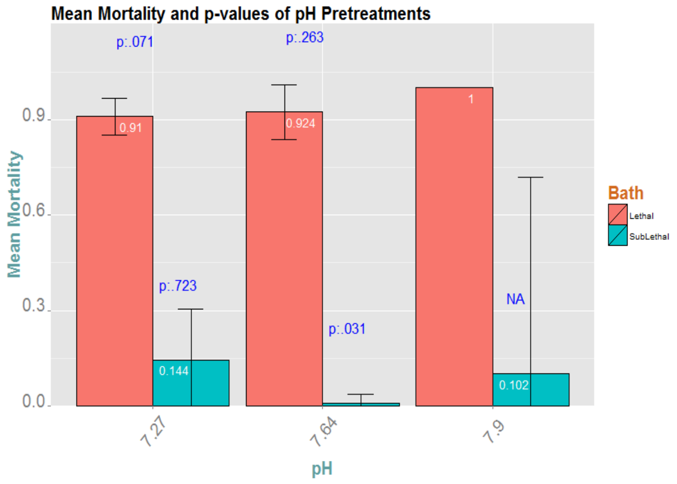


Figure 6 (left): The average amount of mortality in number of individuals changing over time with lethal (red) and sub-lethal (blue) temperatures. There were no deaths on the first day and a large percent of the lethal group died on the second evening. Mortality was checked before each water change every three hours. The final time is at the end of the first observation day.

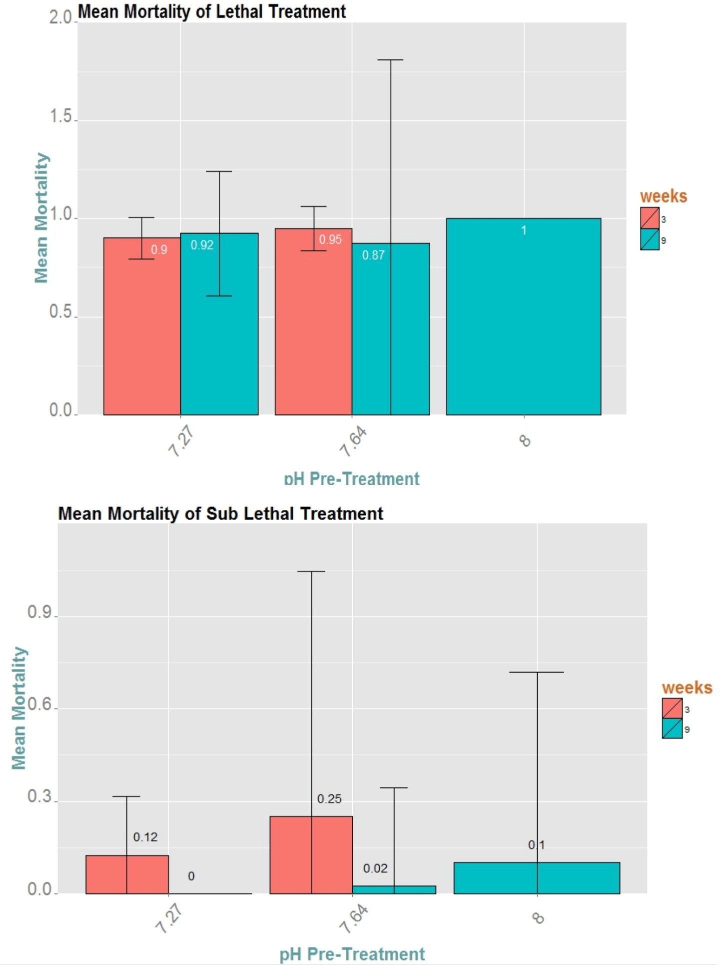


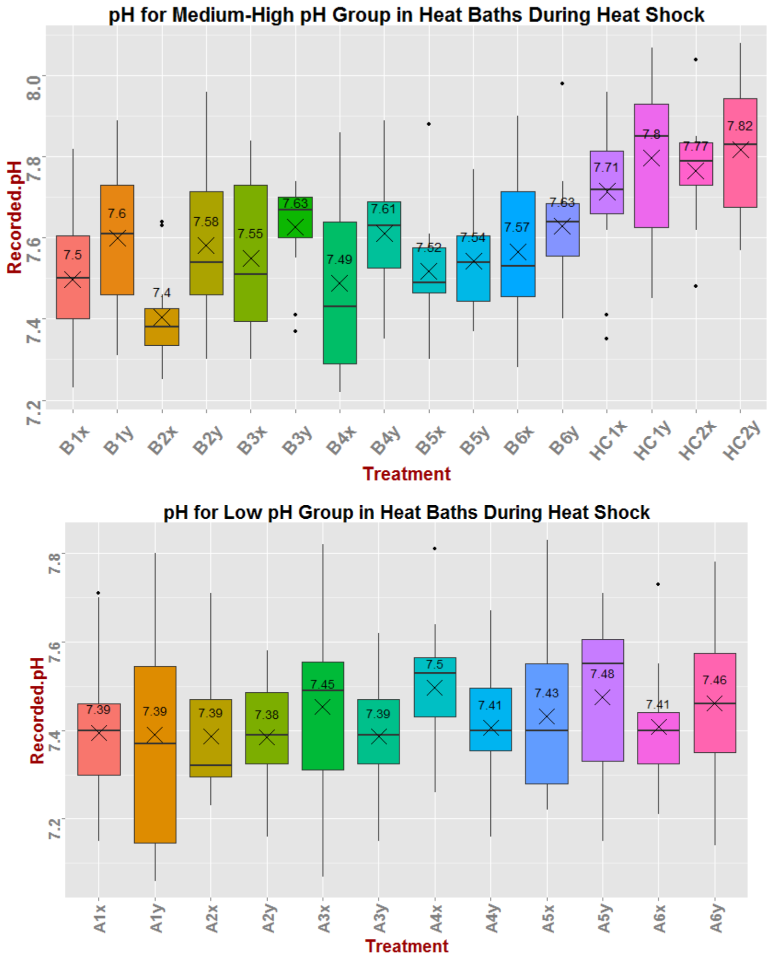
Figure 8: Mean percentage of mortality in our lethal (left) and sublethal (right) treatment groups. Numbers around bars are the means and error bars are the confidence interval. Red bars are three weeks and blue bars are 9 weeks.

Appendix

Additional Figures:

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

Table 2: This table this the raw data about each beaker. Each number represents a different beaker that went through the main heat shock. Mortality shows the percentage of mortality per beaker, Amount lists how many oysters started in each beaker, pH is their cooler’s target pH, and weeks shows how many weeks of pretreatment they had.



pH for Medium-High pH Group in Heat Baths During Heat Shock

pH for Low pH Group in Heat Baths During Heat Shock

Figure 9(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 sublethal 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.