The Interaction of Ocean Acidification and Food Availability on Growth and Metamorphosis in the Gastropod *Crepidula fornicata*

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Blinks/NSF REU/BEACON 2014

Summer 2014

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Keywords: *Crepidula fornicata,* ocean acidification, nutrition,

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Abstract

The earth's oceans are becoming more acidic due to absorption of atmospheric CO_2. Ocean acidification decreases the concentration of carbonate ions in seawater, and imposes increased energy costs on organisms that deposit calcium carbonate shells and skeletons. Larval stages of these organisms are especially vulnerable to stresses of acidification and nutrition, which may have a common energetic basis. We investigated how acidification and nutrition affected pre-competent larval growth of the slipper limpet *Crepidula fornicata* during the first 8 days after hatching. We also asked if a four-day period of food and/or acidification stress, applied to older competent larvae, affected speed of metamorphosis and juvenile growth after stresses were relieved. Treatments in both experiments used all 4 combinations of pH 7.8 or 7.4, and high or low food concentration ($15x10^4$ or $1x10^4$ cells/ml *Isochrysis galbana*). Lower food level in the pH 7.8 treatments resulted in decreased growth rates for pre-competent larvae ($p<0.0001$), while larvae failed to grow in the pH 7.4 treatments regardless of food level. Competent larvae were unaffected by either pH or nutrition level over the course of a four-day stress period indicated by their inability to grow. Increased acidification extended the latency of KCl-induced metamorphosis in the second experiment ($p < 0.0309$), but nearly all larvae completed metamorphosis within 24 hours. Together, acidification and low food availability decreased subsequent juvenile growth in the first 6 days after metamorphosis ($p<0.01$), demonstrating latent effects of larval experience.

Introduction

Anthropogenic atmospheric CO_2 has been increasing at an unprecedented rate for the past 250 years. Oceans have absorbed approximately a third of the anthropogenic carbon that gets released into the atmosphere, causing an increase in pCO_2, which then causes a decrease in the average pH of the ocean (Doney et al., 2009). Ocean acidification also causes a decrease in carbonate ion concentration and can lead to undersaturated waters with respect to calcium carbonate. Current open ocean surface pH levels are approximately 8.1-8.2, and research pertaining to the long-term effects of ocean acidification has predicted that these pH levels will decrease to 7.6-7.9 by the year 2100. By the year 2300, the pH is estimated to further decrease to 7.33-7.5 (Ross et al., 2011). Early development stages of marine species have been thought to be the most susceptible to negative impacts of ocean acidification and therefore the focus of many research endeavors as seen by a compilation of results of past studies in Table 1. Rather than waiting until the year 2100 to observe the effects of these lowered pH levels, however, we can look at areas that are already exhibiting localized pH levels near these future predicted average levels. Upwelling is a naturally occurring phenomenon by which deep-level seawater rich in CO_2 is brought closer to the surface near coastlines. Feely et al. (2008) has recorded lower pH measurements along the Pacific coast of the US due to upwelling of more acidified seawater onto the continental shelf. Upwelling has already been shown to cause insufficient shell development in larval oysters (Barton et al. (2012). Estuarine environments offer another opportunity for studying acidification stress. The seawater in estuaries is less alkaline to begin with due to lower salinity, so an increase in pCO_2 has a more pronounced effect on the pH. High levels of respiratory

CO_2 also contribute to the lower pH levels in estuaries. Twanoh in Washington State is an example of a site where all of these factors contributing to acidification stress are at work: upwelling, anthropogenic CO_2, and estuarine effects. The amount of CO_2 in the Twanoh seawater is extremely variable but has reached up to 1000 umol/mol since 2009 when the seawater was first monitored (PMEL Carbon Group, nd). It is crucial that we examine the species that reside in these areas to see if they have adapted to the lowered pH in their environments, since this might be an indicator that species in other marine environments could gradually become more resilient to ocean acidification.

Decreasing pH levels in seawater is not the only stressor that marine organisms will face in the future. Decreasing food density is another stressor that has been investigated among a variety of invertebrates. It has been shown that larval forms of some invertebrates experience limited densities of food while they are developing in coastal waters (Fenaux et al 1994).

Along with the potential dissolution of the shell under acidified conditions, there could be an energy problem as well. When gastropods calcify and deposit their shell, they must expend a large amount of energy in order to supersaturate the fluid from which they precipitate calcium carbonate. If waters are undersaturated due to acidification, then more energy is required to supersaturate the fluid used to create their shell (Waldbusser et al. 2013).

Ocean acidification also has the potential to disrupt feeding behavior. For example, decreased efficiency with regard to feeding has been observed in the gastropod *Concholepas*, along with a change in food selectivity toward smaller organisms such as dinoflagelleta (Vargas et al. 2013). This is one example of how acidification stress changed the organism's food selectivity and intensity of feeding. When *C. concholepas* was subjected to pH levels of 8.0, 7.8, and 7.6, their feeding intensity dropped by more than 60% and they started to select smaller cells instead of the larger cells. Since ocean acidification has the potential to not only dissolve calcifying organisms shells, but to also disrupt feeding behavior and energy-based activities such as depositing the shell, both pH levels and feeding behaviors should both be examined together.

A great deal of research has been done on attempting to understand the effect of ocean acidification on various marine organisms in different developmental stages. Experiments have been conducted on Cnidaria, Mollusca, Echinodermata, Arthropoda, Annelida, and Chordata (Pechenik, 2006; Byrne and Przeslawski, 2013). Research across a diverse selection of marine invertebrate species has shown a variety of negative impacts in response to decreased pH and combined effects of rising temperature and decreasing pH levels, including slower development, higher mortality, and decreased calcification. Some of the studies also show no effects from lower pH levels.

Marine invertebrates that have a calcium carbonate structure are the most susceptible to ocean acidification (Ross et al., 2011). While acidification appears to have an impact during different life stages of invertebrates, the larval stages of calcifying organisms such as molluscs, cnidarians, and echinoderms are especially vulnerable to lower pH levels. Acidification effects on embryos and larvae have been studied in terms of fertilization, calcification, shell growth and shape, body growth, and survival (Anlauf et al., 2011; Albright and Mason, 2013; Davis et al., 2013; Talmage and Gobler, 2011; Parker et al 2010).

Anlauf et al. (2011) investigated the impact of ocean acidification on the stony coral *Porites panamensis*. Planula larvae of this species were cultured at pH levels of 8.1 and 7.8. There were no negative effects recorded during the time when this species was in a larval state, but reduced calcification and growth were observed when the species were in a pH of 7.8 during their polyp stage. Several research studies have also examined how ocean acidification can affect echinoderms. For example, Byrne et al. (2010b) studied *Heliocidaris tuberculata* that were placed in pH 8.1 and pH 7.4-7.8 from fertilization. They observed that the lower pH samples yielded more abnormalities during development as well as reduced overall length. Other researchers have recorded similar observations in arthropods. Arnberg et al. (2013) investigated *Pandalus borealis* in the larval stage and found that there was an increase in time that it took the larvae to develop at pH 7.6 in comparison to pH 8.1.

Acidified environments do not appear to have the same effects on all species of gastropods and bivalve molluscs; some species are more susceptible to this stressor than others. The gastropod *Bembicium nanum*, when placed as embryos in pH 8.2 and pH 7.6, showed decreased survival at pH 7.6 (Davis et al., 2013). However, in the same study, *Dolabrifera brazieri* showed no detrimental effects under the same conditions, demonstrating that some species are more resistant than others to ocean acidification. Another study done by Crim et al. (2011) studied the effects of lower pH on *Haliotis kamtschatkana.* They found a decrease in shell normality and shell length at pH 7.89-7.95. Similarly, Ellis et al. (2009) studied *Littorina obtusata* in pH 8.09-8.15 and pH 7.59-7.65 and found that the lower of these two seawater pH ranges impeded development and had negative effects on shell morphology.

Marine invertebrates have been shown to be more susceptible to the deleterious effects of ocean acidification during the larval stage than in later stages of development. Talmage and Gobler (2011) researched the effects of ocean acidification on *Argopecten irradians*. This species was placed in pH 8.08 and pH 7.8 as larvae. They experienced decreased survival and growth. However, when placed in acidified conditions as juveniles, these specimens experienced no adverse effects on their development. A study by Parker et al. (2010), examining *Crassostrea gigas* that were placed in pH 8.2 and pH 7.8-8.0 at time of fertilization, showed there were abnormal and smaller larvae at pH 8.0. Kurihara et al. (2007) also examined *Crassostrea gigas* placed in treatments of pH 8.09-8.15 and pH 7.29-7.35 as larvae. These specimens showed a decrease in shell normality and size at the lower pH level. Talmage and Gobler (2011) studied *Crassostrea virginica* cultured at both pH 8.08 and pH 7.8 during the juvenile stage, showing that at pH 7.8 their shell growth decreased. The same study also examined *Mercenaria mercenaria* at both the larval and juvenile stages. Both stages were placed in pH 8.08 and pH 7.8. The larval stage showed effects such as smaller larvae and decreased survival at 7.8, whereas the juvenile stage showed no deleterious effects.

Research into the effects of ocean acidification on *Crepidula fornicata* has yielded additional insight into this area. One of the more recent publications regarding *C. fornicata* has investigated whether encapsulation protected the embryos from the effects of acidification. Adult *C. fornicata* were exposed to elevated pH treatments before and after fertilization until the larvae were released and collected. The researchers examined the larvae for abnormalities and shell morphology such as length and surface area. Noisette et al (2014) found that *C. fornicata* had smaller shell lengths when exposed to high pCO_2. There was also evidence that there were more shell abnormalities found on *C. fornicata* with the more acidified conditions. These results indicated that their encapsulation was unsuccessful in protecting the larvae from the high pCO_2 stress (Noisette et al., 2014).

Ocean acidification is a potential stressor on the larval experience, which, along with delayed metamorphosis, could impact subsequent juvenile and adult life history. While researchers have examined metamorphosis a great deal, they have not always followed up on the effects of metamorphosis and larval experience on the subsequent fate of invertebrates as juveniles (Pechenik 2006). Some of the research has focused on bryozoans, gastropods, polychaetes, crustaceans, and echinoids. Woollacott et al. (1989) researched the bryozoan *Bugula stolonifera* and found that delaying metamorphosis caused latent effects such as slowing the rate at which colonies developed. This was also seen in a study done by Marshall et al. (2003) for the colonial seasquirt species *Diplosoma listerianum*. Also, Pechenik et al. (1993) studied the barnacle *Balanus amphitrite* in which they found that delayed metamorphosis reduced the juvenile growth rate. Gebauer et al. (1999) studied the crustacean *Chasmagnathus granulata* after they delayed their metamorphosis and found there were reduced juvenile survival and growth rates**.** Highsmith and Emlet (1986) studied the latent effects of delayed metamorphosis on two species of echinoids, *Dendraster excentricus* and *Echinarachnius parma*, and found that delaying metamorphosis decreased juvenile growth.

The main research question we plan to investigate is this: what are the combined impacts of ocean acidification and nutrition on larval shell growth, metamorphosis, and juvenile growth? The organism we have selected for this investigation is *Crepidula* *fornicata*. Since the normal developmental stages of *C. fornicata* are well understood, any deviations from these norms due to stressors introduced into the environment are relatively simple to detect. The Washington State *C. fornicata* is particularly appropriate to this study due to Washington's already high elevations of pCO_2. In addition to the wealth of research regarding larval stages of invertebrates and ocean acidification, there have also been studies describing food availability as another stress that occurs in temperate coastal areas (Fenaux et al, 1994). Our investigation will attempt to provide insight into the interaction between these two environmental stressors.

C. fornicata is a benthic gastropod that is native to the east coast but which has become invasive in the west coast of the United States (Blanchard, 1997). During early spring, *C. fornicata* brood their egg capsules and release approximately 10,000-20,000 larvae (Diederich and Pechenik, unpubl.) measuring up to approximately 450 um long (Pechenik and Gee, 1993). From the time they are released, they grow approximately 50-75 um/day (Pechenik and Lima, 1984). Many Caenogastropoda shells are primarily composed of aragonite (Taylor and Reid, 1990) and when they first hatch, their shell is completely made of aragonite (Eyster, 1986). When they are about 12 days old, or approximately 900-1000 um, they reach metamorphic competence (Pechenik and Gee, 1993; Pechenik, unpubl.).

C. fornicata has been shown to metamorphose when a length of approximately 900 to 1000 μm, at an age of 7-11 days, is attained (Pechenik, 2006; Pechenik and Gee, 1993). There are examples of species such as *C. fornicata* that, lacking the right environmental cue, will spontaneously metamorphose (Pechenik and Lima, 1984). There was no difference in juveniles from control individuals (those that metamorphosed earlier) and the juveniles that were delayed in metamorphosis. They were not statistically different in mean survival, growth rate, respiration rate, feeding rate, or effect on juvenile tolerance to high temps or low salinities (Pechenik and Eyster, 1989).

Pechenik et al. (1996) have conducted research on *C. fornicata* to determine if the amount of food that the larvae receive influences the time in which they can metamorphose. They found that for *C. fornicata*, competence to metamorphose is not dependent on growth. However, the starved larvae that metamorphosed grew more slowly as juveniles than the larvae that received regular feeding.

Along with lower pH, other stressors such as food scarcity could contribute negative effects on larval growth and metamorphosis. Fenaux et al (1994) demonstrated that food limitation can inhibit growth and increase mortality in the sea urchin *Paracentrotus lividus*. Another study examined the effects of low food density on polychaetes (Qian and Chia, 1993). They starved both *Capitella sp.* and *Polydora ligni* for approximately 1-3 weeks and observed severely inhibited growth rates in both species. Eckert (1995) examined the echinoid *Encope michelini* under varying feeding conditions. Larvae that had been starved remained in the larval stage up to five times longer than usual, but were unable to metamorphose to the next stage without an adequate food supply (Eckert, 1995).

Methods

Conditioning of Adults

Adult *C. fornicata* were collected from Totten Inlet, Thurston County, Washington State on June 24th, 2014. They were placed in 1-gallon aerated glass jars filled with 2 liters of natural filtered seawater. Jars contained 1-2 stacks of *C. fornicata* depending upon their stack size. The adults were not fed regularly while they were in jars. (Pires, 2014). The jars were changed with unfiltered seawater.

Culturing Larvae to Metamorphic Competence

When the adults released their larvae, the larvae were collected by siphoning them out into a 236 µm Nitex sieve in a beaker. The sieve was lifted with the stranded larvae caught on the bottom, after which they were washed into a small bowl using a squirt bottle filled with their seawater matched to the pH of each experimental treatment.

Each batch of 300 larvae was placed in a jar containing natural seawater conditioned by the ocean acidification system at Friday Harbor Labs. The seawater was first stripped of CO_2 while also filtering the seawater by 0.2µm, and the water was also ultraviolet (UV) sterilized. The specified pH of 7.8 and 7.4 were made in coolers providing a constant reservoir. Using a Honeywell Durafet pH electrode connected with its associated UDA2182 process controller, the chemistry of the water could be monitored. In order to get the desired pH levels of 7.8 and 7.4, the process controller was used to regulate the input of CO_2 and CO_2 free air. There were four replicate jars for each treatment: 7.8 high food, 7.8 low food, 7.4 high food, and 7.4 low food. The larvae were fed *Isochrysis galbana* (clone T-ISO) at a cell density of 15 x 10^4 cells/mL or 1 x 10^4 cells/mL as high and low food concentrations, respectively. Culturing methods for growing the *Isochrysis galbana* are provided in Pires 2014. When the larvae were two days old they were thinned to 150 larvae per jar. Every day, the ASW in each 1-liter jar was replaced with fresh ASW containing their appropriate amount of food. The pH and the temperature were measured at the beginning and end of each water change with a Directline® DL421 pH Transmitter Sensor.

Growth data collection

Twenty larvae were selected randomly and measured nondestructively by pipetting one larva onto a microscope slide and then pipetting the larva into a rescue dish with its corresponding pH condition. The larvae were measured using Motic Images Plus software and ImageJ. The larvae were measured by their longest axis of shell while the larvae were lying on their left side. A two-way analysis of variance was done for the pre-competent larvae to determine the significance of pH and nutrition. All analyses were performed using GraphPad Prism version 6.0e for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com).

Competent Larval Growth

Competent larvae were reared under uncontrolled pH levels ranging from 7.8 – 8.1 and a temperature of 21-23C. They were fed at a 10x10^4 cell density. At the age of 14 days, sets of 25 larvae were placed in each four replicates for the four differently conditioned jars: pH of 7.8 with high food density; pH of 7.8 with low food density; pH of 7.4 with high food density; and pH of 7.4 with low food density. The temperature in each of the four treatments was 20 C. Before they were placed in their treatments, a sample of 20 larvae were measured and found to have an average starting size of 932.4 μm. The water in each jar was changed; and the larvae were fed, once per day for four days. On the fourth day of treatment, the larvae were removed from each jar and the shell length was measured. A One-Way Analysis of Variance was conducted in order to find statistically significant differences within each treatment. We also utilized Tukey's multiple comparisons test, which compares each mean against every other mean in the data set.

Metamorphosis Assay

After cultures were exposed to the four treatments for 4 days, they were taken out of their jars, and then 10 larvae from each treatment were put in six-well plates with pH 8.1 seawater that had been equilibrated with room air. In order to induce metamorphosis, 15 mM of KCl was added to each well in the plate. The six-well plates were checked after 6 hours and 24 hours to determine the frequency to metamorphose. A larva was considered to have metamorphosed when it had completely separated from its vellum. The frequency of metamorphosis was determined by counting the number of larvae that metamorphosed in each six-well plate. The frequency of metamorphosis was calculated and graphed. A Two-Way Analysis of Variance was performed to determine the significant differences in frequency during the both the 6-hour and 24-hour checkpoints. We also utilized Tukey's multiple comparisons test, which compares each mean against every other mean in the data set. The software was used for these analyses, as well as for creating the graphs.

Juvenile Growth

After the metamorphosis assay was complete, the 10 larvae from each treatment that had metamorphosed were placed in individual PETE plastic cups with 50 ml of 1 μm filtered seawater and 10x10^4 cell/ml of T-ISO. Each plastic cup contained 1 individual juvenile that had experienced one of the four larval exposure conditions (pH 7.8 with high food density, pH 7.8 with low food density, pH 7.4 with high food density, or pH 7.4 with low food density). Each individual was measured on the first day after metamorphosis, again at day 3, and finally at day 6. A Two-Way Analysis of Variance was conducted using our measurements, along with Tukey's Multiple Comparisons test, using Prism.

Carbonate Chemistry

In addition to the daily measurements of pH, temperature, and salinity we performed using probes, water samples were collected at about the midpoint of each experiment, both before and after that day's water change, in order to measure pH and total alkalinity. Salinity was recorded on each jar of the water samples along with the temperature at which the sample was taken. The water samples were measured spectrophotometrically using the cresol purple method of Clayton and Byrne (1993), in accordance with standardized procedures defined by Dickson et al. (2007). The alkalinity was measured using the open cell titration method also described in Dickson et al. (2007). The pCO_2, saturation states of calcite and aragonite, and adjusted pH were calculated using CO2Calc version 1.2(1) for Mac OS X (US Geological Survey, www.idn.ceos.org).

Results

Pre-competent larval growth

Growth rates for larvae were affected by both differences in pH level and differences in food concentration. When comparing larval shell lengths from larvae reared in different pH levels while both at low food diets, there were significant differences detected in their size at day 4 (p value <0.05) and at day 8 (p value

<0.0001). Growth rates for larvae reared at pH 7.8 with high food compared to larvae reared at pH 7.8 with a low food diet also had a significant difference at both age 4 (p value <0.01) and at age 8 (p value <0.0001). However, when larvae were reared in pH 7.4, the level of food concentration did not cause a significant difference at either 4 days or 8 days, and as seen in Figure 1, they barely grew. Overall, there was a significant difference when comparing the four treatments (p value <0.0001) and the effects of the stresses became more significant through time.

Competent larval growth

Competent larvae were reared under uncontrolled pH levels ranging from 7.8 – 8.1. They were fed using a 10x10^4 cell density. When placed in their appropriate treatment jars at age 14 days, 20 out of 25 larvae at the beginning of the experiment were measured for reference, measuring to 932.4 microns. After the four days in their respective treatments, there was no significant difference in the amount they grew. In fact, they barely exhibited any measurable growth at all.

Metamorphosis Assay

When larvae from their respective treatments were exposed to KCl, the larvae that received pH and nutritional stress metamorphosed less frequently than the larvae exposed to the lower stress conditions. When comparing the frequency of metamorphosis of the larvae from the pH 7.8 high food treatment to the pH 7.4 high food treatment, the higher pH and high food availability larvae metamorphosed at a faster rate ($p < 0.05$). Overall, the nature of the treatment to which the larvae were exposed during the four-day change influenced their frequency to metamorphose ($p < 0.0309$).

Juvenile Growth

The juvenile larvae were removed from the competent larval growth experiment after the metamorphosis assay to examine latent effects. The only significant values were reached on day 6 of their juvenile life. The larvae exposed to pH 7.8 with high food diet grew significantly more than the larvae exposed to pH 7.4 low food diet (p value <0.01).

Carbonate Chemistry

The ranges of the physical parameters for pH levels 7.8 and 7.4 can be seen in tables 2 and 3, respectively. Of particular interest is the difference in saturation states between the two pH levels, since this parameter has a direct effect on shell dissolution. At pH 7.8, all of the saturation states with respect to aragonite and calcite were oversaturated. At pH 7.4, most of the saturation states with respect to aragonite and calcite were undersaturated.

Discussion

Anthropogenic CO_2 has been increasing at a high rate causing ocean acidification that will continue to increase in severity. The result of rising pCO_2 will potentially have a more severe effect on estuarine environments due to their lower salinity, lower alkalinity, and higher levels of respiratory CO_2 relative to open seawater environments. Food scarcity among marine organisms also adds an energetic stressor to the larvae's development. This study demonstrates the effects of the increasingly lower pH level of the ocean and nutrition on larval and juvenile shell growth and time to metamorphose for *C. fornicata.* Pre-competent larvae reacted negatively to lower pH measurements and lower food diets. Moreover, higher food density was unable to combat the effects of an acidified aquatic environment for *C. fornicata*; in a manner similar to that observed by Vargas et al. (2013) when bivalve larvae in acidified environments changed their feeding intensity and size selection, the *C. fornicata* that we exposed to low pH environments appeared incapable of converting increased food availability into improved shell development. Induction of metamorphosis was significantly slower after a four-day period of combined acidification and low food level stress. Ocean acidification and nutrition have the potential to decrease the rate at which larvae of *C. fornicata* calcify/maintain their shell and could slow down metamorphosis. The results also indicate that latent effects on juvenile growth can be seen when competent larvae are stressed for a short period of time.

Although the population of *C. fornicata* examined in this study was from an area exhibiting varying pH levels due to upwelling and anthropogenic factors, the larvae and juveniles were more resilient to acidification and nutritional stresses than were other larvae. Prior studies have reported differing effects on Mollusca in reaction to lower pH values. For example, a study done by Crim et al (2011), revealed that *Haliotis kamtschatkana* as larvae experience smaller shell lengths at 7.89 -7.95 when compared to larvae grown at pH 8.09-8.15, which is in the same range as the high pH used for our experiment. At pH 7.8, with a high food diet, *C. fornicata* larvae grew slightly below nominal rates at approximately 40 microns/day. When the larvae are not food limited, *C. fornicata* grow approximately

50-70µm/day (Pechenik and Lima 1984). This would suggest that *C. fornicata* are more resilient to ocean acidification, with access to excess food, than other species.

Different species exhibit varying responses to acidified treatments and have different pH thresholds beyond which significant differences in growth occur. Another study conducted by Davis et al. (2013) showed that for the gastropod *Dolabrifera brazier,* there were no detrimental effects at pH 7.6. However, *Littorina obtusata* exhibited results such as negative effects on shell morphology and negative effects on developmental speed (Ellis et al. 2009), which reinforces the conclusion that there are varying tolerances for different gastropod species. Bivalves such as *Argopecten irradians* showed adverse effects such as smaller larvae at pH 7.8 (Talmage and Gobler, 2011). A study done by Kurihara et al. (2007) showed that, for *Crassostrea gigas* at pH 7.29-7.35, the species showed a decrease in shell normality and size, which is close to the pH at which *C. fornicata* started showing significant differences in development. Our finding that larvae grew more slowly after food limitation during the larval period supports the findings of Fenaux et al (1994), who demonstrated that food limitation inhibited growth in the sea urchin *Paracentrotus lividus*.

Most investigations into the effects of ocean acidification on juveniles start with the juvenile life form and do not analyze the carryover effects from the larval life stage to the juvenile life stage. For example, the study done by Talmage and Gobler (2011) researched the effects of ocean acidification, with a pH of 7.8, on *Argopecten irradians* and *Mercenaria mercenaria* juveniles and found that there were no adverse affects. The contrast between this finding and our own results with

C. fornicata reinforces the idea that some species are more resistant to pH level than others. Our data from observing juvenile *C. fornicata* also supports a previous paper in which Pechenik et al. (1996) found that larvae that were not given adequate food during the larval period grew slowly as a juvenile. The study done by Pechenik et al. (1996) also transferred the new juveniles to the full nutrition treatment and saw a decrease in growth for three days. Our experiment looked at juvenile growth for six days, which is twice as long as Pechenik's experiment. However, Pechenik starved the larvae for 6 days while we only had the larvae on a low diet for 4 days. Pechenik found a significant relationship between the amount of time starved and the juvenile growth rate ($p<0.001$). When larvae were starved for 6 days, Pechenik et al. (1996) found that they grew approximately 25 um/day, while larvae that were only starved for two days had a growth rate of about 200 um/day (Pechenik et al. 1996).

Past studies such as a study done by Pechenik et al. (1996) researched the effect of starvation on competence to metamorphose in *C. fornicata*. They found that even though the larvae that were starved stopped growing or grew very slowly, the larvae still metamorphosed while being starved, indicating that competence to metamorphose is not size-dependent for *C. fornicata*. In our study, there was no significant difference in frequency to metamorphose between high food diets and low food diets at the same pH level, while larvae with a low food diet at a lower pH level metamorphosed more slowly than the larvae at a higher pH level and a high food diet.

Future research regarding the effect of ocean acidification on larval forms should take into account whether there is upwelling in the specimen collection area, as in the region in Washington State from which the *C. fornicata* for this study were collected. Additional research should also examine the factors that make some species more tolerant to ocean acidification than others. Another potential line of inquiry is to look into whether the population's location is a factor in their resistance to the combined effects of ocean acidification and nutritional stress. Studies could also look into whether *crepidula* could survive if their shell has been completely or even partially dissolved. If the same research is repeated, mortality should be measured to obtain a larger picture of how ocean acidification and nutrition might affect the *C. fornicata* population size.

Tables and Figures:

Table 1. Compilation of effects on Mollusca of the impacts of ocean acidification

Phylum, Species	**Stage Placed in Treatment**	**pH** (Control, Experimental)	**pCO_2**	**Effect**	**Reference**
Mollusca					
Gastropoda					
Bembicium nanum	Embryos	C: 8.2; E: 7.6		Decrease in Survival	Davis et al. (2013)
Dolabrifera brazier	Embryos	C: 8.2; E: 7.6		No adverse effects	Davis et al. (2013)
Haliotis coccoradiata	From Fertilization	C: 8.2; E: 7.6-7.8	C: 310; E: 1800-1090	Abnormal larvae and higher mortality at pH 7.8	Byrne et al. (2010b)

Haliotis kamtschatkana	Larvae	C: 8.09-8.15 E: 7.89-7.95 E2: 7.59-7.65		Decrease in survival, shell normality and shell length at 7.89-7.95	Crim et al. (2011)
Haliotis laevigata	Juvenile	C: 7.78 E: 7.39		Decrease in growth	Harris et al. (1999)
Haliotis rubra	Juvenile	C: 7.93; E: 7.37		Decrease in growth	Harris et al. (1999)
Littorina obtusata	Embryos	C: 8.09-815; E: 7.59-7.65		Negative Effect on shell morphology and developmental speed	Ellis et al. (2009)
Bivalvia					
Argopecten irradians	Larvae	C: 8.08; E: 7.8	C: 375; E: 772	Smaller larvae and reduced survival	Talmage and Gobler (2011)
Argopecten irradians	Juveniles	C: 8.08; E: 7.8	C: 375; E: 772	No adverse effects	Talmage and Gobler (2011)
Argopecten irradians	Larvae	C: 7.93 E: 7.39		Decreased survival and growth	White et al. (2013)
Crassostrea gigas	From Fertilization	C: 8.2; E: 7.8-8.0	C: 375 E: 1000-800	Increased mortality and abnormal smaller larvae at 8.0	Parker et al. (2010)
Crassostrea gigas	Larvae	C: 8.09-815; E: 7.59-7.65 E2: 7.49-		No adverse effects	Gazeau et al. (2011)

		7.55			
Crassostrea gigas	Larvae	C: 8.09-8.15; E: 7.29-7.35		Decrease in survival, shell normality, and size	Kurihara et al. (2007)
Crassostrea gigas	Juveniles	C: 8.08; E: 7.8	C: 375 E: 772	Smaller larval shell at 7.8	Talmage and Gobler (2011)
Crassostrea virginica	Larvae	C: 8.09-8.15; E: 7.99-8.05; E2: 7.79-7.85		Decrease in shell area at 7.79-7.85	Miller et al. (2009)
Crassostrea ariakensis	Larvae	C: 8.09-8.15; E: 7.99-8.05; E2: 7.79-7.85		No adverse effects	Miller et al. (2009)
Mercenaria mercenaria	Larvae	C: 8.08; E: 7.8	C: 375; E: 772	Smaller larvae and increased mortality at pH 7.8	Talmage and Gobler (2011)
Mercenaria mercenaria	Juvenile	C: 8.08; E: 7.8	C: 375; E: 772	No adverse effects	Talmage and Gobler (2011)
Saccostrea glomerata	From fertilization	C: 8.2; E: 7.8-8.0	C: 375; E: 1000-600	Increased mortality and abnormal and smaller larvae at pH 8.0	Parker et al. (2010)
Tridacna squamosa	Juvenile	C: 8.15; E: 8.01; E2: 7.83	C: 416; E: 622; E2: 1019	Decreased survival under experimental conditions	Watson et al. (2012)
Ostrea lurida	Larvae	C: 8.0; E: 7.9; E2: 7.8		Decrease in shell growth rate at 7.8 and also a decrease in shell area	Hettinger et al. (2012)

Mytilus edulis	Larvae	C: 8.09-8.15; E: 7.59-7.65		Size decreased	Bechmann et al. (2011)
Mytilus edulis	Larvae	C: 8.09-8.15; E: 7.99-8.05; E2: 7.59-7.65		Decrease in size for both experimental pH values	Gazeau et al. (2010)
Mytilus californianus	Larvae	C: 8.09-8.15; E: 7.99-8.05; E2: 7.79-7.85		Decrease in shell area at pH 7.79-7.85; Decrease in shell strength at both experimental values and decrease in shell thickness at pH 7.79-7.85	Gaylord et al. (2011)
Mytilus galloprovincialis	Larvae	C: 8.09-8.15; E: 7.29-7.35		Decrease in size, shell mortality, and developmental speed	Kurihara et al. (2008)
Ruditapes decussatus	Juvenile	C: 8.13-8.18 E: 7.80-7.84 E2: 7.46–7.57		Decreased ingestion and respiration rates. Increased excretion of ammonia	Fernandez-Reiriz et al. (2011)

Table 2. Ranges of physical properties of seawater for the pH 7.8 treatment

	High	**Low**
pH*	7.87	7.72
Salinity	30.2	30.0
Temperature (°C)	20.3	19.7
Total Alkalinity (µmol/kgSW)	2086.531	2082.89

pCO_2 water (µatm)*	832.779	721.172
Ω_a*	1.496	1.320
Ω_c*	2.328	2.190

Table 3. Ranges for the pH 7.4 treatment

	High	**Low**
pH*	7.52	7.42
Salinity	30.2	28.9
Temperature (°C)	20.3	19.7
Total Alkalinity (µmol/kgSW)	2086.531	2082.89
pCO_2 water (µatm)*	2144.355	1709.554
Ω_a*	0.709	0.583
Ω_c*	1.105	0.907

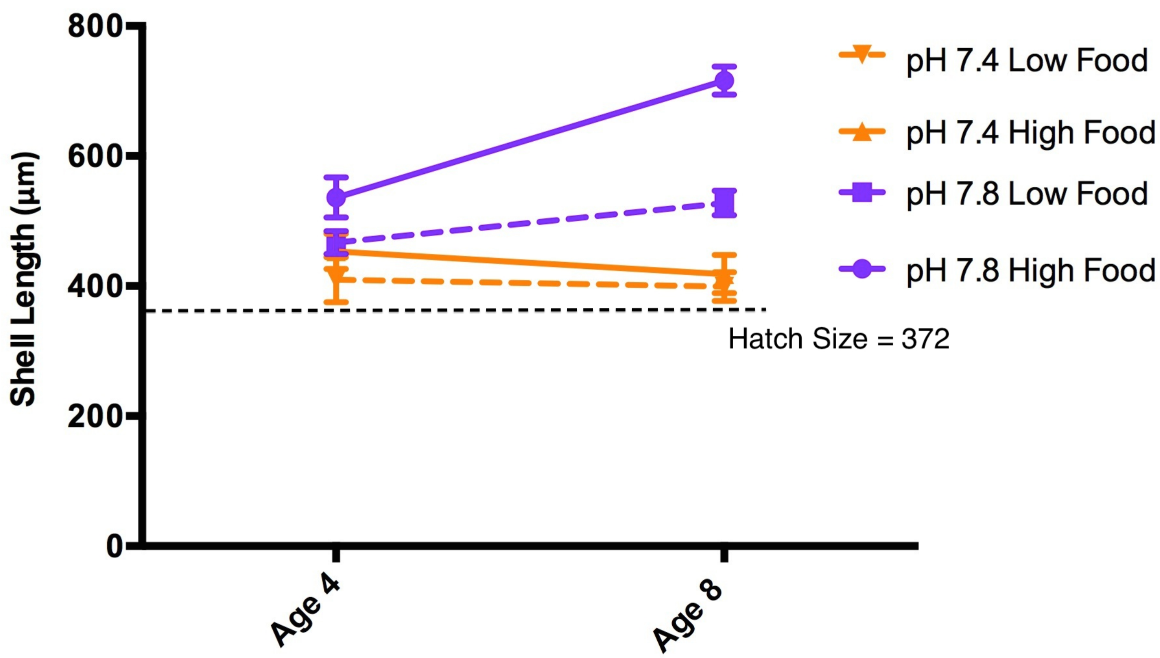

Figure 1. Growth of *Crepidula fornicata* larvae under different acidified conditions and food levels, for 8 days after hatching. Symbols are grand means of 20 larvae sampled from each of 4 replicate cultures of each treatment. Error bars are +/- 1 SEM.

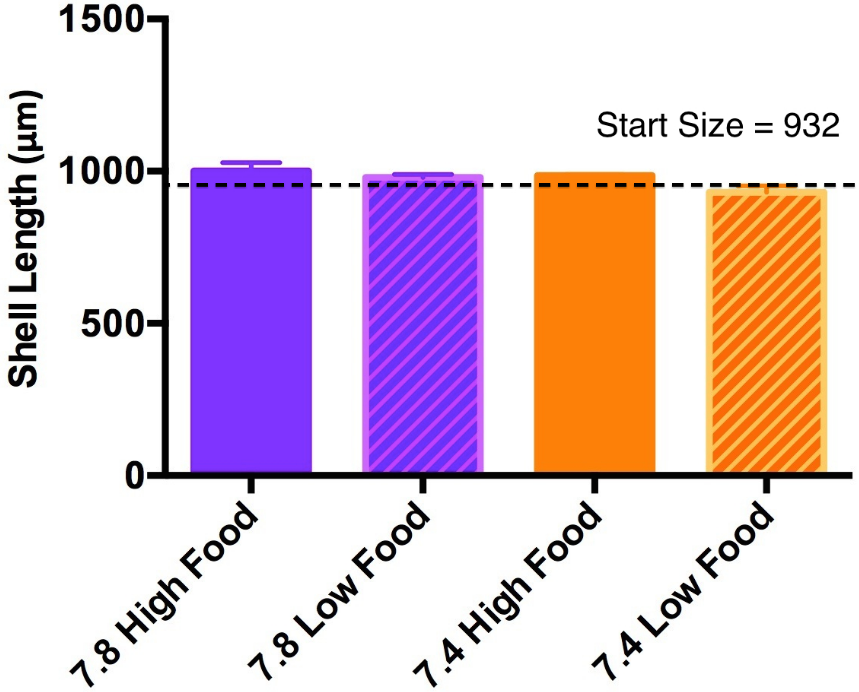

Figure 2. Shell length for *Crepidula fornicata* after four days in either pH 7.8 with a high food concentration, 7.8 with a low food concentration, 7.4 with a high food concentration, and 7.4 with a low food concentration with their respective SEM.

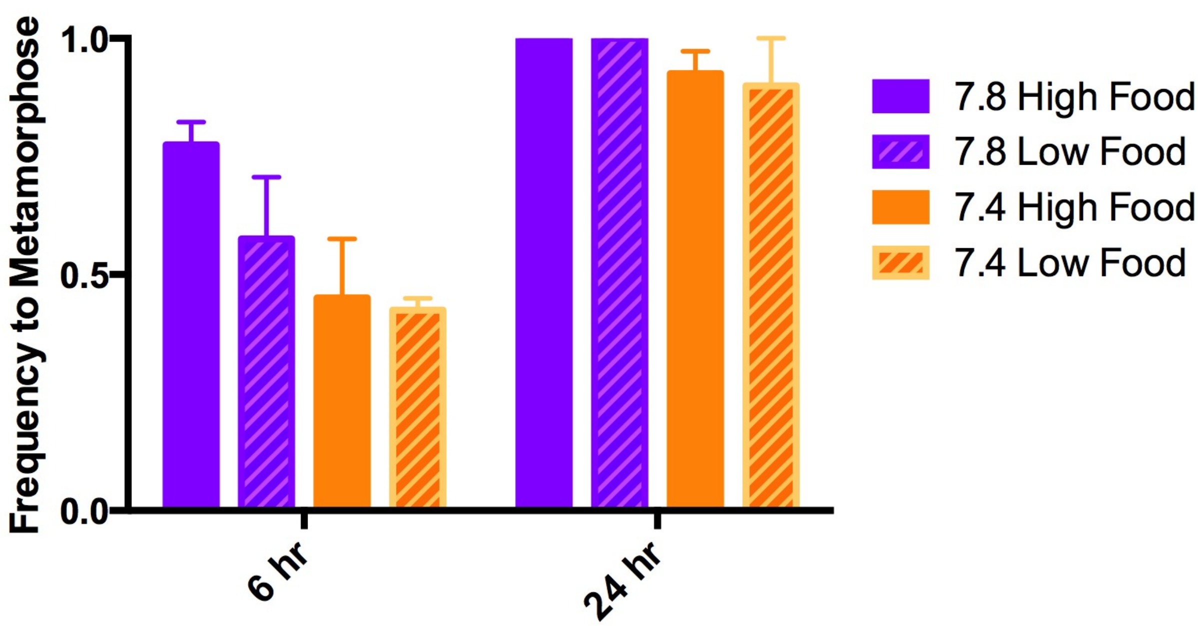

Figure 3. Frequency of metamorphosis for four different conditions at a 6 hour mark and a 24 hour mark.

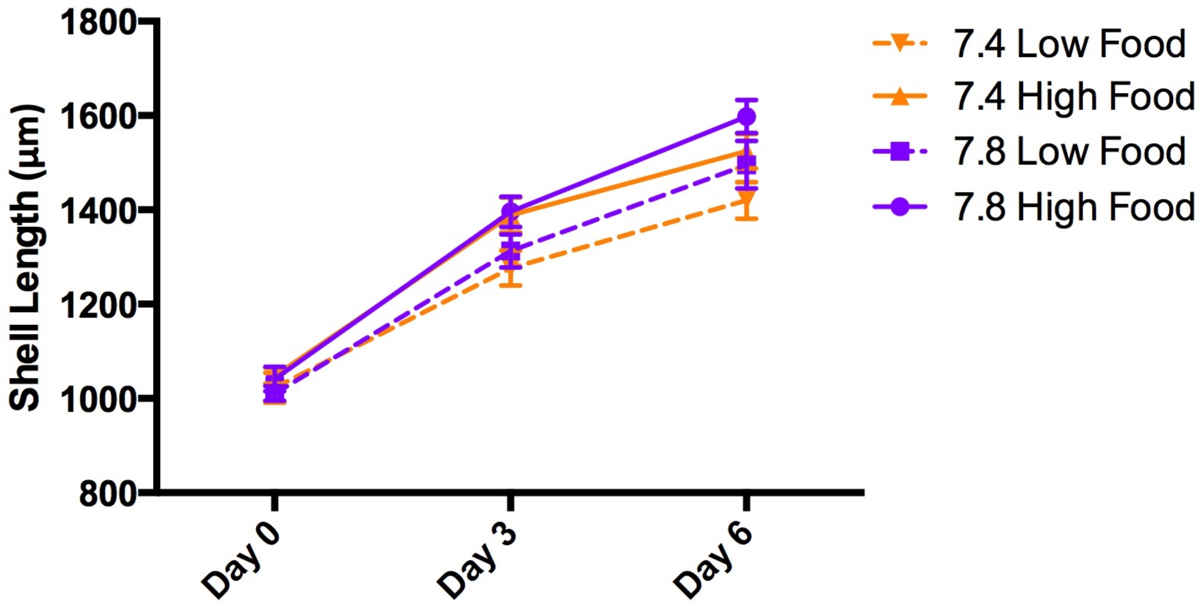

Figure 4. Growth for *Crepidula fornicata* juveniles over 6 days under different acidified conditions and food levels with their associated SEM.

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