

**Interactions of diet quality and pH in growth and mortality in the marine gastropod
Crepidula fornicata.**

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Blinks-NSF REU-BEACON 2017

Summer 2017

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Keywords: Crepidula fornicata, gastropod, pH and diet quality interaction, *Isochrysis galbana*, *Dunaliella tertiolecta*, invasive, shell strength, shell and tissue mass, larval, juvenile, ocean acidification, Totten inlet

Abstract

Ocean acidification metabolically stresses marine organisms, especially those that must expend energy to deposit calcium carbonate shells and skeletons. Nutrition may interact with pH to exacerbate or counterbalance acidification effects in early developmental stages. Previous work had indicated that growth of larvae and juveniles of the caenogastropod *Crepidula fornicata* is resilient to acidification within the pH range of 7.6-8.0 when animals are given a high-quality diet of *Isochrysis galbana* (ISO). We therefore reared larvae and juveniles at two levels of pH (7.6 or 8.0) and on either a diet of ISO or a poorer-quality diet of *Dunaliella tertiolecta* (DUN), and measured larval and juvenile growth and mortality as well as juvenile shell strength. Larvae grew about 30% faster, and juveniles grew about 50% faster, on ISO than on DUN. However, larvae and juveniles grew at similar rates at pH 7.6 and 8.0 within each diet treatment. Survivorship of both larvae and juveniles was not affected by pH, but was higher on a diet of ISO than of DUN. We thus did not find evidence for interaction of diet quality and pH in larval or juvenile growth or mortality. Juvenile shell performance was tested by measuring crushing force after 20 d of postmetamorphic growth in each combination of diet and pH. Shells were 25% weaker at pH 7.6 than at 8.0 for individuals that were fed ISO, and 55% weaker at pH 7.6 than at 8.0 for individuals that were fed DUN, even though mean shell lengths did not differ between pH levels within each diet treatment. Rigorous assessment of the interaction of diet and pH on shell performance will require further study of how shell strength scales with age and size.

Intro

Massive amounts of carbon dioxide (CO₂) have been released into the atmosphere from post-industrial revolution anthropogenic fossil fuel combustion. Due to these emissions, CO₂ levels have risen 40% over the past 250 years and current levels of atmospheric CO₂ are higher than recorded in the last 800,000 years (Doney et al., 2009). Oceanic surface absorbs large amounts of released CO₂, acting as a sink. CO₂ interacts with ocean water and transforms into carbonic acid (H₂CO₃), which dissolves into H⁺ ions and CO₃²⁻ ions. Predicted H⁺ ion increases will result in widespread oceanic pH drops. This phenomenon is known as ocean acidification. Acidification is another hardship for coastal marine ecosystems, which already experience hardships in the forms of constant fluctuations in temperature, and salinity. High nutrient and pollutant runoff levels add organic matter into coastal ecosystems (Halpern, 2008). Due to heterotrophic decomposition of this organic matter, coastal ecosystems experience elevated pCO₂ levels (Melzner, 2013). This factor is expected to increase as overall oceans acidify.

Decreasing oceanic pH levels pose a detriment to many marine species and ecosystems. When organisms are exposed to higher H⁺ concentrations, their reproduction levels, larval survivorship and development are likely to be negatively affected (Parker et al., 2009) (Shirayama, 2005). Organisms that utilize calcium carbonate (CaCO₃) in shell construction are particularly impacted by ocean acidification as lower pH levels reduce calcification rates in many of these organisms, and may change the mineral composition of shells (Fitzer, 2014) (Royal Society, 2005). Molluscs, gastropods, and other calcium-carbonate shell formers must spend more metabolic energy on shell construction/repair and less on growth, resulting in lower growth rates and predicted longer larval stages as

time to metamorphic competence is delayed (Pechenik, 2006). (Kurihara, 2008). Thus, overall metabolic rates are depressed (Melzner et al., 2011) (Lannig et al., 2010). Longer larval stages result in lower survival rates, as planktonic larvae are subject to predation before settlement. Larval stage stresses can also carry over metamorphosis to affect juveniles. This occurs by lowering initial growth rates (Pechenik and Tyrell, 2015).

Mollusc shell characteristics, such as strength and growth rates, have been studied extensively in relation to acidified conditions. Acidification conditions of pH 7.6-7.8, resulted in shells 25% weaker than 8.1-8.2 controls in experiments conducted on a bivalve *Pinctada functa* (Welladsen et al., 2010). Similarly, *Austrocochlea porcata*, a gastropod, experienced reduced shell repair rates and weaker shells between pH 7.7 and 7.9 (Coleman et al., 2014). Tests on *Tripneustes gratilla* indicated acidified conditions resulted in weaker urchin shells (Byrne et al., 2014). Molluscs with compromised shells are more likely to be preyed upon, and unequal responses to acidified conditions has potential to alter predator prey relationships and overall food web stability in coastal marine ecosystems. As a result of these impacts, molluscs, and therefore coastal ecosystems suffer on a global scale (Parker, 2013).

Calcifying organisms experiencing these acidification-related stresses are disadvantaged, posing an overall threat to communities as many of these organisms provide important ecosystem services (Doney et al., 2009). These species play vital roles in nitrogen fixation and oxygen cycling (Zimmerman et al., 1997), food web stabilization and diversity (Hall-spencer et al., 2008), and carbon cycling to deep sea environments (Boyd and Doney, 2002). These services will be negatively impacted as acidification alters ecosystem structure. Global phytoplankton populations suffer from the ocean

acidification-related effects of lower pH and higher water temperatures, shown by a 1% per year reduction in population sizes (Boyce et al., 2010). In addition to contributing more than 50% of the earth's primary production, phytoplankton are a main food source for many filter feeders (Boyce et al., 2010). Decreasing phytoplankton levels are liable to change dietary patterns and success of organisms that consume and interact with them (Putnam, 2017). Studying how ocean acidification affects life histories of organisms living in these ecosystems is vital in learning how these communities will respond to climate change.

Crepidula fornicata, a marine gastropod native to the east coast of the United States, is a model organism to study in relation to ocean acidification (Padilla, 2014) (Henry and Lyons, 2016). *C. fornicata* has spread into Europe and over to the west coast of the US, where it exists as a very successful invasive species (Blanchard, 1997). Most likely this spread has occurred due to adults attaching to oysters that farmers introduced to aquaculture operations (Dijkema, 1997). *C. fornicata* has also been shown to attach to objects in the ocean, migrating large distances as the organisms such as turtles or other presences such as driftwood move across the ocean (Frazier et al., 1985) (Korringa, 1947). Ocean currents also disperse larval *C. fornicata* (Pechenik, 1999). The benthic gastropod is a very efficient suspension feeder with minimal predation pressures in non-native areas. In these ecosystems, *C. fornicata* physically prevent other benthic organisms from settling on the bottom of oceans, alter water flow, and outcompete native species for food, acting as a detriment to ecosystems (Blanchard, 1997) (Decottignies, 2007). They can specifically negatively affect oyster farms and fisheries, reducing economic output of these operations and directly affecting humans (Gazeau, 2013) (Blanchard, 1997).

C. fornicata is a useful organism to study in laboratory environments, as mothers with mature egg sacs will predictably release thousands of larvae within a few days of being brought into benchtop cultures. The larvae are large, allowing them to be measured and reared with low mortality rates (Pechenik, 1987) (Pechenik, 1995). It is easy to manipulate cultures of larval *C. fornicata* to study how they react to various stresses such as varying temperatures, salinities, pH levels, and diet qualities and quantities. Thus, *C. fornicata* is considered a model system in larval development. Pechenik et al. (2002) showed that starvation as a dietary stress negatively impacted growth rates of larval *C. fornicata* for a short duration after the stress period, but soon growth rates returned to normal. Results of these stresses also showed up as latent effects in the juvenile stage, as even with adequate food supplies available, juvenile growth rates for larvae that experienced starvation periods were slowed initially (Pechenik, 1995). This impacted growth rates of the metamorphosed juvenile. Low food quantities affect growth rates and mortalities of many other marine species in addition to *C. fornicata*, including closely related mollusks and echinoderms (His, 1992) (Eckert, 1995) (Wacker, 2002), indicating the overall importance in conducting tests quantifying how dietary stresses on the slipper shell limpet.

As acidifying oceanic conditions affect phytoplankton abundance and distribution, *C. fornicata* diet quantities and qualities are liable to change (Boyce et al., 2010). Possible dietary stresses *C. fornicata* experience include poor diet quality and poor diet quantity. Bogan (unpubl.) showed that diet quality did not interact with pH to affect *C. fornicata* growth. The goal of the current project is to determine whether the stress of a lower quality diet can exacerbate ocean acidification related impacts. Larval *C. fornicata* grow

faster on a diet of *Isochrysis galbana* (Clone T-ISO) than on *Dunaliella tertiolecta* (Clone DUN) (Klinzing and Pechenik, 2000) (Pechenik, 2015), indicating a disparity in algal quality in supporting *C. fornicata* growth. Padilla et al. (2014) tested how differing strains of *Isochrysis* impacted *C. fornicata* survivorship and time to metamorphic competence under varying conditions. However, the study found that algal quality did not have significant effects on larval survivorship or time to metamorphic competence. Diet composition is a large factor in how well marine creatures can mitigate ocean acidification stresses. Studies have been conducted on other mollusks to observe how diet interacts with acidifying conditions to affect success of organisms (Vargas et al., 2013). *Concholepas concholepas* larvae altered diet composition under low pH conditions to adjust to ocean acidification related impacts. Increased diet quantity also increases lipid synthesis in the Pacific Oyster *Crassostrea gigas* (Kheder et al., 2010).

Recent develops have called into question whether mineral content or energetic rates of calcifying organisms play a larger role than diet in overcoming low pH scenarios (Pan et al., 2015) (Waldbusser et al., 2013) (Waldbusser et al., 2016). *C. gigas*, minimizes ocean acidification effects through makeup of inorganic minerals and metabolic rate alteration (Waldbusser et al., 2013). *C. gigas* also presented slower shell growth rates under acidified scenarios, potentially minimizing acidification hardships (Waldbusser et al., 2016). *Strongylocentrotus purpuratus* larvae allocate energy from growth to ion transport and protein synthesis under acidified scenarios (Pan et al., 2015). *Ostrea ludria* showed no reaction to acidified scenarios (Pan et al., 2015). Understanding how molluscs react to acidifying scenarios is tough, as there are variable responses between species and even within species (Gazeau, 2013). As *C. fornicata* has had success in an acidifying ocean, it

is important to explore how diet interacts with acidification to alter growth characteristics. Tests conducted in this experiment on how diet quality and pH interact to affect growth rates, shell strength, shell and tissue mass, and mortality rates of larval and juvenile *C. fornicata* expanded current knowledge on how marine organisms function under acidified conditions.

Materials and Methods

Adult Crepidula fornicata Collection and Care

Adult *Crepidula fornicata* were collected from Totten Inlet in the south part of Puget Sound four times throughout summer 2017 to provide larvae for culture. During low tide, several stacks of adult *C. fornicata* females were transported to Friday Harbor Laboratories. At the labs, six stacks were placed into 1-gallon aerated jars that were filled with 3L of unfiltered seawater. Every day, the water was completely changed and 1 mL an algal mixture comprised of *Isochrysis*, *Pavlova*, and *Tetraselmis* was added to each jar as food. This was continued until there was a large enough hatch for experiments.

Larval Culture Setup

Larvae were collected and placed into individual culture jars shortly after they were hatched. This marked day 0 of each experiment. 20 of these larvae were imaged. Initially, 200 larvae were introduced into an 800mL culture of 1 μ m filtered seawater solution in each jar. These jars were constructed from airtight 800 mL cylindrical glass jars, retrofitted with a plexiglass lids and gas inputs so each contents of each jar could receive lighting from above while simultaneously experiencing controlled atmospheric conditions. Each jar was then fed either an *Isochrysis galbana* clone T-ISO or *Dunaliella*

tertiolecta clone DUN, depending on test group. In total, sixteen jars were set up, eight fed with DUN and eight fed with T-ISO. Concentrations of T-ISO and DUN to feed *C. fornicata* larvae were determined to be 15×10^4 cells/mL (Pechenik and Tyrell, 2015). This concentration allows for maximum growth rates for groups with both diet sources. Food was prepared by centrifuging T-ISO and DUN and resuspending algal cells in filtered seawater to minimally affect culture pH and alkalinity. Phytoplankton were counted on a hemocytometer before each feeding.

The groups were further subdivided, with four jars of each diet exposed to a pH 8.0 gas mix and four groups of each diet exposed to a pH 7.6 gas mix (Bogan, unpubl.). These pH values were chosen due to their status as an ideal value and a low value of the pH range that supports maximum growth rates in larvae of *C. fornicata*. Each grouping had 4 replicate jars. These jars were kept at 20° C. Jars were rotated around in coolers to minimize cooler-based location impacts on results. The gas mixes were created by mixing scrubbed air, free of CO₂, with pure CO₂, at rates regulated by Aalborg mass-flow controllers.

Brood 36 was the first group of larvae used in this experiment, hatching the night of July 1. This brood was carried until metamorphic competence, when metamorphosis was induced. These larvae were utilized as juveniles in a temperature – pH interaction experiment. Brood 38 was the second larval brood in this experiment, hatching the night of July 22. Due to high mortality rates, DUN groups from brood 38 were terminated early. T-ISO groups from brood 38 were utilized for a temporary larval stress experiment.

Larval Testing and Measuring

On day 2, populations of larvae in each jar were reduced to 150 surviving individuals. Water was changed and food was added into each jar. On day 4, water was changed and 20 individuals from each replicate group were imaged using microscopes under 12x to 50x magnification, depending on larvae size. Maximum shell lengths of *C. fornicata* larvae were measured from each image using imageJ. This process was repeated: every other day the jars would be fed and given a water change, and every fourth day 20 *C. fornicata* from each jar were imaged. After day 4, food concentrations of both DUN and T-ISO were lowered to 10×10^4 cells/mL as initial concentrations were too high. Larvae were raised until they metamorphosed into juveniles with exposure to KCl (Pechenik, 2015).

Brood 36 DUN replicates were terminated after ten days, as unidentified protists were visibly growing on larval shells in 7.6 DUN and 8.0 DUN groups, obscuring edges of shell. For fear of contamination, the DUN culture that had been providing food for the larvae was terminated. A new culture of the same clone was used for feeding after this discovery. These protists did not impact growth rates, as similar trends in Brood 36 DUN replicates were observed in Brood 31 Juvenile trials and in previous studies done on how diet quality, specifically T-ISO vs DUN, impacts *C. fornicata* growth (Klinzing and Pechenik, 2000) (Pechenik and Tyrell, 2015). Another hatch of larvae was collected and put into culture to collect more data on growth rates of *C. fornicata* fed DUN vs. T-ISO, initially following the same protocol for the first hatch group. These larvae were from Brood 38.

Juvenile Testing and Measuring

Fifty newly-metamorphosed juveniles were placed into individual 40-mL cups. These juveniles were from Brood 31, hatched on June 20 2017. These larvae were reared in room temperature (21-23°) 2L benchtop cultures and fed a diet of T-ISO at a density of 10×10^4 cells/mL daily. Benchtop cultures consisted of rearing larvae in gallon jars until metamorphosis. These jars were subject to ambient pH and temperature conditions in addition to daily water changes and feedings. Metamorphosis was induced in these larvae on July 11 2017, after almost three weeks of unmeasured growth. These fifty juveniles were separated into four distinct testing groups. 12 individuals were fed DUN at 10×10^4 cells/mL in 7.6 filtered seawater (group 7.6 DUN), 12 individuals were fed DUN at 10×10^4 cells/mL in 8.0 filtered seawater (group 8.0 DUN), 13 individuals were fed T-ISO at 10×10^4 cells/mL in 7.6 filtered seawater (group 7.6 T-ISO) and 13 individuals were fed T-ISO at 10×10^4 cells/mL in 7.6 filtered seawater (group 8.0 T-ISO). Water was changed and juveniles were fed the same concentrations of respective algal culture daily. Every few days, juveniles were imaged to measure growth rates and shell lengths. Over the course of the experiment, several juveniles died and were removed from the experiment.

All juveniles from each grouping were crushed to test shell strength at Friday Harbor Labs. Shell strength was quantified in Newtons in regards to how much force juvenile shells withstood before fracturing. A 20% load reduction event was criterion for determining that a shell had fractured. Larvae and all shell fragments were collected after crushing and preserved in a 10% formalin solution to allow for later testing of shell and tissue mass (Pechenik, 2015). Final shell size measurements were taken before crushing

to compare with prior growth rate data. Juveniles were crushed using an Instron 5965 materials testing system.

Results

Water Chemistry: While pH, temperature and salinity of all cultures were intended to remain constant, fluctuations occurred. pH, salinity, and temperature for all groups were in line with expected values for both Brood 31 Juveniles and Brood 36 larvae (Table 1, 2). Ω aragonite values were higher in test groups at 8.0 pH than 7.6 pH (Table 1, 2), but no values were below 1.00. pCO₂ values averaged 1172±52.54 for the 7.6 jars and averaged 414.3±40.72 for the 8.0 jars across all experiments (Table 1, 2).

Mortality: Mortality was higher in groups fed DUN than groups fed T-ISO (Figure 1,2). For the brood 31 juveniles, out of 24 individuals, only 17 of 24 (71%) original individuals fed exclusively DUN survived past day 14, compared to 26 of 26 (100%) individuals that survived on a diet of pure T-ISO. Diet quality significantly impacted mortality – Brood 31 Juveniles fed DUN were less likely to survive than individuals fed T-ISO ($P = 0.0029$). pH did not significantly impact mortality rates in either larval or juvenile *C. fornicata* experiments. Brood 36 larval cultures were counted on day six to observe how many of the 150 original larvae survived. The highest percentage of larvae survived in the 8.0 T-ISO group, while the lowest percentage of larvae survived in the 7.6 DUN group ($P = 0.0062$). pH and diet did not interact to impact growth rates. For brood 38 larvae, cultures were opened on day two for a scheduled water change and feed, revealing that under 20% of larvae in DUN all eight replicates (both 7.6 and 8.0 pH) survived, compared to expected survival rates in T-ISO groups. Due to the high mortality

rates in the DUN replicates, the DUN cultures were terminated after two days of growth. Surviving larvae in the Brood 38 DUN replicates were disposed of.

Larval Shell Size: Larval shell size for Brood 36 was calculated by comparing grand means of all four replicates of each test group. Each replicate had the same pH and algal diet. These two variables differed between test groups. Groups fed pure T-ISO grew larger on average than groups fed pure DUN (Figure 7). Largest replicate mean size at day 8 was 8.0 T-ISO at 821.84um. Smallest replicate mean size at day 8 was 7.6 DUN at 557.53um. Largest individual larval shell size at day 8 was 8.0I-2 at 929.60um. Smallest individual larval shell size at day 8 was 7.6D-12 at 450.58 um.

Larval Growth Rates: Larval growth rates for Brood 36 were calculated by comparing differences between measuring days in grand means of all four replicates of each test group. Each replicate had the same pH and algal diet quantity and quality. These two variables differed between test groups. All groups grew at similar rates between 0 and 4 days. Between days 5 and 8, groups fed pure T-ISO grew faster on average than groups fed pure DUN (Figure 8, 9).

Juvenile Shell Size: Juveniles in the T-ISO groups grew faster and larger than DUN groups (Figure 3). Individual juvenile 31.22I had the largest shell size 13 days after metamorphosis, 4182 µm. Individual juvenile 31.35D had the smallest shell size 13 days after metamorphosis, 1588 µm. 31.22I grew in the 7.6 pH box while 31.35D grew in the 8.0D box. These patterns of shell size were reflected in mean shell lengths of DUN and T-ISO treatment groups. Out of the four test groups, the group with the largest mean size was 7.6 T-ISO (mean shell length = 3840 µm) and the group with the smallest mean size was 8.0D (2231 µm). Group 8.0 T-ISO followed 7.6 T-ISO in shell size closely (mean

shell size = 3800 μm), while group 7.6 DUN grew slightly larger than 8.0DUN (mean shell size = 2413 μm).

Juvenile Growth Rates: Shell growth rates were differentiated between groups based on diet quality. Groups 7.6 T-ISO and 8.0 T-ISO had higher mean growth rates than groups 7.6 DUN and 8.0 DUN between every measuring interval (Figure 4, 5, 6). Growth rate peaked for 7.6I at 242 $\mu\text{m}/\text{day}$ and for 8.0I at 270 $\mu\text{m}/\text{day}$. Growth rate for 7.6 DUN peaked at 112 $\mu\text{m}/\text{day}$ and for 8.0 DUN at 121 $\mu\text{m}/\text{day}$. Individual 31.15I experienced the single largest growth rate of 336 $\mu\text{m}/\text{day}$ between days 5 and 8, while individual 31.1D experienced the slowest growth rate of 9 $\mu\text{m}/\text{day}$ between days 8 and 13.

Juvenile pH and diet quality did not interact in any trials to alter growth rates (Figure 4, 5, 6). Diet quality significantly impacted growth rates during all three measurement intervals ($P < 0.001$), as groups fed T-ISO grew at much faster rates than groups fed DUN. pH did not significantly affect growth rates during any of these stages, indicating no interaction between the two factors ($P > 0.005$).

Juvenile Shell Strength: Juvenile shell strengths were determined by crushing individuals and observing when a 20% load reduction occurred, indicating a shell suddenly breaking. Juveniles reared in 7.6 pH had significantly weaker shells than juveniles reared in 8.0 pH across both diet treatments (Figure 10). ($P < 0.0001$). Juveniles reared at 7.6 pH and 8.0 pH were of similar size within each diet group, so unconfounded shell strength comparisons could be made between pH levels within each diet. However comparisons between ISO juveniles and DUN juveniles could not be made as ISO juveniles were larger than DUN juveniles across the same pH. In order to interpret these results, a scaling factor comparing size and shell strength will need to be constructed.

Juvenile Shell and Tissue Mass: Tests calculating juvenile shell and tissue mass have not yet been conducted. After termination, Brood 31 juveniles were sent to Dr. Jan Pechenik at Tufts University, where he will calculate these values.

Only two four-day growth intervals were measured for both DUN and T-ISO groups. Over both growth periods (0-4 days and 4-8 days), diet significantly affected growth rates in both DUN and T-ISO groups (Figure 7, 8). Groups fed DUN grew significantly slower than groups fed T-ISO during both growth periods ($P = 0.0017$ and $P < 0.001$, respectively) (Figure 7, 8). pH did not significantly affect this value, and there was no interaction between pH and diet quality in affecting growth rates.

Tables and Figures:

	pH (total)	Salinity(ppt)	Temp(°C)	TA corr	Ω Aragonite	pCO ₂
8.0 DUN	7.96±0.04	29.9±0.61	23.9±1.11	2086±12.90	2.46±0.24	465±47.5
7.6 DUN	7.59±0.02	29.7±0.80	24.0±1.02	2086±12.90	1.16±0.07	1207±71.73
8.0 T-ISO	7.97±0.04	29.9±0.65	24.0±1.10	2086±12.90	2.47±0.25	459±50.7
7.6 T-ISO	7.59±0.02	29.9±0.67	24.1±1.05	2086±12.90	1.16±0.07	1210±56.64

Table 1: Brood 31 Juvenile Carbonate Chemistry. Values represent means and \pm S.D. from the filtered seawater added to cultures during water changes over the course of Brood 31's existence. TA = total alkalinity in $\mu\text{mol/kg}$, Ω Aragonite = Aragonite saturation (dimensionless), pCO₂ = CO₂ partial pressure in μatm . Sample size (N) for each value is 14.

	pH (total)	Salinity(ppt)	Temp(°C)	TA corr	Ω Aragonite	pCO ₂
8.0 DUN	8.05±0.03	29.2±0.41	20.18±0.10	2080±24.25	2.47±0.15	366.7±30.70
7.6 DUN	7.62±0.02	29.3±0.26	20.13±0.12	2080±24.25	1.03±0.05	1114±40.43
8.0 T-ISO	8.05±0.02	29.3±0.46	20.18±0.12	2086±21.98	2.49±0.14	365.7±24.25
7.6 T-ISO	7.61±0.02	29.4±0.45	20.07±0.18	2086±21.98	1.00±0.05	1158±41.36

Table 2: Brood 36 Larvae Carbonate Chemistry. Values represent means from the filtered seawater added to cultures during water changes over the course of Brood 31's existence. \pm S.D. are present in addition to water property values. TA = total alkalinity in $\mu\text{mol/kg}$, Ω Aragonite = Aragonite saturation (dimensionless), pCO₂ = CO₂ partial pressure in μatm . N = 8.

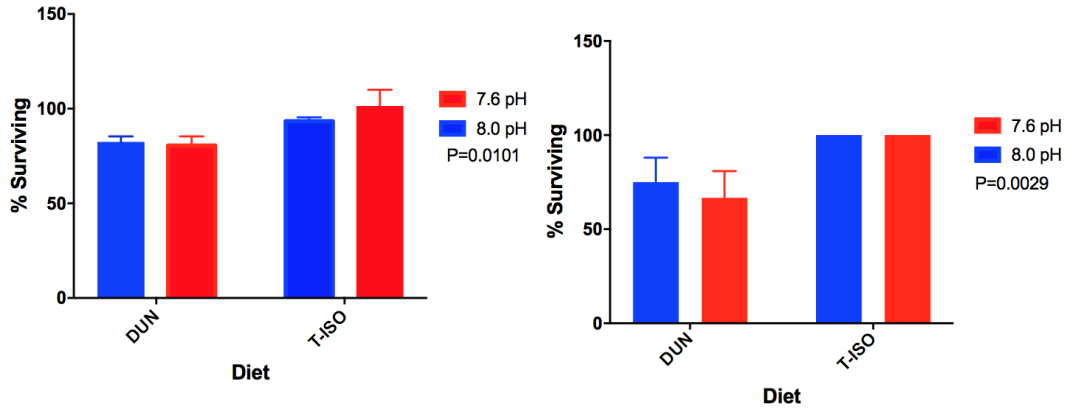


Figure 1, 2: Brood 36 and Brood 31 survival rates, respectively. Survival rates were significantly higher in groups fed T-ISO than DUN. There were quantifiable but insignificant effects of pH on survival rates. Error bars represent \pm SEM in trials. P values show that trials were significant and show relationship of diet quality on *C. fornicata* survival. Graph 1 N = 12 for DUN, N = 13 for ISO. Graph 2 N = 200.

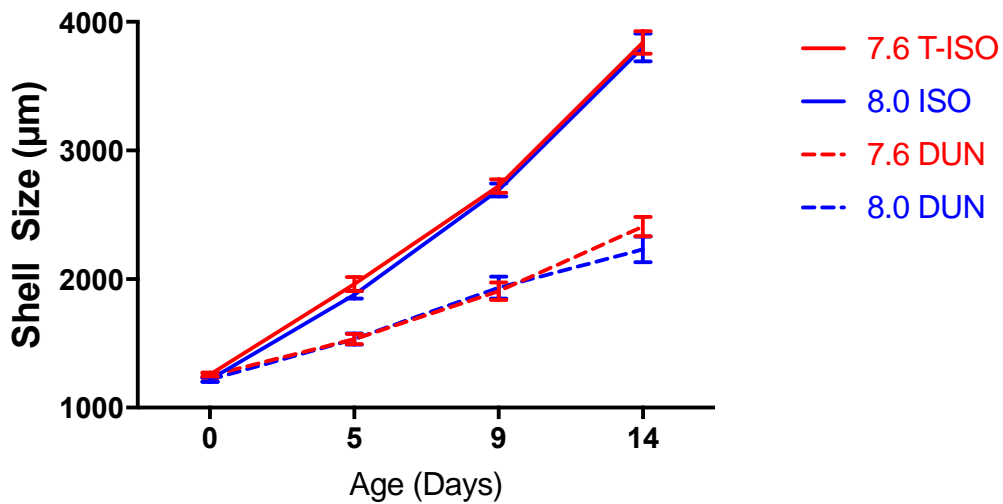


Figure 3: Showing shell size in Brood 31 Juvenile *C. fornicata* over a period of 14 days. Groups fed T-ISO grew significantly larger than groups fed DUN ($P < 0.0001$). Error bars represent \pm standard deviation values of means of up to 13 individuals at each data point. N = 12 for DUN and N = 13 for ISO.

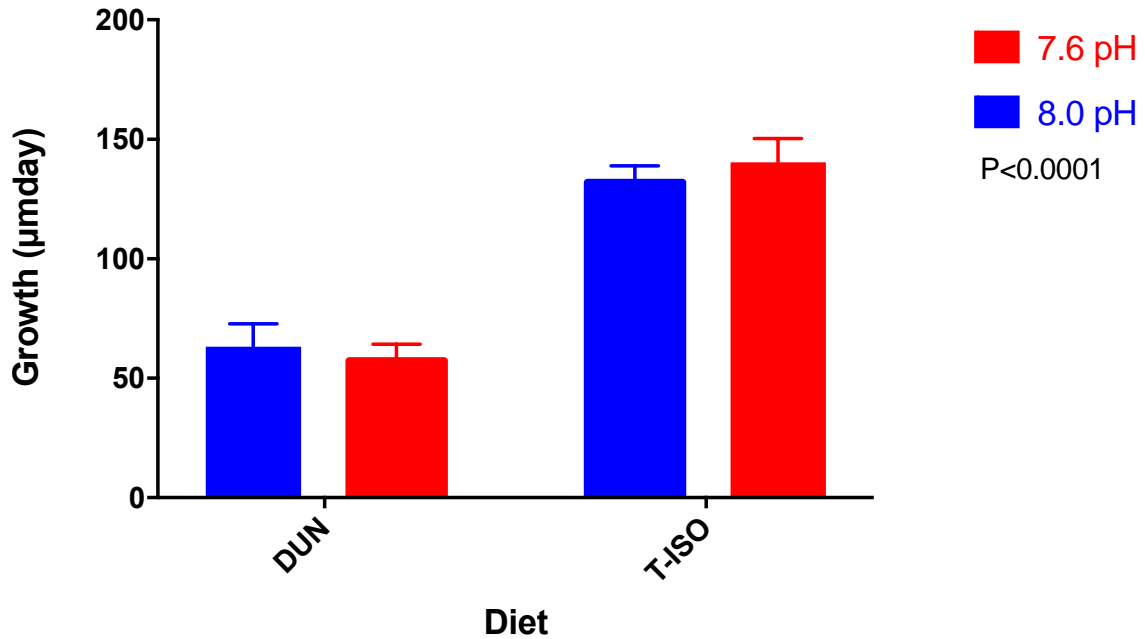


Figure 4: Brood 31 Juvenile growth rate in um/day between days 0 and 5. Juveniles fed T-ISO grew significantly faster than Juveniles fed DUN ($P < 0.0001$). Error bars represent \pm SEM values of means of the juveniles measured in this experiment. pH had no effect on juvenile growth rates during this time period. $N = 12$ for Dun and $N = 13$ for ISO.

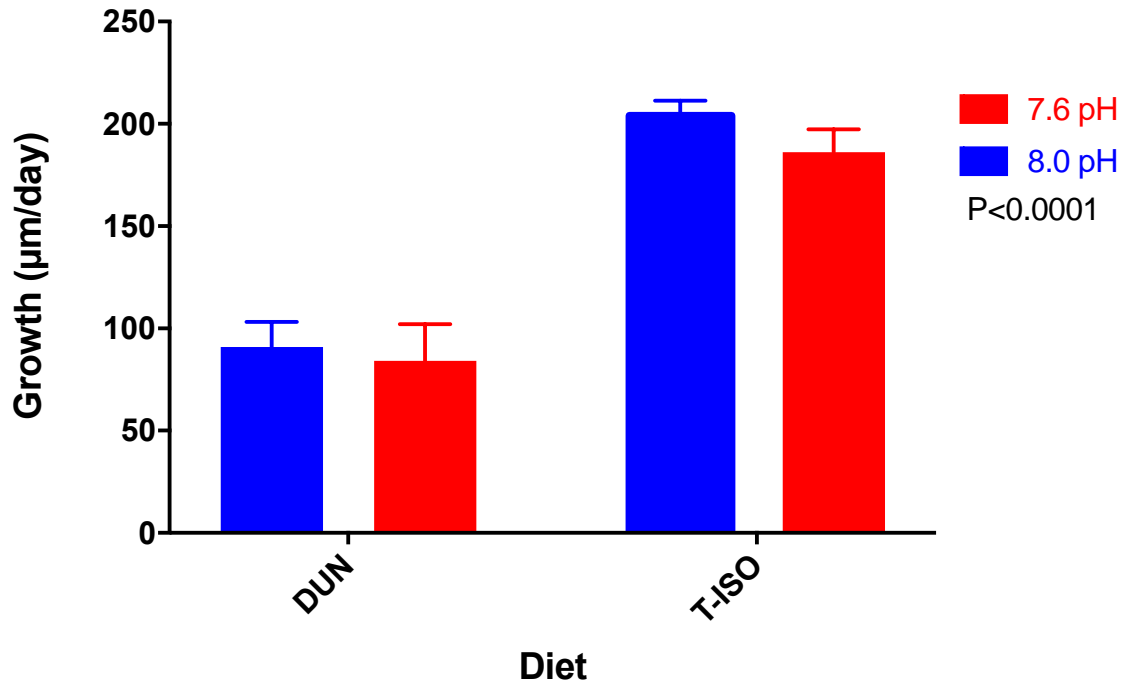


Figure 5: Brood 31 Juvenile growth rate in um/day between days 5 and 9. Juveniles fed T-ISO grew significantly faster than Juveniles fed DUN ($P < 0.0001$). Error bars represent \pm SEM values of means of the juveniles measured in this experiment. pH had a

quantifiable but insignificant effect on trials. Juveniles subjected to an 8.0 pH regime grew quantifiably but insignificantly faster than individuals under the 7.6 pH regime. N = 12 for DUN and N = 13 for ISO

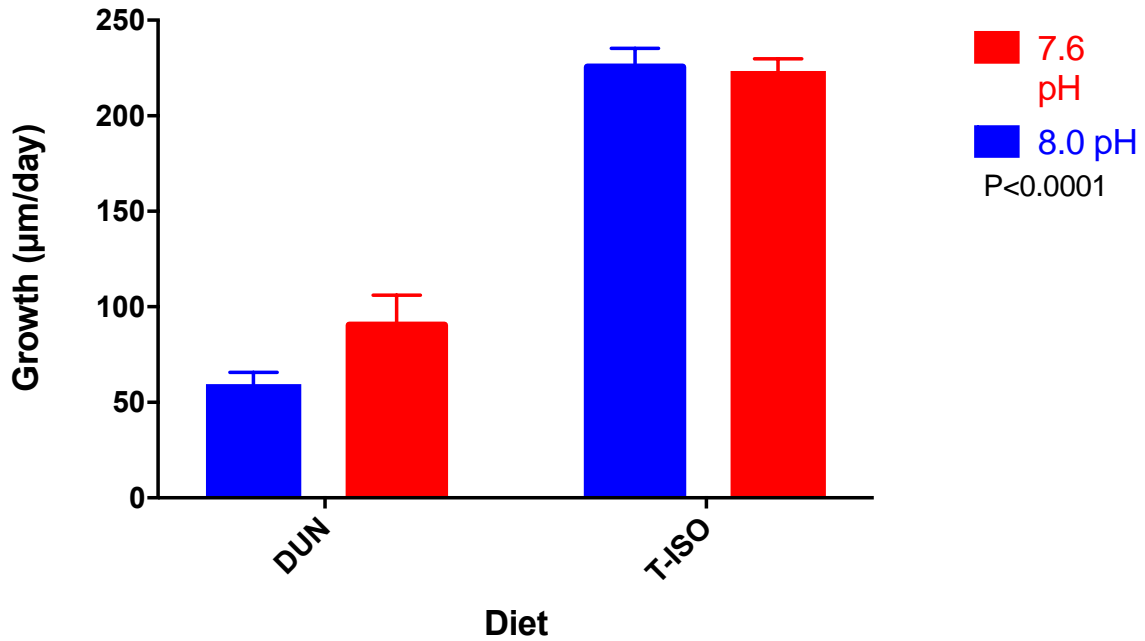


Figure 6: Brood 31 Juvenile growth rate in um/day between days 9 and 14. Juveniles fed T-ISO grew significantly faster than Juveniles fed DUN ($P < 0.0001$). Error bars represent \pm SEM values of means of the juveniles measured in this experiment. pH had no effect on growth rates during this time period. N = 12 for DUN and N = 13 for ISO.

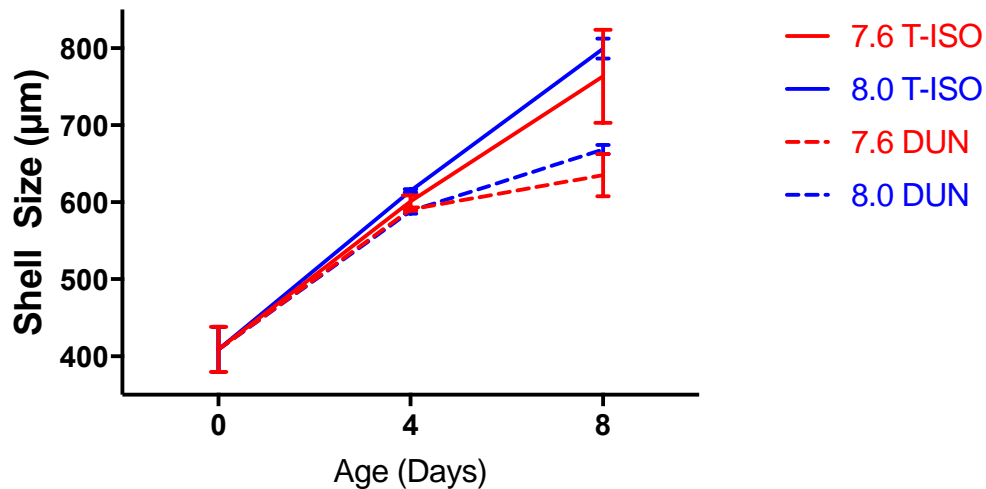


Figure 7: Showing shell size in Brood 36 larval *C. fornicata* over a period of 8 days. Groups fed T-ISO grew significantly larger than groups fed DUN ($P < 0.0001$). Error bars represent \pm SEM values of means of up to 13 individuals at each data point. N = 20.

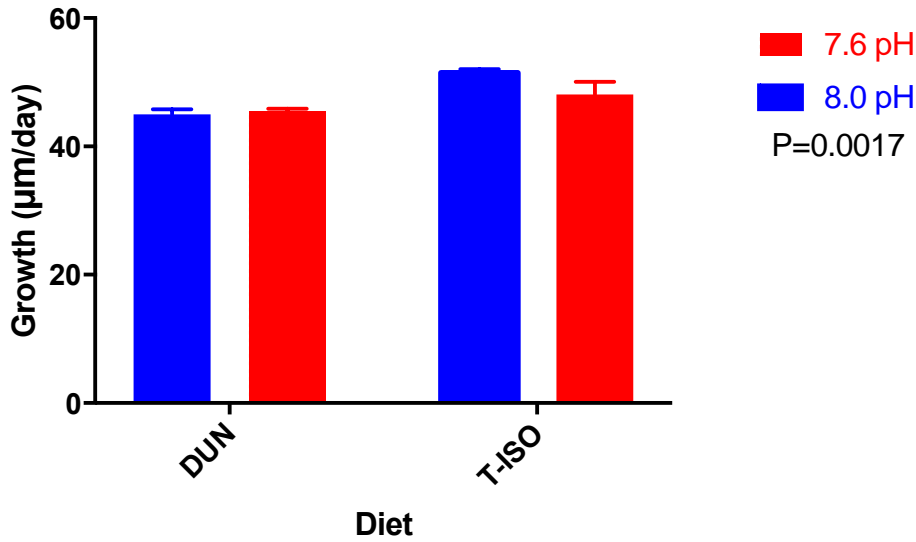


Figure 8: Brood 36 larvae growth rate in $\mu\text{m}/\text{day}$ between days 0 and 4. Data represents grand means of 20 larvae from each of four replicates sampled. Larvae fed T-ISO grew significantly faster than Juveniles fed DUN ($P < 0.0001$). Error bars represent \pm SEM values of means of the juveniles measured in this experiment. $N = 20$.

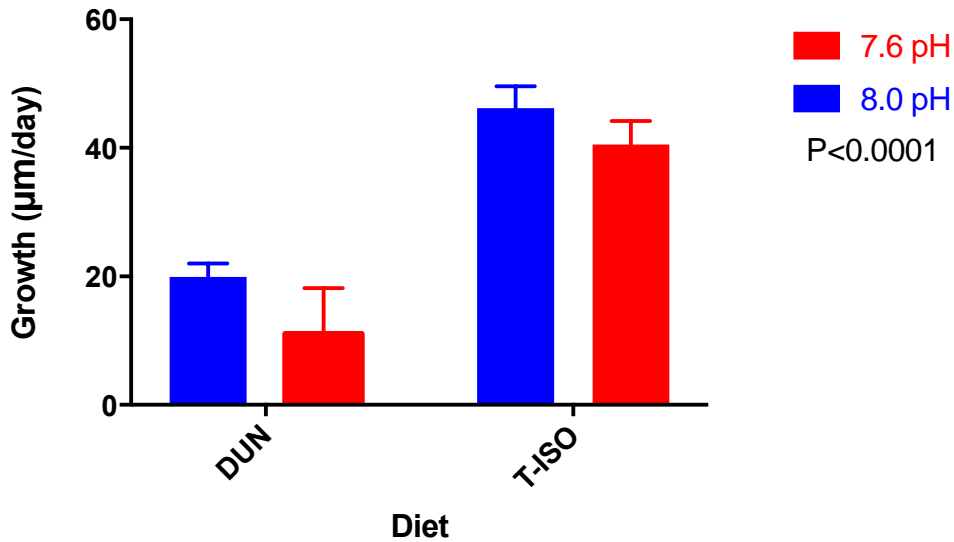


Figure 9: Brood 36 larvae growth rate in $\mu\text{m}/\text{day}$ between days 4 and 8. Data represents grand means of 20 larvae from each of four replicates sampled. Larvae fed T-ISO grew significantly faster than Juveniles fed DUN ($P < 0.0001$). Error bars represent \pm SEM values of means of the juveniles measured in this experiment. $N = 20$

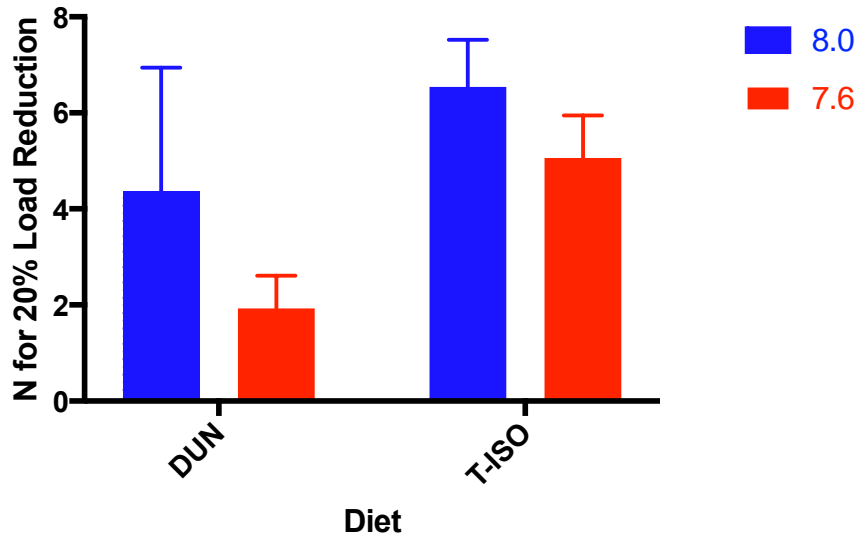


Figure 10: Brood 31 Juvenile Shell Strength in Newtons. Data represents the mean force required to cause a 20% decrease in applied load, which is how a shell was determined to be fractured. Juveniles fed T-ISO had shells that withstood more applied force before breaking than juveniles fed DUN. In both diet treatments, juveniles raised at pH 8.0 environments had significantly stronger shells than juveniles raised at pH 7.6. Error bars represent \pm SEM values of the measured force values. N = 8 for DUN, N = 13 for ISO.

Discussion

We conducted this study to observe how diet as a stress affected development of *Crepidula fornicata* at lower pH limits of optimal growth rates. *C. fornicata* are intertidal creatures and thus experience additional pH fluctuations that are present in biologically productive coastal ecosystems, such as nutrient loading, upwelling, and respiration (a (Wootton et al., 2008) (Halpern, 2008) (Melzner, 2013). Observing whether a poor-quality diet can exacerbate effects brought on by an overall lowering of water pH due to ocean acidification is key in determining interactions between the two.

Our results fell in line with results from Klinzing and Pechenik (2000) showing that T-ISO is a more nutritive food than DUN in fostering growth of *C. fornicata*. In both larval and juvenile experiments, *C. fornicata* that were fed T-ISO grew larger and faster than ones fed DUN. Survivorship of Brood 31 juveniles, Brood 36 larvae, and Brood 38 larvae

was much higher in replicates fed ISO than DUN Larvae and young juveniles potentially metabolize ISO at higher rates than DUN due to the smaller particle sizes, resulting in higher growth rates and lower mortalities on ISO.

Diet quality influenced growth rates of *C. fornicata* at a higher rate than environmental pH. Diet did not significantly interact with pH in any conducted experiment. pH may have stressed some individual experiments, for instance causing larval groups fed T-ISO to grow slightly faster and larger in 8.0 pH conditions than in 7.6 pH conditions. This indicates that pH conditions close to the lower end of *C. fornicata*'s ideal growth rate range (7.6) are stresses that are slightly worsened by inadequate nutrition. Similar patterns were expressed in Brood 36 larval groups fed DUN between days 4 and 8, Brood 31 juvenile groups fed T-ISO between days 5 and 15, and Brood 31 juveniles fed DUN between days 0 and 9. None of these interactions were significant.

Diet and pH likely interacted to affect shell strength in juvenile *C. fornicata*. ISO replicates had higher mean shell strength than DUN replicates across the same pH. Juveniles grown in 8.0 conditions also had stronger shells than juveniles grown in acidified conditions across the same diet. Both diet quality and pH impacted shell strength. Further analyses will need to be conducted to assess how shell strength scales with size to make more direct comparisons between diets: DUN shells were much smaller than ISO shells, which may account for some degree of weakening.

To overcome acidified conditions, molluscs allocate resources in different ways (Kroeker, 2014). *Mercenaria mercenaria* sacrifices shell thickness for shell size (Dickinson et al., 2013), while *Littorina littorea* maintains shell thickness, especially

when exposed to predatorial cues (Bibby et al., 2007). Shell strength is a key component of predator-prey interactions: molluscs with weaker shells are more susceptible to predation pressures (Amaral et al., 2012), (Sanford et al., 2014). Acidifying coastal waters can cause intertidal molluscs to be preyed upon more as their protective shells are altered, disrupting food webs and altering trophic cascades in these ecosystems. *C. fornicata* juveniles and larvae reared at 7.6 pH grow at similar rates to ones grown at 8.0 pH. However, the large disparity in shell strength between these two cohorts indicates that *C. fornicata* prioritizes growth over defense in term of metabolic allotment. This comparison can be made as shell sizes were comparable within each diet group. In native environments, *C. fornicata* are under minimal predation pressures. (Pechenik, 2010). Pechenik also postulates that, in invasive areas, *C. fornicata* experiences even less predation than in native areas (2010). Possessing weaker shells might not act as a detriment to *C. fornicata*, as strong protective shells are not needed to ward off predators. Thus, *C. fornicata* likely behaves differently under acidified scenarios than the calcifying molluscs that Waldbusser et al. and Pan et al. studied (2013) (2016) (2015). Instead of slowing shell growth and allocating resources to other processes to minimize acidification impacts, the gastropod prioritized shell growth. Determining how low pH impacts other metabolic processes within *C. fornicata* would add to this narrative. *C. fornicata* has already been a very successful invasive species, spreading over vast regions including the Pacific Coast of the United States and Northern Europe. The species adapts to a variety of conditions, including wide temperature and salinity ranges. The gastropod's response to acidified conditions has potential to explain its current spread in addition to predict future performance as ocean pH drops.

Further tests should be conducted to assess latent or carry-over effects of diet quality of stresses *C. fornicata* larvae experience on *C. fornicata* juveniles. Both latent effects and carry over effects are stresses experienced during larval growth stages that manifest effects on post-metamorphic juveniles in different ways. Documented experiments show that latent effects are widespread among *C. fornicata* and related species in terms of salinity, food concentrations, and pH (Pechenik, 1996; Hettinger et al., 2012; Ng and Keough, 2003) No latent effects were tested in this study. Surveys to observe *C. fornicata*'s natural diet composition in coastal ecosystems would be useful in determining if diet selection is a factor of the gastropod's response to acidification. It would also allow for more direct tests to compare interactions between diet and pH. Lastly, calculating a method for scaling shell strength by size is key in comparing DUN vs. ISO shell strengths.

Acknowledgements

I would like to thank Dr. Tony Pires for mentoring me and offering continual feedback and constructive criticisms through the project and Dr. Vikram Iyengar for offering support and feedback. I would like to thank Dr. Rebecca Guenther in the FHL Ocean Acidification Environmental Laboratory for keeping up with infrastructure and functionality of testing systems. I would also like to thank Dr. Jan Pechenik for additional testing and feedback in the synthesis of this project idea. I thank Taylor Shellfish companies for allowing us access to the *Crepidula fornicata* collection site in Totten Inlet. I would like to thank fellow researchers Carrie Gillespie, Jack Litle, Morgan Levy, and Jamie Trudel for assistance in measuring and culture maintenance. This study was funded by National Science Foundation award OCE-1416690 to Dickinson College.

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