Effects of Multiple Climate Change Related Stressors on Larval Development of the Red Abalone, *Haliotis rufescens*.

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ABSTRACT

Global climate change is driving three principle shifts in ocean conditions, including: warming of surface waters, and acidification and deoxygenation of deeper waters. These changes create stressful conditions within marine ecosystems and are projected to increase through this century and beyond. Seasonal events known as upwelling bathe coastal ecosystems in colder, nutrient-rich waters from deeper areas of the ocean, but the acidic, deoxygenated water due to climate change is brought to nearshore-coastal communities from these seasonal events and can be challenging for species inhabiting these areas. To assess the impacts of current and future upwelling-related ocean conditions, we selected the red abalone, *Haliotis rufescens*, as a model organism affected by upwelling dynamics. Few studies have been done to test the effects of simultaneous exposure to low pH and low dissolved oxygen on developing embryos. In this novel study, we measured the effects a multi-stressor event, such as upwelling, would have on development of calcium carbonate dependent organisms, such as *H. rufescens*. We compared the developmental stages until hatching for abalone embryos through the exposure to two treatments (low pH/low oxygen and low pH/high oxygen) and a control

treatment of high pH/high oxygen. This experiment evaluates whether these potentially harmful conditions would slow and/or impede the transition from early development stages to free-swimming planktonic larvae. Preliminary data suggests development in a low pH/low oxygen environment will slow hatching of *H. rufescens* embryos.

INTRODUCTION (Normal, Times New Roman, 12 pt, bold)

Rising carbon dioxide levels have been threatening global populations, and will continue to threaten marine organisms in the near future (Doney et al., 2009; Orr et al., Hoegh-Guldberg et al., 2007; Kim et al., 2013). Burning of fossil fuels contributes to the increase in atmospheric carbon dioxide. This CO_2 makes its way to deeper areas of the ocean through the carbonic cycle and the biological pump. This abundance of CO_2 causes a drop in the ocean's pH – resulting in exposure of organisms to potentially stressful conditions. Global climate change also affects the saturation of oxygen in areas of the ocean, and stratification impedes thorough mixing of oxygenated surface layers with deeper, colder, and denser areas of the ocean. The combination of acidic and hypoxic seawater in deeper areas is brought to shallow nearshore ecosystems (Grantham et al., 2004) and threatens early development and settlement of marine organisms (Ross et al., 2011). There has been concern about the stability and conservation of marine organisms that are already exposed to these stressful environmental conditions that are projected to worsen in the future. *Haliotis rufescens,* along with several other species of abalone, have been crucial stock for commercial fisheries, but declined to alarming numbers by the beginning of the century due to a combination of disease, climate change conditions, and overfishing. Choosing *H. rufescens* as a model organism to study the effects of factors related to upwelling and climate change will give a better understanding of how shell-forming, calcifying organisms will be impacted in a future of potentially stressful environmental conditions.

Efforts to stabilize populations of *H. rufescens* have been successful in some no-takes zones that were implemented as part of conservation efforts, but with new

environmental threats linked to rising atmospheric carbon dioxide levels and upwelling (cold, hypoxic and acidic water brought to nearshore coastal ecosystems), *H. rufescens* and other marine organisms face stressful conditions that may affect larval development (Barry et al., 2011; Ross et al., 2011). Larval growth and development of calcifying organisms is an important topic to hatching success of *H. rufescens* embryos and is affected during these periods of projected extremes (Dupont et al., 2010). Many studies focus on one aspect of climate change (i.e. temperature, oxygen concentration and pH). This study aims to understand how two of these factors (pH and O_2 concentration) will affect developing larvae. *H. rufescens* larvae were exposed to a control and two treatments. The control replicates conditions seen in the Monterey bay (pH of 7.9/O_2 concentration of 190 µmol/kg). The two treatments used consisted of a pH of 7.2 and O_2 concentrations of either 60 µmol/kg or 190 µmol/kg. I hypothesize the combination low pH/low O_2 will affect hatching more than the low pH/high O_2 treatment. These values simulate current oceanic conditions vs future conditions brought to nearshore ecosystems by upwelling. Results from these simulations will enhance the understanding of how future marine populations will fare during stressful upwelling events.

MATERIALS AND METHODS

ACQUISITION

Gravid abalone were acquired from American Abalone Farms in Davenport, CA two weeks before spawning. Once newly-purchased abalone were brought back to the lab, each was marked with a unique number by a tag that was glued to the shell. Once tagged, weighed, and measured for shell length, broodstock were placed in gender-specific tubs with a closed lid that were maintained under stable conditions in 24 hour darkness, kept at 13ºC, and fed *ad libitum* with *Macrocystis pyrifera*. This experiment also aimed to answer the question of whether post-spawners in the lab would be able to spawn again with chemical inducement. Six post-spawners (3 of each gender) were introduced into the tubs along with eight newly-purchased

abalone (4 of each gender) – resulting in 14 broodstock total. Only red light was used during this acclimation period as to prevent premature spawning due to sudden change in light.

SPAWNING

After two weeks, gravid females were removed first from the tubs. Each female was weighed, measured for shell length and placed in a 6 L tube with one other abalone of the same gender. The newly-purchased broodstock were placed with another newly-purchased abalone, whereas the post-spawning broodstock were organized by two in a single tub and the other in a tub by itself. These tubs were filled to 5 L of control seawater (8.0 pH and 190 µmol/kg O_2) with a starting temperature of 13ºC. There were eight 6 L tubs containing control seawater and aeration placed on a water table, which was filled with fresh water. This fresh water served as the temperature ramp that would be used to aid in spawning. This temperature ramp was achieved by adding 5 L of warm freshwater (18ºC) to the water table but not the 6 L seawater tubs every 45 minutes in order to raise the temperature to 17ºC before spawning occurred.

Thirty minutes after females were introduced to the spawning tubs, each tub received 40 ml of Tris buffer. Once 15 minutes had passed, 25 ml of H_2O_2 was added (Morse *et al.*, 1977). An hour and a half after females were separated into spawning tubs, males were then removed from their stable environment, weighed, measured, and separated into 6 L tubs on the freshwater table with the same organization and conditions as the females. The same protocol for Tris buffer and H_2O_2 was followed for the male broodstock. By the end of the addition of chemical solutions, the temperature inside the 6 L tubs had arrived at 17ºC.

All 8 tubs were drained, rinsed three times and refilled to 5 L with control seawater. If any spawning occurred before or during the rinsing process, these gametes were marked unviable and were not used in the development experiment. Once tubs were refilled, spawning timeframe was about two hours. Abalone were watched closely during this time.

COLLECTION

Once spawning occurred, sperm was collected with a pipet before eggs because sperm dilutes rapidly once it leaves the respiratory holes. Sperm was placed in a clean 250 mL plastic beaker. Eggs were collected after sperm collection from the bottom of individual tubs with a turkey baster and were placed in a clean 500 mL glass beaker.

Density calculations were done for both egg and sperm once enough gametes were collected to fill one 500 mL beaker with eggs and two 250 mL beakers with sperm. 3 samples of 500 µl from beaker containing eggs were sampled after sufficient mixing. Eggs were counted under the Olympus SZH10 dissecting scope and density was calculated to be 1200 eggs/mL. Two samples of 10 µl volume from sperm were sampled after mixing. Sperm were dyed and diluted with Lugol's solution and counted under a compound scope. We calculated a sperm concentration of 10^6 sperm/mL.

FERTILIZATION

Using the most successful density for fertilization, (10^6 for sperm/10^5 for eggs) we used five clean 6 L tubs and eggs were added with the goal of creating a thin layer on the bottom of the tub for maximum fertilization success. We divided the amount of sperm equally between the 5 tubs and allowed gametes to fertilize in control seawater (7.9 pH and oxygen concentration of 190 µmol/kg) for 30 minutes.

SEPARATION

After this period of time, the total pool of developing embryos was divided into 9 experimental glass jars for the remainder of the experiment. Three conditions were used (n=3) to test which factor associated with climate change (ocean acidification or hypoxia) would impact hatching of *H. rufescens* embryos. The control replicates common conditions seen in the Monterey Bay, which is a pH of 7.9 and an O_2 concentration of 190 µmol/kg. This control will be referred to as HH. Two treatments were chosen to compare pH to oxygen. Three jars contained seawater with a pH of 7.2 and an O_2 concentration of 190 µmol/kg. This will be denoted as

LH. Three other jars contained seawater with a pH of 7.2 and an O_2 concentration of 60 µmol/kg, which will be denoted at LL. The 1 ½ L experimental jars were engineered to have a small inlet tube opening an outlet tube opening of the same diameter to minimize gas exchange. The inlet tube extended to the bottom of the jar to allow for a circular flow and suspend embryos during development. The flow rate was enough to suspend embryos during development while avoiding massive impingement on the outlet filter. Our effort to minimize impingement was done by creating a circular filter wrapped in 65 micron Nytex mesh and capped with falcon tube lids on either end. These caps were super glued and left to soak in freshwater then seawater to remove harmful chemicals that may affect embryo development. This filter was connected to the outlet tube through a hole in the center of one of the caps.

SAMPLING

Sampling was done directly after fertilized embryos were introduced to separate jars. Both live and fixed samples were taken. Fixing was done by sieving 2-4 mL of embryos from each jar and transferring to 20 mL scintillation vials. If a 2 mL sample was taken, 8 mL of 10% Phosphate buffered formalin was added. If a 4 mL sample was taken, 16 mL was added. Fixing and imaging was successful until 41 hours post fertilization due to fixative issues. The exploded embryos were excluded from total counts, and larvae that were intact were quantified and analyzed. The schedule used for continued sampling was 0, 4, 8, 16, 22, 25 and 41 hours post fertilization (HPF). During each of these time points, fixed samples were taken and live samples were photographed afterwards using CellSentry software. Counts of each developmental stage seen were taken during each sampling point and were done using the Olympus SZH10 dissecting scope. Live counts and micrographs were only done up to 16 hours post fertilization due to technical difficulties with the camera and microscope. Using a depression slide, fixed counts were taken for all sampling points. 100 embryos per fixed sample were counted during each sampling period. Any remaining embryos in the sample past 100 counts were

excluded from the data. For 41 hours post fertilization, sample size dropped drastically in all jars except jar 4.

The stages of development that were of interest for this project were: 1 cell, 2 cell, 4 cell, 4+ cell/gastrula, pre-trochophore and trochophore (figure 1). Defining 4+ cell and gastrula became difficult past 8 HPF and were grouped together to avoid confusion and/or error. Categorizing pre-trochophore from trochophore relied upon the vitelline envelope still encompassing the larvae. Once larvae did not have this envelope, larvae was considered successfully hatched.

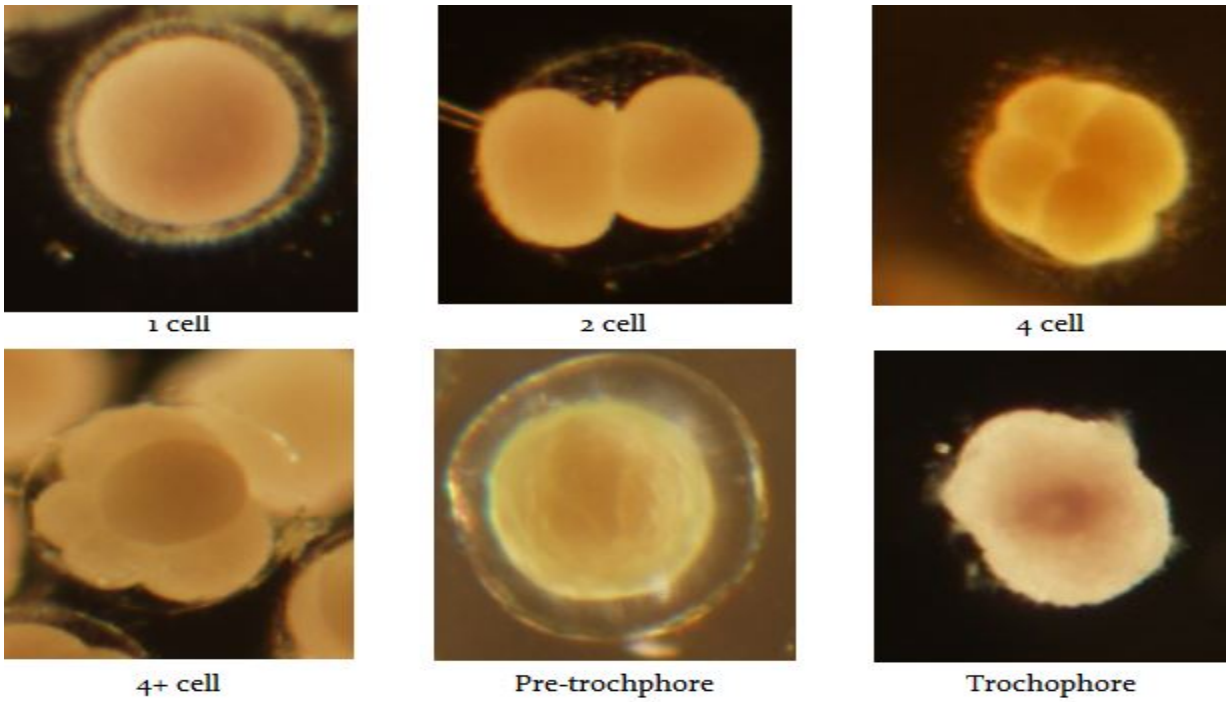

Figure 1. The above six stages were used to create a timeline for development of *H. rufescens* larvae. Quantifying stopped after the trochophore stage.

RESULTS

The metric used to test success relied on hatching of *H. rufescens* larvae from the vitelline envelope they were encased in since fertilization. From this experiment alone, we were able to determine, through using ANOVA, with a pairwise

comparison (Figure 2) and a Tukey test (Figure 3), there is significance between HH and LL; however, there is no significance between HH vs. LH and LH vs. LL. For 25 HPF, the averages for larvae hatched is as follows: HH=25%, LH=56% and LL=34%. For 41 HPF, hatched larvae averages were HH=96%, LH=82% and LL=50%. Comparing 25 HPF to 41 HPF, there is a large difference between for HH (Figure 4). The low percentage hatched may be due to human error/handling error or may be a statistical outlier. Due to the small sample size (n=3), we were not able to fully understand what occurred. A larger sample size in future experiments may be able to answer this question.

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
HH	LH	34.333	15.128	.064	-2.684	71.351
	LL	47.417*	15.128	.020	10.399	84.434
LH	HH	-34.333	15.128	.064	-71.351	2.684
	LL	13.083	15.128	.420	-23.934	50.101
LL	HH	-47.417*	15.128	.020	-84.434	-10.399
	LH	-13.083	15.128	.420	-50.101	23.934

Figure 2. Pairwise comparisons for HH (high pH/high O_2), LH (low pH/low O_2) and LL (low pH/low O_2). The mean difference is significant at the .05 level.

	Treatment	N	Subset 1	Subset 2
Tukey HSD[a,b]	LL	3	49.3567	
	LH	3	62.4400	62.4400
	HH	3		96.7733
	Sig.		.680	.137

Figure 3. Tukey test results showing significance between LL and HH. $\alpha = .05$

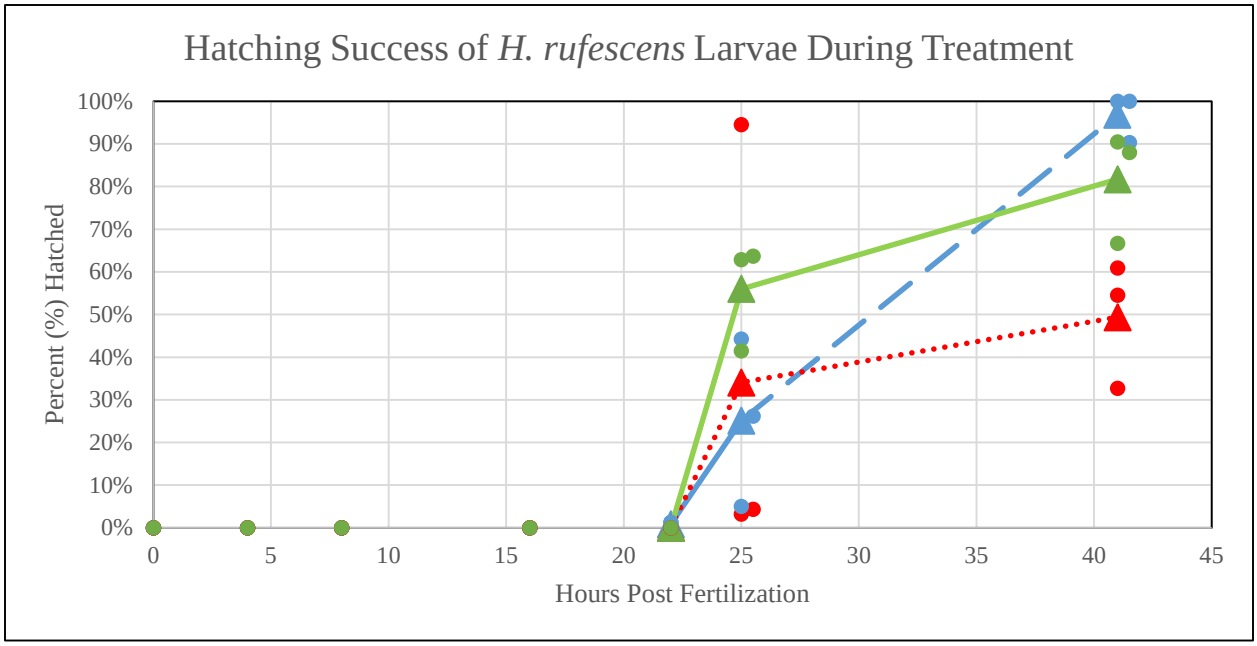

Figure 4. Hatching success of H. rufescens larvae for each condition. Long dashes line refers to the high pH/high O_2 control. The solid line refers to the low pH/high O_2 treatment. The dotted line refers to the low pH/low O_2 treatment. From the graph, there is visible variability in hatching success for 25 hours post fertilization and 41 hours post fertilization. Ultimately, the order of hatching success followed the hypothesis, with low pH/low O_2 having the most effect on hatching.

DISCUSSION

From our findings, we found there is a significant difference between hatching success of H. rufescens larvae between the HH treatment and the LL treatment. We suggest there may be a more significant role played by pH on embryo development than oxygen saturation, however, a fully factorial experimental design will provide better insight as to which factors have more impact. Further data analysis from this experiment may also provide insight for separate developmental stages as opposed to only hatching success. Comparing each sampling time point with the development levels seen for each treatment would create a development story to be used for purposes regarding conservation efforts, as well as a fundamental understanding of how stressful conditions related to climate change affect developing embryos. Future experiments aim to understand how climate change conditions affect development during the entire abalone life cycle, as well as implementing better conservation efforts in the future.

CONCLUSIONS/RECOMMENDATIONS

Although climate change is a topic that is being heavily researched, there is still much work to be done. Implementing the combined condition method (low pH and low O_2 as factors) will enable our society to make more accurate predictions of how our oceans will change.

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References:

Barry, J.P., Widdicombe, S., Hall-Spencer, J. (2011). Effects of ocean acidification on marine biodiversity and ecosystem function. Pages 192-209 in, Ocean Acidification, J.P. Gattuso, ed., Oxford Univ. Press,

Booth, J., McPhee-Shaw, E., Chua, P., Kingsley, E., Denny, M., Phillips, R., Bograd, S., Zeidberg, L., and Gilly, W.: Natural intrusions of hypoxic, low pH water into nearshore marine environments on the California coast, Cont. Shelf Res., 45, 108–115, doi:10.1016/j.csr.2012.06.009, 2012.

Doney, S.C., W.M. Balch, V.J. Fabry, and R.A. Feely. 2009. Ocean acidification: A critical emerging problem for the ocean sciences. Oceanography 22(4):16–25, http://dx.doi.org/10.5670/oceanog.2009.93.

Feely, Richard, Christopher Sabine, Kitack Lee, Will Berelson, Joanie Kleypas, Victoria Fabry, and Frank Millero. "Impact of Anthropogenic CO2 on the CaCO3 System in the Oceans." Science, 305.5682 (2004): 362-366.

Grantham, B., Chan, F., Nielsen, K., & Fox, D. (2004). Upwelling-driven nearshore hypoxia signals ecosystem and oceanographic changes in the northeast pacific. Nature, 429(6993), 749.

Kim, T. W., Barry, J. P., & Micheli, F. (2013): The effects of intermittent exposure to low pH and low oxygen conditions on the mortality and growth of red abalone. Biogeosciences. 10: 7255-7262. (Special Issue for The Ocean in a high-CO2 world III) SCIE IF: 3.754

S Dupont, O Ortega-Martínez, M Thorndyke: Impact of near-future ocean acidification on echinoderms. Ecotoxicology 19 (3), 449-462

Hoegh-Guldberg, O., Mumby, P., Hooten, A., Steneck, R., Greenfield, P., et al. (2007). Coral reefs under rapid climate change and ocean acidification. Science, 318(5857), 1737-1742.

Morse DE, Duncan H, Hooker N, Morse A. Hydrogen peroxide induces spawning in molluscs, with activation of prostaglandin endoperoxide synthetase. Science 1977;196:298-300.

Ross, P., Parker, L., O'Connor, W., & Bailey, E. (2011). The impact of ocean acidification on reproduction, early development and settlement of marine organisms. Water, 3(4), 1005-1030.