Low pH effects on early development and toxin transporter activity in sea urchins

(Strongylocentrotus Purpuratus, Tripneustes gratilla, Echinometra mathaei)

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Abstract

This study explores both the direct effects of ocean acidification on early embryology and the indirect effects of a low pH environment on the embryos ability to expel toxins through membrane-bound transporters. This work was done on three different species of sea urchins, *Strongylocentrotus Purpuratus, Tripneustes gratilla*, and *Echinometra mathaei,* each collected at a different location (Monterey, CA, Hawaii, and Washington Island, respectively). Cell counts in individuals were performed to assess impact on early development. Surface membrane toxin transporters (mrp, pgp) were blocked, and accumulation of a toxin proxy, calcein-am, was noted by measuring the fluorescent levels as calcein-am hydrolyzed into fluorescent calcein in the embryo. The results indicate negative effects of low pH on early development of *Echinometra*, while the toxin transporters in all three species appear to work more effectively at low pH. There is some evidence that specific types of transporters may work more effectively until a threshold pH, below where the transporter no longer functions as well. In general, *Echinometra* had less transporter activity than *Tripneustes*.

Introduction

The atmospheric concentration of CO2 has risen from pre-industrial levels of 280 ppm to 380 ppm in the past 200 years, and this CO2 increase is not only raising temperatures and causing large shifts in climate it is also projected to have a large impact on ocean chemistry. The ocean absorbs from 30% to 50% of atmospheric CO2, and as it diffuses into the oceans it acidifies them. The IPPC projects a decrease in global surface average pH of between 0.14 and 0.35 units over the 21st century, in addition to the 0.1 pH change that has already occurred since pre-industrial times (IPCC, 2007). While this decrease in pH may not seem significant, it is important to remember that the pH scale is logarithmic, so every one-step change on the pH scale equates to a ten-fold change in acidity. This change in pH can result in large perturbations of the animals living in the oceans, and we are just beginning to understand what may happen to a few of the many ocean species as pH decreases.

Much of the research on ocean acidification has so far focused on the effects on calcifying marine organisms. Many marine organisms have calcium carbonate shells that protect them, but as CO2 diffuses into the oceans, the following reactions can accelerate, causing the dissolution of calcium carbonate and depriving organisms of their protective shell.

$CaCO_3 + CO_2 + H_20 \leftrightarrow Ca^{2+} + 2HCO_3^-$ (Takahashi, 2004)

Though it is written here as CaCO3, this same chemistry affects a variety of forms of marine carbonates, including magnesium calcites (produced by coralline algae), aragonite (produced by corals and pteropods), and calcite (produced by coccolithophorids and foraminifora) (Feely et. al., 2004). Scientists are beginning to study these species, and have observed decreases in shell thickness and shell stability in coccolithophorids, pteropods, foraminfora, and others.

In addition to work on adult calcifying organisms, scientists have just started researching the effects of a decreased pH on early development. Sea urchin embryos have been used to investigate the effect of pH on fertilization success, cleavage rates, and larval development (Kurihara et. al., 2004). Sea urchins have calcium carbonate spines, so the larval development section of the study investigated whether the decreased pH had a negative effect on the length of the larval spicules, the calcium carbonate components of the skeleton that form first in the sea urchin. The fertilization success and cleavage rates do not relate to calcium carbonate disintegration, but simply test the effect of a low pH on fertilization and early development. Kurihara et. al. conducted this study on *Hemicentrotus pulcherrimus* and *Echinometra mathaei* off the coast of Japan, and found that with decreasing pH, fertilization success dropped, cleavage rates dropped, and spicule length decreased (Figure 1).

A drop in fertilization success may be due to several factors. The sea urchin sperm is not active until it interacts with sea water, pumping Na^+ into the cell in order to pump H^+ out. In low pH treatments, there may be so many protons in the surrounding seawater that the sperm cannot pump more protons out and so does not activate. Additionally, after the sperm fertilizes the egg, there is another Na^+/ H^+ pump exchange,

pumping H^+ out of the cell and raising the pH. Again, if it is too acidic outside the cell the zygote may not be able to pump the H^+ out, and will not proceed to later fertilization events such as the fusion of the egg and sperm pronuclei, activation of specific enzymes, and increased DNA and protein synthesis (Biology 44X).

In my experiment, fertilization for all pH treatments was performed in ambient seawater in order to guarantee that the ratio of sperm to eggs was consistent for all treatments. Thus, any pH effects on sperm or eggs directly were not relevant, and only post-fertilization effects of pH were tested.

While the Kurihara *et. al.* study is a good start to understanding the effects of ocean acidification and the range of pH's where significant problems begin to arise for sea urchin development, much remains to be explored. Like most past work on biological effect of ocean acidification, the Kurihara *et. al.* study looked only at the direct effects of changing the pH on the organism and did not explore the indirect effects of how ocean acidification may affect an organisms ability to respond to heat shock or toxins in the environment.

Recognizing this gap in our understanding, the goal of the present study was to examine how pH effects sea urchin embryos' ability to respond to toxins in the environment, while also replicating the Kurihara *et al*. experiment. I expected to see the same results as were found in the Kurihara *et. al.* experiment, with decreased cleavage rates at lower pHs.

In order to examine how well the embryos can get rid of toxins under different pHs, I wanted to examine transporter activity. The primary mechanism embryos use in

exporting toxins are several transporters on the surface of the cell membrane. These transporters are more prevalent in early embryology than in adulthood and are extremely important for protecting the embryo in its early stages. There are two main types of transporters: the mrp transporter, which exports most toxins, and the p-gp transporter, which exports fewer toxins. I can test the prevalence of these transporters by the following method. C-am here simulates a toxic chemical. When c-am is in the cell, it becomes hydrolyzed by the cell and turns into calcein, a florescent molecule. If instead it is transported out of the cell it remains c-am and does not fluoresce (Figure 2). Thus, by blocking various transporters and looking a florescent levels the prevalence of the transporters in different samples becomes clear. A high florescent value indicates a low prevalence of transporters and a low florescene value indicates high prevalence of transporters. (Hamdoun, 2004).

Methods

I ran the experiment three times on three different species of sea urchins collected from three distinct locations. The first round of the experiment was run at Hopkins Marine Station in Monterey, California on the species *Stronglyocentrotus purpuratus;* the second portion of the experiment was run on *Tripneustes gratilla* collected near Kealakekua Bay on Hawaii, HI; and the third stint of the experiment was run on *Echinometra mathaei* collected at Washington Island in the Line Islands. From each of these locations, I collected several individuals and injected a few before identifying a gravid male and gravid female.

The methods for each portion of the experiment involved the same steps but slightly different dilutions and measurements and thus the methods for each of the three

runs will be outlined separately though there are many similarities. The Line Island experiment will be explained first as this was the most complete procedure.

Washington Island, Line Islands: Echinometra

Once collected, urchins were injected with .55 M KCl in a syringe using a 18 gauge needle, taking care that there were no bubbles. I inserted the needle angled away from the mouth into the edge or the disc of soft tissue surrounding the mouth. The KCl stresses the urchins to the point that they think they are dying, and, as an evolutionary response, they shed their gametes as one last chance at reproduction before death. When the gonopores located on top of the urchin shed milky-white sperm, I used a glass pipet to transfer the dry semen to a test tube. If the gonopores shed yellowish orange eggs, I inverted the female over a small beaker filled with seawater. The *Echinometra* were injected with approximately 1.5 mL as they did not shed very many gametes but I was hesitant to give them a lethal dose of 2 mL. I had to inject five urchins in order to get one gravid urchin of from each sex.

In addition to collecting gametes, I also prepared three different pH treatments. I filtered seawater from the area sampled through .45 μm 100/PK filter (Pall Corporation), then placed water in liter containers. To each container I added 2.4 g of HEPES, which I expected to buffer the seawater with a pka of 7.5 and to drop the pH of the seawater. I then added NaOH to each of the three tanks, adding the appropriate amount to raise the pH to the desired level: 8.0, 7.8, and 7.4. The 8.0 pH was chosen because this is the ambient pH. The 7.8 pH is .3 pH below the 8.1 and the IPCC predicts a decrease in pH on the order of 0.14 to 0.35 by the end of the century, so the 7.8 pH is an experiment in conditions we may see in the next 100 years (IPCC, 2007). The 7.8 pH and 7.4 pH were

also used in the Kurihara et. al. study, affording a good comparison in results. The 7.4 pH is also important in establishing a gradient in pH rather than comparing only two samples.

Because the *Echinometra* were not very prolific, I used all of the eggs from the female I sampled, allowing the eggs to settle on the bottom due to their negative buoyancy before pipetting off the seawater on top. This procedure was repeated for a total of three rinses to expel the egg's jelly coat before the eggs were resuspended in 100 mL. I prepared a 5% sperm suspension by taking 5 μL of sperm and suspending it in 15 mL of seawater. I took 3 mL of the sperm solution and added it to the 100 mL egg solution, stirred it, and left it to incubate in a seawater bath (t=0). Two hours and fifty minutes later (t=2:55), I drained off the top portion of the 100 mL, such that only visible egg particulate remained, mixed this uniformly and then I evenly divided it into three test tubes, filling the test tubes to 50 mL with the altered pH seawater. I tested these tubes to make sure the pH was consistent, which they were.

I then began the fluorescent tests on the embryos. The c-am solution was prepared by adding 50 μL DMSO to 50 μg c-am. The two transporter blocks I used were 1 mM MK571 and .1 M verapamil. The set-up allows each of the three pH treatments to be tested for transporter activity with no blocks (control), an pgp block (verapamil), and an mrp block (MK571). The DMSO aids in transport across cell membranes. Table 1 shows the treatments used for each of the samples.

I staggered my samples because the test is time sensitive. That is, c-am is transported out of the cell over time and fluorescence will go up over time, so if cells are tested at significantly different times, this could skew the results. I added the c-am and

verapamil to the samples at t=2:08, first to the 7.4, then to 7.8, and finally to 8.0. At t=2:46, I added the c-am and MK571 to the samples, in the same order. At t=3:27, I added the c-am to final set of the samples, in the same order. After each sample had run for approximately two hours (verapamil t=4:10; MK571 t=4:41; control t=5:25), samples were pipetted off from the bottom of the tubes where the negatively buoyant embryos were concentrated, and photographed on a Zeiss Axiostar epifluorescent microscope at 400X. As many cells as could be found on a single slide were photographed, before moving on to the next sample. These images were then imported into Image J, where the mean grey value was computed for the cell and this data was used for the fluorescence.

In addition to the fluorescence aspect of the experiment, I also examined embryo development at 3, 9, and 22 hours. At each of these time intervals, I counted the number of individuals that were unfertilized, had not divided, had 2 cells, 4 cells, 8 cells, or more, and finally identified those individuals which appeared to reached the blastula stage. In each of the counts I photographed at least one individual I felt was representative in order to qualitatively compare morphological differences.

Hopkins, Monterey: S. purpuratus

Many of the same steps were used in the Hopkins experiments, though there were only two pH treatments, 8.0 and 7.5, at Hopkins, and I did not do early embryology observations. The measurements and timing varied as well. *S. purpuratus* was more prolific than *Echinometra* though they were injected with only .4 mL of KCl as opposed to the 1.5 mL with which I injected the *Echinometra*. Because *S. purpuratus* was so prolific, there was a layer of eggs and I sampled 2.25 mL from here and rinsed this three times in 300 mL. The sperm was diluted in a similar manner, and 3 μL of 5% sperm was

added to the 300 mL for fertilization. The fertilized eggs sank to the bottom and the seawater was drained off the top such that 60 mL remained.

The fluorescent experiment was very similar to that in the Line Islands, but instead of verapamil, PSC833 was used to block the pgp transporter. These chemicals work on the same transporter and should be roughly equivalent. For PSC833 I started with a 10mM solution and added 5 μL to my 10 mL sample. Table 2 shows my set up for this portion of the experiment.

Three hours and 10 minutes (t=3:10) after fertilization, the fluorescent samples were prepared. At t=4:40, all of the samples were put on slides and photographed. The fluorescence was analyzed using MetaMorph software.

Hawaii, HI: Tripneustes

In the Hawaii run of the experiment the pH treatments used the same procedure outlined earlier, but because of a miscalibrated pH meter, the actual pH treatments were 7.8, 7.4, and 7.0. In this run of the experiment I also did not observe embryo development because I set aside samples in formalin and these samples did not have enough individuals in them to sample.

The *Tripneustes* was also a very productive species, and I was able to collect ample eggs and sperm. Unfortunately, I only added .6 mL of pure egg to 300 mL because I felt this provided a good monolayer of eggs on the bottom of the container. This deviation from my original procedure requiring 1. 75 mL of egg caused my samples to be very dilute. I then rinsed the eggs three times and added 3 μL of 5% sperm to the 300 mL of egg. At t=0:30 I drained the seawater off the top to get down to 60 mL, which I split

evenly between the three treatments before filling each to 150 mL with the different pH treated waters.

The c-am set-up was equivalent to that shown in Table 1 for the Line Islands, except that my pH treatments were in fact lower, as previously mentioned. At t=1:14 I ran the three control samples; at t=1:30 I ran the three MK571 samples; at t=1:45 I ran the three verapamil samples. These were photographed at t=3:02, t=3:24, and t=3:33, respectively. The images were analyzed in Image J.

Results

My experiments yielded results in two areas concerning pH effects on sea urchin embryos: direct effects on embryo development and effects on multidrug efflux transporters working to protect the embryo from toxins. This section will first outline the results from the early embryo effects before discussing the results related to the toxin transporters.

The only run in which I successfully sampled the sea urchin embryo was the one performed in Washington Island. My results in this portion therefore consist of several images used for qualitative morphological comparisons and cell counts used to evaluate the stage of the different embryos (Figure 3). In the photos taken at 9 hours, the main differences consist of differing amount of pigment in the dorsal layer of the embryo. The 8.0 sample is darkened throughout the cell, perhaps indicating later development than in the 7.8 and 7.4 treated samples. In the 22 hour photos, the 8.0 treated cell appears to be at the blastula stage. In the acid treatments, the individuals appear not only slowed down in development but also fail to show a circular pattern of cells like those exhibited in the pH

8.0 treatment. The 7.4 treatment appears to be less detrimental than the 7.8 treatment based on the previous observations, but this may be a random difference between the two samples.

In a more quantitative analysis of the pH effect on sea urchin embryos, I also found some evidence that pH has a negative effect on sea urchin embryos. Figures 4 and 5 show the results of the 9 hour and 22 hour counts. Note that no individuals had progressed beyond the single cell stage at 3 hours. At both time intervals the 8.0 treatment had the most individuals in the two most developed stage recorded, and had few individuals in the early stages.

I ran a Kruskal-Wallis Test on the early development data because the data does not have a normal distribution and has high variance, making ANOVA results slightly unreliable. The test indicated that the result was not significant 9 or 22 hours, with a 9 hour $p=.351$ and a 22 hour $p=.419$.

The results from the pH effects on the transporter activity are shown in Figures 6 and 7. The control results are not graphed because there is little variation that shows up in these runs. In almost all species and all blocks, there appears to be a decrease in fluorescence, indicating that, transporter activity seems to increase in lower pH conditions. The results from the mrp block (Figure 6) show that pgp and other transporter activity increased with decreasing pH, while the results from the pgp block (Figure 7) show that mrp and other transporter activity increased initially before decreasing. This v-shaped curve is seen in both the Hawaii and Line Island legs of the experiment while the Hopkins portion shows an increase in fluorescence, indicating

increased transporter activity. It is important to note a few results of questionable validity. I did not graph my control results because changes in total transporter activity are small and difficult to observe without transporter blocks. However, the values for my mrp block in my Hawaii run are in the same range as my control results, either indicating that the mrp transporter in *Tripneustes* is extremely unimportant or indicating that for some reason my block was not working in this run.

For the transporter portion of the experiments, I ran two-factor ANOVAs in order to examine the significance of my results. I ran these separately for both the Hopkins and Line Island portions. Hawaii data was not included due to lack of replication. The results from Hopkins ($p=.107$) indicate a trend but do not show a significant effect of pH on transporter activity. The results from the Line Islands ($p=.564$) do not show any significant effect of pH on transporter activity. However, in all runs except the Hopkins pgp block, there is a drop in fluorescence with decreasing pH, lending some further credibility to the idea that decreasing pH upregulates transporter activity.

Discussion

The aim of this study was to replicate work already done in the area of pH effects on embryo development and to forge new ground by looking at effects of pH on toxic transporter activity. Based on previous work, I hypothesized that pH would have a negative effect on the rate of cleavage development and on the overall development morphology (Kurihara et. al, 2004). I hypothesized that the individuals would be fitted to their environments and would thus suffer a decrease in transporter activity at higher pHs.

For the embryo development portion of the experiment, the observed negative effect of the low pH water water on the embryos supports the hypothesis, although it was not a statistically significant result. The 9 hour sample had more individuals than the 22 hour sample, and it shows that the 8.0 treatment yielded individuals with both 4 and 8 cells, stages to which the acid treated cells had not yet progressed. Though the data is a bit more scattered for the 22 hour sample due to low sample size, there are more multicellular and blastula cells in the 8.0 treatment than in either of the other two lower pH treatments. Though not statistically significant, the results do follow my prediction, with the 8.0 treatment yielding individuals that have progressed further than the acid treated individuals.

In comparing this study's results to the Kurihara *et. al*. results, I find that although my individuals trended in the same direction as those in their study, my individuals developed significantly more slowly (Figure 1 b, c versus Figures 4, 5). Figure 1 b, c shows the 105 min (1h:45m) and 210 min (3h:30m) progression of the cells. After 3 hours, only single cell fertilized embryos and unfertilized embryos and did not observe any four or eight cell embryos in any of the treatments. In the Kurihara *et. al*. data, the first division had taken place in some embryos by 1:45, and the embryos had progressed to the four and eight cell stage by 3:30.

I cannot easily explain this difference in development time, but suspect it may have to do with the rinsing of the jelly coat on the eggs. The eggs naturally have a jelly coat to protect them as they drift in the water column. In lab, this is rinsed off. I rinsed my eggs three times, and in the Kurihara *et. al.* study they rinsed theirs "several times" but perhaps there was a difference in how much jelly was removed that prevented my

embryos from being fertilized as easily. Alternatively, there may have been some negative effect of the HEPES and NaOH on the embryos which might have slowed down their progression rather than just the initial lag until fertilization. Finally, I had some trouble evaluating how many cells were in each of the individuals and perhaps my counts themselves were inaccurate. Unfortunately, the Kurihara *et. al*. paper does not specify the time at which the blastula developed, so it is difficult to say whether delayed fertilization can account for this difference. It seems odd to me that there were still some single and double celled embryos when the blastula was forming in other individuals, and this may be an indication there was a problem after fertilization.

In addition to the one run of pH effects on embryo development, I ran three tests on transporter activity on three different species in three different locations. The florescence I measured in the cells is a measure of the calcein build-up in the cell, essentially showing the amount of a toxin remaining in the cell. Thus, more fluorescence is indicative of less transporter activity, and less fluorescence is indicative of more transporter activity.

The results from the mrp block portion of the experiment show an increase in transporter activity. This increase could either be due to development of more transporters or it could be due to more rapid transport out of the existing transporters. The results from the pgp block portion of the experiment show an increase in transporter activity at moderate pHs, and a decrease in transporter activity at lower pHs in both the Hawaii and Line island runs. This increase in transporter activity may indicate that the transporters speed up at moderate pHs and then decrease in prevalence at lower pHs.

Alternatively, moderate pHs may increase the number of transporters and then further reductions in pH cause the transporters to slow down.

Little is known about the mrp and pgp transporters, so it is difficult to know by what mechanism pH may alter transporter activity. Many biological pumps work with H+ ions and it is possible that the mrp and pgp transporters are coupled to a H+ ion pump, exchanging H+ for toxins. If this were the case, a higher external concentration of H+ would make it easier for the transporters to pump and would accelerate the transporter activity. Alternatively, with increased H+, more H+ will diffuse into the cell, perhaps causing the cell to start pumping out H+ and toxins alike at higher rates. Because pH seemed to negatively effect cell development, it seems that if pH were to cause a change to the number of transporters it would have a negative effect. The effect of having fewer transporters may overwhelm the increased pumping rate at some threshold pH. These posited mechanisms support the idea that transporters may be speeding up at lower pHs though certain transporters may have decreased prevalence at lower pHs.

It is also notable that the transporter activity in *Echinometra* from the Line Islands was lower than the transporter activity in *Tripneustes* from Hawaii, under both transporter blocks. I did not analyze the significance of this as I only had fluorescent values from one cell per treatment for the Hawaii run. *S. Purpuratus* from Hopkins unfortunately cannot be compared because the cells were analyzed on a different program.

These conclusions, however, are drawn from only a few experiments performed with lower densities than desired and resultantly only providing a few individuals for each condition. The strength of fertilization experiments is usually the large sample size,

but unfortunately due to dilution problems in Hawaii and unprolific urchins in the Line Islands, I was unable to get enough individuals for a good run of the experiment. Future studies could easily rectify this problem with a prolific species and correct dilution of gametes. Less prolific species can be used, but I would suggest gathering eggs from more than one individual in order to get enough eggs. This crucial step of getting a large enough sample size would help greatly in confirming or disproving the data trends presented here.

Conclusion

With the onset of global climate change it is extremely important to understand the impact we will have on the ocean. While scientists have begun to examine direct impacts of CO2 and ocean acidification on a few organisms, no one has yet investigated how ocean acidification might impact an organism's ability to respond to other stresses in the environment. Toxins are becoming an increasingly large problem as humans dump toxins into the aquatic environment from sewers, agriculture, and other sources. Thus, investigating the impact of ocean acidification on the transporters that allow embryos to export these toxins is a good first step in investigating one of the indirect impacts of ocean acidification on the marine community.

As I expected, embryo development was negatively affected by decreased pH conditions, but counter to my hypothesis, toxin transporter activity was upregulated under more acidic conditions. These results suggest that while sea urchin embryos will have to toil with developmental problems in a more acidic oceans, they will be able to continue ridding themselves of toxins, and may actually even be able to get rid of toxins more

easily. Mrp and pgp transporters are widespread transporters, found in other invertebrates like *C. elegans* and even humans (Hamdoun et. al., 2004) and so this result may indicate that other animals will also be spared the problem of compromised defense as they face the many other challenges of global climate change.

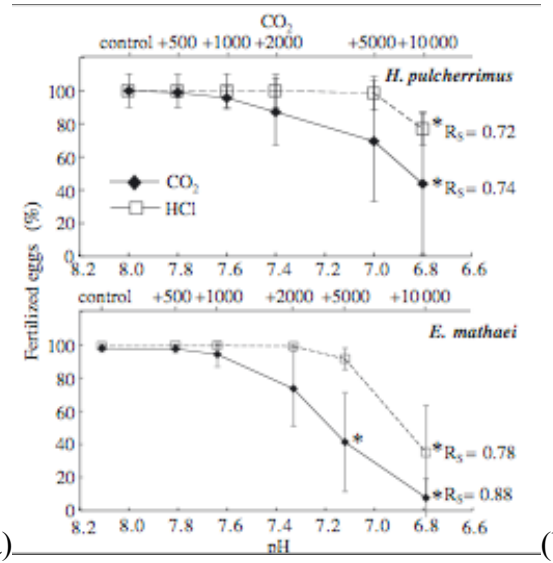
Though I had not anticipated comparing differences in species' transporter activity, my results do indicate higher activity in *Tripneuses* than in *Echinometra* though I was unable to compare these species with *S. Purpuratus* because of different fluorescence analyses.

Though sea urchin transporters may be a convenient place to start in order to investigate some of the indirect effects of pH on marine organisms, future research should delve into this broader question, exploring additional ways in which pH might compromise various organisms' abilities to respond to a wide variety of unrelated environmental stressors. If an organism is killed by their inability to deal with a common virus under low pH conditions, for example, the effects of pH on the organism's calcium carbonate shell may be of very little consequence. For the two transporters I examined in sea urchins, pH seemed to upregulate rather than compromise the activity of the transporters, aiding the embryo in protecting itself from toxins with the onslight of global warming. This shows that in this case, indirect effects do not appear to overwhelm direct effects, and indeed the developmental problems seem a more significant problem for the sea urchin embryos. Testing possibly detrimental indirect effects is a crucial step, and without exploring these other options we could be missing large pieces of the puzzel as to what the affects of global climate change really are.

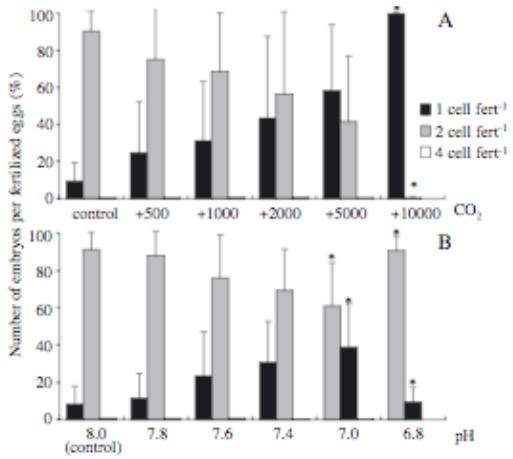
Figures

Figure 1: Kurihara et. al., 2004 Results

(a) Percent fertilized eggs decreases with decreasing pH in two studies species of sea urchins. CO2 treatment has a worse effect on the fertilization rates than HCl. (b) Cleavage rates decrease with decreasing pH for both CO2 treatment and HCl treatment. This is seen as the number of embryos with two cells falling as those with one cell rise as the pH decrease. This graph is drawn for t=105 min. (c) Body length decreases with decreasing pH, measured along three axes. (Kurihara et. al. 2004)



(a)



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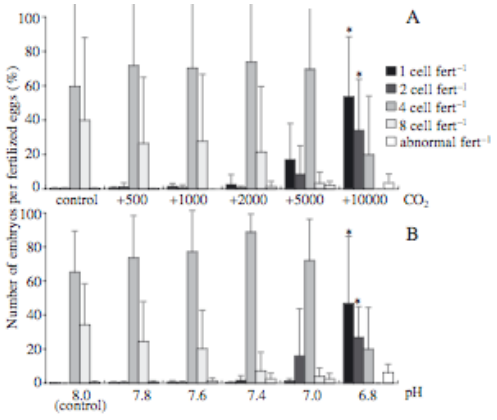

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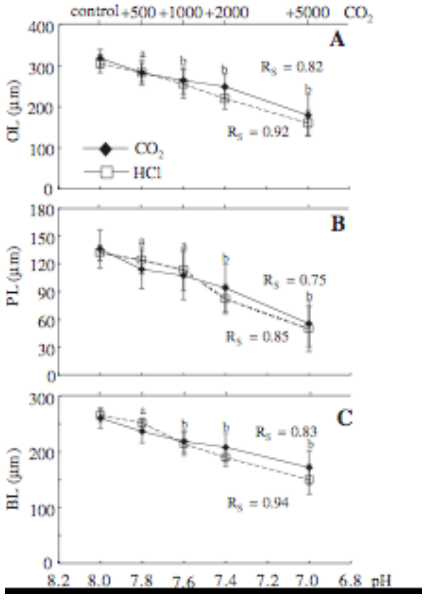


(d)

Figure 2: Transporter and c–am molecular system.

This is a cartoon of the transporter and c-am molecular system.

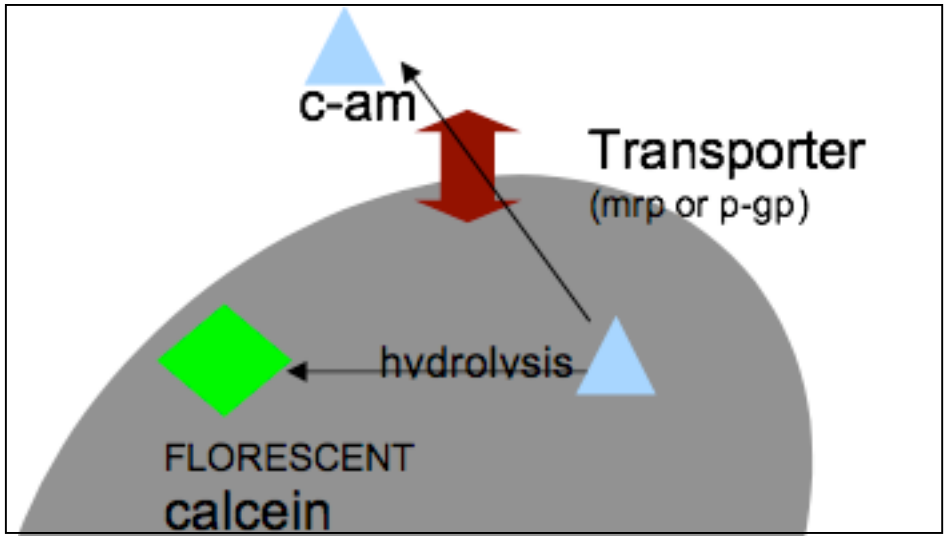
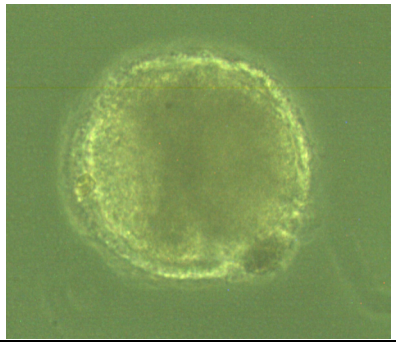
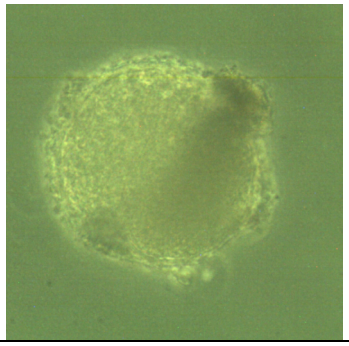
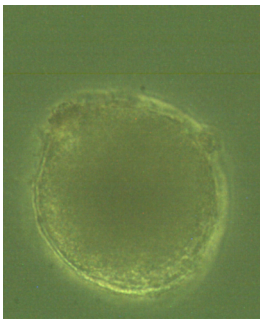


Figure 3: Pictures of sea urchin embryos under different pH conditions

These are pictures taken at 400X of *Echinometra* sea urchins taken at 9 hours and 22 hours under different pH treatments, as indicated. Note that darkening of the individuals at 9 hours, with the most darkness in the 8.0 embryos. Also note, in the 22 hour individuals the distinct shape of the 8.0 embryo and the lack of circular formation in the 7.8 and 7.4 embryos.

	pH= 8.0	pH=7.8	pH=7.4
9 hours			

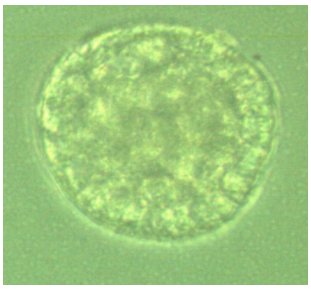
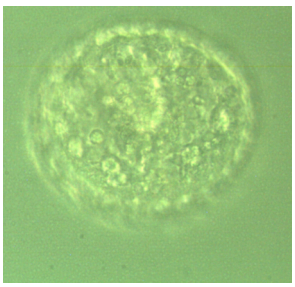
22 hours			

Figure 4: pH effects on embryo development at 9 hours in *Echinometra*

This graph shows percent of fertilized embryos at different stages: 1, 2, 4, and 8 cells at 9 hours. The number of individuals counted in each sample is indicated. Note that the 8.0 sample has some individuals at four and eight cells and has fewer individuals earlier in development who have not yet undergone division.

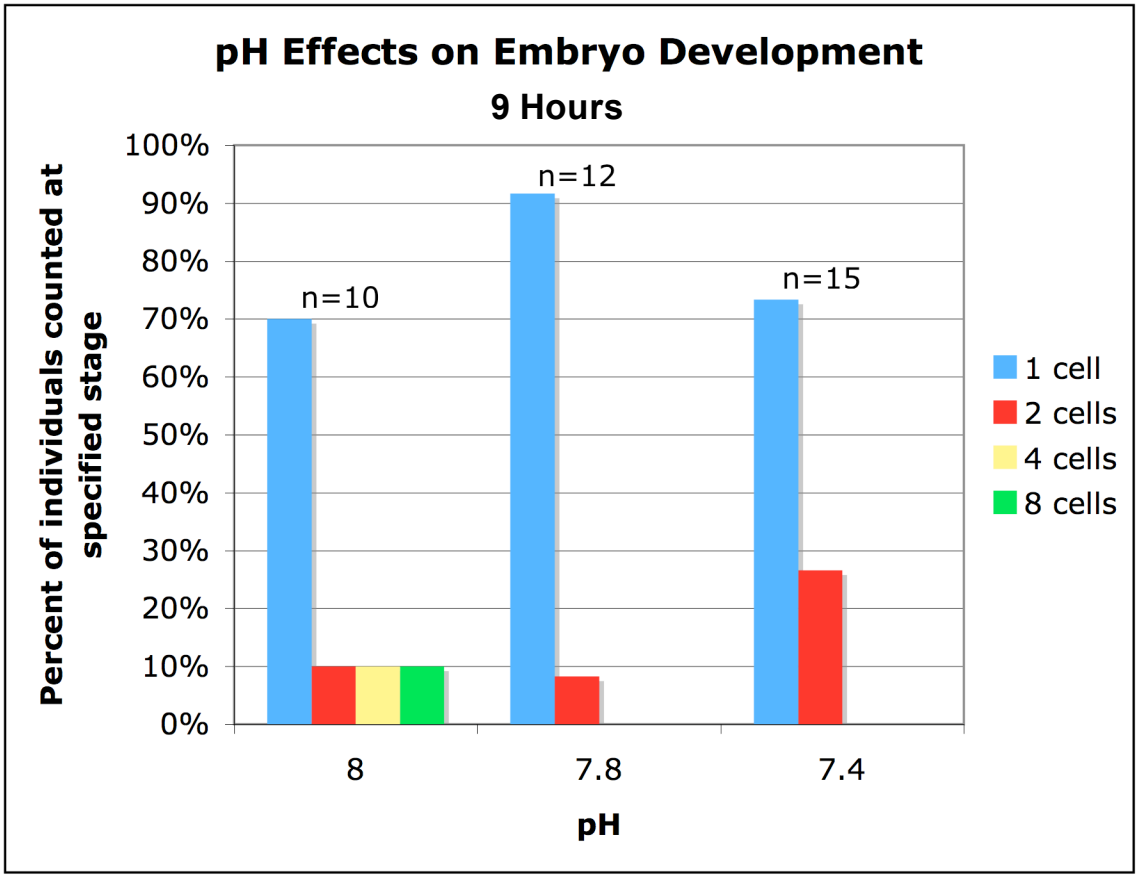


Figure 5: pH effects on embryo development at 22 hours in *Echinometra*

This graph shows percent of fertilized embryos at different stages: 1 cell, 2 cells, 4 cells, mulitcelluar, and blastula, observed at 22 hours. The number of individuals counted in each sample is indicated. Note that the 8.0 sample has some more mulicelluar and blastula individuals than the other two treatments.

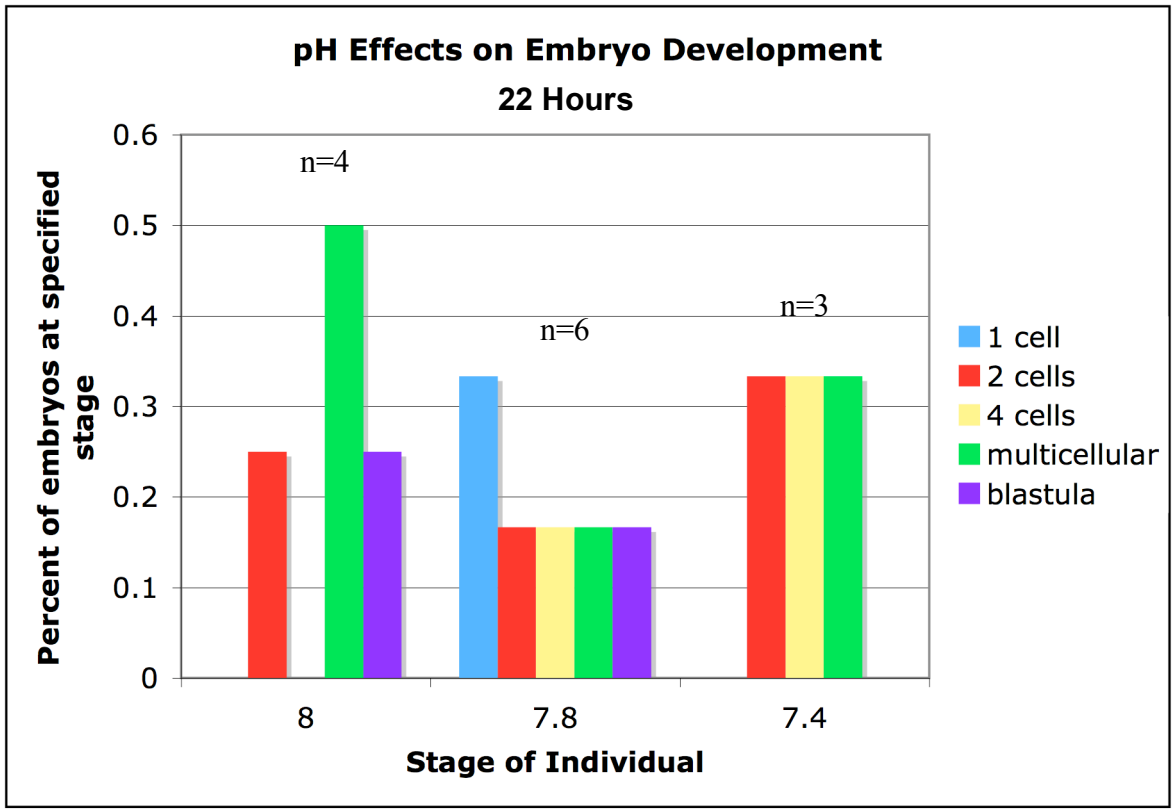


Figure 6: Calcein build-up with mrp transporter block

This graph shows the fluorescence results for all three experiments, with the Hopkins run on a different axis because the results were evaluated using a different program. When the mrp transporters were blocked, there was an increase in transporter activity in the other channels, indicated by a drop of fluorescence with decreasing pH. This is consistent for all three species in all three locations. Samples sizes are shown for each set of data, and the ANOVA p-values for the Hopkins and Line Island analyses. Error bars represent one standard error.

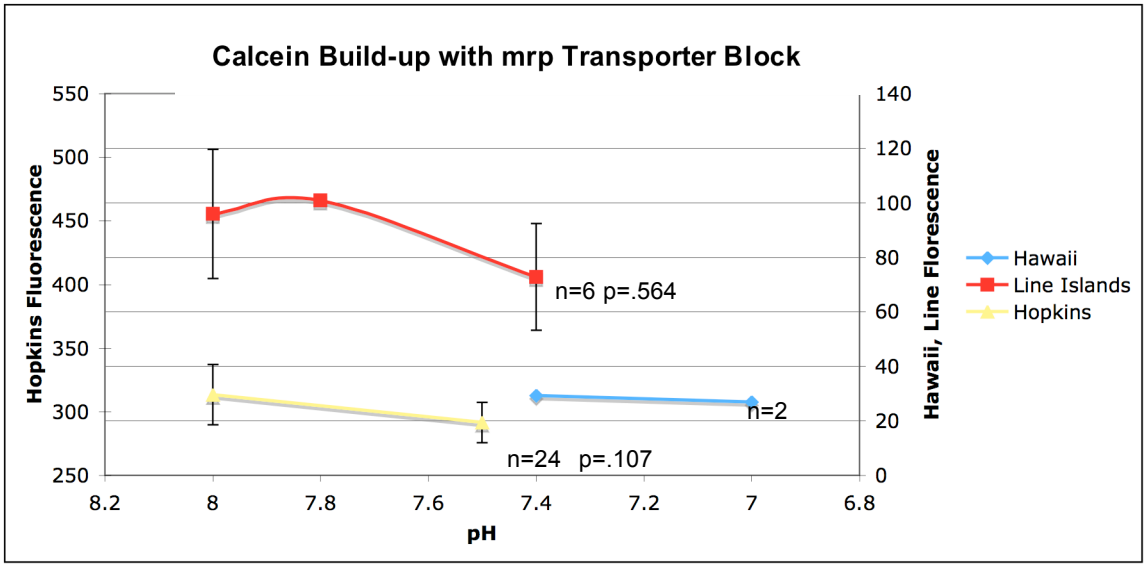
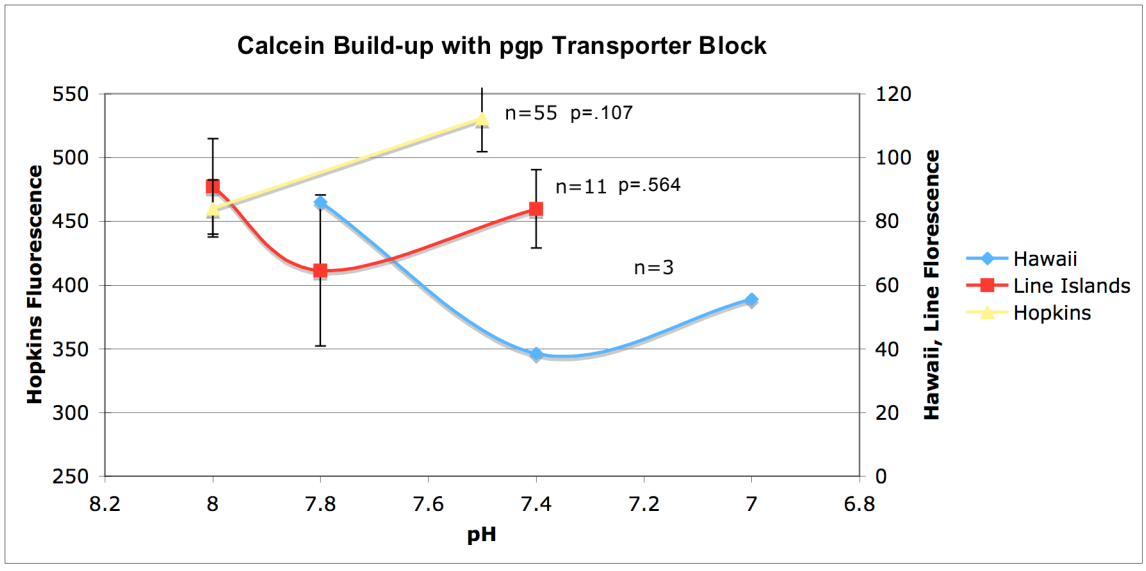


Figure 7: Calcein build-up with pgp transporter block

This graph shows the fluorescence results for all three experiments, with the Hopkins run on a different axis because the results were evaluated using a different program. Both the Hawaii and Line Island runs show an initial decrease in calcein accumulation and then an increase in calcein accumulation. The Hopkins data shows an increase in calcein accumulation. Samples sizes are shown for each set of data, and the ANOVA p-values for the Hopkins and Line Island analyses. Error bars represent one standard error.



Tables

Table 1: C-am experiment set-up for Line Islands
This shows the experimental set-up for the Line Islands fluorescent run. Essentially this same set-up was used in Hawaii as well, though 8.0 was substituted by 7.8, 7.8 by 7.4, and 7.4 by 7.0.

Condition (label on test tube)	pH	mL from pH treated sample	c-am solution	MK571 (1mM)	Verapamil (.1M)
pH= 8.0, p-gp inhibitor	8.0	10	2.5 μL		5 μL
pH= 7.8, p-gp inhibitor	7.8	10	2.5 μL		5 μL
pH= 7.4, p-gp inhibitor	7.4	10	2.5 μL		5 μL
pH = 8.0, mrp inhibitor	8.0	10	2.5 μL	100 μL	
pH = 7.8, mrp inhibitor	7.8	10	2.5 μL	100 μL	
pH = 7.4, mrp inhibitor	7.4	10	2.5 μL	100 μL	
pH= 8.0, control	8.0	10	2.5 μL		
pH= 7.8, control	7.8	10	2.5 μL		
pH= 7.4, control	7.4	10	2.5 μL		

Table 2: C-am experiment set-up for Hopkins

This shows the experimental set-up for the fluorescent experiments run at Hopkins. Note that PSC833 was used to block the pgp transporter rather than verapamil.

Condition (label on test tube)	pH	mL from pH treated sample	c-am solution	MK571 (1mM)	PSC833 (.1M)
pH= 8.0, p-gp inhibitor	8.0	10	2.5 μL		5 μL
pH= 7.5, p-gp inhibitor	7.5	10	2.5 μL		5 μL
pH = 8.0, mrp inhibitor	8.0	10	2.5 μL	100 μL	
pH = 7.5, mrp inhibitor	7.5	10	2.5 μL	100 μL	
pH= 8.0, control	8.0	10	2.5 μL		
pH= 7.5, control	7.5	10	2.5 μL		

Appendix

Notes on Collection:

I had planned to collect *Echinometra mathaei* in both Hawaii and the Line Islands, but this species is found in well-protected holes in the reef and is extremely difficult to access. I therefore opted for *Tripneustes* in Hawaii as these urchins, colloquially known as collector's urchins, are extremely easy to collect and can simply be picked up off the reef. While the Line Islands do sometimes have *Tripneustes*, these urchins were not present in late May when we were there and thus I reverted to extracting the *Echinometra,* with some difficulty. *Echinometra* are fragile and first attempts and

prying them out of their holes often resulted in breaking their shells and killing the individuals. At the advice of Steve Polumbi, a scientist at Hopkins who has collected *Echinometra* before, I switched my collection method from prying to using a hammer and chisel to break away the dead parts of the reef protecting the urchin before freeing it from the substrate. This collection method worked much better and we were able to extract the urchins and use them for experimentation. It should be noted that *Diadema* were also collected, with great care because of their venomous spines, but appeared not to be gravid in late May and were not used for experimentation.

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