Carry-over effects of fertilization in stressful conditions on red abalone (*Haliotis rufescens*) larval development

A.L. San [1, 2, 3], C. Boch [1, 2], S. Litvin [2], F. Micheli [2], G. D. Leo [2], C. Lovera [1], J. P. Barry [1]

[1] *Monterey Bay Aquarium Research Institute, 7700 Sandholt Rd, Moss Landing, CA 95039, USA*
[2] *Hopkins Marine Station, Stanford University, 120 Oceanview Blvd, Pacific Grove, CA 93950, USA*
[3] *California State University Monterey Bay, 100 Campus Center, Seaside, CA 93955, USA*

Abstract

Significant levels of anthropogenic carbon dioxide released into the atmosphere cause global warming, which increases upwelling in coastal areas, bringing cold waters low in pH and oxygen to the surface. These changes may threaten coastal ecosystem function—including species important for commercial and recreational fisheries. For example, red abalone (*Haliotis rufescens*) larvae have been found to develop abnormally in low water temperatures. It is unclear though if any "carry-over" effects will occur when embryos are fertilized in cold, low pH seawater then allowed to develop under "normal" conditions with higher pH and temperature. We tested whether such a "carry-over" effect would occur for red abalone larvae using a laboratory experiment. Gravid red abalone were induced to spawn, and gametes were collected. Eggs and sperm were combined in syringes filled with either high pH (7.85) and high temperature (18˚C) seawater, low pH (7.2) and low temperature (9˚C) seawater, high pH (7.85) and low temperature (9˚C) seawater, or low pH (7.2) and high temperature (18˚C) seawater. The samples (n=64) were then transferred into culture tanks to develop in ambient (pH 8.0, 11.0˚C) seawater conditions, and were sampled at four time periods (4 h, 24 h, 48 h, 72 h). After collecting the samples at the end of each period, the level of development for all larvae and eggs were measured. We saw carry-over effects in the larvae, most notably the high rate of abnormal development in larvae fertilized in LL conditions and a slower developmental rate in larvae fertilized in low pH.

Introduction

The massive rise in fossil fuel use since the initiation of oil extraction has led to a rapid rise in atmospheric CO2 levels, of the ~9 gTC emitted per year, about 26% is absorbed through the ocean surface (Sabine et al. 2004) where it reacts with seawater, reducing the pH of ocean surface waters. Atmospheric CO_2 concentrations have increased from 280 parts per million (ppm) to over 400 ppm since the industrial revolution began, with about 50% of the increase occurring in the past 30 years (Feely et al. 2009; cdiac.ornl.gov). Normally, upwelling can last from less than 24 hours to over several days off the California coast, bringing colder, more acidic seawater with low oxygen content to the ocean surface. But higher atmospheric CO_2 levels will increase continental temperatures faster than ocean temperatures, strengthening offshore winds that can start upwelling events earlier, and end later in the year (Bakun. 1990). This prolonged upwelling may increase the exposure of coastal marine species to stressful ocean conditions, leading to reduced rates of growth and development.

Many marine organisms are experiencing the effects of a changing ocean, and calcifying organisms are predicted to be significantly impacted. Organisms that form calcium carbonate shells or skeletons for survival may have difficulty constructing these structures in ocean waters with a low pH. A variety of experiments have been performed to examine rates of calcification in a more acidic ocean, including studies on corals (Jokiel et al. 2008), sea urchins (Byrne et al. 2010), mussels (Fitzer et al. 2014), and other species. These studies have shown that these organisms can still create their calcium carbonate structures in more acidic water conditions, but the rate of calcification is reduced.

The red abalone (*H. rufescens*), which was historically an economically important species along California, has experienced a decline in populations worldwide due to disease, overfishing, habitat loss, and poor government management (Gordon and Cook. 2004). Red abalone are

found from Oregon to Baja California on the coast of North America (Morris et al. 1980). Gametes are broadcast into the water column, after which fertilized gametes develop into a free-swimming stage over 3 -10 days before settling on substrate (Leighton 1972). Such early life history phases of abalone and many other marine species may be particularly vulnerable to environmental stressors such as low ocean pH and cold temperatures. Although the effects of ocean acidification on fertilization have been investigated (Boch et al, in prep) up to the 4-cell stage, the consequences for larval development after early exposure to environmental stress remains unknown. We used *Haliotis rufescens* as a model organism representing many coastal species, to determine if fertilized embryos exposed to stressful pH and ocean temperatures will have additional negative consequences in later stages of larval development.

Materials and Methods

Experimental preparation

Six brood stock abalones (3 male, 3 female) were purchased from American Abalone Farms in Davenport, CA and were transferred to the Monterey Bay Aquarium Research Institute (MBARI) in Moss Landing, CA. Each abalone was measured for shell diameter and then placed into gender-specific bins with a cover and filtered seawater flowing through the system. The bins are kept under 24-hour darkness in 11˚C, and allowed to feed *ad libitum* on fresh giant kelp (*Macrocystis pyrifera*). Seawater temperature is checked twice daily using a Taylor 9842 digital thermometer and the tanks were cleaned each week.

Gamete Collection

Each *H. rufescens* were induced to spawn using methods described by Morse et al. 1977. Sperm was collected upon release then transferred into a 250 mL beaker, and eggs were collected after they settled to the bottom of the container then transferred into a 500 mL beaker. A 2 mL glass pipette was used to collect sperm and a turkey baster for collecting eggs. Collection of seawater was kept at a minimum to create concentrated sperm stock samples.

The density of egg and sperm was measured and stock solutions holding eggs and sperm were prepared. Three 50 µL aliquots were taken from the egg stock, each aliquot transferred to a depressed microscope slide and eggs counted under an Olympus SZH10 Stereo Microscope. Our egg concentration was determined to be 1,163 eggs per mL. Sperm concentration was determined from four 10 µL aliquots. Each aliquot was diluted with 90 µL of seawater and 100 µL of Lugol's dye solution, creating a 20x dilution. A 10 µL subsample of the diluted solution was then placed in a hemocytometer and counted under a compound scope. Our estimated sperm concentration was $7.18 * 10^7$ sperm per mL.

Fertilization

Three mL of eggs and 2 mL of sperm from stock solutions were transferred into a gas-tight glass syringe holding 35 mL of seawater from 1 of 4 treatments, and held for 10 minutes at 13 ^{o}C to allow fertilization to occur (64 syringes total). Each treatment reflected either a target high pH of 7.95 or low pH of 7.2, and either a target high water temperature at 18.5˚C or a low water temperature of 8.59˚C. To validate these measurements, we used Sentron ISFET 8100 to measure pH and a Taylor 9842 digital thermometer for temperature measurements. Syringes labeled with an odd number contained high pH seawater while the even numbered syringes contained low pH seawater. The first pair of syringes were placed in the 8.5˚C water bath, then

the next pair was placed in the 18.5˚C water bath, alternating placement of syringe pairs between the high and low temperature water baths. The methods have been established by previous experiments (Boch et al., in prep). We will test 16 replicates for each treatment. We injected 3 mL of eggs and 2 mL of sperm into each syringe, and eggs were allowed to fertilize for 600 seconds.

Once the fertilization period was finished, the contents of the syringes were transferred into modified falcon tubes where the bottoms were cut off and replaced with 60-micron mesh. The eggs were then rinsed to remove any debris and excess sperm. Following rinsing, all 64 falcon tubes were placed in plastic bins with a flow-through system with seawater at 11˚C at a pH of 8.00 where the eggs developed into larvae.

Sampling

Sets of sixteen samples were collected at four time periods to assess larval development. Each sample set contained 4 replicates of each treatment and development was stopped at 4 hours post fertilization (HPF), 24 HPF, 48 HPF, and 72 HPF. Eggs were rinsed from the modified falcon tubes into 20 mL scintillation vials with fresh seawater then preserved with formaldehyde to stop development. Micrographs of samples of eggs and larvae were collected immediately to determine developmental rates at the particular time period.

In samples taken at 4 HPF, micrographs were analyzed for 1-cell, 2-cell, and 4-cell development as well as damaged and abnormal eggs. The eggs were rinsed into scintillation vials to the shoulder of the container then we added 500 µL of 37% Formaldehyde solution to preserve the samples to obtain a final concentration of 10% formalin. From each vial, at least 100

eggs were removed for subsampling, and were then photographed using an Olympus SZH10 Research Stereo Microscope with an Olympus DP71 Microscope Camera.

In sampling periods of 24 HPF and later, larval development was analyzed in addition to egg development and were analyzed under an Olympus SZH10 Research Stereo Microscope at 4x zoom. Samples collected at 24 HPF were transferred into 20 mL scintillation vials to about 10 mL. The eggs were allowed to settle to the bottom of each vial before seawater was removed with a pipette until about 3 mL remained. The larvae were relaxed with 150 µL of $MgCl_2$, gently swirled to ensure mixing, then left for 20 minutes. After 20 minutes, we then added 10 mL of Buffer A, which was 90% of 37% Formaldehyde solution. We counted the number of cells at the 4+ to gastrula, pre-hatching and hatching larvae in addition to egg development stages. Eggs in the 4+ to gastrula stage were determined as eggs with multiple cleavages, pre-hatched larvae were defined as trochophores surrounded by an egg envelope, and hatched larvae lacked the egg envelope.

Larvae collected at 48 HPF and 72 HPF were rinsed into 20 mL scintillation vials with fresh seawater, then we added 150 µL of $MgCl_2$ as a relaxer. After 20 minutes, 5 mL of 10% Formaldehyde solution was added to each tube. Once the solution in the vials turned a milky-white and the larvae dropped to the bottom of the vial, the milky solution was pipetted out until about 3 mL remained. 10 mL of 10% Formaldehyde solution was added to each vial then left to sit for 5 minutes, when the solution in the vial turned milky white. With a pipette, 10 mL of the solution was disposed of, taking care to avoid collection of larvae. Another 10 mL of 10% Formaldehyde solution was added in to clear up the preservation solution, then at least 100 cells were removed from each vial for micrographs.

After micrographs were taken, we counted the number of larvae at specific development stages, and any that were damaged or developed abnormally. At 4 HPF, eggs were assessed for development at the 1-cell, 2-cell, and 4-cell stages. At 24 HPF, eggs and larvae were assessed for development to the 4 cell+ to gastrula, pre-hatching trochophore, and hatched trochophore stages.

Results

At 4 HPF, eggs fertilized in low pH and low temperature conditions experienced the lowest fertilization success among the four treatments (Figure 1), as established in previous experiments performed by Boch et al (in preparation). At 24 HPF, we expected to see larvae at the pre-hatching stage and as trochophores without a protoconch. Development to the pre-hatching and trochophore without a protoconch stages were 35.7% and 45.4% in HH conditions 40.0% and 45.6% in HL conditions, 45.9% and 32.7% in LH conditions, and 47.7% and 37.0% in LL conditions, respectively (Figure 2). We saw a slower development in eggs fertilized in low pH conditions and a higher rate of abnormal development than eggs fertilized in high pH. At 48 HPF, we expected to see trochophores with a protoconch. Development to this expected stage was 79.6% in HH, 75.9% in HL, 71.8% in LH, and 19.7% in LL (Figure 3). There was a low rate of development to the expected stage but a high proportion of larvae developed abnormally in low pH and low temperature conditions. Of the larvae fertilized in the other three treatments, at least 70% developed to the trochophore with a protoconch stage. After 72 HPF, the larvae were expected to have gone through torsion. Development to this stage was 79.6% in HH fertilized

larvae, 75.9% in HL fertilized larvae, 71.8% in LH fertilized larvae, and 19.7% in LL fertilized larvae (Figure 4).

From our results, there is a carry-over effect in development when larvae are fertilized in stressful conditions. The most significant effects are seen in eggs fertilized in LL conditions, which experienced low fertilization rates and, of those that fertilized, developed normally at later time periods. Across all four treatments at 24 HPF, development was slowed when fertilized in either low pH, low temperatures, or both. However, a longer exposure to ambient conditions helped the larvae develop at a normal rate, as seen in the larvae fertilized in either low pH or low temperatures then left to develop in ambient conditions for at least 48 HPF. In larvae fertilized in LL though, there was a sharp increase of abnormal development 48 HPF, which further increased 72 HPF. We also see a difference in development rates at 24 HPF between eggs fertilized in low pH conditions and high pH conditions. A higher proportion of larvae hatched and became trochophores without protoconches in high pH than those in low pH.

Discussion

From our results, we see a carry-over effect in larvae fertilized under stressful conditions, particularly eggs fertilized in low pH and low temperature. When eggs are fertilized for short periods of time in conditions with only one stressful element—in seawater that either has a low pH or low temperature—abalone larvae seem able to recover and develop normally. Low pH slows development in larvae, but after an exposure of more than 24 HPF to ideal conditions, developmental rate recovers to normal levels. This slower rate of development could result in prolonged exposures to stressful conditions, while larvae are in a developmental stage more vulnerable to environmental stressors. However, when eggs are fertilized in low pH and low

temperature, we see few larvae reaching the expected protoconch development stage and a high proportion of abnormal larvae development. Unless red abalone learn to adapt to these stressful conditions, it could possibly lead to a collapse in the population.

This experiment sets the foundation for future experiments that aim to develop the story about how climate change conditions can affect abalone development after the larval stage, and to develop a better understanding of ocean acidification effects on calcifying organisms. This can include experiments focusing on effects of long-term exposure to stressful conditions at different life stages or if abalone are able to adapt to stressful conditions. The effects of ocean acidification are currently being studied, but there is much more to learn. Further research can help scientists better predict how populations of calcifying organisms will fare in future predicted ocean conditions.

Tables and Figures

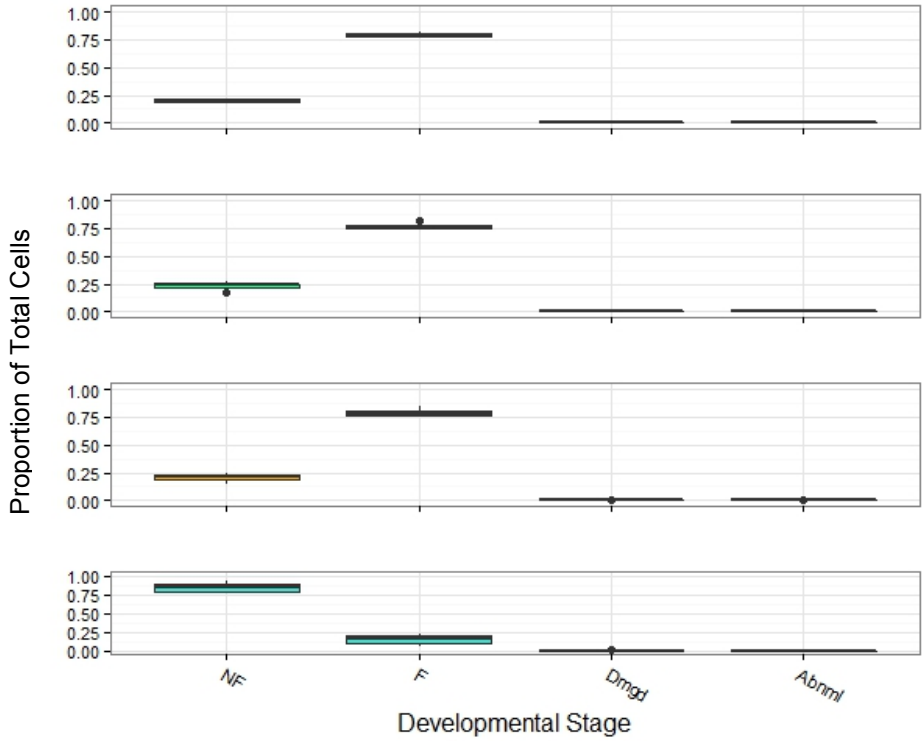

Figure 1. Fertilization success rates 4 hours post fertilization (HPF) across 4 treatments

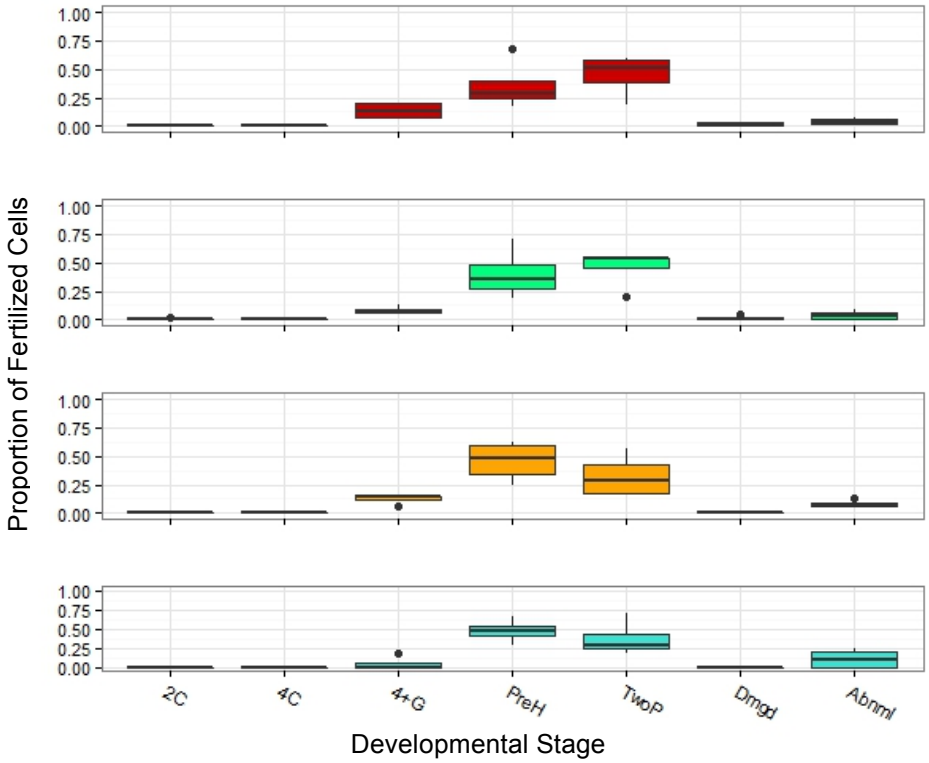

Figure 2. Larval development rates of fertilized eggs at 24 HPF

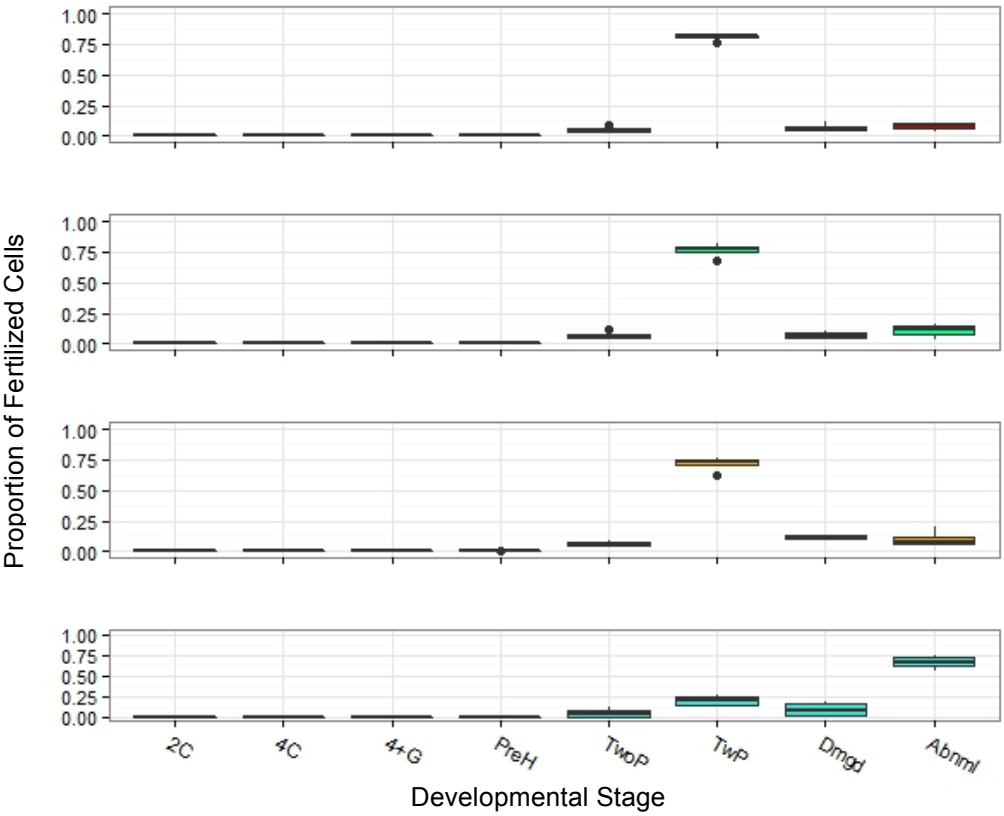

Figure 3. Larval development rates at 48 HPF

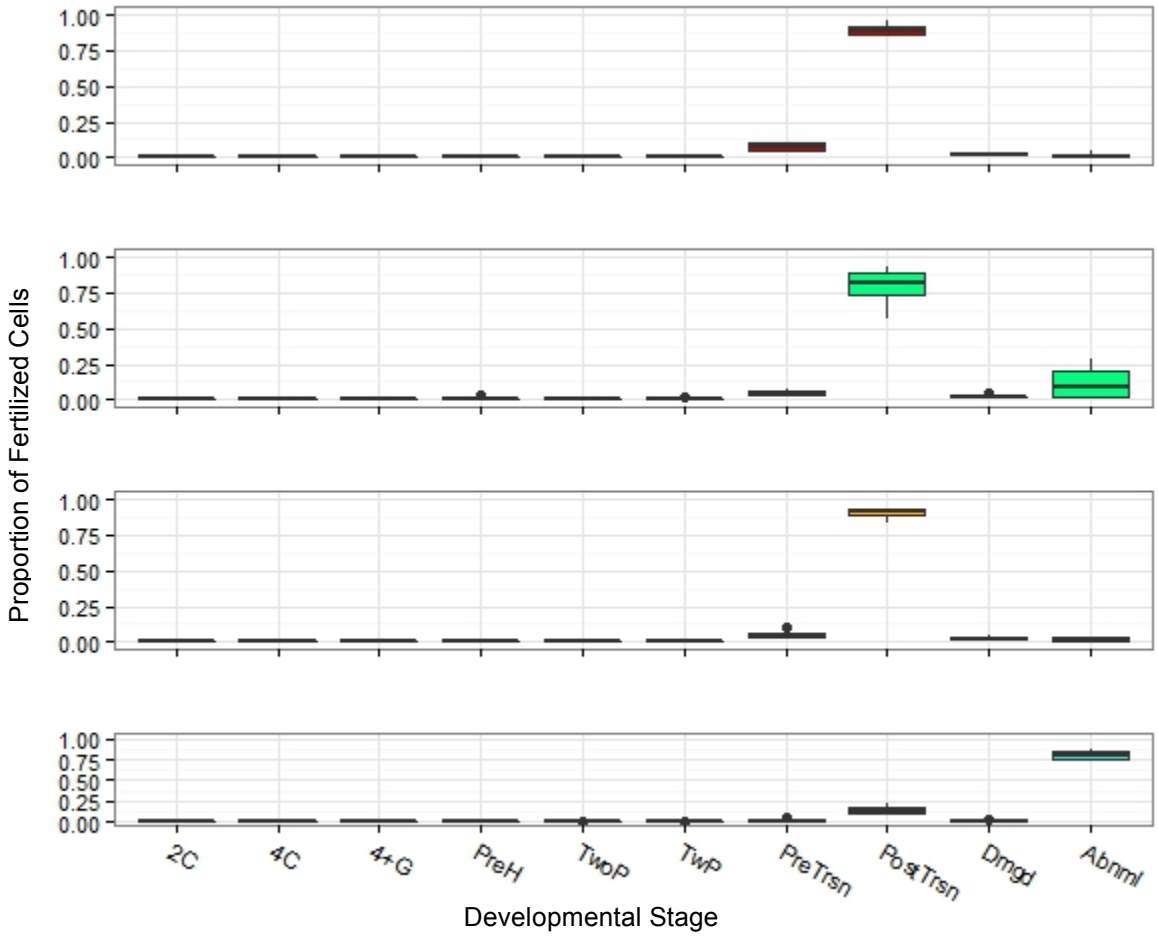

Figure 4. Larval development at 72 HPF

Acknowledgements

Thank you to American Abalone Farms for providing us with our abalone, and the National Science Foundation and the Monterey Bay Regional Ocean Science REU Program for funding our project. I would also like to thank the REU staff and my fellow REU interns for all your support throughout this intense 10-week program. Thank you to the Monterey Bay Aquarium Research Institute and Hopkins Marine Station for having me, as well as my partners Tiffany Thisner and Daryll Carson for your willingness to stay late at MBARI with me as well as your endless support. Thank you to my mentors Charles Boch, Jim Barry, and Giulio De Leo for

your seemingly-endless patience in helping me grow as a researcher. Without your guidance, I would not have made any progress. Last, but not least, thank you to George Matsumoto and Linda Kuhnz for all you have done for us MBARI interns throughout our ten weeks here.

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