Low salinity stress experienced by larvae does not affect post-metamorphic growth or survival in three calyptraeid gastropods

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A B S T R A C T

Marine larvae that experience some sub-lethal stresses can show effects from those stresses after metamorphosis, even when they seem to recover from those stresses before metamorphosis. In this study we investigated the short and long-term effects of exposing the larvae of three calyptraeid gastropods (Crepidula fornicata, Crepidula onyx, and Crepipatella fecunda) to temporary reductions in salinity. Larval of all three species showed slower larval growth rates, longer time to metamorphic competence, and substantial mortality after being stressed in seawater at salinities of 10, 15, and 20 for less than 48 h. Larval tolerance to low salinities varied widely within and among species, but longer stresses at lower salinities were generally more harmful to larvae. However, larvae in nearly all experiments that were able to metamorphose survived and grew normally as juveniles; there were no documented “latent effects.” For all three species, starving larvae in full-strength seawater was not as harmful as exposing larvae to low salinity stress, indicating that detrimental effects on larvae were caused by the salinity stress per se, rather than by an indirect effect of salinity stress on feeding. C. fornicata that were stressed with low salinity as juveniles were more tolerant of the stress than larvae: all stressed juveniles lived and showed reduced growth rates for no more than 3 days. Our data suggest that even though reduced salinity is clearly stressful to the larvae of these 3 gastropod species, metamorphosis seems to generally provide individuals with a fresh start.

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1. Introduction

Larval experience can cause latent effects for juveniles and adults (reviewed by Pechenik, 2006). For example, Phillips (2002) temporarily stressed larvae of the marine bivalve Mytilus galloprovincialis with decreased food concentrations and monitored the performance of juveniles both in the lab and after transplanting some to the field. In both cases, juveniles that had been stressed as larvae showed significantly slower growth for at least the next 14 days compared to control individuals, even under conditions of abundant food. Thus larval experience can dramatically affect juvenile performance long after individuals have returned to benign conditions. Most studies to date have involved depriving larvae of food, exposing larvae to pollution, or delaying metamorphosis and documenting the effects on juvenile growth and survival (e.g., Cebrian and Uriz, 2007; Jacobs et al., 2008; reviewed by Pechenik, 2006). However, larvae may also experience rapid and substantial fluctuations in salinity, particularly in intertidal, estuarine, and other shallow water environments (Richmond and Woodin, 1996). For example, offshore surface waters may drop from salinity of over 30 to 15 during heavy rains (Allen and Pechenik, 2010), estuarine waters may fluctuate from salinity of 35 to 10 in under 12 h (Chaparro et al., 2008), and tide pools may reach near fresh water conditions after heavy rains (Pechenik, 1982). The gastropod family Calyptraeidae contains more than 90 species, many of which live in estuaries or intertidal environments that may be periodically exposed to temporary reductions in salinity (Collin, 2003). The pelagic larvae of such species are likely to experience low salinity exposures intermittently during development. Previous studies have demonstrated latent effects of nutritional stress on juvenile growth rate for some members of the family, including Crepidula fornicata and Crepidula onyx (Pechenik et al., 2002; Chiu et al., 2007, 2008), and some data have been published on the salinity tolerance of C. fornicata juveniles (Pechenik and Oyster, 1989) and Crepidula plana larvae (Zimmerman and Pechenik, 1991). However, the salinity tolerance of most calyptraeid larvae and the consequences of short-term exposure to sub-lethal salinities on subsequent larval development and juvenile performance have not been reported.
In this study we examined the effects of temporary salinity stress during early larval development for three calyptraeid gastropods: C. fornicata from New England, C. onyx in Hong Kong (native to west coast of the United States), and Crepipatella fecunda (formerly Crepidula fecunda, see Collin, 2003) from Chile. These three species live in environments that are likely to experience substantial fluctuations in salinity. At our collection sites, all 3 species can be found in the intertidal zone. Depending on tidal cycles, specific location of animals (e.g., tide pool), and weather patterns (e.g., heavy rains at low tide), individuals may be exposed to rapid drops in salinity over short periods of time (e.g., Pechenik, 1982; Chaparro et al., 2008). During the monsoon season in Hong Kong and the wet season in Chile, periods of time (e.g., Pechenik, 1988; Pechenik et al., 2002) before use.

The larvae used in each experiment were released by one female, but probably had multiple fathers (e.g., Dupont et al., 2006; Le Cam et al., 2009). In all experiments, when larvae were to be exposed to low salinity they were first transferred to a bath of seawater at that low salinity, and then pipetted from there into another set of dishes or 6-well (10 ml) microplates for the actual exposures, thus maintaining the desired final salinity in all treatment dishes. In all studies, salinity was reduced by adding appropriate amounts of deionized water to 0.45 µm-filtered seawater.

All phytoplankton cell concentrations were determined using Hauser Ultraplane hemacytometers, after the cells in 1 ml samples were killed with 0.05 ml of Lugol’s iodine or a Coulter counter with aperture tube diameter of 100 µm.

2. Methods

2.1. Collecting and maintaining adults and larvae

Adults were collected at low tide from Wickford, Rhode Island in 1999, 2000, and 2009 and from Westbrook, Connecticut in 1999 and 2000 (C. fornicata); Victoria Harbor, Hong Kong in 2009 (C. onyx); and Puerto Montt, Chile in 2009 and 2010 (C. fecunda). C. fornicata and C. fecunda were maintained at room temperature (~23 °C) and at a constant temperature of 18 °C, respectively, in the laboratory in glass aquaria of aerated seawater (full-strength salinity, approximately 30). Those temperatures correspond to typical ambient sea temperatures at the time of collection. C. onyx were maintained at ambient seawater temperatures in a flow-through sea table. Adults of all 3 species were fed phytoplankton once or twice each day, mostly Isochrysis galbana (clone T-ISO) and Dunaliella tertiolecta (clone DUN), and water was changed every other day until larvae were released. Upon their release, larvae were collected on 150 µm mesh filters, rinsed with seawater, and transferred to one-gallon glass containers of filtered seawater (0.45 or 0.22 µm). Larvae were used in some experiments within 12 h of hatching, without any feeding (see below), or for other experiments were fed for up to 5 days on T-ISO at approximately 18 × 10⁴ cells ml⁻¹ (e.g., Pechenik and Lima, 1984; Pechenik et al., 2002) before use.

2.2. Effect of reduced salinities on larval feeding rates for C. fornicata

Experiments with larvae of C. fornicata were conducted at room temperature (~23 °C) in dim light (to reduce the likelihood of phytoplankton fission during experiments) to determine whether larvae continued to feed at reduced salinities. Larvae were pipetted into a bath at the lowered salinity for 10 min prior to being tested. Larvae were then pipetted into test tubes containing a final volume of 5 ml seawater at salinities of 30, 20, 15 or 10, with initial phytoplankton concentrations (T-ISO) of 18 × 10⁴ cells ml⁻¹ (Eyster and Pechenik, 1988; Pechenik and Eyster, 1989). Each tube contained 5 ml T-ISO suspension and 20 larvae, with 3 replicates per treatment. Tubes containing phytoplankton suspension but no larvae served as controls. One milliliter samples of T-ISO suspension were taken from each tube after 3 h, to determine feeding rates (Pechenik, 1980). To determine if larvae in low salinity treatments fed initially and then stopped feeding or if they fed continually at slower rates throughout the experiment, the experiment was later repeated with extra replicates from which 1 ml samples of T-ISO suspension were taken after 1 h and 6 h of elapsed feeding time.

2.3. Larval stress experiments

2.3.1. Stress conditions and larval growth rates

Pilot studies revealed differences in salinity tolerance among species and sometimes within a species, so we often needed to use different levels of stress in different experiments. Our original goal was to determine the magnitude of latent effects, not to document variation in salinity tolerance. Table 1 summarizes the experiments conducted for each species and gives the corresponding figure number for the results. For most experiments, larvae were pipetted

<table>
<thead>
<tr>
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<th>Day after</th>
<th>Stress</th>
<th>% of</th>
<th>Larval</th>
<th>Mortality</th>
<th>Metamorphic</th>
<th>Larval</th>
<th>Juvenile</th>
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<td></td>
<td></td>
<td>(hrs)</td>
<td>(hrs)</td>
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<td>stressed</td>
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<td>assessed</td>
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<td>3.5 or 7.1</td>
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<td>No</td>
<td>Yes (Fig. 3B)</td>
<td>No</td>
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</tr>
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</table>

* All larvae in the experimental treatment died.

b All larvae died before metamorphosis, so juvenile growth rates could not be measured.
into 80–100 ml phytoplankton suspensions in glass dishes (10–20 larvae per dish; see Results for details); for the experiment whose results are shown in Fig. 6, however, larvae were pipetted into 10 ml phytoplankton suspensions in microplates (1 larva per well). Larvae of particular ages were stressed in salinities ranging from 10 to 20 for 12–48 h with or without food, as detailed in Results. Control larvae were kept in seawater at a salinity of 30. All larvae were fed T-ISO at concentrations of approximately 18 × 10^4 cells ml^−1 and cell concentrations never fell below 13 × 10^4 cells ml^−1, except in starvation experiments.

Since it has been shown that larval nutritional stress can cause latent effects on juvenile growth rates even when larval growth rates return to normal before metamorphosis (Pechenik et al., 1996a, 2002), we starved larvae in some experiments to distinguish between the effects of salinity stress and possible indirect effects of starvation stress induced by low salinity. Salinities were determined to the nearest practical salinity unit (psu) with a salinometer or handheld refractometer. After the stress period (12–48 h), larvae were returned to filtered seawater at a salinity of 30 with phytoplankton, with seawater and food replaced every other day for the duration of the study. Larvae were monitored for survival at intervals for the duration of the experiments (see Results). Using methods found in Untersee and Pechenik (2007), larvae that lacked a heartbeat (the heart is visible through the shell), swimming activity, and muscular activity were scored as dead. Larvae were periodically (see Results) either measured non-destructively (Pechenik et al., 1996a) at 40× or 63× using a calibrated ocular micrometer or photographed and examined using standard image-analysis software until metamorphosis. These measurements allowed us to determine initial larval growth rates after stress and, when the growth rates of stressed larvae were initially lower than those of the controls, the extent to which larval growth rates recovered over the next 2–6 days.

2.3.2. Time to metamorphic competence

In several experiments, we determined whether salinity stress prolongs the time to metamorphic competence (i.e., slows developmental rate). When larvae in control treatments reached an average shell length of approximately 900 μm or when at least 80% of larvae in control treatments formed posterior shell brims— a crude proxy for metamorphic competence (Pechenik, 1986; Pechenik and Heyman, 1987)—all larvae were exposed to 20 mM excess KCl in seawater for 6 h to quantify the number of individuals competent to metamorphose (Pechenik and Heyman, 1987; Pechenik and Gee, 1993). Inducing metamorphosis through exposure to excess KCl does not affect juvenile growth rates (Eyster and Pechenik, 1988). Average larval size at exposure to excess KCl was not standardized because growth and onset of metamorphic competence are uncoupled in C. fornicata (Pechenik et al., 1996a). Larvae that did not metamorphose were returned to seawater at a salinity of 30 with phytoplankton and tested again for metamorphic competence after 5 days. In all experiments larvae were stressed for 12–48 h within the first 5 days after hatching. In one experiment with C. fecunda, cumulative spontaneous metamorphosis was recorded for 24 days after the end of the stress. Remaining larvae were then transferred to dishes containing adult-conditioned seawater to induce metamorphosis.

2.3.3. Juvenile growth rates after larval stress

Juvenile growth rate measurements allowed us to determine if there were latent effects due to salinity stress or starvation stress experienced in the larval stage. After metamorphosis, juveniles were transferred to 50 ml phytoplankton suspensions in glass dishes and reared individually at T-ISO concentrations of approximately 18 × 10^4 cells ml^−1, with water changed daily; cell concentrations never fell below 13 × 10^4 cells ml^−1 between water changes in spot checks. Juveniles were measured immediately after metamorphosis and 3–5 days later to estimate growth rates. In one experiment, some juveniles of C. fornicata from each treatment were reared in the laboratory for 4 days and then transferred to the field to determine how fast juveniles could grow under natural conditions (e.g., Phillips, 2002). Individual juveniles were pipetted into 250 ml screw-cap Nalgene bottles that had two 7 cm × 5 cm openings covered with 500 μm mesh to allow for water exchange. Bottles with juveniles were haphazardly placed in the top two 20 cm tall compartments of a 5 compartment shellfish bag (40 cm × 40 cm × 100 cm). The bag was suspended from a floating dock at Outer Island in Branford, CT such that the juveniles were always in the upper 1 m of the surface water.

2.4. Juvenile stress experiments

The direct impact of low salinity stress on juveniles was examined for C. fornicata. In this set of experiments larvae were reared to metamorphosis in full-strength seawater; juveniles were later exposed to low salinity or to starvation stress. Several hundred juveniles were obtained from larvae that were reared in the lab and induced to metamorphose using 20 mM excess KCl as previously described; 70 of those juveniles were haphazardly chosen for the experiment. The juveniles were pooled from 3 different mothers that had released larvae within 1 day of each other. Following metamorphosis, the 70 juveniles were maintained in filtered seawater on a diet of T-ISO (about 18 × 10^4 cells ml^−1) for one day. They were then measured at 40× or 63× using a calibrated ocular micrometer and transferred to individual containers with 50 ml of phytoplankton suspension at approximately 18 × 10^4 cells ml^−1, one juvenile per container, 10 juveniles per treatment. Juveniles were stressed as follows: a salinity of 15 for 6, 12, 24, or 48 h with food; a salinity of 15 for 48 h without food; a salinity of 30 for 48 h without food. Control juveniles were maintained at full-strength seawater (salinity of 30) for 48 h, with food. After stress exposure, all animals were re-measured and returned to full-strength seawater and phytoplankton suspension for another 6 days. Juvenile shell lengths were determined 3 and 6 days after the stress ended to assess growth. During this period of growth, phytoplankton was replenished daily. Phytoplankton cell concentrations never fell below approximately 13 × 10^4 cells ml^−1 in spot checks.

2.5. Statistical analyses

Growth rates were calculated from changes in shell length for both larvae and juveniles. For experiments in which larvae were stressed, all treatments in each experiment were compared using one-way analysis of variance (ANOVA) with Bonferroni post-tests to compare individual treatments (GraphPad Prism Software Version 4.03). Data for percent metamorphosis and percent survival at single time points

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**Fig. 1.** Influence of decreased salinity on Crepidula fornicata larval feeding rate. Mean ± S.E.M. shown for all treatments; N=6 tubes, with 20 larvae per tube feeding for 3 h. Means that have the same letter are not significantly different (p>0.05, Bonferroni multiple comparisons test). Phytoplankton samples taken after 1 h and 6 h of feeding show that larvae continue to feed the entire time they are in the low salinity treatments.
were arcsine transformed before using one-way ANOVA with Bonferroni post-tests to compare individual treatments. Where appropriate (Figs. 6C, 7A, 11D) treatments were compared using two-way ANOVA with salinity and starvation as independent variables. Kaplan–Meier survival curves (Kaplan and Meier, 1958) were analyzed using a log-rank test to determine if the curves were significantly different. For experiments in which juveniles were stressed, because shell lengths were determined on different days after stress, individual t-tests were used to compare each treatment to the control treatment at a particular time.

3. Results

3.1. C. fornicata larval feeding rates at reduced salinities

Phytoplankton concentrations in control tubes containing no larvae did not change during the 3 h experiments; i.e., reduced salinities of 20, 15, or 10 did not cause measurable cell rupture or cell

Fig. 2. Influence of salinity or starvation stress on larval survival. Mean ± S.E.M. shown for all treatments. Day 0 is the day that larval hatched. Bar color indicates the salinity at which larvae were stressed (black—control, dark gray—20, light gray—15, and white—10). Within each experiment, means that have the same letter are not significantly different (p>0.05, Bonferroni multiple comparisons test). N=3 dishes for all treatments. (A) Crepidula fornicata larval survival on day 10; 12 larvae per dish. All larvae stressed in a salinity of 10 eventually died. (B) Crepidula onyx larval survival on day 11; 20 larvae per dish; treatment 'a' reflects a 12-hour stress 1 day after hatching. (C) Crepipatella fecunda larval survival on day 8; 20 larvae per dish; treatment 'b' indicates larvae that were starved during the stress period; treatment 'c' reflects a 12-hour stress 2 days after hatching.

Fig. 3. Influence of salinity and/or starvation stress on larval survival. Each point represents mean cumulative survival on a given day ± S.E.M. Symbols indicate the type of stress and the salinity at which larvae were stressed (black circle—control, light gray circle—15, black triangle—starvation only, light gray triangle—starvation at a salinity of 15, light gray square—15 for 12 h, and white square—15 for 24 h). N=3 dishes for all treatments; 20 larvae per dish at day 0. Within each graph curves are significantly different (p<0.0001, Kaplan–Meier survival curves with log-rank test) (A) Crepipatella fecunda stressed on day 2 for 48 h. (B) Crepipatella fecunda stressed on day 2 for 12 or 24 h. (C) Crepidula onyx stressed on day 0 for 48 h.
division. At these reduced salinities, larvae of *C. fornicata* continued to feed, albeit at substantially lower rates than in full-strength seawater (Fig. 1). Feeding rates at low salinities determined 1 h, 3 h, and 6 h after the start of the experiment were statistically equivalent (two-way ANOVA, \( p < 0.0001 \) for salinity, \( p = 0.11 \) for time, \( p = 0.55 \) for salinity \( \times \) time), indicating that stressed larvae fed at nearly constant rates for the duration of the experiment.

### 3.2. Larval and juvenile survival after salinity stress

Low salinity stress often increased larval mortality substantially, with the magnitude of the effect varying with both salinity and species (Figs. 2 and 3). Larval age when stress was applied also affected larval mortality at a salinity of 10 in one experiment (Fig. 2A), but all larvae in these treatments (salinity of 10) from this experiment eventually died. Of the 3 species tested, *C. fecunda* larvae were the least tolerant to decreased salinities: A 12-hour stress at a salinity of 15 killed nearly all *C. fecunda* larvae within the following 8 days (Fig. 3B); the same stress killed only 4% of *C. onyx* larvae by day 11 (Fig. 2B) and a 24 hour stress at a salinity of 15 did not kill any *C. fornicata* larvae by day 8 (Fig. 2A). Although larvae of *C. fecunda* also exhibited substantial mortality under control conditions in the laboratory, over 50% of control larvae were still alive after 12 days (Fig. 2B). Exposing larvae of *C. fecunda* to a salinity of 20 was less stressful than exposing them to a salinity of 10, but mortality was still significantly higher than that of control larvae (Fig. 2C).

All *C. fornicata* larvae stressed at a salinity of 10 for 24 h (Fig. 2A) or at 15 for 48 h (data not shown, \( n = 36 \)) eventually died within 21 days.
after the stress ended, though none died within the first 5 days after the stress; no control larvae died. Larvae of *C. onyx* seemed the most tolerant of low salinity stress in our experiments: they experienced substantial mortality after a 48 h exposure to a salinity of 15 (Fig. 3C), but not to the extent shown by *C. fecunda* (Fig. 3A) or *C. fornicata* (all *C. fornicata* larvae died, data not shown). Starving larvae at full-strength salinity, on the other hand, did not affect larval survival in any of the species tested (Figs. 2 and 3, data for *C. fornicata* not shown), suggesting that mortality was due to the salinity stress per se, rather than to an indirect effect of low salinity on ability to feed. No juveniles died in any treatment for those individuals that survived to metamorphose.

### 3.3. Larval growth rates after salinity stress

The larvae of all 3 species showed no detectable growth while experiencing salinity or starvation stress. After larvae were returned to full-strength seawater with food, the effect of the salinity stress on growth rates varied widely, even within species. Mean larval growth rates for *C. fornicata* were either unaffected (Fig. 4—salinities of 10 and 20, all treatments; Fig. 5A and C—salinity of 20; and Fig. 6B—salinity of 15 for 12 h), slowed for a number of days but then returned to control rates before metamorphosis (Fig. 5B—salinity of 20, Fig. 6A—salinity of 15, Fig. 6B—salinity of 15 for 24 h), or slowed dramatically and then never returned to control growth rates (Fig. 5—salinity of 10, all treatments; Fig. 6A—salinity of 10; Fig. 6C—salinity of 15).

The effect of reduced salinity on subsequent larval growth rate depended on the strength and duration of the stress, with exposure to lower salinities for longer periods generally slowing growth rates more dramatically (e.g., see Fig. 5A—salinity of 20 vs. 10 and Fig. 6B and C—salinity of 15 for 24 h vs. 15 for 48 h). Interestingly, the effect that the stress had on larval growth rate for *C. fornicata* also varied among experiments, probably reflecting genetic differences among parents.

Mean larval growth rates also varied with duration of the low salinity stress for *C. onyx* (Fig. 7).

*C. fecunda* larvae stressed at a salinity of 20 grew normally after the stress was removed (Fig. 8B), but larvae stressed at a salinity of 15 grew significantly more slowly than control larvae for 2 days after the end of the stress; moreover, the larvae were all dead within 5 days after the stress ended (Fig. 8A).

Starving larvae did not slow larval growth rate like salinity stress did for *C. fornicata* (Fig. 6C, Table 2), *C. onyx* (Fig. 7A, Table 2), or *C. fecunda* (Fig. 8A, B; *C. fecunda* data were not analyzed by two-way ANOVA because they did not meet the requirements of that statistical test).

The amount of time that *C. fornicata* larvae were allowed to grow under control conditions before being subjected to salinity stress (i.e., the date that larvae were stressed after hatching) had little effect on growth rate following stress (Figs. 4–6). For larvae of all 3 species, exposure to lower salinity and longer exposure duration depressed larval growth rates to a greater extent, but the magnitude of effects varied with species and among experiments within a species.

### 3.4. Juvenile growth after stress to larvae

In nearly all experiments, larvae that were able to metamorphose had juvenile growth rates statistically equivalent to those of controls, even following the most severe salinity stress, regardless of whether or not larval growth rates recovered to control levels before metamorphosis (Figs. 9 and 10). Only when larvae of *C. fornicata* were stressed at a salinity of 10 for 48 h starting on day 4 after hatching did juveniles grow significantly more slowly than the parents.
controls following metamorphosis (Fig. 9A). In addition, no juveniles died in our study for any of the three species tested.

For *C. fecunda*, starving larvae for 48 h appeared to reduce juvenile growth rates: mean growth rate (± standard deviation) of control juveniles was 93.1 ± 33.3 μm juvenile⁻¹ day⁻¹ while mean growth rate for juveniles starved for 48 h as larvae was only 61.9 ± 35.9 μm juvenile⁻¹ day⁻¹ (Fig. 10C). However, the difference was not significant and a more comprehensive study needs to be done for *C. fecunda* to determine if starving larvae causes latent effects on juveniles, as it often does for *C. fornicata* and *C. onyx* (Pechenik et al., 2002; Chiu et al., 2007, 2008).

3.5. Impact of salinity stress on time to metamorphosis

Subjecting larvae to salinity stress for 12 to 48 h prolonged the pre-competent period for all 3 species (Figs. 11 and 12). In all experiments with *C. fornicata* and *C. onyx*, nearly all control larvae metamorphosed on the day that they were exposed to the metamorphic stimulant, excess KCl (Pechenik and Heyman, 1987; Pechenik and Gee, 1993). However, subjecting larvae to salinity stress earlier in development reduced the number of larvae competent to metamorphose on those same days, relative to controls; the magnitude of the delay in becoming metamorphically competent varied with the strength and duration of the salinity stress (Fig. 11). For *C. fornicata* the age at which larvae were stressed had no significant effect on the rate at which larvae became competent to metamorphose (Fig. 11A, B). For *C. onyx* subjecting larvae to starvation did not affect the duration of the pre-competent period (Fig. 11D, Table 2).

Although we did not assess the effect of salinity stress on time to metamorphic competence in *C. fecunda*, we did assess the effect of salinity stress on time to spontaneous metamorphosis. Control larvae

### Table 2
Summary of two-way ANOVAs performed on all experiments in which data met the requirements of the statistical test.

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* Indicates significantly slower growth rates than control larvae of *C. fornicata* in Fig. 6C.

b Indicates significantly slower growth rates than control larvae of *C. onyx* in Fig. 7A.

c Indicates significantly longer duration of pre-competent period than control larvae of *C. onyx* in Fig. 11D.
of *C. fecunda* began to metamorphose spontaneously 12 days after hatching, while larvae that were exposed to low salinity stress for 12–48 h took significantly longer to reach that point in development (less than 10% had metamorphosed by day 28 after hatching, Fig. 12).

Larvae of *C. fornicata* and *C. onyx* that did not metamorphose when first tested were allowed to grow under control conditions for 5 more days and then re-assessed for metamorphic competence. Stressed larvae from some treatments metamorphosed successfully after this second exposure to the metamorphic stimulus (Fig. 11D: 33% of larvae subjected to a salinity of 15 and 78% of larvae subjected to a salinity of 15+starvation metamorphosed after the second exposure), while larvae from other treatments (Fig. 11B—salinity of 10) again failed to metamorphose and in fact never produced a living juvenile. For treatments in which larvae never metamorphosed, larvae did not die immediately after the stress, but typically lived with minimal growth as long as several weeks before dying.

### 3.6. Impact of salinity stress on *C. fornicata* juveniles

Juveniles of *C. fornicata* that were stressed for 12, 24, or 48 h at a salinity of 15 had significantly slower growth rates for 3 days after the end of the stress (Fig. 13A). However, mean growth rates returned to
control levels for juveniles from all treatments during the following three days (Fig. 13B), although stressed individuals remained smaller than control individuals during this time. Even when juveniles were exposed to a salinity of 15 for 48 h, juvenile growth rates were only slowed for 3 days; exposing larvae to the same stress halted growth and eventually killed all the larvae. No juveniles died following any treatment. Although juveniles stopped growing while subjected to starvation stress, the juveniles that were starved for 48 h resumed normal (control) growth immediately after the stress was removed (Fig. 13A).

4. Discussion

The direct effect that low salinity stress may have on osmoconformers like the calyptraeid species studied here may vary depending on the organism's ability to manipulate intracellular solute concentrations in order to regulate cell volume (Bradley, 2009). When faced with hypoosmotic stress, osmoconformers must “dump” solutes or increase protein synthesis (converting many osmotically active amino acids into one osmotically active protein molecule) to prevent the cells from swelling and bursting (Bradley, 2009). Even if volume regulation is successful, cells may have discarded molecules important for cellular function, causing long-term or permanent damage (Bradley, 2009). Thus, the degree to which the salinity in the environment is reduced should be of considerable importance to the animal: the more the salinity drops, the more likely that the animal will be permanently injured. Indeed, C. fornicata larvae were often not able to recover from the lowest salinities that we subjected them to (e.g., a salinity of 10 for as little as 24 h) (Figs. 5 and 6), but were able to recover from more moderate stresses (e.g., salinities of 20 and 15 for 12–48 h) (Figs. 4–6). Similarly, although larvae of C. fecunda were less tolerant of salinity stress than those of C. fornicata, they were also able to recover from a stress at a salinity of 20, although not from a stress at a salinity of 15 (Fig. 8).

Survival of organisms in reduced salinity and the immediate effects of low salinity stress on characteristics such as growth rate have been widely studied, especially in estuarine species (e.g., Lance, 1963—copepods; Lyster, 1965—polychaete larvae; Griffith, 1974—killifish; Brand, 1984—phytoplankton; Dunson and Seidel, 1986—reptiles; Deaton et al., 1989—bivalves; Kirst, 1990—algae). Some of the larvae that we tested could not even tolerate a salinity of 15 for 12 h (C. fecunda, Fig. 3B), while those of other species were able to tolerate that same reduction in salinity for 48 h (C. onyx, Fig. 3C), or even a reduction to a salinity of 10 for 48 h (C. fornicata, Fig. 4). The focus of the present study, however, was on the possibility of latent effects from salinity stress, not the determination of larval salinity tolerance per se. Though larvae likely had multiple fathers (e.g., Dupont et al., 2006; Le Cam et al., 2009), they came from a limited number of females and some (or much) of the variation seen among species in this study may actually reflect among-female variation; additional work is needed to explore the variation in salinity tolerance and the genetic basis for that variation.

Indeed, for C. fornicata we found a surprising amount of within-species variation in salinity tolerance for larvae released by different females (e.g., compare Figs. 4–6), suggesting that there is a substantial amount of genetic variation for traits conferring resistance to the detrimental effects of reduced salinity. A genetic effect has previously been seen in C. fornicata: Hilbish et al. (1999) showed highly significant differences in both growth rate and swimming speed among larvae from different C. fornicata families. In our experiments, mean (+standard deviation) growth rates of control larvae from different parents ranged from 28.4 ± 3.5 μm/larva/day (Fig. 4B) to 87.9 ± 1.6 μm/larva/day (Fig. 6A), which is within the range reported by Hilbish et al. (1999).

However, mean growth rates of control larvae within an experiment stayed fairly constant over time, which agrees with Pechenik’s (1980) finding that larvae of C. fornicata grow at a constant rate after hatching.

Larvae of all three species that recovered from low salinity stresses generally took longer to become competent to metamorphose than control larvae (Figs. 11 and 12). Since all 3 of the species that we examined live in environments that may experience rapid and prolonged reductions in salinity (Schmidt et al., 2006; Pechenik et al., 2007), these results suggest that their current abundances and distribution in nature may be partially determined by the degree to which they encounter water of reduced salinity as larvae.
However, our data suggest that if individuals of *C. fornicata* encounter reduced salinity after they metamorphose, they will be largely unaffected after the stress is removed. Although juvenile growth rates slowed considerably for up to 3 days after juveniles were stressed at a salinity of 15 for 48 h, they returned to control growth rates after full-strength salinity was restored, and none of the stressed juveniles died (Fig. 13). In contrast, all *C. fornicata* larvae died after exposure to the same stress for the same amount of time (data not shown). It is possible that juveniles are able to seal themselves off from their external environment better than larvae, decreasing the duration of exposure to low salinity. Alternatively, there may be a physiological shift during metamorphosis that provides the individual with an increased ability to tolerate salinity changes. Indeed, some marine animals do become more tolerant of environmental stress as they develop (e.g., Bambang et al., 1995; Anger, 1996; Schreiber and Specker, 1999; Anger and Charmantier, 2000), but this has not yet been documented for *C. fornicata*.

Although many marine animals demonstrate latent effects (e.g., Pechenik et al., 1996b; Wendt, 1998; Pechenik et al., 2002; Emlet and Sadro, 2006; Giménez, 2010) or "carry-over effects" (e.g., Marshall et
treatments. Periodically for spontaneous metamorphosis. On day 24 larvae were transferred to
24 h, and white circle—20 for 48 h). N = 3 dishes for all treatments; 20 larvae per dish. Asterisks indicate a signifi-
cant difference in mean metamorphosis on a particular day (p < 0.05, one-way ANOVA for each day). From hatching to day 23 larvae were checked periodically for spontaneous metamorphosis. On day 24 larvae were transferred to adult-conditioned seawater prompting an increase in metamorphosis of larvae in all treatments.

Mean ± S.E.M. shown for all data points. Symbols indicate the type of stress and the salinity at which larvae were stressed (black circle—control, black triangle—starvation only, dark gray circle—20 for 12 h, light gray circle—20 for 24 h, and white circle—20 for 48 h). N = 3 dishes for all treatments; 20 larvae per dish. Asterisks indicate a significant difference in mean metamorphosis on a particular day (p < 0.05, one-way ANOVA for each day). From hatching to day 23 larvae were checked periodically for spontaneous metamorphosis. On day 24 larvae were transferred to adult-conditioned seawater prompting an increase in metamorphosis of larvae in all treatments.

Crepipatella fecunda stressed on day 2

Fig. 12. Influence of salinity or starvation stress on time to metamorphosis of Crepipatella fecunda larvae. Mean ± S.E.M. shown for all data points. Symbols indicate the type of stress and the salinity at which larvae were stressed (black circle—control, black triangle—starvation only, dark gray circle—20 for 12 h, light gray circle—20 for 24 h, and white circle—20 for 48 h). N = 3 dishes for all treatments; 20 larvae per dish. Asterisks indicate a significant difference in mean metamorphosis on a particular day (p < 0.05, one-way ANOVA for each day). From hatching to day 23 larvae were checked periodically for spontaneous metamorphosis. On day 24 larvae were transferred to adult-conditioned seawater prompting an increase in metamorphosis of larvae in all treatments.

A) Days 0-3 after stress

B) Days 3-6 after stress

Fig. 13. Influence of juvenile salinity or starvation stress on growth rate in Crepidula fornicata. Mean ± S.E.M. shown for all treatments. Bar color and pattern indicate the type of stress and the salinity at which larvae were stressed (black—control, light gray—15, striped—starvation only, and cross hatch—starvation at a salinity of 15). Asterisks indicate a significant difference between control and experimental treatments (p < 0.05, t-test). N = 10 individually reared juveniles per treatment. (A) Growth rates for the 1st three days after the end of the stress. (B) Growth rates for the 2nd three days after the end of the stress.

The reasons why some stresses cause latent effects and others do not are unknown, largely because the mechanisms responsible for those effects are also unknown (see Pechenik, 2006). Some have offered a simple morphological hypothesis: larval stress reduces the size of the juvenile feeding apparatus, which in turn decreases the animal’s ability to feed after metamorphosis, causing slower growth rates later in life (Wendt, 1996; Pechenik et al., 2002; Marshall et al., 2003). Others have suggested that stresses experienced early in development may interfere with transcriptional or translational processes that will show effects across life history stages (Pechenik et al., 1998, 2006). Also, it is thought that some stresses may alter DNA or damage enzymes directly, causing effects long after the stress is gone (Heintz et al., 2000). Recently, advances in the understanding of epigenetic effects across cell lineages (including cellular inheritance of changes in DNA methylation patterns) has provided a possible mechanism through which environmental stress experienced at one life history stage might affect organisms later in their lives (Jablonka and Raz, 2009). These hypotheses are not mutually exclusive and much work is needed to determine the specific mechanisms causing latent effects in particular species.

Why such a clearly stressful event for the larvae of these 3 calyptraeid species did not impact juvenile growth rate when at least some other stresses do cause such latent effects is unknown. Crepidula spp. (especially C. fornicata) have recently been suggested as a

affected juvenile growth or survival for any of the three calyptraeid gastropod species that we investigated in this study (Figs. 9 and 10), even when the salinity stress caused substantial larval mortality or substantially reduced mean larval growth rate and increased time to achieving metamorphic competence (Figs. 2–8, 11, 12). This is surprising since short-term exposure to low salinity was clearly stressul to the larvae and since C. fornicata and C. onyx have previously been shown to exhibit latent effects in response to short-
term nutritional stress experienced by larvae. However, the larvae of these species were affected by the salinity stress to various degrees, and the effects differed both among species and among individuals within a species. Some larvae in all 3 species never recovered from the stress and died before metamorphosing (Figs. 2, 3, 5, 6, 7, 8), while others showed initially reduced growth rates and reduced rates of development but nevertheless recovered to normal growth rates before metamorphosis (Figs. 2–7). Similarly, Pechenik et al. (2001) found no latent effects for juveniles of Capitella sp. For larvae exposed to cadmium, even though some of the larvae were killed at the higher cadmium concentrations tested and even though this species showed latent effects when larvae were exposed to reduced salinity.

Many marine animals exhibit latent effects after metamorphosis from some larval stresses but not from others. For example, Thiyagarajan and Qian (2003) found that juvenile growth rates of the solitary ascidian Styela plicata were reduced when larvae were exposed to low salinity and low temperature, but not when the larval swimming period was prolonged. Of the species that we investigated, latent effects have also been shown to vary with the stress applied. In C. fornicata, juvenile growth rates were depressed following starvation stress in the larval stage (Pechenik et al., 1996b, 1996c, 2002), but latent effects were not detected when larvae were exposed to sub-
lthal concentrations of cadmium (Pechenik et al., 2001) or when metamorphosis was delayed (Pechenik and Eyster, 1989). In C. onyx, juveniles had slower growth rates if those individuals had experi-
cenced nutritional stress as larvae in studies by Chiu et al. (2007, 2008), but did not grow more slowly in our study (Fig. 10A). Since our experiments and those of Chiu et al. (2008) were similarly designed, the difference in results suggests that there may be variation in susceptibility among offspring from different parents. There have been no previous studies on latent effects in C. fecunda, though the present study suggests that starving larvae may cause slower growth rates as juveniles, while low salinity stress as larvae appears to have no effect on juvenile growth rate.

The reasons why some stresses cause latent effects and others do not are unknown, largely because the mechanisms responsible for those effects are also unknown (see Pechenik, 2006). Some have offered a simple morphological hypothesis: larval stress reduces the size of the juvenile feeding apparatus, which in turn decreases the animal’s ability to feed after metamorphosis, causing slower growth rates later in life (Wendt, 1996; Pechenik et al., 2002; Marshall et al., 2003). Others have suggested that stresses experienced early in development may interfere with transcriptional or translational processes that will show effects across life history stages (Pechenik et al., 1998, 2006). Also, it is thought that some stresses may alter DNA or damage enzymes directly, causing effects long after the stress is gone (Heintz et al., 2000). Recently, advances in the understanding of epigenetic effects across cell lineages (including cellular inheritance of changes in DNA methylation patterns) has provided a possible mechanism through which environmental stress experienced at one life history stage might affect organisms later in their lives (Jablonka and Raz, 2009). These hypotheses are not mutually exclusive and much work is needed to determine the specific mechanisms causing latent effects in particular species.

Why such a clearly stressful event for the larvae of these 3 calyptraeid species did not impact juvenile growth rate when at least some other stresses do cause such latent effects is unknown. Crepidula spp. (especially C. fornicata) have recently been suggested as a
promising model system for developmental biology (Henley et al., 2010); as more information on the genomes of these species and information on the genetic, epigenetic, enzymatic, and cellular effects of sub-lethal stress become available we may ultimately be able to understand the mechanisms underlying latent effects in these organisms, and why some stresses produce them while others do not.

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