Larval diet alters larval growth rates and post-metamorphic performance in the marine gastropod *Crepidula fornicata*

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Abstract

Global climate change and ocean acidification are expected to decrease phytoplankton concentrations and alter both phytoplankton species composition and nutritional characteristics. In this study we examined the impact of 3 phytoplankton species (*Isochrysis galbana*, clone T-ISO; *Pavlova lutheri*, clone MONO; and *Dunaliella tertiolecta*, clone DUN) differing in nutritional quality on larval growth and subsequent post-metamorphic fitness in the slippersnail *Crepidula fornicata;* once larvae metamorphosed, the juveniles were all reared on the diet that produced the fastest growth, T-ISO, to look for latent effects of larval diet on subsequent juvenile growth. In all experiments, larvae grew most quickly on T-ISO; diet did not affect relative rates of shell and tissue growth. In 2 of the 4 experiments conducted, larvae reared on T-ISO metamorphosed into juveniles that grew significantly faster than those that had been raised on the other phytoplankton species, indicating clear latent effects of dietary experience and suggesting parent-related genetic variation in susceptibility to this type of stress. Rearing larvae at a very low food concentration of T-ISO (1×10^4 cells ml^{-1}) until metamorphosis also produced severe latent effects on juvenile growth, reducing juvenile growth rates by more than 30%. These data indicate yet another way in which stresses experienced during larval development can influence post-metamorphic performance, and add another level of complexity to attempts at predicting the future consequences of environmental change on marine community structure and species interactions.

Introduction

The microscopic, planktonic larval stages of many benthic marine invertebrates must feed on phytoplankton for days or weeks before becoming competent to metamorphose (reviewed by Thorson 1950; Crisp 1974; Morgan 1995). The effects of food concentration and diet on larval growth rates and time to metamorphic competence have been studied for a number of marine invertebrate species (Pechenik 1987; His and Seaman 1992; Hansen 1993; Anger 1995; Basch 1996; Klinzing and Pechenik 2000; McEdward and Qian 2001), in part because the extent to which diet slows larval growth and prolongs larval life should increase the extent of planktonic mortality (Rumrill 1990; Morgan 1995; Vaughn and Allen 2010; Byrne 2011) and dispersal away from the parental habitat (Pechenik 1999; O'Connor et al. 2007; Cowen and Sponagle 2009). However, an inadequate food supply during larval development can also have detrimental effects following metamorphosis, so-called "latent effects": post-metamorphic consequences of stresses experienced during embryonic or larval development (reviewed by Pechenik 2006). For example, juveniles of the marine gastropod *Crepidula fornicata* reared under ideal conditions typically grew at least 30% more slowly for at least the first several days after metamorphosis if the larvae had been starved for even just 2 d earlier in development (Pechenik et al. 1996a, b, 2002). Similarly, larvae of the related species *C. onyx* grew significantly slower as juveniles if they had been reared at a low food concentration as larvae (Chiu et al. 2007), as did the mussel *Mytilus galloprovincialis* (Phillips 2002). To the extent that there is an escape in size from predation, competition, or bulldozing following metamorphosis (Gosselin and Qian 1997; Hunt and Scheibling 1997; Pechenik et al. 2010; Jennings and Hunt 2011), such reduced juvenile growth rates would likely increase the extent of early post-metamorphic mortality.

Larval growth rates can also vary substantially with the species of phytoplankton provided as food (Pechenik and Fisher 1979; Klinzing and Pechenik 2000). In particular, in previous studies (Klinzing and Pechenik 2000) the larvae of *C. fornicata* grew much more rapidly on a diet of *Isochrysis galbana* (clone T-ISO) than on either *Pavlova lutheri* (clone MONO) or *Dunaliella tertiolecta* (clone DUN). The impact of different larval diets on post-metamorphic performance has not previously been reported. Such information is becoming increasingly relevant, however, as climate change and ocean acidification have the potential to alter phytoplankton species ranges and cause shifts in both species composition (Hinga 2002; Tortell et al. 2002; Hayes et al. 2005; Kim et al. 2006; Leu et al. 2013) and the nutritional content of individual microalgal species (Hoogstraten and Timmermans 2012; Rossoll et al. 2012; Wynne-Edwards et al. 2014).

In this study, we sought to determine whether phytoplankton species that alter larval growth rates can also produce latent effects on subsequent post-metamorphic growth rates in the marine gastropod *Crepidula fornicata*. We also asked whether diet can alter not only rates of larval shell growth, but also relative rates of shell and tissue growth. Finally, we examined the impact on juvenile development of rearing larvae on an ideal diet (T-ISO) at a low phytoplankton concentration. Two prior studies with *C. fornicata* (Pechenik et al. 1996a, 2002) found no latent effects on juvenile development after feeding larvae at a low food concentration (1×10^4 cells ml^{-1}) of T-ISO for 2 or 4 d; in the present study we reared larvae at that same low-food concentration from hatching until metamorphosis. Such treatment did produce latent effects in the related species *C. onyx* (Chiu et al. 2007). In our study, we also examined the effects of this treatment on relative rates of shell and tissue growth during larval development.

Crepidula fornicata is ideal for such studies: larvae hatch at shell lengths of about 450 µm and grow quickly (typically 50-100 µm day^{-1}: Pechenik et al. 1996c; Hilbish et al. 1999; Klinzing and Pechenik 2000) making them easy to measure non-destructively (Pechenik et al. 2002); larvae typically become competent to metamorphose within 2 weeks at room temperature at large sizes, at shell lengths approximately 850-1200 µm (Pechenik and Heyman 1987; Pechenik et al. 1996c); larval shell length and organic tissue weight are linearly related for this species (Pechenik 1980), so that shell growth is also an accurate measure of tissue growth; competent larvae can be triggered to metamorphose within 6-8 h, simply by elevating ambient K^+ concentration by 15-20 mM (Pechenik and Gee 1993); and both larvae and juveniles can be reared in the laboratory with negligible mortality, so that any latent effects encountered cannot be due to selective mortality for particular genotypes (Pechenik et al. 1996c, 2002; Klinzing and Pechenik 2000). Finally, although *C. fornicata* is native to the East Coast of the United States, it is now widely distributed among coastal communities in many parts of the world (Blanchard 1997; Bohn et al. 2012), making it potentially useful as a biomonitor of climate change.

Materials and methods

General procedures

Several stacks of adults were collected intertidally at Nahant, Massachusetts (collecting permit issued by the Commonwealth of Massachusetts, Division of Marine Fisheries) in September 2013 (Experiments I and 2), early November 2013 (Experiment 3), March 2014 (Experiment 4), and May 2014 (Experiment 5), and brought to the laboratory where they were maintained on a mixed diet of *Isochrysis galbana* (clone T-ISO) and *Dunaliella tertiolecta* (clone DUN) (Pechenik et al. 1996a, b) until larvae were released. Larvae were then retained on a 150 µm

Nitex mesh filter and transferred to seawater that had been filtered to 0.45 µm (Pechenik et al. 1996a).

Five experiments were conducted. The first 4 experiments were designed to detect latent effects resulting from rearing larvae on different algal diets: larvae were raised on a diet of either T-ISO (*Isochrysis galbana*), MONO (*Pavlova lutheri*), or DUN (*Dunaliella tertiolecta*); cell sizes range from about 3-5 µm for T-ISO to about 8-10 µm for DUN (Pechenik and Fisher 1979). Phytoplankton was raised at 18 °C using f/2 medium and standard techniques (Guillard and Ryther 1962), and harvested at concentrations of ~ 1-3 x 10^6 cells ml^{-1}. Larvae (14-19 per dish) were reared in 45 ml of 0.45 µm filtered seawater (salinity 30) at room temperature (~ 23 $^{\circ}$C), with phytoplankton at ~18 x 10^4 cells ml^{-1} as in previous studies with this species (Klinzing and Pechenik 2000; Pechenik et al. 1996a, b, c; Pires et al. 2000). Five dishes of larvae were established for each treatment. Three dishes of larvae were used to monitor larval growth and to obtain juveniles after metamorphosis. One additional dish of larvae was used for tissue and shell weight analyses, to determine if rates of shell and tissue growth were affected by diet to the same degree, while the fifth dish of larvae was used to monitor the onset of metamorphic competence. Seawater and food were changed every 2 to 3 d; in spot checks, phytoplankton concentrations in Experiments 1-4 never fell below ~ 10 x 10^4 cells ml^{-1}, well above the critical concentration below which ingestion rates are impacted (Pechenik and Eyster 1988).

In a final experiment (Experiment 5), we reared larvae at high (18 x 10^4 cells ml^{-1}) or low (1 x 10^4 cells ml^{-1}) concentrations of the diet on which larvae grew the fastest (T-ISO) for the entire larval period, to determine whether low concentrations of a nutritious microalga produces latent effects on post-metamorphic growth in this species as it does in *C. onyx* (Chiu et al. 2007). The

two phytoplankton concentrations were chosen based on the results of previous studies (Pechenik et al. 1996a; Klinzing and Pechenik 2000; Chiu et al. 2007).

For all experiments, treatments were begun on Day 0, 1-2 d after the larvae hatched, at which time mean initial shell length was determined for at least 12 larvae at 50 X or 63 X (Table 1), using a dissecting microscope equipped with an ocular micrometer; there is little within-brood variation in shell length at hatching for this species (Pechenik et al. 1996a; 2002) so that these measurements suffice for estimating initial larval size. Larvae were then measured at least 2 more times during development to determine growth rates. Final larval shell lengths were determined when larvae had reached an average size of ~850 μm, after different lengths of time depending on larval growth rates (e.g., in Experiment 1, after 9 d for larvae reared on T-ISO and after 13 d for larvae reared on MONO).

Within each experiment, once larvae reached shell lengths of ~ 850 μm, the larvae from one replicate dish were tested periodically for metamorphic competence by raising the concentration of KCl in seawater by 20 mM; competent larvae typically metamorphose in response to this treatment within 6-8 h (Pechenik and Gee 1993), and without any impact on post-metamorphic growth rates (Eyster and Pechenik 1988). If more than 50% of the tested larvae metamorphosed within 6-8 h of exposure to the inducer, metamorphosis was then induced in the other replicates of the same diet on the following day. Larvae that failed to metamorphose within the 6-8 h exposure periods ("pre-competent" larvae—Pechenik and Gee 1993) were preserved in 10% formalin buffered to a pH of approximately 8.0 with sodium borate, for later determinations of shell and tissue weights. Larvae that had been reared expressly for determining shell and tissue weight were also preserved at this time.

Following metamorphosis, 12-15 juveniles haphazardly chosen from each treatment (up to 5 from each replicate) were reared individually in 45 ml of filtered seawater (salinity 30, ~23 °C) on a diet of T-ISO at ~18 x 10^4 cells ml^{-1}, which generally supports rapid juvenile growth (typically 100-200 µm day^{-1}) with negligible mortality (Pechenik et al. 1996b, c, 2000; Klinzing and Pechenik 2000); young juveniles of this species grow at constant rates above food concentrations of about 7 x 10^4 cells ml^{-1} (Eyster and Pechenik 1988; Pechenik and Eyster 1989). Note that all juveniles were reared on T-ISO, regardless of what the larval diet had been. Water was changed every 1-2 d. On days that the water was not changed, juveniles were given enough additional T-ISO to restore the total cell concentration to approximately 18 x 10^4 cells ml^{-1}. In spot checks, cell concentrations never fell below 10 x 10^4 cells ml^{-1}.

Juvenile shells were measured at least twice (at 12-50 X magnification, depending on their size): once on the day following metamorphosis and once again 3-8 d later.

Shell and tissue weights were determined as follows. Weights were determined for both larvae and juveniles in Experiments 1 and 2, but only for larvae in Experiments 3, 4, and 5. Preserved larvae and juveniles were quickly rinsed in deionized water to remove adhering salts and preservative and then dried in small pre-weighed aluminum foil cups at 50 ^oC for at least 48 h before being weighed to the nearest 0.001 mg on a Mettler Toledo balance, to determine total dry weight. For larval weight determinations in Experiments 1-4, we had 2-4 replicates per diet, each with 4-10 individuals per replicate; for Experiment 5, we had 3 replicates with 11-14 individuals per replicate. For juveniles, we used 3-9 replicates per treatment, with one individual per replicate. After weighing, samples were combusted at 500^oC for at least 12 hours to determine organic (tissue) weight by quantifying weight loss following combustion (Paine 1964; Pechenik 1980; Pechenik and Eyster 1989). Control foil containers did not lose weight

during this process. Shell (inorganic) weights were determined by subtracting the empty container weight from the container's post-combustion weight. Mean shell lengths of larvae and juveniles are given in Table 1.

Details of particular experiments are given below.

Experiments 1-4: Impact of larval diet on juvenile growth

In Experiment 1, larvae were raised on a diet of T-ISO, MONO, or DUN. Larval survival on DUN was very poor in this experiment (see Results); surviving larvae were transferred to a diet of T-ISO after 18 d, but none of those larvae survived to metamorphosis. Following the metamorphosis of larvae in the other 2 treatments, 15 juveniles (5 from each replicate) that had been reared as larvae on T-ISO and 15 juveniles that had been reared as larvae on MONO were reared on a diet of T-ISO for another 15 d, again at room temperature (23 ºC). Juveniles were changed to new seawater and phytoplankton daily. Shells were measured at 16 X or 32 X, to determine juvenile shell growth rates. After making the final shell measurements, juveniles were preserved in 10% buffered formalin as described above, for later analysis of shell and tissue weight.

For Experiments 2 and 3, larvae were raised on the same 3 diets used in Experiment 1, all at approximately 18 x 10^4 cells ml^{-1}, as in Experiment 1. Because larvae had survived poorly on a diet of DUN in the previous experiment, we raised groups of larvae on DUN in Experiment 2 for 3, 6, or 9 d and in Experiment 3 for 5 d or 10 d, and then switched them to a diet of T-ISO for the rest of larval development. Larvae were measured every few days throughout larval development in Experiment 2 and on days 0, 5, and 8 in Experiment 3.

Toward the end of larval rearing in Experiment 2 we noticed that our T-ISO phytoplankton culture had become mildly contaminated with DUN. Following metamorphosis, we reared juveniles for only 4 d, during which time T-ISO cells still dominated the phytoplankton culture.

Experiment 4 was similar to the previous experiments except that larvae were reared on T-ISO for the entire larval period or on MONO for 3 (treatment MONO-3), 6, or 9 d, after which they were transferred to a diet of T-ISO for the duration of the study. For the T-ISO and MONO-3 data, larvae were measured on days 0, 3, and 6. For the MONO-6 and MONO-9 data, larvae were only measured twice, on days 0 and 6.

Experiment 5: Impact of rearing larvae at a low T-ISO food concentration

In this experiment, larvae were raised at two different concentrations of T-ISO (18×10^4 cells ml^{-1} or 1×10^4 cells ml^{-1}) until metamorphosis, to determine whether rearing larvae of this species on a high-quality diet at a low food concentration produces latent effects on juvenile growth. Larvae reared at the high food concentration were measured on days 0, 3, and 9, while those reared at the low food concentration were measured on days 0, 4, and 10.

Statistical analyses

The effects of larval diet on mean larval shell growth rates, mean juvenile shell growth rates, mean larval tissue weight as a percentage of overall mass, and mean juvenile tissue weight as a percentage of overall mass were analyzed using: (1) ANOVA followed by Tukey's multiple comparisons test if there were more than two groups being compared; (2) Student's t-test if there

were only two groups being compared and data for both groups were normally distributed with similar variances; (3) Welch's t-test if the variances of the two groups being compared were unequal; or (4) Mann-Whitney U test if either of the two groups being compared had a non-normal distribution. There were no significant differences in mean larval growth rates among replicates (ANOVA, $p > 0.10$), so that growth rate data from replicates were combined for further analysis. Data on percent larval survival were arc-sine transformed before analysis. Data were analyzed using GraphPad Prism 4.0.

Results

Experiment 1

Larval growth rates were linear in all treatments (T-ISO $R^2 = 0.973$; MONO $R^2 = 0.98$; DUN $R^2 = 0.884$). Larvae grew significantly faster on T-ISO than on either MONO or DUN, and grew significantly more slowly on DUN than on MONO ($F_{(3,131)} = 54.3$, $P < 0.0001$; Tukey's tests, $P < 0.05$) (Fig. 1A). After 9 d of being reared on a diet of DUN, larval shell length plateaued at about 650 μm; larvae grew even more slowly on this diet during days 9-20 (Tukey's test, $P < 0.05$) (Fig. 1A), despite having been transferred to a diet of T-ISO after day 10. Mortality was 98% by day 21 for larvae being reared on DUN, but was less than 7% in any replicate for larvae in other treatments. No larvae raised on DUN survived to become juveniles. Such poor performance on DUN was not seen in subsequent experiments (see below).

Larvae raised on MONO grew nearly 38% more slowly than those raised on T-ISO (Fig. 1A). Despite this large difference in larval growth rates there were no significant differences in juvenile growth rates, either for the first 3 d after metamorphosis (Fig. 1B) ($t = 0.04$, df = 26, $P = 0.97$) or for the first 15 d after metamorphosis (Fig. 1C) (Mann-Whitney U test, $U = 79.0$, $P =$

0.61), for juveniles that had been raised as larvae on either T-ISO or MONO. Juvenile mortality ranged between 0 and 13% (N = 15 individuals per treatment)

Experiment 2

Larval mortality was generally below 7% in all treatments. Larvae grew at constant rates over time on all diets (R^2 for length versus time varied only from 0.93 to 0.99) (Fig. 2) but growth rates differed on different diets (see below). Growth rates for larvae reared on DUN for only 3, 6, or 9 d never recovered to control levels even after larvae had been transferred to T-ISO for the remainder of the larval rearing period (Fig. 2).

Although our T-ISO algal culture became contaminated with DUN toward the end of larval rearing in this experiment, larvae reared on a diet of "T-ISO" exhibited higher mean growth rates over the course of the 15-19 d larval period than larvae reared in any of the other treatments, including those in which larvae were reared purely on DUN for up to 9 d (Figs. 2, 3A) ($F_{(3,131)}$ = 54.3, $P < 0.0001$; Tukey's tests, $P < 0.05$), as in Experiment 1. Larvae reared on MONO grew about 33% more slowly than those reared on T-ISO, and about 18% more slowly than those reared on DUN for either 3 or 6 d, but at equal rates to those reared on DUN for 9 d (Tukey's tests, $P > 0.05$) (Fig. 3A).

Over the first 4 d after metamorphosis, juveniles from larvae that had been raised on DUN for 9 d (treatment DUN-9) grew significantly more slowly than those that had been reared on T-ISO or MONO, or on DUN for only 6 d (DUN-6) ($F_{(4,57)}$ = 5.13, P = 0.0013; Tukey's tests, $P <$ 0.05) (Fig. 3B). Indeed, those juveniles grew about 35% more slowly than those that had been reared on T-ISO as larvae. There were no other significant differences in juvenile shell growth rates among treatments (Tukey's tests, $P > 0.05$) (Fig. 3B). Juvenile mortality was 0% for

treatments T-ISO, DUN-3, and DUN-9; 7% for DUN-3; and 20% for DUN-9 (N = 15 individuals in each treatment).

Experiment 3

Larval mortality was less than 5% in all treatments and growth was linear on all diets (DUN-10 $R^2 = 0.97$; $R^2 > 0.99$ for larvae in all other treatments). Larvae again grew substantially more quickly when reared on T-ISO than when reared on the other two diets ($F_{(6,128)} = 73.0$; $P = 0.006$); in particular, larvae reared on MONO grew about 40% more slowly than those reared on T-ISO (Fig. 4A). Larvae also grew significantly more slowly on a diet of DUN than on T-ISO (Fig. 4A; Tukey's test, $P < 0.05$), and growth rates for larvae reared on DUN for 10 d were as low as those for larvae reared on MONO (Tukey's test, $P > 0.10$).

Juveniles that had been reared as larvae on MONO grew significantly more slowly than those that had been reared on T-ISO as larvae, for at least the first 5 d after metamorphosis (Tukey's test, $P < 0.05$) (Fig. 4B). Indeed, 5 individuals (of the 12 monitored) that had been reared on MONO as larvae grew at less than 32 μm day^{-1} as juveniles, on a diet of T-ISO. There were no significant differences in juvenile growth rates for individuals that had been reared as larvae on T-ISO or on DUN, even for those that had been reared on DUN for 10 d (Tukey's test, $P > 0.10$), despite the difference in larval growth rates.

Experiment 4

Larval mortality was below 5% in all treatments, and larvae grew at constant rates (T-ISO $R^2 = 0.993$; MONO-3 $R^2 = 0.990$; larvae were only measured at two different times for the other two treatments). As in all previous experiments, larvae grew significantly faster on a diet of T-ISO

than on MONO (ANOVA $F_{(5, 108)} = 42.9$, $P < 0.0001$; Tukey tests, $P < 0.05$) (Fig 5A), even though individuals raised on MONO were transferred to a diet of T-ISO after 3, 6, or 9 d. Larvae grew significantly more slowly when reared on a diet of MONO for 6 or 9 d (treatments MONO-6, MONO-9) and then transferred to T-ISO than when reared on MONO for only 3 d (MONO-3) before their transfer to T-ISO (Tukey test, $P < 0.05$). Nevertheless, juvenile growth rates were not affected by larval diet, regardless of the time period over which growth rates were measured (Fig. 5B). Note that although a significant *P*-value was obtained by ANOVA for juvenile growth rates over the first 4 d after metamorphosis (Fig. 5B), no paired comparisons differed significantly (Tukey's tests, $P > 0.05$ for all comparisons).

Experiment 5

Larvae grew significantly more slowly on a diet of T-ISO at 1 x 10^4 cells ml^{-1} than at 18 x 10^4 cells ml^{-1} (days 0-4, $t = 10.8$, df = 80, $P < 0.0001$; days 0-9, Mann-Whitney $U = 23.0$, $P < 0.0001$) (Fig. 6). All larvae exhibited linear growth (high food $R^2 = 0.999$, low food $R^2 = 0.995$), and mortality was under 5% in all replicates. Following metamorphosis, when all juveniles were reared at the high food concentration, juveniles that had been reared as larvae at the low food concentration showed latent effects for at least the first 6 d after metamorphosis, growing 34-43% more slowly than individuals that had been raised as larvae at the high food concentration (days 0-3, Mann-Whitney $U = 37.0$, $P = 0.01$; days 0-6, $t = 7.00$, df = 25, $P < 0.0001$) (Fig. 6).

Influence of diet on relative rates of shell and tissue growth

In none of the experiments did phytoplankton species affect the relationship between shell and tissue weight, either for larvae or for juveniles, even when shell growth rates differed significantly and substantially within an experiment (Fig. 7). Results were quite different for larvae that had been reared at different concentrations of T-ISO, however. Although larvae reared at the two T-ISO concentrations had similar mean shell weights shortly before metamorphosis (t = 0.708, df = 4, P = 0.52), mean individual tissue weight for larvae reared at the low T-ISO concentration was only 38.6% that of larvae that had been reared at the high T-ISO concentration (t = 11.15, df = 4, P = 0.0004) (Fig. 8).

Discussion

As expected from previous research (Klinzing and Pechenik 2000), larvae of *C. fornicata* grew most rapidly on a diet of T-ISO in all experiments. Similarly, larvae of the marine mudsnail *Nassarius obsoletus* (= *Ilyanassa obsoleta*) also grew more quickly when reared on *Isochrysis galbana* (clone ISO) than on *Dunaliella tertiolecta* (DUN) (Pechenik and Fisher 1979); however, those larvae did not grow more slowly when reared on a diet of *Thalassiosira pseudonana* (MONO). Food-specific growth rates could be caused by differences in ingestion rates, digestive rates, or assimilation efficiency, or by subtle differences in nutritional chemistry (Pechenik and Fisher 1979). Based on studies with mudsnail larvae (Pechenik and Fisher 1979), in which growth rates, ingestion rates, and assimilation efficiencies were measured, differences in nutritional content seem the most likely sources of the diet-related differences in mean growth rates seen in the present study. T-ISO is known to be especially rich in certain essential fatty acids (Yoshioka et al. 2012), which play a critical role in determining the nutritional value of phytoplankton (Rossoll et al. 2012). Phytoplankton species also vary substantially in vitamin

content (Brown et al. 1997). The dietary requirements of *C. fornicata* larvae have yet to be determined.

There is no reason to suspect that either of the 2 poorer microalgal diets used in this study are toxic to *C. fornicata.* Larval mortality was generally low (< 7%) on all diets, and larval growth rates were linear over time (e.g. Fig. 2). Moreover, the highest mean juvenile growth rates recorded in Experiment 2, in which the T-ISO culture had become contaminated with DUN towards the end of the larval rearing period, were comparable to those recorded in Experiments 3 and 5, in which the T-ISO cultures remained pure.

Mean larval growth rates on each diet varied considerably among experiments in our study. Such differences cannot be due to differential larval mortality, as larval mortality was generally low, as already noted, but instead are likely to have a substantial genetic basis; when larvae from 90 families of *C. fornicata* were reared on *Isochrysis galbana*, mean larval growth rates varied from 35 μm d^{-1} to over 90 μm d^{-1}, with a highly significant family effect on larval growth (Hilbish et al. 1999). The remarkably poor growth and survival of larvae on a diet of DUN in our Experiment 1 suggests that larvae from that hatch had unusually high requirements for some particular micronutrient that was either missing from that diet or present at very low concentrations. What those micronutrients are remains to be determined. There was negligible mortality for larvae being reared on the other 2 phytoplankton species in that experiment, indicating that the larvae were generally healthy, and larval mortality was negligible when we reared larvae on DUN in our other experiments. On the other hand, the variation in mean larval growth rates seen among our experiments on a given diet could also reflect differences in algal chemistry among experiments; the fatty acid composition of particular phytoplankton species is known to vary with culture conditions and with the timing of algal harvesting (Chuecas and

Riley 1969; Brown et al. 1997). However, we drew phytoplankton from several cultures of each species over the course of each experiment, and cell concentrations at harvesting only varied between ~1 and 3 x 10^6 cells ml^{-1}. Larvae grew at constant rates within experiments (e.g., Fig. 2), suggesting that algal chemistry was stable.

Latent effects of larval diet on juvenile growth were seen in a number of our experiments, including Experiment 2. Juvenile growth rate data from that experiment are potentially complicated by the contamination of our T-ISO culture with DUN late in larval development, as noted earlier. However, the reduced growth rates of juveniles from larvae that had been reared on DUN in that experiment were not likely due to contamination of the juvenile growth media with DUN. Individuals that had been reared as larvae on MONO were stimulated to metamorphose 2-4 d after larvae in any of the other treatments, due to especially slow larval growth on that diet; those individuals would therefore have been exposed to even higher DUN concentrations during their 4 d of juvenile growth than juveniles from any of the other treatments, and yet their mean juvenile growth rates were the same as those of juveniles that had been raised as larvae on T-ISO. Moreover, as mentioned earlier, mean juvenile growth rates on the contaminated T-ISO in that experiment were comparable to those reported in Hilbish et al. 1999 and other studies. The data suggest, then, that the reduced juvenile growth rates recorded for the DUN treatments in Experiment 2 reflect latent effects of algal diet during larval development, as in Experiment 3, rather than contamination of the T-ISO culture with DUN during the period of juvenile growth.

Perhaps the most interesting finding from this study is that dietary differences during larval development significantly influenced juvenile growth rates in some experiments, but not in others. In Experiment 3, for example, feeding larvae on a diet of MONO through

metamorphosis produced latent effects on juvenile growth (Fig. 4B), whereas that was not the case in Experiments 1, 2, or 4, even though larvae grew substantially and significantly more slowly on a diet of MONO than on T-ISO in all of those experiments. Similarly, rearing larvae on a diet of DUN for 9 d influenced post-metamorphic growth rates in Experiment 2 (Fig. 3B), but had no effect on post-metamorphic growth rates in Experiment 3. In previous studies, several days or more of starvation during larval development also produced latent effects on juvenile growth rates in some experiments but not in others (Pechenik et al. 1996a, b, 2002).

Overall, our data suggest that the effects of larval diet on juvenile development seen in the present study are somehow due to subtle deficiencies in one or more key nutrients being provided to the larvae, and that the larvae of some parents may have somewhat higher requirements for particular fatty acids or other micronutrients than the larvae of other parents. It is important to remember that in our studies, documented latent effects on juvenile growth could not have been caused by genotype-specific differential mortality, since juvenile mortality was generally quite low (less than 7%), as in our previous studies with this species (e.g., Klinzing and Pechenik 2000; Pechenik et al. 1996c, 2002).

Note that the larvae in our experiments were triggered to metamorphose soon after they became competent to do so. However, competent larvae of this species can delay their metamorphosis for a considerable time (Pechenik 1980; Pechenik and Eyster 1989), the potential delay period varying inversely with rearing temperature (Zimmerman and Pechenik 1991). Thus larvae could experience nutritional stress for an even longer period than that which was examined in this study, the consequences of which could be explored in future work.

Despite the diet-related differences in larval growth rates that were seen in Experiments 1-4, and at least sometimes in subsequent juvenile growth rates as well, the proportional contribution

of tissue to total dry weight was not altered in any of those experiments. In contrast, differences in the concentration of T-ISO during larval rearing *did* alter that relationship--not by altering individual shell weight, but rather by altering individual tissue weight. In studies by Bashevkin and Pechenik (in review), rearing larvae of this same species at different salinities (20 or 30) did not affect relative rates of shell and tissue growth at 25 °C, despite dramatic differences in mean shell growth rates, but did impact relative rates of shell and tissue growth at 20 °C. We are not aware of any research that examines how the growth of shell and tissue is coordinated during molluscan development, but the topic seems worth exploring.

This study shows that feeding larvae on different microalgal species can indeed negatively impact growth after metamorphosis. Rearing larvae on the best diet (T-ISO) at the low concentration of 1 x 10^4 cells ml^{-1} throughout larval development also resulted in pronounced reductions in mean juvenile growth rates following metamorphosis, lasting for at least 6 d (Fig. 6). Such latent effects have now been demonstrated for a variety of animals from many phyla in response to a variety of stresses experienced during larval development, including thermal and salinity stress (Pechenik et al. 2001; Thiyagarajan et al. 2007; Wu et al. 2012; Hartmann et al. 2013; Hopkins et al. 2014; Jonsson and Jonsson 2014), heavy metal exposure (Ng and Keough 2003), ocean acidification (Hettinger et al. 2012, 2013), hypoxia (Li and Chiu 2013), nutritional stress (Pechenik et al. 1996b, 2002, present study; Merilä and Svensson 1997; Phillips 2002; Gardner et al. 2009; Van Allen and Rudolf 2013; Jonsson and Jonsson 2014), delayed metamorphosis (Pechenik et al. 1993; Gebauer et al. 1999; Marshall et al. 2003; Wendt 1998; De Block and Stoks 2005; Thiyagarajan et al. 2007; Graham et al. 2013), and the presence of predators (Relyea and Hoverman 2003; Nicieza et al. 2006; Tejedo et al. 2010) (reviewed by Pechenik 2006; Jonsson and Jonsson 2014). Some of these stresses have produced latent effects

in some species but not in others. For example, exposing larvae to reduced salinity caused latent effects for barnacles (Thiyagarajan et al. 2007) and for the polychaete *Capitella teleta* (Zimmerman and Pechenik 1991), but not for *Crepidula fornicata*, even when larval growth rates were severely depressed (Diederich et al. 2011). Similarly, delayed metamorphosis produced latent effects for the polychaete *Capitella teleta,* the bryozoan *Bugula neritina* (Wendt, 1998), the barnacle *Balanus amphitrite* (Thiyagarajan et al. 2007), and the colonial seasquirt *Diplosoma listerianum* (Marshall et al. 2003), but not for *Crepidula fornicata* (Pechenik and Eyster 1989) or for the solitary ascidian *Styela plicata* (Thiyagarajan and Qian 2003). In addition to the latent effects that have been so widely documented as resulting from some stresses experienced during larval development, it now seems that even stresses experienced by sperm prior to fertilization can influence offspring performance (Ritchie and Marshall 2013). The mechanisms accounting for such latent effects, and for the higher or lower vulnerability of some species--and apparently of the offspring of some parents within a species--to certain stresses, are not yet clear. For juvenile *Crepidula fornicata* (Pechenik et al. 2002) and *C. onyx* (Chiu et al. 2007, 2008), reduced juvenile growth seems to be at least partly caused by reduced individual filtration rates. Molecular studies may help us to determine why some stresses experienced during larval development produce latent effects while others do not, and why the responses differ among species.

The results of these studies also raise another question: how much of the variation that we see in the natural world is caused by direct genetic differences among individuals and how much reflects the impact of experiences in early development? Our results also add an intriguing note of complexity to attempts at predicting future consequences of environmental change on marine community structure and species interactions, as worldwide phytoplankton concentrations

decline (Montes-Hugo et al. 2009; Boyce et al. 2010) and as phytoplankton species composition and nutritional characteristics change (Burkhardt and Riebesell 1997; Rossoll et al. 2012).

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