Effects of Temperature and pH on Larval and Juvenile Development in the Marine Gastropod, *Crepidula fornicata*

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Abstract. Rising atmospheric CO_2 levels are associated with warming and acidification in coastal marine ecosystems, with impacts that are especially acute for marine calcifiers. We have investigated the interactive effects of pH and temperature on larval growth, time to metamorphic competence, and juvenile growth in the marine gastropod *Crepidula fornicata.* Larval growth rates and acquisition of competence for metamorphosis were measured in 4 replicate cultures in each of 4 treatment groups, representing all combinations of pH 7.6 and 8.0, at either 20° or 24°C. Higher pH and higher temperature additively increased larval growth, and larvae became competent for metamorphosis sooner at higher temperature and at higher pH, but there was no significant interaction of pH and temperature on larval growth rates or on frequencies of metamorphosis . After metamorphosis, juveniles from each larval condition were individually cultured at either pH 7.6 or 8.0. Temperature was not controlled during juvenile growth; all individuals were exposed to the same ambient laboratory temperatures of 21°-23°C. Juveniles grew at similar rates in both pH conditions. However, juveniles reared at 24° as larvae grew more slowly than their siblings that had been reared at 20° C as larvae, during the first 7 days of post-metamorphic growth. This difference was no longer apparent by 11 days after metamorphosis for juveniles derived from most larval conditions. However, slower growth persisted in juveniles that had been reared as larvae at 24° and pH 7.6, and kept at pH 7.6 after metamorphosis. This result is consistent with related studies that show persistent effects of larval pH experience that emerge under some juvenile growth conditions

Introduction. Rising CO_2 levels in coastal marine ecosystems lower pH of the waters that marine animals inhabit, affecting many aspects of their life. NRDC reports that one quarter of human-generated CO_2 emissions is absorbed into oceans (NRDC 2014), making for a drop in pH. Because of this large amount of CO_2 being absorbed into oceans, atmospheric CO_2 levels are much less than they would be without oceans. However, CO_2 rise in oceans comes at a cost for marine life, especially for animals with shells that require calcification to form properly. The effects of ocean include but are not limited to growth rates, fertilization success, reproduction, and embryonic development (Parker et al., 2009, Gaylord et al., 2011; reviewed by Byrne, 2011).

Organisms with calcareous shells are at the forefront of the ocean acidification phenomenon because reduced seawater pH is closely tied to reduced calcium carbonate saturation (Doney et al., 2009). Calcification of the snail's shell is essential to metamorphosis and development of the organism. This process is hindered in acidic conditions, causing elevated risks to animals with calcareous shells. A study by Rebecca Guenther at Friday Harbor Labs found that "ocean acidification negatively affected spore adhesion in two species of red algae, suggesting that reduced pH may affect the life cycles of both calcified and fleshy red seaweeds" (Guenther 34). Acidified conditions causing poor pore adhesion in these algae shows another sort of influence that ocean acidification can have on marine invertebrates while keeping with the idea of the fragility of marine life cycles and how they are altered by shifts in environmental conditions.

Temperature and pH are two factors, inextricably tied because when temperature is altered, pH also changes. Their recent alarming fluctuations in coastal and wider ocean ecosystems are affect the development of the marine gastropod *C. fornicata* at several points in its development. The two factors have been studied in tandem because it is thought that they may have interactive effects on growth rates of marine organisms. Studies have been done to test effects of temperature and acidification independently and as co-occurring factors. Byrne and Przeslawki set up nine combinations of temperature and pH stressors on development of *Patiriella regularis* at various life stages. They found that with increased temperature, embryos progressed through developmental stages more quickly. In addition, they found at 60% mortality rate of larvae reared at 7.6 and in increased 4 degrees Celsius (Byrne et al. 2011). This suggest the extremes of culture conditions for this species and shows that there is a reasonable range in which to study thermal and acidified stressors. For this study, our lowest extreme is 7.6 pH and 24 degrees Celsius.

I studied the effect of the temperature-pH interaction on growth rates in *Crepidula fornicata*, a gastropod colloquially known as the common slipper snail.
C. fornicata are an appropriate species to use for this investigation first because of their efficient reproductive strategy. Crepidula are protandrous hermaphrodites, meaning the larvae are male and only become female after metamorphosis. Crepidula females stack one on top of each other and brood large egg masses typically containing between 5,000 and 10,000 eggs (EMBRC-France). The larvae have survival rates close to 100% (Pechenik 2002) which allows for consistent numbers of larvae per trial and sufficient

growth rate data in each culture. In addition, larvae are a measurable size when they hatch and can be seen with the naked eye, making culture maintenance and handling the animals possible.

Previous studies have investigated the interaction between salinity and temperature and how it affects larval and juvenile growth. Results showed that elevated temperatures cause increased growth rates and that decreasing salinity caused slower growth rates in both larvae and juveniles (Bashevkin, Pechenik 2015). Therefore, increasing temperature causing melting glaciers and therefore decreasing the salinity of oceans makes for non-ideal conditions for Crepidula development. Effects of acidification can be exacerbated or weakened at higher temperatures (Byrne et al., 2011). In addition, it has been proven that decreased carbonate saturation and interactive effects of ocean warming and acidification are likely to impair skeletogenesis in unshelled abalones and urchins (Bryne et al., 2011). This poses the question of whether there are interactive effects of temperature and pH in coastal acidification ecosystems that could affect development of our marine gastropod, *C. fornicata.*

In this study, I manipulated larval temperature and pH and juvenile pH to see the effect of these two factors *Crepidula fornicata* metamorphic competence and development. I looked for independent effects of varying pH and temperature as well as interactions between pH and temperature.

Materials and Methods:

Collection

Crepidula fornicata were collected in the Totten Inlet in South Puget Sound, WA in June and July 2017. This gastropod is invasive to Friday Harbor and the surrounding area. Crepidula were brought back to Friday Harbor Laboratories, WA where larval hatches were collected for study. Individual stacks of Crepidula were placed in benchtop culture. Each stack was fed 2-3 mL of Shellfish Diet 1800 from Reed Mariculture daily. Jars were emptied and filled with fresh water daily while waiting for a hatch to occur.

Culture Setup and Maintenance

When a hatch occurred, the air stone was removed and larvae were syphoned out. 200 larvae were put into culture with four replicates at each pH: 7.6, and 8.0. Culture jars were set up in the ocean acidification lab at Friday Harbor Laboratories. Larval cultures were set up in coolers with temperature and pH regulated by a gas mix of CO2 and scrubbed air generated by Aalborg GFC mass-flow controllers. Each jar had a screw on cap with two pieces of flexible plastic tubing attached by hard plastic fittings. Coolers contained a submersible aquarium heater that sits on the bottom of the cooler to maintain desired temperature. A bucket of seawater conditioned to the appropriate pH and temperature using a gas mix containing CO2 and scrubbed air was prepared.

Larvae were fed *Isochrysis galbana* Tahitian strain, or T-ISO, algae at 10 x 10^4 cells / mL in each 800mL jar every other day. Every two days when feeding occurred, we also changed culture water. At the start of each pH culture group, we measured pH,

temperature and salinity of individual culture jars as well as of the conditioned seawater. Every four days, 20 larvae from each culture jar were imaged using Motic microscope camera and measured, taking maximum shell length with ImageJ software.

In the first set of 8 jars, I varied pH with 4 replicates at 7.6 and four replicates at 8.0 while holding the temperature at a constant 20 degrees Celsius. In the other set of 8 jars, I elevated the temperature to 24 degrees Celsius and maintained the same two pH levels: 7.6 and 8.0. From these two sets of jars I compared larval growth rates. This experiment allows for variation of both temperature and pH to observe the interaction between these two factors.

A: 20 degrees, pH 7.6	Rep 1	Rep 2	Rep 3	Rep 4
B: 20 degrees, pH 8.0	Rep 1	Rep 2	Rep 3	Rep 4
C: 24 degrees, pH 7.6	Rep 1	Rep 2	Rep 3	Rep 4
D: 24 degrees, pH 8.0	Rep 1	Rep 2	Rep 3	Rep 4

Above is the layout of culture jar conditions used in experiment to test pH-temperature interaction.

This is a two-layer interaction experiment involving two levels of pH and two temperatures with four replicates for each condition resulting in a total of 16 culture jars. I tested pH levels 7.6 and 8.0, both under 24 degree Celsius conditions with four replicates each. I maintained the same two pH levels, 7.6 and 8.0 under 20 degree Celsius conditions with four replicates each. To obtain the desired culture conditions for my experiment I used the spectrophotometer to calibrate the pH electrodes and took our pH data with these calibrated electrodes. Salinity was measured by a conductivity probe. In

addition, “CO2Calc” software was used to calculate the appropriate amounts of each gas to obtain the desired pH at a given temperature.

pH will be measured and maintained using spectrophotometric calibration using m-Cresol Purple dye. Samples of culture water were assessed frequently to ensure accurate pH and temperature probe readings. To deliver a gas mix that contains the desired pCO2 to make the 24-degree pH-8.0 condition, I used a mass flow controller with CO2-free air and air with the pCO2 at pH 8.0 and 20 degrees. I did the same calculations for the other pH and temperature conditions. Once larvae from both temperatures and pH conditions had been maintained in culture and imaged for 12 days of the larval life stage, shell length was plotted.

Inducing Metamorphosis

Metamorphic competence was gauged by putting larvae into FSW with 20mM excess KCl (Pechenik et al 2002). Metamorphosis was assessed by the absence of the ciliated velum. Presence of any ciliated velar structure meant that the animal was still in the larval stage. A metamorphic competence test was performed on day 8 and day 12 for each of the larval groups. Larvae were exposed to elevated KCl and the number of metamorphosed juveniles was noted after 8 and 12-hour time points. Then, a mass metamorphic induction was done for 24-degree cultures on day 13 (7/14/17) and for the 20-degree cultures on day 15 (7/16/17).

Juvenile Culture Maintenance and Setup

Juveniles were grown in individual 40-mL plastic cups in a plastic atmosphere box with the appropriate pH gas line attached. Temperature for all juveniles was approximately 23-25° C due to the ambient temperature of the ocean acidification lab. Therefore, only pH was varied at the juvenile stage. Twelve juveniles were selected from each of the larval treatments to be grown in the 7.6 box and twelve in the 8.0 box. Juveniles were fed 10x10^4 cells / mL T. isochrysis and received water changes daily.

Results

Larval growth data is shown in Figure 1 with the day 12 shell sizes being significantly different in pH 7.6 and 8.0 treatments. Growth rates in the 24°C cultures were greater from day 0-3 and 3-7 than those of the 20°C cultures, though the difference is not significant. Growth rates were comparable across all 4 treatments from days 8-11 with a slightly lower rate in the 7.6 20°C culture (Figure 1).

Metamorphic competence was tested at 8 and 12 days of larval growth. Significantly higher frequency of metamorphosis was observed in larvae reared at 24°C than those raised at 20°C. Similarly, at 8.0 pH a significantly higher frequency of larvae was metamorphically competent than were at the 7.6 pH (Figure 2).

Latent effects were found when analyzing juvenile growth data. A latent effect is a result that appears in the juvenile or adult stages because of some larval condition. These latent effects prove that the effect of larval conditions doesn't disappear after metamorphosis

(Bashevkin, Samuel M., Pechenik, J.A. 2015). Figures 4 and 5 show juvenile growth in the 7.6 and 8.0 atmosphere boxes, respectively. Latent effects of warm larval culture conditions were clear in the 7.6 juveniles. Growth rates were significantly lower in the animals with a 24°C larval history than those reared at 20°C.

In conclusion, significant results observed in this study include:

1. pH and Temperature affect larval growth.
2. pH and Temperature affect metamorphic competence.
3. Latent effects of larval temperature on juvenile growth.

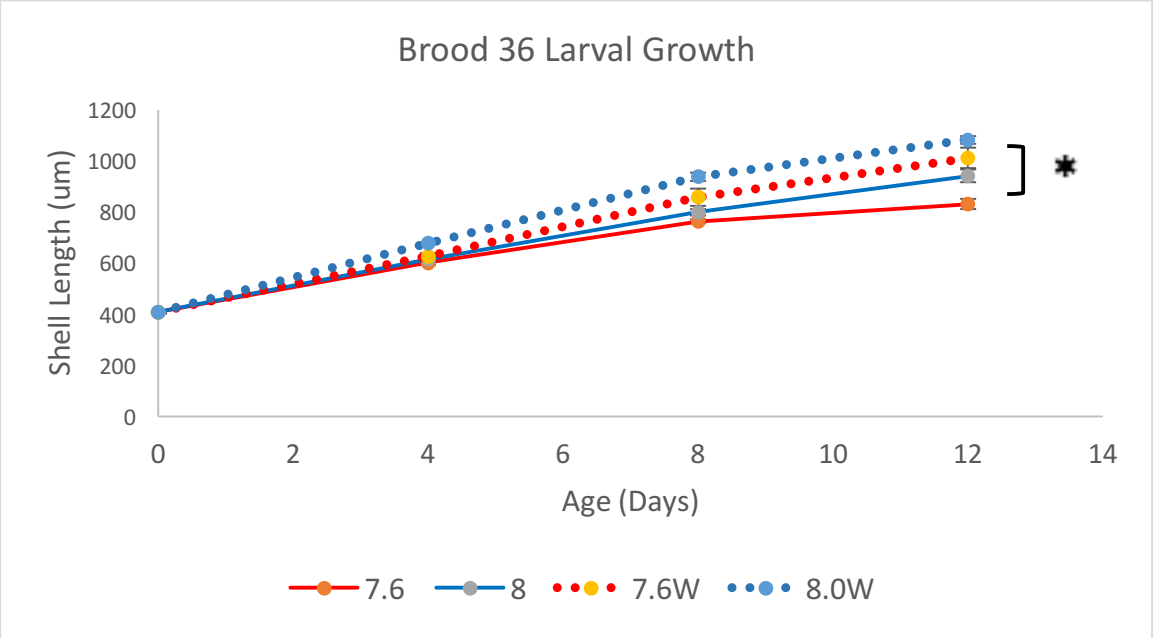


Figure 1. Brood 36 shell length (um) from day 0-12. Significant difference between day 12 sizes in pH 7.6 and 8.0 treatments.

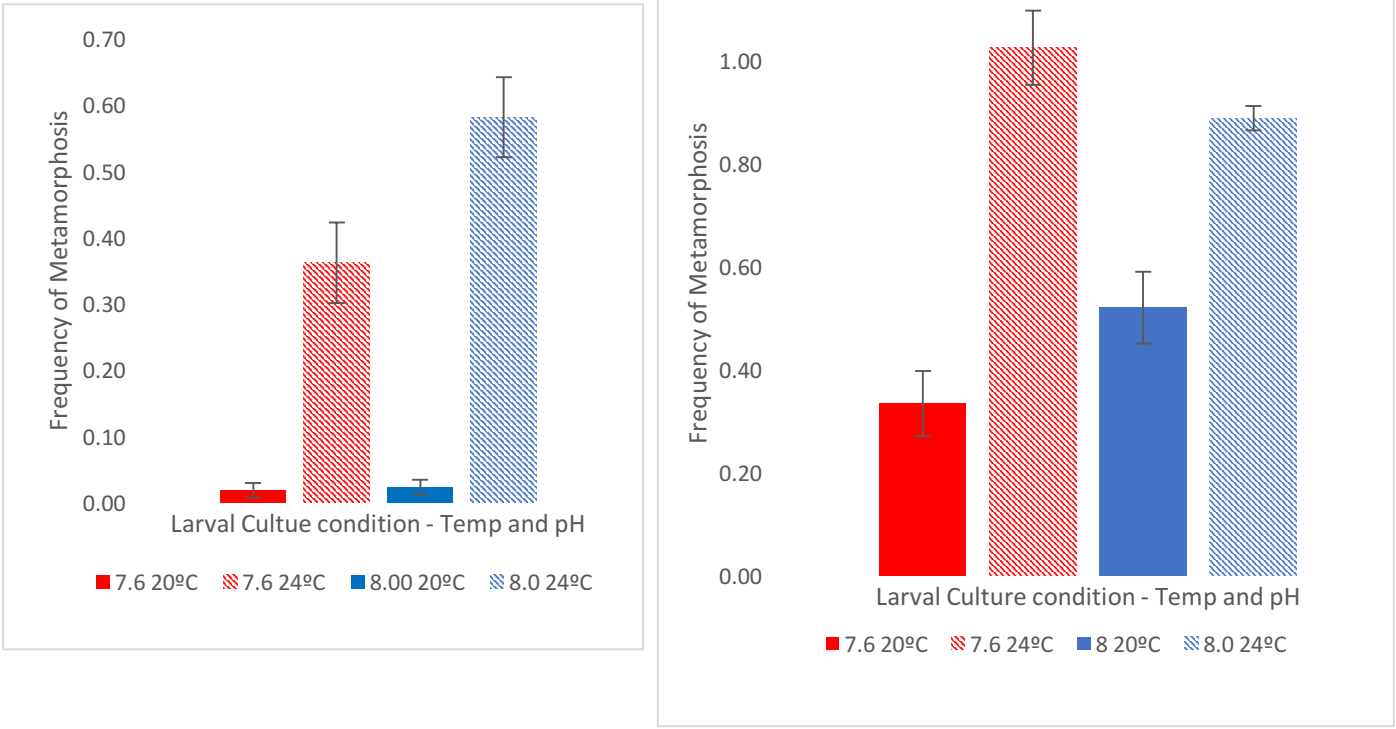


Figure 2. Average frequency of metamorphosis at day 8 (left) and day 12 (right). Significant difference in frequency noted between 20°C and 24°C treatment. Error bars reflect standard errors of the mean with a sample size of 4 replicates.

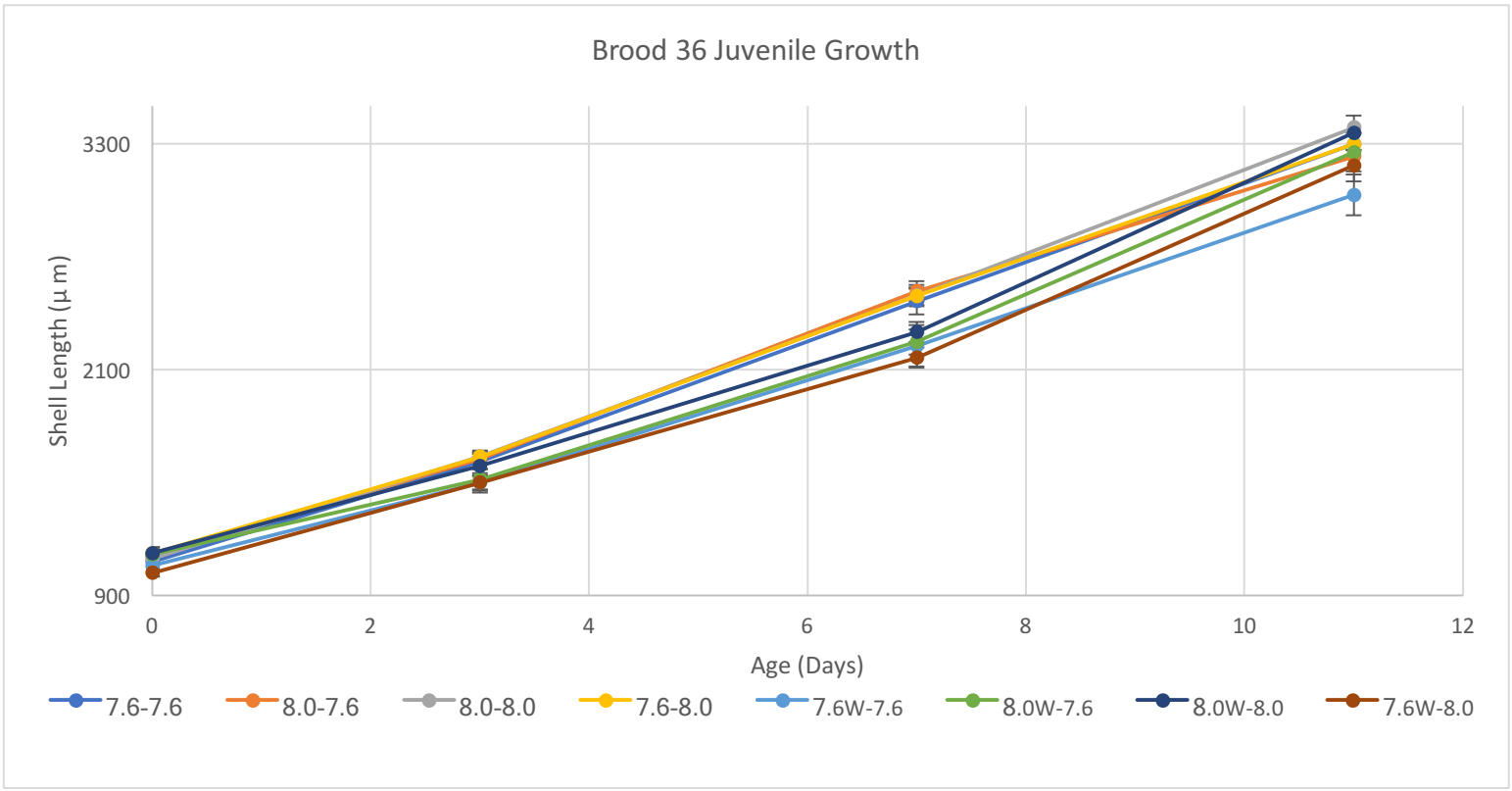
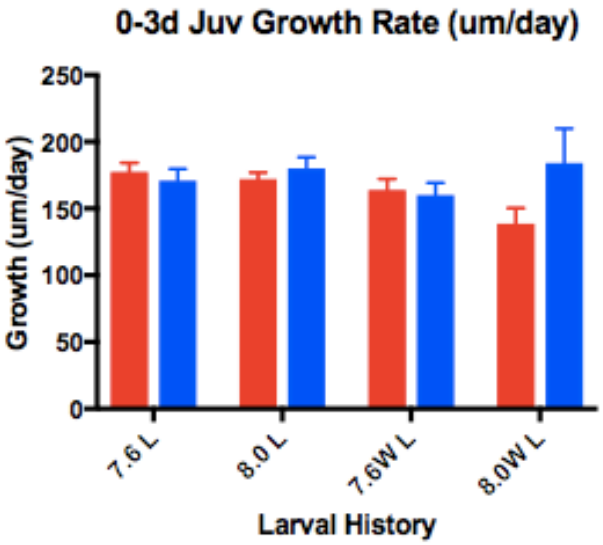


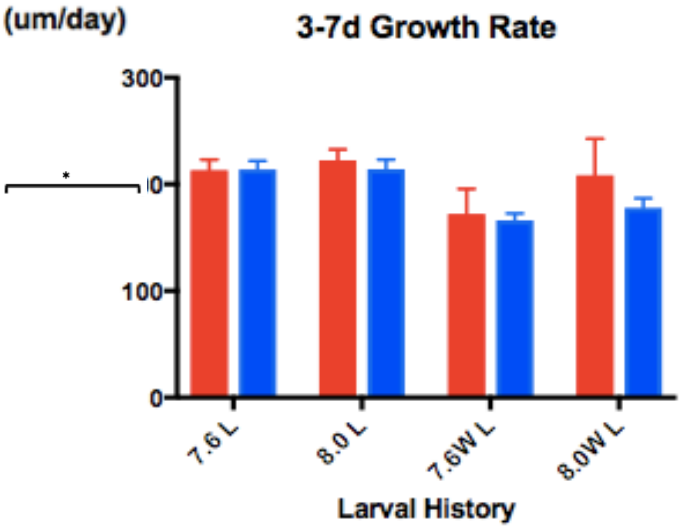
Figure 3. Brood 36 juvenile growth in 8 different condition groups. Juveniles reared at 24°C larval cultures had larger shell length than those reared at 20°C.

Measurements of shell growth were broken down in to three separate time frames to isolate when stressors began affecting growth rates. These periods were: 0-3 days, 3-7 days, and 7-11 days. The data show significant difference in growth rates between juvenile who had different larval conditions (either temperature or pH) in the 3-7-day time frame (4b), and a significant difference in larval history and juvenile pH from 7-11 days (4c). No significant difference was found in the earliest time frame from 0-3 days.

(a)



(b)



(c)

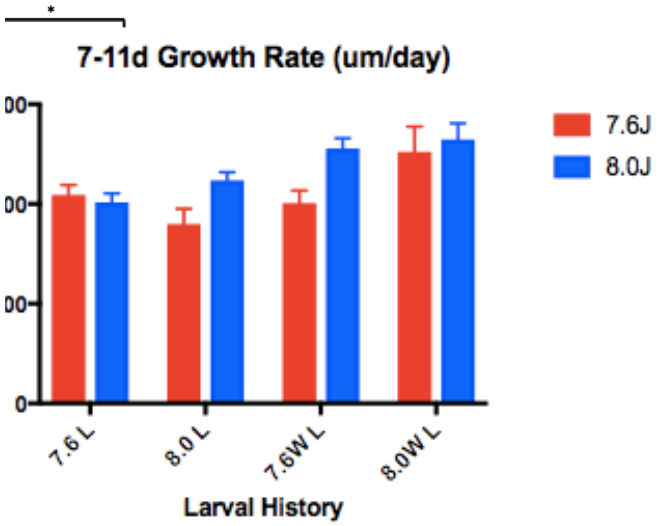


Figure 4. Juvenile growth rates from 0-3 days (a), 3-7 days (b) and 7-11 days (c) of animals with different larval histories and juvenile conditions.

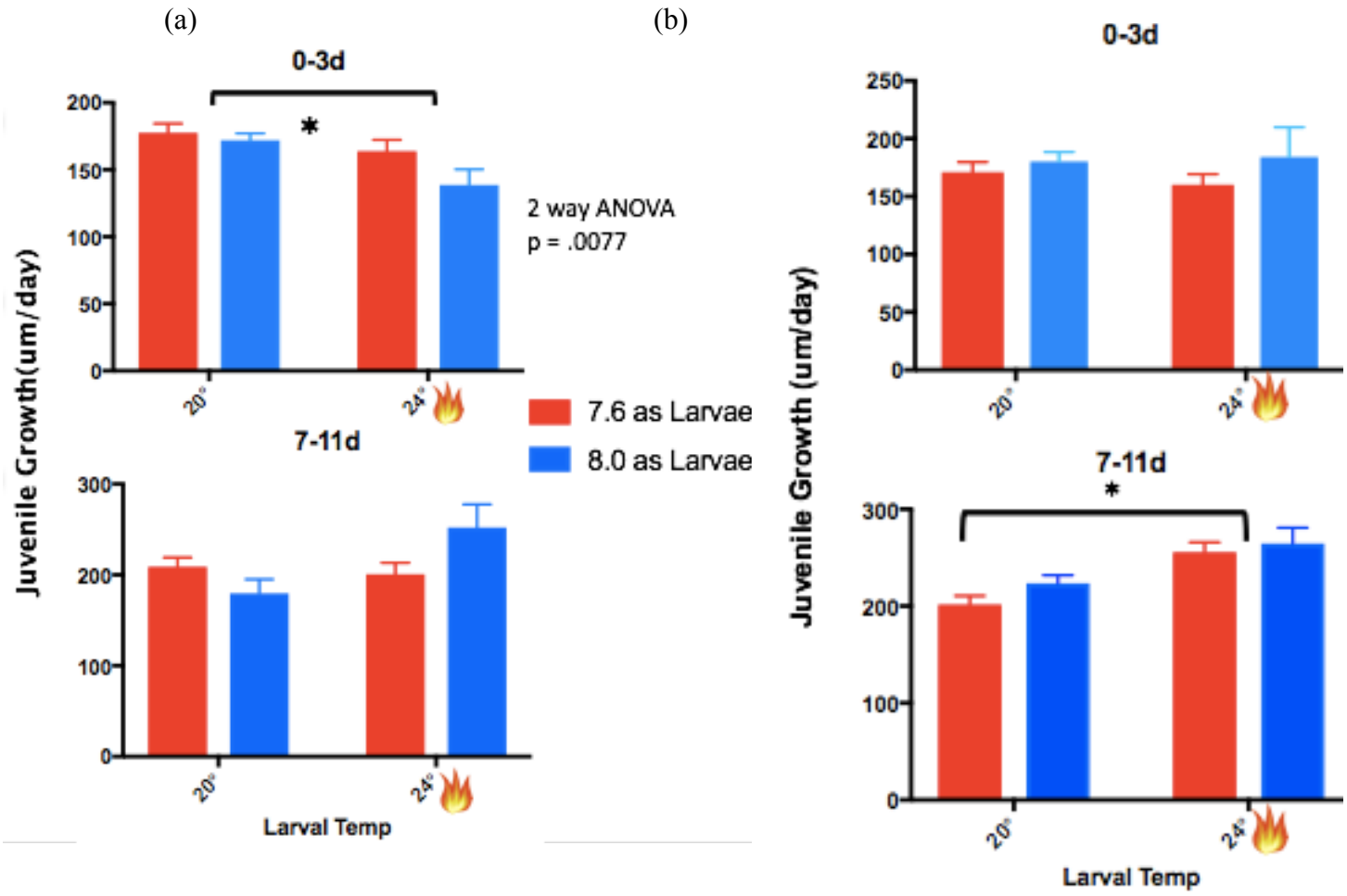


Figure 5. Juvenile growth in (a) 7.6 and (b) 8.0 atmosphere box of animals with 4 different larval histories - 20°C and 24°C.

Discussion. This study exemplifies the many effects of both temperature and pH on the development of *C. fornicata*. First, a significant difference in larval size by day 12 was noted in animals raised at different temperatures and pH levels (Figure 1). This means that both factors affected growth of *C. fornicata* as larvae. Metamorphic frequency was significantly higher at days 8 and 12 for larvae reared at warmer temperatures (Figure 2). Overall juvenile growth is shown in Figure 3 to highlight the larger final size of larvae reared at 24°C than those raised in the cooler cultures. In addition, thermal and pH stressors as larvae significantly affected juvenile growth during 3-7 and 7-11 days of

growth (Figure 4). This shows the latent nature of pH and temperature conditions on these animals. Lastly, when looking at isolated juvenile growth at 7.6 and 8.0 pH, juveniles grown at the lower pH showed a significantly higher growth rate for cooler temperatures in the first 3 days after metamorphosis (Figure 5a). More importantly, though, Figure 5b shows that for the 8.0 juvenile box, a latent effect of high temperature stress caused higher growth rates during the 7-11-day time frame.

None of the results showed an interaction between the two factors, however, and it can therefore be said from this study that these two factors affect development independently of one another but could be having additive effects on growth rates. Further investigations would be necessary to confirm this. Future projects could still look out for an interaction between the two factors, however, as this could add insight into the development of knowledge on coastal acidification.

Manzello et. al studied the combined effects of thermal and acidification stressors on Pacific corals (Manzello 2010), and found that branching corals were more tolerant to increased temperature than to increased acidity and found opposite effects in massive corals. Therefore, the study species does dictate the sort of response to temperature and pH stressors. In the Byrne and Przeslawski study investigating multiple stressors, data showed additive negative effects of warming and acidification but no interaction between the two. This is very much like the results we found in this study. *C. fornicata* is a highly successful invasive species. Originating on the east coast of the United States and spreading to the west coast, as well as many European coastal waters, this marine

gastropod has found multiple ecosystems to thrive in. In future projects, temperature and pH conditions in nature could be observed and mimicked in an experimental setting, for in nature the pH and temperature are not held steady as they were in laboratory settings. Fluctuations in water flow could also be simulated instead of growing the animals in stagnant water jars to mimic the tides.

Effects of various larval histories, notably elevated temperature and higher pH, resulted in significantly higher growth rates as juveniles. Future investigations should continue to look for these latent effects in *C. fornicata* who have experiences similar temperature or pH stress as larvae

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