The interactive influence of temperature and salinity on larval and juvenile growth in the gastropod *Crepidula fornicata* (L.)

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**A B S T R A C T**

Sea surface temperatures have been rising and are predicted to continue rising in coming years because of global warming. In addition, salinity has been decreasing in high latitudes and is expected to continue decreasing due to altered precipitation patterns and glacial melting caused by climate change. Many marine organisms that are adapted to the present conditions may be drastically affected by these oceanic changes. Early life stages (larvae and juveniles) should be especially susceptible, since they do not yet have the fully developed morphological defenses of adults. This study investigated the effects of reduced salinity (20 compared to a control of 30) and altered temperature (15, 20, 25, and 29 °C) on the growth, percent inorganic content (representing shell calcification), and feeding rates of larvae and juveniles of the widespread coastal snail *Crepidula fornicata*. Both larval and juvenile growth rates were significantly depressed at low salinity and lower temperatures. In addition, the salinity that snails were exposed to as larvae significantly impacted their juvenile growth rates in 4 out of 6 treatments, an example of latent effects. The magnitude and direction of this effect were strongly impacted by temperature and parentage. Juvenile feeding rates were significantly depressed following a rapid change in salinity but then gradually recovered to near initial levels over 3 h. There was little difference in the percent inorganic content of juveniles but for larvae that had been reared at 20 °C, percent inorganic content was 27% lower for those reared at a salinity of 20 compared with those reared at 30. In conclusion, the early life stages of *C. fornicata* may experience more favorable conditions in a warmer future: growth rates will increase, probably making larvae and juveniles less vulnerable to size-specific predation. Conversely, in regions where salinity is decreasing, *C. fornicata* larvae and juveniles will likely grow more slowly, thereby increasing predation risk by forcing them to spend more time at more vulnerable smaller sizes. Thus, the future of *C. fornicata* and their potential to continue invading new habitats will depend greatly on the specific salinity and temperature conditions they will be exposed to throughout larval and juvenile development.

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1. Introduction

Sea surface temperatures have been increasing at the alarming rate of 0.11 °C per decade since at least 1971 (IPCC, 2013). These rising temperatures have altered precipitation patterns and increased the rate of glacial melting, causing salinity to decrease in high latitude waters since the 1950s (IPCC, 2013). While these salinity changes are not large in magnitude (~0.5 over 50 years along the Eastern US Coast since the 1950s (IPCC, 2013), they could cause the frequency or duration of salinity fluctuations to increase in near-shore ecosystems like estuaries, where even now salinity can drop by 33–67% in a matter of hours (Chaparro et al., 2008b; Khangaonkar et al., 2011). These documented changes are expected to continue in the future, or worsen, with a potentially large impact on many marine ecosystems. Species ranges will likely shift (Harley et al., 2006; Scavia et al., 2002), some species will go extinct (Thomas et al., 2004), while others will persevere (Moritz and Agudo, 2013), and some invasive species may even do better under the new conditions (Stachowicz et al., 2002).

The early life stages of marine organisms which lack the fully developed defenses of the adult (*Gosselin and Qian, 1997; Hunt and Scheibling, 1997*) will be especially affected by climate change. These early life stages have a huge influence on the future distribution and abundance of adult populations, which are often limited by recruitment, a product of larval supply and juvenile survival (*Gosselin and Qian, 1997; Hunt and Scheibling, 1997; Keough and Downes, 1982*). Both larvae and juveniles are subject to extremely high mortality rates, reaching as high as 87% over 2 min in ascidian larvae (Olson and McPherson, 1987) and over 90% for juveniles of a number of species in the first few days or weeks after metamorphosis (reviewed in *Gosselin and Qian, 1997*). With such high background mortality, any
additional disturbance caused by anthropogenic temperature or salinity changes could push recruitment below minimum levels required for population sustainability.

In addition to direct mortality, the non-lethal effects of climate change could be just as devastating. Juveniles (Paine, 1976; Vermeij, 1972) and possibly larvae (Morgan, 1995; Pechenik, 1999; Rumrill, 1990) are typically more vulnerable to predation and environmental stresses at the smaller sizes of each life stage; thus, decreased growth rates may indirectly increase mortality by making larvae and juveniles spend more time at the more vulnerable smaller sizes and, in the case of larvae, forcing them to spend more time in the plankton subject to pelagic predation (Rumrill, 1990). Reduced feeding rates could be a major driver for reduced growth rates in low salinity since some species completely seal themselves off by clamping to the substrate when salinity drops too low (e.g. the gastropod Crepipatella dilatata: Chaparro et al., 2008a). Also, if larvae spend more time in the plankton their dispersal patterns and therefore their chance of ending up in appropriate habitat would likely be affected, which could reduce the supply of larvae to adult populations (Pechenik, 1999).

Another non-lethal effect of salinity reduction could be reduced shell calcification in the larvae and juveniles of oysters, gastropods, and other calcifying species since low salinity seawater, with a lower concentration of calcium, is known to slow calcification in such calcifying invertebrates as adult mussels (Malone and Dodd, 1967) and bristle tunicates (Donachy and Watabe, 1986). Therefore, these individuals may take even longer to fully develop adult defenses, many of which depend on calcification.

Further complicating the biological response to climate change is the phenomenon of latent effects, wherein stresses experienced during the larval stage impact juvenile performance or growth (Padilla et al., 2001; the barnacle Balanus balanoides: Klimas and Pechenik, 1976a,b), ocean acidiﬁcation, and late metamorphosis (the oyster Ostrea lurida: Hettinger et al., 2012), low salinity (the polychaete Capitella teleta: Pechenik et al., 2001; the barnacle Balanus amphitrite: Thiagajaran et al., 2007), copper (the bryozoan Watersipora subtorquata: Ng and Keough, 2003) and delayed larval metamorphosis (the colonial ascidian Diplosoma listerianum: Marshall et al., 2003; B. amphitrite: Pechenik et al., 1993; the bryozoan Bugula stolonifera: Woodlaccott et al., 1989). Therefore, it is not sufficient to only consider the conditions directly experienced by juveniles when attempting to predict their response to environmental changes; the conditions experienced as larvae must be considered as well.

One widespread, calcifying, osmoconforming, marine invertebrate likely to be affected by climate change is the marine gastropod Crepidula fornicata. Native to the Eastern U.S. coast, C. fornicata is an extremely successful invader of Northern Europe, Japan, and Western U.S. coastal habitats (Blanchard, 1997; Thieltges et al., 2003). Females release free-swimming, shelled veliger larvae in hatches usually fathered by multiple males (Dupont et al., 2006). The larvae then spend from 7–27 days in the plankton before settling and metamorphosing (Pechenik, 2006). This is an ideal species for studying the impact of environmental change on early life stages since the juveniles and larvae can be reared in the lab with very low mortality and once metamorphic competence is reached, larvae can easily be induced to metamorphose by simply raising the ambient concentration of KCl (Pechenik and Gee, 1993; reviewed by Henry et al., 2010). In addition, by studying the effects of climate change on this species, researchers may be able to predict how both native and invasive populations will be affected and where C. fornicata will be able to invade in the future.

Previous research has demonstrated that C. fornicata juveniles can separately tolerate salinities as low as 15 and temperatures as high as 32 °C (Pechenik and Eyster, 1988), while larvae can tolerate salinity at least as low as 15 (Diederich et al., 2011) and temperatures up to at least 30 °C (Lucas and Costlow, 1979; Pechenik and Lima, 1984). A number of studies have documented that C. fornicata larvae grow faster at higher temperatures (Klinzing and Pechenik, 2000; Lucas and Costlow, 1979; Pechenik, 1984; Pechenik and Heyman, 1987; Pechenik and Lima, 1984) but no studies have investigated the interactive effects of temperature and salinity on the growth of C. fornicata larvae and juveniles.

Latent effects have been observed in this species in response to larval nutritional stress (Pechenik et al., 1996a,b), but no latent effects were found from larval exposure to low salinity stress (Diederich et al., 2011). In the latter study, Diederich et al. (2011) reared the post-metamorphic juveniles in stress-free, control salinity conditions. No studies have investigated the effect of larval exposure to low salinity on juvenile performance under stressful (low salinity) conditions, or how this effect may be modulated by temperature.

In molluscs, larval and juvenile growth rates are usually measured by changes in shell length or total dry weight, assuming a constant relationship between shell length, shell weight, and tissue biomass. While previous studies on C. fornicata have investigated the relationship between biomass and shell length for individuals reared under a particular set of conditions (Pechenik, 1980; Pechenik and Eyster, 1989), no studies have considered the effects of environmental factors on relative rates of shell and tissue growth. Given the detrimental effects of decreased salinity on calcification, rates of shell mass increase may be more strongly affected than those of shell length or tissue biomass. Therefore, this study also examined the potential impact of temperature and salinity on the ratio of tissue to shell mass during both larval and juvenile development.

The following questions were addressed: 1) What are the interactive effects of salinity and temperature on C. fornicata larval and juvenile growth? 2) How does larval exposure to low salinity impact juvenile growth, and how is this effect modulated by a) the temperature and b) the salinity that juveniles are subsequently reared in? and 3) Are reduced juvenile growth rates at low salinity caused in part by reduced feeding rates? To address these questions, this study measured the growth rates (both in terms of shell length and shell mass/tissue mass) of juveniles and larvae in a number of temperature and salinity treatments, investigated the effects of larval exposure to low salinity on subsequent juvenile growth both at low and normal salinity and at 3 different temperatures, and measured the effect of salinity change on juvenile feeding rates.

2. Materials and methods

2.1. Adult collection and maintenance

Adult C. fornicata were collected from Nahant, MA in September 2012, September 2013, and January 2014 and maintained in the lab with aeration and frequent water changes until larvae were released. Adults collected in January were slowly acclimated to lab temperature (about 23 °C) to prevent mortality from heat stress. All adults were kept in a mixture of Instant Ocean artificial seawater (ASW) and natural seawater at a salinity of 30 and were regularly fed the microalgae Isochrysis galbana (clone T-ISO) and Dunaliella tertiolecta (clone DUN). Algal cultures were started from cells ordered from the National Center for Marine Algae and Microbiota at the Bigelow Laboratory for Marine Sciences, East Boothbay, Maine.

2.2. Larval and juvenile maintenance

Released larvae were collected on 130 μm mesh, transferred to 1-gallon glass jars half-filled with aerated 0.45 μm filtered natural seawater (FSW) at a salinity of approximately 30, and fed I. galbana until needed for experiments (see Sections 2.4–2.6 for specific durations). Larvae to be used in experiments were kept in glass bowls filled with 45 ml FSW adjusted to the desired salinity with deionized water. Larvae were reared in groups of approximately 15 per bowl and were...
fed *I. galbana* every 1–2 days at approximately 1.8 × 10⁵ cells/ml (Diederich et al., 2011; Pechenik and Eyster, 1989). Temperature was maintained with incubators that were set to a cycle of 12 h light and 12 h dark.

When larvae were competent to metamorphose (see Section 2.5), they were transferred to ASW with 20 mM excess KCl to induce metamorphosis (Pechenik and Gee, 1993; Pechenik and Heyman, 1987). Juveniles were reared exactly as described for larvae except they were kept individually in 45 ml of 50% PSW and 50% ASW, adjusted to the appropriate salinity with deionized water. Juveniles were fed *I. galbana* and occasionally *D. tertiolecta* at 1.8 × 10⁵ cells/ml. Food concentrations remained above 7 × 10⁴ cells/ml, so that feeding rates should have remained constant during the experiment (Pechenik and Eyster, 1989).

Water was changed and new phytoplankton was added every 2–3 days. Larvae were transferred to clean bowls at each water change but bowls containing juveniles were cleaned and refilled with new water. Desired salinity for each treatment was confirmed with a handheld refractometer.

### 2.3. Growth measurements

Shell length measurements for growth rate determinations were obtained for larvae and juveniles using a dissecting microscope fitted with an ocular micrometer. Larvae were measured at magnifications of 50 or 63× and juveniles were measured at 16–63×, depending on their size.

Additionally, organic (tissue) and inorganic (shell) masses were determined for both larvae and juveniles. Juveniles were measured individually while larvae were measured 5–9 to a sample in order to get accurate measurements despite their small size. All masses were determined to the nearest 0.001 mg using a Mettler Toledo MTS balance. Samples were quickly rinsed in deionized water to remove any adhering salt or formalin, placed on pre-weighed foil, dried at approximately 80 °C for at least 48 h, and weighed to obtain the total dry mass. Percent inorganic content (i.e., shell mass) of larvae and juveniles was calculated by dividing inorganic mass by total dry mass. Cumulative growth rates were calculated for larvae and juveniles using a dissecting microscope equipped with a hand-held refractometer.

### 2.4. Experiment 1: long-term effects of temperature and salinity on juvenile growth

This experiment was conducted using juveniles reared from larvae released by 2 females in September 2012. Larvae were reared to metamorphosis at room temperature (23 °C) and a salinity of 30. Juveniles were distributed among treatments as soon as they had metamorphosed. There were 6 treatments, comprising every combination of the temperatures 15, 20, and 25 °C and the salinities 20 and 30, and each treatment had 12 replicate juveniles. Replicate juveniles were reared individually in separate bowls as described above. Juveniles were measured every 2–4 days until they reached approximately 6 mm in length, at which point they were sacrificed for determinations of organic/inorganic mass. Cumulative growth rates were calculated for each day that juveniles were measured by subtracting initial size from the size at that day and dividing by the number of days elapsed.

#### 2.5. Experiment 2: effects of larval exposure to low salinity on juvenile growth

Experiments were started 1–4 days after larvae hatched and on three hatches of larvae from different females (Hatch A: September 2013, Hatch B: January 2014, Hatch C: October 2013). First, 12 larvae were measured from each hatch to get initial shell lengths. Initial larval shell lengths were 398.7 ± 17.1 μm for Hatch A, 481.5 ± 16.8 μm for Hatch B, and 442.7 ± 41.9 μm for Hatch C (mean ± SD). Larvae were then distributed among a total of 6 treatments consisting of all combinations of the temperatures 20, 25, and 29 °C and the salinities 20 and 30 (Fig. 1). Larvae from each hatch were reared at 2 of the 3 temperature treatments. Larvae from Hatches A and B were reared at 20 and 25 °C while larvae from Hatch C were reared at 25 and 29 °C. Each treatment had 4 replicate bowls filled with 45 ml algal suspension (see Section 2.3), with approximately 15 larvae per bowl. Three replicates were measured for larval growth rates and a 4th was used to test for metamorphic competence.

Larval shell lengths were determined periodically at least 3 times before metamorphosis was induced. When the maximum shell lengths of larvae were close to 800 μm (Pechenik and Heyman, 1987), 7–8 larvae from the 4th replicate were tested for competence by transferring them to ASW + 20 mM KCl for 6 h. If at least 75% of the larvae metamorphosed, larvae from that treatment were determined to be metamorphically competent. Or, if larvae were larger than 800 μm before testing for competence, they were assumed to be competent (Pechenik and Heyman, 1987). Once competent, larvae from replicates 1, 2, 3, and any from replicate 4 that had not previously been exposed to excess KCl were transferred to ASW + 20 mM KCl for 6 h to induce metamorphosis (Pechenik and Heyman, 1987).

After larvae metamorphosed into juveniles, each larval treatment was split into 2 new salinity treatments: 20 and 30, with 12 replicate juveniles per treatment (Fig. 1). Temperatures were kept constant for the entire experiment. Thus, there were a total of 12 different juvenile treatments (3 temperatures × 2 larval salinities × 2 juvenile salinities). All larvae from each treatment were mixed and randomly subsampled for the new juvenile treatments. Juveniles were reared individually on a diet of 1.8 × 10⁵ cells/ml *I. galbana* and occasionally *D. tertiolecta* as described above. Juveniles were initially measured 1–2 days after metamorphosis, then every 4–5 days after that. Once juveniles reached 4–4.5 mm in length, they were sacrificed for organic/inorganic mass measurements.

#### 2.6. Experiment 3: effects on larval inorganic content

An experiment was conducted to determine the effects of salinity and temperature on the percent inorganic content (i.e., shell mass) of larvae. Treatments consisted of the temperatures 20 and 25 °C and the

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**Fig. 1.** Experimental design for Experiment 2. The larval hatches exposed to each temperature treatment are indicated below the temperature.
salinities 20 and 30. Each treatment had 5 replicate bowls with 15 larvae per bowl in 45 ml algal suspension. Larvae were put into treatments 2 days after hatching. First, 12 larvae were measured from each hatch to get initial shell lengths. Once larvae reached about 800 μm in length, the approximate size at which larvae reach metamorphic competence (Pechenik and Heyman, 1987), larvae in the first 3 replicates were measured and then all larvae from the 5 replicates (~75 total) were fixed and used for determinations of inorganic and organic mass. Larvae from all 5 bowls were mixed, fixed in 10% buffered formalin for a minimum of 30 min, randomly divided into 10–11 groups of 5–9 larvae each (larvae were too small to weigh singly), and then processed as described above for organic/inorganic mass measurements.

2.7. Experiment 4: feeding rate experiments

To determine the effects of short-term salinity changes on juvenile feeding rates, juveniles from the 25 °C treatments of Experiment 1 were also used for feeding rate determinations. Feeding rates were measured by periodically monitoring cell concentrations with a Coulter Electronic Particle Counter. The Coulter Counter was configured to draw 500 μl samples through the 140 μm diameter aperture and count only cells greater than 3.522 μm in diameter. Samples of 1 ml were first diluted in 10 ml of 0.45 μm filtered Isoton Diluent, shaken, and then sampled 3 times using the Coulter Counter. The mean of these 3 values was used in subsequent calculations. Initial cell concentrations were 20–24 × 10^4 cells/ml for all feeding and pre-feeding steps (Pechenik and Eyster, 1989). These experiments were conducted in standard 6-well plates, with round wells of 35 mm diameter and ~17 ml total volume.

2.7.1. Experiment 4a

At 30 days of age, feeding rates of the 10 surviving juveniles reared at 25 °C and a salinity of 30 were measured at salinities of 20 and 25. Juvenile shell lengths were 6235.1 ± 396.2 μm (mean ± SD). First, juveniles were removed from their glass bowls and distributed among the wells of two 6-well plates. They were then pre-fed in 10 ml of I. galbana suspension at a salinity of 30 for 1 h. At the beginning of the pre-feeding period, juvenile shell lengths were measured as described above, and then the animals were placed in a dark, 25 °C incubator for the remainder of the pre-feeding hour to slow the rate of potentially confounding effects and all 2-way interactions of temperature, salinity and larval hatch, and with replicate nested within the interaction of temperature, salinity, and hatch. Then, to determine the effects of salinity and temperature within each larval hatch, larval growth rates from each of the 6 treatments to determine whether the treatments had significantly diverged in size. In addition, a two-way ANOVA was run on arcsine-transformed proportion inorganic contents to determine the effects of salinity and temperature. Multiple comparisons were also performed after all 2-way ANOVAs (Tukey's test).

For Experiment 2, larval shell growth rates were calculated from changes in shell length from the start of the experiment to the day that larvae were induced to metamorphose. Since individual larvae were not followed through the experiment, growth rates were calculated as the mean of shell lengths measured at the end of the experiment. Larval growth rates were analyzed with a 4-way nested ANOVA (fixed factors: temperature, salinity; random factors: larval hatch, replicate), including the main effects and all 2-way interactions of temperature, salinity and larval hatch, and with replicate nested within the interaction of temperature, salinity, and hatch. Then, to determine the effects of salinity and temperature within each larval hatch, larval growth rates from each of the 3 larval hatches were analyzed separately by nested, three-way ANOVAs (fixed factors: temperature and salinity; random factor: replicate) with replicate nested within the interaction of temperature and salinity. Replicates diverged significantly only for Hatch A (nested ANOVA, F_{1,8} = 4.430, p = 0.0001). Subsequent analyses by one-way ANOVAs and Tukey post-hoc tests on each temperature and salinity.
combination from Hatch A were conducted to identify which replicates diverged significantly. There was a significant difference between two replicates in the treatment at 20 °C and a salinity of 30 (one-way ANOVA, F2,44 = 4.644, p = 0.0148; Tukey test, p = 0.013), and one replicate from the treatment at 20 °C and a salinity of 20 diverged significantly from the other two replicates (one-way ANOVA, F2,31 = 8.860, p = 0.0099; Tukey tests, p = 0.0014, 0.0057).

Juvenile growth rates in Experiment 2 were calculated from changes in shell length from day 1 or 2 after metamorphosis to day 16 after metamorphosis. Juvenile growth rates were analyzed with a 4-way ANOVA (fixed factors: larval salinity, juvenile salinity, temperature; random factor: larval hatch). The ANOVA included all main effects, all 2-way interactions, and the 3-way interaction temperature × larval salinity × juvenile salinity. Then, to decompose the results and investigate the specific effects of larval and juvenile salinity exposures on juvenile growth rates within each temperature and parentage combination, juvenile growth rates were analyzed with 6 two-way ANOVAs run on each of the 6 combinations of temperature and parentage conducted in this study (Hatch A 20°, Hatch A 25°, Hatch B 20°, Hatch B 25°, Hatch C 25°, Hatch C 29°). The ANOVAs tested the effects of the factors larval salinity and juvenile salinity. Juvenile percent inorganic content was analyzed the exact same way after data were arcsine transformed. Multiple comparisons were also performed after all 2-way ANOVAs (Tukey’s test).

Table 2
Summary of 2-way ANOVAs performed on larval and juvenile growth and percent inorganic content. Larval salinity is the salinity in which larvae were reared while juvenile salinity is the salinity in which juveniles were reared. Highlighted cells indicate significant differences, stars represent the degree of significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), and “ns” means “not significant.” Up arrows (↑) indicate a positive effect and down (↓) a negative effect of an increased level of the independent variable (temperature or salinity) on the dependent (growth rate or percent inorganic content).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Temperature treatments (°C)</th>
<th>Larval salinity p-value</th>
<th>Juvenile salinity p-value</th>
<th>Temperature p-value</th>
<th>Interaction p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval growth A</td>
<td>20, 25</td>
<td>0.0008***</td>
<td>0.0013**</td>
<td>0.1601 ns</td>
<td>0.0030** ns</td>
</tr>
<tr>
<td>B</td>
<td>20, 25</td>
<td>0.1104 ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>25, 29</td>
<td>↑ &lt;0.0001***</td>
<td>↑ &lt;0.0001**</td>
<td>↑ &lt;0.0001**</td>
<td>0.0031** ns</td>
</tr>
<tr>
<td>Juvenile growth A</td>
<td>20</td>
<td>↑ &lt;0.0001***</td>
<td>↑ &lt;0.0001***</td>
<td>↑ &lt;0.0001**</td>
<td>0.0143 ns</td>
</tr>
<tr>
<td>A</td>
<td>25</td>
<td>0.4257 ns</td>
<td>↑ &lt;0.0001***</td>
<td>0.3986 ns</td>
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</tr>
<tr>
<td>B</td>
<td>20</td>
<td>↑ 0.0311*</td>
<td>↑ &lt;0.0001***</td>
<td>0.4031 ns</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>↑ 0.0357*</td>
<td>↑ &lt;0.0001***</td>
<td>0.8618 ns</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>29</td>
<td>0.1355 ns</td>
<td>↑ &lt;0.0001***</td>
<td>0.1374 ns</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>15, 20, 25</td>
<td>↑ &lt;0.0001***</td>
<td>↑ &lt;0.0001***</td>
<td>↑ &lt;0.0001**</td>
<td>0.0116* ns</td>
</tr>
<tr>
<td>Larval relative inorganic content</td>
<td>20, 25</td>
<td>0.0036**</td>
<td></td>
<td>0.3186 ns</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
<td>0.0513 ns</td>
<td>0.7653 ns</td>
<td>0.0016** ns</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>↑ &lt;0.0001***</td>
<td>0.1709 ns</td>
<td>0.4270 ns</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>0.3639 ns</td>
<td>↑ &lt;0.0001***</td>
<td>0.5037 ns</td>
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<tr>
<td>D</td>
<td>25</td>
<td>0.8175 ns</td>
<td>↑ &lt;0.0001***</td>
<td>0.7686 ns</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>29</td>
<td>0.0103 ns</td>
<td>↑ 0.0001**</td>
<td>0.0237* ns</td>
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<tr>
<td>Juvenile relative inorganic content</td>
<td>15, 20, 25</td>
<td></td>
<td>↑ &lt;0.0001***</td>
<td>0.0110* ns</td>
<td></td>
</tr>
</tbody>
</table>

Data for larval percent inorganic content were first arcsine transformed and then analyzed with a two-way ANOVA to determine the effects of temperature and salinity, followed by a Tukey’s test.

For each feeding rate experiment, a one-way repeated measures ANOVA (factor: time) with Tukey post-hoc tests was run to compare the feeding rates in each of the four 1-hour increments (see Fig. 2). A few negative feeding rates occurred due to error; these were changed to 0 since juvenile cannot actually exhibit negative feeding. Juvenile shell lengths at the time of feeding rate experiments were compared with independent, 2-tailed t-tests.

3. Results

Because of the sheer number of experiments, all p-values are presented in Table 2 rather than in the text. When results of multiple
experiments are discussed, the most conservative test statistics and p-values are reported. Full ANOVA tables from all analyses are presented in supplementary Table S1.

3.1. Experiment 1: long-term effects of temperature and salinity on juvenile growth

Juvenile survival was high during the 33-day experiment (91.7%); a few juveniles perished by crawling out of the water and desiccating. Differences in juvenile size and growth rates among all 6 treatments (larval salinity was not manipulated in this experiment) were apparent by day 13 after metamorphosis (Fig. 3). Juveniles continued growing in all experiments for the duration of the experiment, even at 15 °C and low salinity (20), and shell growth rate gradually increased for animals in most treatments over the duration of the experiment (Fig. 3). There was one major exception: juveniles reared at 25 °C and low salinity that initially had growth rates similar to those of juveniles reared at 25 °C and normal salinity (30), slowed their growth dramatically after day 7 (Fig. 3B). The growth rates of the individuals reared at 25 °C and low salinity decreased rapidly for the next week before beginning to gradually increase.

Low salinity significantly depressed juvenile growth rates (2-way ANOVA, $F_{1,60} = 100.71, p < 0.0001$) and higher temperatures over the range tested significantly increased juvenile growth rates (2-way ANOVA, $F_{2,60} = 101.37, p < 0.0001$). In addition, the interaction of temperature and salinity was significant (2-way ANOVA, $F_{2,60} = 4.81, p = 0.0116$), indicating that salinity had a more pronounced effect on growth rates at higher temperatures (Fig. 3, Table 2). Juveniles in the low salinity treatments generally experienced reduced growth rates between days 8–15 followed by increasing growth rates for the remainder of the experiment. Juveniles reared at the highest temperature and in full-strength seawater (25 °C, 30) grew about 4 times faster than

![Fig. 4](image_url)

**Fig. 4.** The effects of salinity and temperature on larval *C. fornicata* shell lengths over time (left side) and mean growth rates (right side). Hatches from 3 different mothers (A, B, C) were used in these experiments. Shell lengths were determined at the start of the experiment and every few days after until larvae reached metamorphic competence. Growth rates were calculated as changes in shell length from the initial measurement to the day of metamorphosis. Data points indicate the mean of 3 replicate bowls with 15 larvae per bowl and error bars show standard error. Salinity is denoted by color (black: 30, clear: 20), and, for the left column, temperature is denoted by shape (triangle: 20 °C, circle: 25 °C, square: 29 °C). Different letters above points indicate significantly different means (Tukey test; $p < 0.05$).
those reared at the lowest temperature in seawater of reduced salinity (15 °C, 20; Fig. 3).

3.2. Experiment 2: effects of larval exposure to low salinity on juvenile growth

3.2.1. Mortality

Larval loss in all treatments was between 0–13%, mean 2.3%, with all larvae surviving in over 2/3 of replicates. Empty shells were never found, so most of the larval disappearance was likely due to actual losses during water changes rather than mortality. The only exception was for one replicate from Hatch C at 25 °C and normal salinity, whose larvae became infected by an unknown microbial growth that killed all but 4 individuals (73% mortality); these larvae were excluded from analyses. Juvenile mortality was also very low, and the few juvenile deaths that occurred were usually due to suicide as in Experiment 1. Overall juvenile mortality over the 16 day experiment was 13% for larval Hatch A, 5% for Hatch B, and 16% for Hatch C.

3.2.2. Larval growth

Larval hatch significantly interacted with the effect of salinity (4-way nested ANOVA, $F_{2,26} = 19.144, p < 0.0001$) on larval growth, but did not significantly interact with the effect of temperature (4-way nested ANOVA, $F_{1,25} = 0.914, p = 0.3483$).

Larvae grew 34–108% faster at 25 °C than at 20 °C, but 1–23% more slowly at 29 °C than at 25 °C; the effect of temperature on larval growth rates was significant for all 3 larval hatches (nested 3-way ANOVAs, $F_{1,8} > 16.51, p < 0.0034$; Table 2). For Hatches A and C (with the temperature treatments 20 and 25 °C, and 25 and 29 °C respectively), low salinity significantly decreased larval growth rates by 20–48% (nested 3-way ANOVAs, $F_{1,8} > 27.38, p < 0.0008$), but for Hatch B (with the temperature treatments 20 and 25 °C), salinity had no effect on growth rate (nested 3-way ANOVA, $F_{1,8} = 1.172, p = 0.3104$; Table 2). There was a

![Fig. 5. The effects of larval rearing salinity on juvenile growth rates of C. fornicata at 3 different temperatures. Growth rates were calculated as changes in shell length from day 1 or 2 after metamorphosis to day 16 after metamorphosis. Hatches from 3 different mothers (A, B, C) were used in these experiments. Larvae were reared at salinities of 20 or 30 from a few days after hatching until they reached metamorphic competence. Metamorphosis was then induced and the resulting juveniles were distributed among two new salinity treatments of 20 or 30. Temperature was held constant throughout larval and juvenile development for each treatment and is indicated by labels on each graph. Error bars indicate standard error and sample sizes are indicated below each point. Different letters above points indicate significantly different means (Tukey test; p < 0.05).](image)
significant interaction of temperature and salinity for larvae from Hatch B (nested 3-way ANOVA, $F_{1,8} = 17.48$, $p = 0.0030$) because of the trend of decreased growth by low salinity at 20 °C but slightly increased growth by low salinity at 25 °C. The interaction was also significant for larvae from Hatch C (nested 3-way ANOVA $F_{1,8} = 11.43$, $p = 0.0093$), indicating that salinity had a stronger effect on larval growth at the higher temperature (Fig. 4, Table 2).

Abnormal larval development was observed in the 20 °C low salinity treatment of Hatch A, but abnormal development was not observed for larvae from this hatch in other treatments. These abnormal larvae grew shells that curled upwards around the brim, resembling a bowler hat. Generally, the larval body could not fit inside the abnormal shell.

3.2.3. Juvenile growth

Larval hatch significantly interacted with the effects of larval salinity (4-way ANOVA, $F_{1,256} = 8.617$, $p = 0.0002$) and temperature (4-way ANOVA, $F_{1,256} = 6.780$, $p = 0.0098$) to affect juvenile growth rates, but had no significant interaction with juvenile salinity (4-way ANOVA, $F_{1,256} = 0.406$, $p = 0.6666$). Temperature significantly interacted with the effects of larval salinity (4-way ANOVA, $F_{1,256} = 4.433$, $p = 0.0128$), but not juvenile salinity (4-way ANOVA, $F_{1,256} = 2.039$, $p = 0.1322$), to affect juvenile growth rates.

In all 6 temperature by parentage treatments, juveniles rear at low salinity grew from 8–75% more slowly than those reared at normal salinity (2-way ANOVAs, $F_{1,38} > 20.05$, $p < 0.0001$). In 4 out of 6 treatments (20 °C Hatches A and B, 25 °C Hatches B and C), rearing larvae at low salinity significantly affected juvenile growth rates (2-way ANOVAs, $F_{1,38} > 4.687$, $p < 0.0357$). For both 20 °C treatments and for Hatch B of the 25 °C treatment, rearing larvae at low salinity decreased growth rates of juveniles rear at both low and normal salinity. For the Hatch C 25 °C treatment, the interaction of juvenile and larval salinities was significant (2-way ANOVA, $F_{1,39} = 8.401$, $p = 0.0061$); in this case, rearing larvae at low salinity increased the growth rates of juveniles reared at low salinity, but had no effect on the growth rates of juveniles rear at normal salinity (Tukey test, $q_{39} = 0.1224$, $p > 0.05$; Fig. 5, Table 2).

3.3. Percent inorganic content

3.3.1. Experiment 3: larvae

Salinity, but not temperature, significantly affected percent inorganic content of larvae (2-way ANOVA, $F_{1,39} = 9.585$, $p = 0.0036$). At 20 °C, low salinity depressed inorganic content by 27% relative to that of control larvae; salinity had no effect on percent inorganic content at 25 °C so the interaction of salinity and temperature was significant (2-way ANOVA, $F_{1,39} = 32.02$, $p < 0.0001$; Fig. 6, Table 2).

3.3.2. Experiments 1 and 2: juveniles

Temperature, but not salinity, significantly affected juvenile percent inorganic content in Experiment 1 (2-way ANOVA, $F_{1,53} = 17.48$, $p < 0.0001$). At low salinity, juveniles had a higher percent inorganic content at higher temperatures. At normal salinity the order of increasing juvenile percent inorganic content by temperature was 20 °C > 15 °C > 25 °C. Therefore, the interaction of salinity and temperature was significant (2-way ANOVA, $F_{1,53} = 4.916$, $p = 0.0110$; Fig. 7, Table 2).

In Experiment 2, larval hatch significantly interacted with the effect of larval salinity (4-way ANOVA, $F_{2,229} = 9.069$, $p = 0.0002$), marginally significantly interacted with the effect of temperature (4-way ANOVA, $F_{2,229} = 3.857$, $p = 0.0507$), but did not interact significantly with the effect of juvenile salinity (4-way ANOVA, $F_{2,229} = 0.892$, $p = 0.4112$). In addition, temperature significantly interacted with larval salinity (4-way ANOVA, $F_{2,229} = 7.663$, $p = 0.0006$) and with larval salinity and juvenile salinity in a 3-way interaction (4-way ANOVA, $F_{2,229} = 7.061$, $p = 0.0011$), but not with juvenile salinity alone (4-way ANOVA, $F_{2,229} = 1.420$, $p = 0.2437$).

For juveniles from Experiment 2, the salinity in which larvae were reared significantly affected juvenile percent inorganic content in only 1 of 6 treatments (25 °C Hatch A; 2-way ANOVA, $F_{1,43} = 40.73$, $p < 0.0001$). In this treatment, larval exposure to low salinity depressed juvenile inorganic content, but only by about 2.5%. The salinity in which juveniles were reared significantly affected juvenile inorganic content in 3 treatments (25 °C Hatch B, 20 °C Hatch B, 29 °C Hatch C; 2-way ANOVAs, $F_{1,32-41} > 12.72$, $p < 0.0012$), but the direction of the effect was not consistent (Fig. 7). The interaction of larval and juvenile salinities was significant for 2 treatments (20 °C Hatch A, 29 °C Hatch C; 2-way ANOVAs, $F_{1,32-33} > 5.641$, $p < 0.0237$). All differences in juvenile percent inorganic content from Experiments 1 and 2 were less than 6% (Figs. 7, 8, Table 2).

3.4. Experiment 4: feeding rates

A rapid change in salinity in either direction resulted in a trend of substantially decreased juvenile feeding rates followed by gradual recovery over the next several hours in all 3 experiments.

![Fig. 6. The effects of temperature and salinity on larval C. fornicata inorganic content. Larvae were distributed among 4 temperature and salinity treatments 2 days after hatching and sacrificed when they reached metamorphic competence. Larvae were combined into groups of 5–9 per sample in order to obtain accurate weight measurements. Inorganic mass was determined by combusting samples at 500 °C for at least 12 h; percent inorganic content was then calculated by dividing inorganic mass by total dry mass. Each point represents the mean value of 10–11 samples and error bars indicate standard error. Different letters above points indicate significantly different means (Tukey test; $p < 0.05$).](image)

![Fig. 7. The interactive effects of temperature and salinity on juvenile C. fornicata inorganic content. Larvae had all been reared in the lab at room temperature (~23 °C) and full strength seawater. Juveniles were distributed into treatments 1 day after metamorphosis. Inorganic mass was determined by combusting snails at 500 °C for at least 12 h; percent inorganic content was then calculated by dividing inorganic mass by total dry mass. All juveniles were approximately the same length (~6–6.5 mm) when sacrificed for these measurements. Error bars indicate standard error and sample sizes are indicated below each point. Different letters above points indicate significantly different means (Tukey test; $p < 0.05$).](image)
3.4.1. Experiment 4a
Juveniles reared at normal salinity (30) fed fastest in the initial hour of feeding in that salinity, but feeding rate dropped dramatically (by 86%) after animals were immersed in low salinity seawater. Feeding rate gradually recovered over the 3 h of feeding at low salinity to 75% of the initial rate at normal salinity. There were significant differences between feeding rates at each of the four 1-hour feeding periods (repeated measures ANOVA followed by Tukey test, $q_{27}^2 = 4.684, p < 0.05$; Fig. 9).

![Graph showing feeding rates](image)

3.4.2. Experiments 4b and 4c
Juveniles reared at low salinity in Experiment 4b fed fastest in the initial hour of feeding in that salinity, but feeding rate dropped dramatically (by 68%) after the juveniles were transferred to normal salinity. The feeding rate at normal salinity gradually recovered over the 3 h of feeding at normal salinity until the 3rd hour when there was no longer a significant difference between feeding rates at normal and low salinities (repeated measures ANOVA followed by Tukey test, $q_{27} = 0.9970, p > 0.05$; Fig. 10a). When this part of the experiment was repeated (Experiment 4c), mean feeding rate dropped slightly following transfer of juveniles to normal salinity seawater, but then recovered quickly. There were no significant differences between feeding rates at any of the four 1-hour feeding periods in that second experiment (repeated measures ANOVA, $F_{3,8} = 1.010, p = 0.4056$; Fig. 10b).

For the initial 1 hour feeding period in their rearing salinity, juveniles reared at low salinity (Experiments 4b and 4c) fed at 70% the rate of those that had been reared at normal salinity (Experiment 4a). Yet, juveniles reared at low salinity were 85% the size of those reared at normal salinity at the time of the feeding rate experiments and the size differences between juveniles in Experiment 4a and those in Experiments 4b and 4c were significant (2 t-tests, $t_{17.18} > 3.032, p < 0.0075$).
Fig. 9. Juvenile *C. fornicata* feeding rate in response to a rapid drop in salinity. Juveniles were reared at 25 °C and a salinity of 30 from larvae reared at room temperature (~23 °C) and a salinity of 30. Points represent mean plus standard error of 10 juveniles with shell lengths of 6235.1 ± 396.2 μm (mean ± standard deviation). Juveniles were fed the alga Isochrysis galbana. Juveniles were first pre-fed at a salinity of 30, then feeding rates were measured for 1 h at the same salinity followed by 3 h at a salinity of 20 (sampled every hour). Water was changed whenever food concentrations dropped below 50% of the initial concentration. Different letters above points indicate significantly different means (Tukey test; p < 0.05). The absence of letters indicates no significant differences (p > 0.05).

Fig. 10. Juvenile *C. fornicata* feeding rate in response to a rapid increase in salinity. Juveniles were reared at 25 °C and a salinity of 20 from larvae reared at room temperature (~23 °C) and a salinity of 30. Two replicated experiments were conducted 4 days apart. Points represent mean plus standard error of 10 juveniles with shell lengths of A) 5124.3 ± 618.9 μm and B) 5519.4 ± 619.7 μm (mean ± standard deviation). Juveniles were first pre-fed at a salinity of 20, then feeding rates were measured for 1 h at the same salinity followed by 3 h at a salinity of 30 (sampled every hour). Water was changed whenever food concentrations dropped below 50% of the initial concentration. Different letters above points indicate significantly different means (Tukey test; p < 0.05). The absence of letters indicates no significant differences (p > 0.05).

4. Discussion

These experiments demonstrated that rearing larvae at low salinity can influence subsequent juvenile growth in the gastropod *C. fornicata*. The magnitude and direction of this influence depended strongly on the rearing temperature and parentage. In addition, juvenile growth rates were elevated by increased temperature and depressed by low salinity, but the effect was synergistic, such that salinity had a greater effect on growth at higher temperatures.

4.1. Temperature

Both larvae and juveniles generally grew more quickly at the higher temperatures, agreeing with many other studies on the growth of larval and juvenile molluscs (*e.g.*, *C. fornicata* larvae: Klinzing and Pechenik, 2000; Lucas and Costlow, 1979; Pechenik, 1984; Pechenik and Heyman, 1987; Pechenik and Lima, 1984; juvenile clams: Laing et al., 1987). In contrast, Zhao (2002) found no significant effect of temperature on juvenile growth rates in the related species *Crepidula onyx*. The only exception is that larvae in our study grew a little more slowly at 25 °C than at 29 °C, which could indicate that optimal larval growth rates occur close to 25 °C.

4.2. Salinity

Larvae grew more slowly in low salinity seawater in 2 out of 3 larval hatches, and at all 3 temperature levels tested: 20, 25, and 29 °C. In Hatch B, however, there was no significant effect of salinity on larval growth rates. The fact that our larvae grew at a salinity of 20 is in contrast to a study by Diederich et al. (2011), which found that larvae of *C. fornicata* and 2 closely related species (*Crepipatella fecunda* and *C. onyx*) showed no detectable growth at salinities of 10, 15, and 20. While larvae were only exposed to low salinity for 12–48 h in their experiments, the larvae in our experiments grew detectably even in the first 2 days of low salinity exposure (see Fig. 4, Hatch B). In agreement with our results, Zimmerman and Pechenik (1991) found that larvae of the related species *Crepidula plana* grew at significantly slower rates at a salinity of 20 than at a salinity of 30, but grew nonetheless. Studies on other molluscan larvae have also found reduced growth at low salinity (Kingston, 1974; Richmond and Woodin, 1996; Scheltema, 1965).

While previous studies have observed constant growth rates in *C. fornicata* larvae (Klinzing and Pechenik, 2000; Pechenik, 1980, 1984; Pechenik and Lima, 1984), this study found that this is not always the case. Larvae in a number of treatments grew at different rates over time, especially at the low salinity. Since this is the first study to look at the effects of constant exposure to low salinity on the growth of *C. fornicata* larvae and most larvae growing at varying rates were reared at low salinity, it seems likely that the low salinity exposure was responsible for varying growth rates.

Juveniles also grew more slowly at low salinity in all experiments. Similarly, Diederich et al. (2011) found that juvenile *C. fornicata* growth was slowed in the 3 days following a short exposure to a salinity of 15. Low salinity has been shown to decrease juvenile growth in other species as well (*e.g.*, the shrimp *Farfantepenus brasiliensis*; Brito et al., 2000; the crab *Callinectes sapidus*; Cadman and Weinstein, 1988; the shrimp *Panaeus vannamenei*: Ponce-Palafox et al., 1997; the scallop *Argopecten irradians*: Shriver et al., 2002).

The effects of salinity on larval and juvenile growth could be due to changes in rates of feeding, assimilation, and/or respiration. *C. fornicata* larvae at a salinity of 20 fed at 50% of the rate of control larvae in a previous study (Diederich et al., 2011); in our study juvenile feeding rates dropped dramatically (by up to 86%) following transfer to a new salinity but gradually recovered substantially over the next 3 h. For juveniles reared at low salinity, feeding rates at normal salinity gradually recovered to the same level as their initial feeding rates at their low rearing salinity of 20. On the other
hand, for juveniles reared at normal salinity, feeding rates after 3 h at low salinity only reached a maximum of 75% the initial feeding rate at their control rearing salinity of 30. This likely indicates that juveniles maintained since metamorphosis at the low salinity acclimated and as a consequence were more responsive to salinity change than those kept at normal salinity throughout development. Such acclimation to low salinity could incur a metabolic cost that contributed to decreased rates of growth. For example, low salinity caused standard metabolic rate (measured as resting oxygen consumption) to increase in juveniles of the clam Mercenaria mercenaria (Dickinson et al., 2013). A combination of decreased feeding rate with increased energy demand would very likely lead to the decrease in growth rates at low salinity observed in all juvenile and most of our larval growth experiments.

4.3. Inorganic content

Percent inorganic content can serve as a proxy for percent shell weight in these experiments since all samples were rinsed with deionized water before processing, so the vast majority of inorganic matter would be from the shell.

While percent inorganic content of juveniles was significantly affected by temperature and, in 3 of the 7 treatments, by the salinity at which juveniles were reared, the directions of the effects were not consistent and the biological significance is not clear. Differences were in the range of only 3–4%, but comparisons with other species are difficult to make, as few other studies have looked at the effects of environmental factors on relative rates of shell and tissue growth or attempted to correlate organismal inorganic content to function. Two studies by Dickinson et al. (2012, 2013) found no significant effect of low salinity exposure on juvenile shell hardness and fracture resistance for the clam M. mercenaria and the oyster Crassostrea virginica. Therefore, the differences observed in our study may be more relevant from an energy budget and energy partitioning perspective than in terms of the protection afforded by the shell. Or, since the differences observed in our study were so small, these results could indicate a strict regulation of relative rates of shell and tissue growth that results in a relatively constant ratio of inorganic to organic content in juveniles.

Larval percent inorganic content was strongly influenced by rearing salinity, but not by rearing temperature. Percent inorganic content was reduced by 27% for larvae reared at low salinity, but only at the lowest temperature tested (20 °C). This likely reflects a synergetic reaction between slowed calcification at lower temperature and salinity, which has been observed before in the mussel Mytilus edulis (Malone and Dodd, 1967). Such a large difference in inorganic content is likely to have biological significance by making larvae more vulnerable to predation. In addition, it could explain the abnormal development observed in larvae from Hatch A reared at 20 °C and low salinity. If tissue growth was outpacing shell growth in these larvae, the oversized tissue could have exerted too much pressure on the relatively weak shell, forcing it to “pop” upwards into a bowler hat shape. Kingdom (1974) also observed deformed development in calcifying clam larvae at a salinity of 20, but the deformation was not described nor was any explanation proposed. In addition, Byrne et al. (2011) found that the combination of warmer temperatures and lower pH increased the incidence of deformed, unshelled abalone veligers by suppressing calcification, similar to the effect of low salinity.

While a number of studies have investigated the effects of reduced salinity on adult mollusc growth in terms of increases in shell mass (Almada-Villela, 1984; Brown and Hartwick, 1988; Malone and Dodd, 1967; Nagarajan et al., 2006), the effects on increases in larval or juvenile shell mass have been little studied. Since larval and juvenile shells are much smaller and more vulnerable, it is during these stages that organisms are adding functional protection at the greatest rate. Future studies could investigate the effects of climate change on vulnerability to predation in early life stages.

The differences in percent inorganic content in larvae observed in this study could be indicators of energy partitioning in response to unfavorable conditions. If these snails were facing reduced energy supply and increased energy demand at lower salinity (as observed in juvenile clams by Dickinson et al., 2013), they may have devoted more of their resources to growing tissue in order to increase the size of the feeding apparatus and thereby increase rates of energy uptake. In fact, food-limited C. Fornicata larvae have been shown to increase the size of their velar lobes (used for both feeding and swimming) relative to shell length (Klinzing and Pechenik, 2000). The large difference in larval percent inorganic content at 20 °C between larvae reared at low and normal salinity could be a dramatic example of this, since it has been shown that feeding rates of C. Fornicata larvae are significantly reduced at low salinity (Diederich et al., 2011). In addition, the smaller differences noted in percent inorganic content of juveniles could also be indications of this type of compensation for an energy deficit.

4.4. Latent effects of larval exposure to low salinity

It is becoming increasingly clear that transitions to new life stages are not necessarily a new beginning; each successive life stage can be strongly influenced by events and influences that acted on previous stages (Padilla and Miner, 2006; Pechenik, 2006). The results of our experiments provide further support for this idea.

Juvenile shell growth was significantly affected when larvae were reared at low salinity in 4 out of 6 of our treatments, when larvae and juveniles were reared at either 20 °C or 25 °C. Therefore, low salinity stress experienced by larvae caused latent effects on juvenile growth rates of C. Fornicata, in contrast to the results of a previous study (Diederich et al., 2011) that found no latent effects from low salinity stress experienced in the larval stage of the same species. Although Diederich et al. (2011) only reared juveniles in stress-free, normal salinity conditions while juveniles in this study were reared in both normal and low salinity, the present results demonstrate latent effects for juveniles reared at both salinities so this would not account for the difference in results. Diederich et al. (2011) exposed larvae to low salinity for only 12–48 h while in our study larvae were reared in the low salinity from 1–4 days after hatching until metamorphosis. In addition, Diederich et al. (2011) measured juvenile growth rates over only the 4 days following metamorphosis while in this study growth rates were measured from day 1–2 after metamorphosis until day 16 after metamorphosis. It is possible that either latent effects do not manifest themselves in this situation unless larvae are exposed to low salinity for more than 48 h or unless juveniles are measured for more than 4 days after metamorphosis. As this study detected latent effects within the first 6 days of juvenile growth (unpublished data), it seems likely that larvae must experience some benchmark level of salinity stress (e.g. more than 48 h of exposure to a salinity of 20) before latent effects will become apparent in juvenile growths. This is further supported by the fact that Diederich et al. (2011) did find latent effects for C. Fornicata in one experiment, but only when larvae were exposed to the very low salinity of 10 for 2 days.

Similarly, latent effects resulting from exposing larvae to low salinity have been documented in the polychaete C. teleta (Pechenik et al., 2001) and the barnacle B. amphitrite (Thiyagarajan et al., 2007). Adding the results of our study to these previously documented cases of salinity-induced latent effects lends weight to the necessity of considering the environmental conditions larvae are exposed to when attempting to predict the responses of juvenile or adult populations. Juveniles in stress-free conditions would be expected by conventional methods to exhibit normal growth and mortality rates. Yet if they were exposed as larvae to low salinity during a substantial portion of their dispersal phase, these juveniles could grow much more slowly than expected, which would likely increase mortality by predation or abiotic stresses (Paine, 1978; Vermeij, 1972), thereby limiting the supply of adults in the future.
The mechanisms behind latent effects are not well understood. Since the stress was experienced in a previous life stage, it is less likely to have the same sort of direct effects that stresses experienced during the juvenile stage have on juvenile growth. Studies have shown that latent effects can sometimes manifest themselves as damages to juvenile feeding structures (Marshall et al., 2003; Wendt, 1998). Other possibilities could be reduced energy reserves, effects on transcriptional or translational processes, altered patterns of DNA methylation, and/or damaged DNA or functionally important enzymes (Pechenik, 2006).

4.5. Interactions between environmental factors

Stresses experienced by organisms due to changing environmental conditions will not be felt in isolation. This study revealed a number of significant interactive effects of salinity and temperature on larval and juvenile growth and inorganic content.

In 2 of the 3 larval hatches used in our study (Hatches B and C), the effect of salinity on larval growth varied with temperature. In Hatch B low salinity (20 °C) depressed larval growth at the lower temperature (20 °C) but increased larval growth at the higher temperature (25 °C), while in Hatch C low salinity depressed larval growth more at the higher temperature (29 vs. 25 °C). As for juveniles, low salinity had a greater effect on growth at higher temperatures. Moreover, temperature significantly modulated the effects that larval exposure to low salinity had on juvenile growth. The salinity that larvae were reared affected juvenile growth rates at 20 °C in both treatments and in 2 out of the 3 treatments at 25 °C. In contrast, larval rearing salinity had no significant effect (p > 0.10) on juvenile growth rates at 29 °C. Thus, larval exposure to low salinity may have a greater effect on juvenile growth at lower temperatures. This is in contrast to the interaction of temperature and juvenile salinity, where exposure to low salinity had a greater effect on juvenile growth at lower temperatures. In fact, larval salinity had a significant interaction with temperature in the overall analysis, indicating that temperature does modify the effect of larval salinity on juvenile growth.

Finally, the impact of salinity on larval percent inorganic content was strongly temperature dependent. At the lower temperature (20 °C), larvae reared at lower salinity had a much lower inorganic content than those reared at normal salinity, while at the higher temperature (25 °C) there was little difference in inorganic content between larvae reared at the different salinities. Therefore, these experiments demonstrate the importance of temperature not only in its direct effects on growth rates, but also in the way it modulates the effects of other variables such as salinity, agreeing with many previous studies (juveniles of the snail Littorina rudis: Berry and Hunt, 1980; juveniles of the crabs Menippe adina and M. mercenaria: Brown and Bert, 1993; larvae of the abalone Haliotis coccoideum: Byrne et al., 2011; larval clams and oysters: reviewed by Calabrese and Davis, 1970; juveniles of the sea star Patriirilla pseudoexigu: Chen and Chen, 1993; the oyster C. virginica: Heilmayer et al., 2008; the oyster Ostrea edulis: Hutchinson and Hawkins, 1992; juveniles of the shrimp P. vannamei: Ponce-Palafoux et al., 1997; embryos of the snail Siphonaria denticulata: Przeslawski et al., 2005; embryos and larvae of the mussel Mytilopsis leucophaeata: Verween et al., 2007).

4.6. Hatch variability

Our experiments demonstrated a wide variability in responses to salinity and temperature and in overall growth rates among larval hatches. For example, larvae reared at 20 °C and low salinity from Hatch A developed abnormally into a bowler hat shape, while larvae from Hatch B developed normally under the same conditions and grew 44% faster than those from Hatch A (Fig. 4B). In addition, juveniles reared at 25 °C and low salinity actually grew 40% faster after prior exposure to low salinity in one hatch (Hatch C; Fig. 5) but that effect was not observed for juveniles in the other two hatches.

Statistical analyses revealed significant interactions between larval hatch and larval salinity exposure in all dependent variables analyzed (larval growth rates, juvenile growth rates, and juvenile inorganic content). Also, larval hatch significantly interacted with temperature to affect juvenile growth rates, and the interaction of hatch and temperature was marginally significant for juvenile inorganic content.

Previous studies have also documented substantial genetic or heritable differences in the responses of larvae from different hatches in C. fornicata (Diederich et al., 2011; Hilbish et al., 1999). The effect of this hatch variability in our experiments seems to indicate that larvae from different parents will be differentially affected by temperature and salinity conditions. The larvae produced by some parents may express latent effects from low salinity exposure during larval development while the larvae produced by other parents will be unaffected (e.g. compare the results for 25 °C Hatch A with those from Hatches B and C at the same temperature; Fig. 5) and larvae from some parents may experience decreased growth rates at low salinity while those from other parents will not (larval Hatch B vs. Hatches A and C; Fig. 4). Hence, natural selection may be able to select for the more hardy individuals as environmental conditions continue to change. In addition, this variability could help to explain the great success of C. fornicata as an invasive species (Blanchard, 1997), since such a large range of temperature and salinity tolerances within the population could make it easier for the species to invade habitats with a wide range of abiotic conditions. Hatch variability in temperature and salinity sensitivity also lends more weight to the importance of factors that influenced larval or juvenile growth rates in every single hatch, such as the effect of the salinity in which juveniles were reared on juvenile growth. In all cases, there was no significant interaction between larval hatch and juvenile salinity (p > 0.4). Juvenile salinity exerted a large influence for which the amount of genetic variation considered here could not compensate.

4.7. Implications and conclusion

These experiments demonstrate the complexity of temperature–salinity effects on the growth of C. fornicata larvae and juveniles. Temperature, salinity, and parentage were related with a number of interactive effects. The most confident conclusions of this study are that C. fornicata larvae and juveniles grew faster at higher temperatures (although larval growth rates began decreasing at 29 °C) and C. fornicata juveniles grew more slowly at the low salinity of 20 than under control conditions (30). In addition, while prolonged larval exposure to low salinity influenced rates of both larval and subsequent juvenile growth, the effect varied with temperature and parentage. Being raised at low salinity decreased the percent inorganic content of larvae at competence, but only at the lowest temperature tested (20 °C).

In the absence of salinity stress, then, C. fornicata would be expected to grow faster as oceanic temperatures continue to increase. Both larvae and juveniles should grow faster, and so would therefore be more likely to escape size-selective predation or mortality from abiotic factors (Morgan, 1995; Paine, 1976; Pechenik, 1999; Rumrill, 1990; Vermeij, 1972), and thereby increase the supply of new adults to the population.

In regions with frequent exposure to low salinity, or where salinity is decreasing due to the altered precipitation patterns or glacial melting caused by global warming (IPCC, 2013), C. fornicata may not fare as well. Low salinity (20) reduced larval growth rates by 20–50% in 2 out of 3 larval hatches in our study, reduced the percent inorganic content of competent larvae (~900 μm) by 27% at 20 °C, and reduced juvenile growth rates in all experiments. In addition, juvenile growth rates were affected by larval exposure to low salinity in 4 out of 6 treatments with the largest effect (seen for juveniles reared at 20 °C and low salinity) causing a 4-fold reduction in growth rates. Therefore, low salinity exposure will likely have an effect that is opposite to that of increasing water temperatures. Larvae and juveniles exposed to low salinity will spend more time at smaller sizes and possibly face increased size-
selective mortality as a result. Furthermore, larvae may be less likely to reach suitable habitats if they spend abnormally long in the plankton, since currents could carry them far from suitable sites before they become competent to metamorphose. This could severely limit the size of adult populations. Alternatively, increased time in the plankton could increase the dispersal potential of C. fornicata and aid its invasion of new habitats. Also, our feeding rate experiments seem to indicate that prolonged exposure to low salinity as juveniles can result in acclimation and greater adaptability to future salinity changes. In addition, larvae and juveniles survived and grew at low salinity in combination with all temperatures tested, demonstrating the ability of C. fornicata early life stages to tolerate the fluctuating salinity conditions common to estuaries and many other near-shore environments. In fact, C. fornicata populations have been recorded in estuaries (e.g. Chipperfield, 1951).

Given the conflicting effects of increasing temperature and decreasing salinity on the early life stages of C. fornicata, it is difficult to predict how this species will be impacted by changing conditions in the future. This study emphasizes the importance of including larval experience in models attempting to predict the effects of climate change on species distributions. As shown in this study, larval exposure to low salinity can significantly affect juvenile growth rates and these effects can persist for at least 16 days. Effects of exposing oyster larvae to ocean acidification on juvenile growth rates can persist for months in both lab and field (Hettinger et al., 2012, 2013). Latent effects have been shown in a variety of animals from a variety of taxonomic groups (reviewed by Pechenik, 2006; Pechenik et al., 1998). But they may be even more common than previously thought. In cases where latent effects have not been observed in laboratory studies (e.g., Diederich et al., 2011; Thiagarajan and Qian, 2003) the present study suggests that they might appear after metamorphosis under different conditions (e.g. different temperatures and parentage).

Other molluscs (including commercially important species) may also face latent effects from exposure to low salinity in the larval stage. More research is needed in order to determine how widespread these effects are. This study highlights the complexity of environmental influences on the larval and juvenile stages of C. fornicata. These early life stages determine the eventual distribution and abundance of adults, so that any effects of climate change on their growth or survival will determine the future success, spread, or decline of this species.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2015.05.004.

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References


Dickinson, G.H., Ivanina, A.V., Matoo, O.B., Pörtner, H.O., Lannig, G., Bock, C., Beniash, E., Sokolova, I.M., 2012. Laboratory-reared juvenile blue crabs (Callinectes sapidus) are more common than previously thought. In cases where latent effects have not been observed in laboratory studies (e.g., Diederich et al., 2011; Thiagarajan and Qian, 2003) the present study suggests that they might appear after metamorphosis under different conditions (e.g. different temperatures and parentage).

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References


Zhao, B., 2002. Larval Biology and Ecology of a Non-Indigenous Species, the Slipper Limpet Crepidula onyx (Ph.D.). The Hong Kong University of Science and Technology, Hong Kong.