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Legacy of Multiple Stressors: Responses of Gastropod Larvae and Juveniles to Ocean Acidification and Nutrition

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Abstract. Ocean acidification poses a significant threat to calcifying invertebrates by negatively influencing shell deposition and growth. An organism's performance under ocean acidification is not determined by the susceptibility of one single life-history stage, nor is it solely controlled by the direct physical consequences of ocean acidification. Shell development by one life-history stage is sometimes a function of the pH or pCO_2 levels experienced during earlier developmental stages. Furthermore, environmental factors such as access to nutrition can buffer organismal responses of calcifying invertebrates to ocean acidification, or they can function as a co-occurring stressor when access is low. We reared larvae and juveniles of the planktotrophic marine gastropod *Crepidula fornicata* through combined treatments of nutritional stress and low pH, and we monitored how multiple stressors endured during the larval stage affected juvenile performance. Shell growth responded non-linearly to decreasing pH, significantly declining between pH 7.6 and pH 7.5 in larvae and juveniles. Larval rearing at pH 7.5 reduced juvenile growth as a carryover effect. Larval rearing at pH 7.6 reduced subsequent juvenile growth despite the absence of a negative impact on larval growth, demonstrating a latent effect. Low larval pH magnified the impact of larval nutritional stress on competence for metamorphosis and increased carryover effects of larval nutrition on juvenile growth. Trans-life-cycle effects of larval nutrition were thus modulated by larval exposure to ocean acidification.

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Abbreviations: Ω_{Ar}, saturation state of aragonite; FDR, false discovery rate; HF, high food; LF, low food; OA, ocean acidification; TA, total alkalinity; T-ISO, Tahitian *Isochrysis galbana*.

Introduction

Increases in atmospheric CO_2 since the pre-industrial era are driving ocean acidification (OA) at a global scale. Hydration of dissolved CO_2 yields carbonic acid, which dissociates into free H^+ and HCO_3^-. Increased H^+ protonates dissolved CO_3^{2-}, generating additional HCO_3^-. The net effect of increased pCO_2 is decreased pH, decreased $[CO_3^{2-}]$, and increased $[HCO_3^-]$ (Doney *et al.*, 2009). Broadly, physiological effects of low seawater pH include acidosis, hypercapnia, and oxidative stress (Michaelidis *et al.*, 2005; Miles *et al.*, 2007; Fabry *et al.*, 2008; Tomanek *et al.*, 2011). OA also slows the biomineralization of calcium carbonate in the shells and skeletons of calcifying invertebrates such as corals, molluscs, and echinoderms (Orr *et al.*, 2005; Kurihara, 2008; Kroeker *et al.*, 2013). The relative roles of physicochemical and biological factors controlling calcification have been debated (Jokiel 2011a, b; Cyronak *et al.*, 2015; Thomsen *et al.*, 2015; Waldbusser *et al.*, 2015; Von Euw *et al.*, 2017); but the reallocation of energy between calcification, ion transport, and other physiological processes under OA has clear consequences for the growth and development of marine invertebrates (Waldbusser *et al.*, 2013; Pan *et al.*, 2015; Brunner *et al.*, 2016; Frieder *et al.*, 2017).

Organisms often exhibit stage-specific responses to OA throughout their life histories (Zippay and Hofmann, 2010; Talmage and Gobler, 2011; Ceballos-Osuna *et al.*, 2013; Dupont *et al.*, 2013; Kapsenberg and Hofmann, 2014; Small *et al.*, 2015). Investigations into the effects of OA on marine invertebrates have paid particular attention to larval development (Kurihara *et al.*, 2007; Kurihara, 2008; Miller *et al.*, 2009; Sunday *et al.*, 2011; Doropoulos *et al.*, 2012) because

the fate of larvae is linked to settlement and population distribution (Jenkins, 2005; Johnson and Geller, 2006). While the effects of OA on larvae are important, these effects are not independent of other life-history stages. Several recent studies have documented both positive and negative effects of parental exposure to OA on the resilience of larvae to acidification stress (reviewed by Ross *et al.*, 2016). Within a generation, larval experience of OA may have consequences for later life-history stages (Byrne *et al.*, 2011; Dupont *et al.*, 2013; Gobler and Talmage, 2013). For example, in the bivalve *Ostrea lurida*, the restriction of larval shell growth by OA can persist across metamorphosis and can continue to reduce juvenile growth rate even when acidified conditions are no longer present (Hettinger *et al.*, 2012, 2013). "Carryover" effects of larval experiences on juvenile outcomes have been observed in many marine invertebrates in the contexts of a broad range of environmental stressors (Pechenik *et al.*, 1998; Pechenik, 2006, 2018).

A calcifying invertebrate may experience the costs of past acidification stress in the form of "carryover" or "latent" effects tied to their larval experience. Latent effects, as they are discussed here, are consequences of a stressor experienced during the larval period that (i) arise during later life-history stages following the recovery of larvae, or (ii) arise during later life-history stages despite the absence of a larval response (Pechenik, 2006, 2018). For example, transient nutritional deprivation of gastropod veligers can impact post-metamorphic juvenile performance even after recovery of normal larval growth rates (Pechenik *et al.*, 1996a, b, 2002). Similarly, maintaining larvae of the polychaete *Capitella* sp. at 10–12 ppt salinity for 24–48 h decreased postmetamorphic survival and growth rate, even when reduced salinity showed no effect on larval mortality (Pechenik *et al.*, 2001).

Performance under OA is also contingent on additional biotic and abiotic factors (Breitburg *et al.*, 2015; Kroeker *et al.*, 2017). A multitude of environmental stressors co-occur in coastal environments where pCO_2 is most variable (Reum *et al.*, 2014; Baumann *et al.*, 2015), and these co-occurring stressors can shape responses to OA in many taxa and levels of biological organization (Boyd and Hutchins, 2012; Byrne and Przeslawski, 2013; Todgham and Stillman, 2013; Ko *et al.*, 2014; Gaylord *et al.*, 2015; Griffith and Gobler, 2017; Parker *et al.*, 2017). Adequate food supply can ameliorate the effects of high pCO_2 and low pH in larval and juvenile bivalves (Hettinger *et al.*, 2013; Sanders *et al.*, 2013; Thomsen *et al.*, 2013; Ramajo *et al.*, 2015), as well as in corals, echinoderms, and crustaceans (Ramajo *et al.*, 2016). Conversely, low access to nutrition has been shown to exacerbate effects of OA in larval and juvenile bivalves (Thomsen *et al.*, 2013; Cole *et al.*, 2016) or to additively decrease performance in an adult bivalve (Melzner *et al.*, 2011).

Interactions between pH and nutrition may persist as carryover or latent effects across life-history stages in marine invertebrates undergoing indirect development. We tested this hypothesis through a series of experiments using veliger larvae and juveniles of the slipper limpet, *Crepidula fornicata*. This species is a brooding planktotrophic littoral gastropod with a wide thermal tolerance (Thieltges *et al.*, 2004; Diederich and Pechenik, 2013), pH tolerance (Noisette *et al.*, 2014, 2015, 2016), and geographic distribution (Blanchard, 1997, 2009; Bohn *et al.*, 2012; JNCC, 2015). Developmental, physiological, and behavioral responses to a number of environmental factors have been studied in larvae, juveniles, and adults of this species (Pechenik, 1984; Pechenik *et al.*, 1996a, b, 2001; Diederich *et al.*, 2011; Noisette *et al.*, 2014, 2015, 2016; Padilla *et al.*, 2014; Bashevkin and Pechenik, 2015). Under optimal laboratory culture conditions, veligers of *C. fornicata* grow at about 50 μm d^{-1} with little mortality, become competent for metamorphosis at about 800-μm shell length, and are easily induced to metamorphose by exposure to 15–20 mmol L^{-1} elevated $[K^+]$ (Pechenik and Gee, 1993; Pechenik *et al.*, 1996b; Hilbish *et al.*, 1999; Padilla *et al.*, 2014). Methods for evaluating effects of nutrition on larval and juvenile development are well established in this species (Pechenik *et al.*, 2002; Pechenik and Tyrell, 2015).

We carried out four independent experiments (Table 1) in which we reared larvae and juveniles of *C. fornicata* under several different combinations of food availability and pH. We measured larval shell growth, larval survivorship, acquisition of competence for metamorphosis, and juvenile shell growth in order to understand the relationship of responses to nutrition and pH across the larva-to-juvenile transition.

Materials and Methods

Adult collection and larval culture

Two experiments (Experiments 1 and 2) were carried out at the University of Washington's Friday Harbor Laboratories in Friday Harbor, Washington. For these experiments, brooding adults of *Crepidula fornicata* (Linnaeus, 1758) were collected during their reproductive season from the intertidal of Totten Inlet, Thurston County, Washington. Stacks of approximately 4–6 adults were housed in separate aerated 3-L glass jars containing 2 L of room temperature unfiltered seawater, which was changed daily. After adults had acclimated to the laboratory for at least 1 d, naturally hatched veligers were collected by siphoning onto a 150-μm sieve shortly after release by adults. Larvae used in Experiments 1 and 2 were derived from two separate broods. Larvae were distributed across 3 pH treatments of 8.0, 7.5, and 7.6 in Experiment 1, and 2 pH treatments of 8.0 and 7.5 in Experiment 2 (Table 1). Each pH treatment consisted of 4 replicate 800-mL jars each containing 150 larvae (Experiment 1) or 4 replicate 400-mL jars each containing 20 larvae (Experiment 2). Larvae were fed 15×10^4 cells mL^{-1} of Tahitian *Isochrysis galbana* (T-ISO), a diet that supports maximal growth rates in larvae of *C. fornicata* (*e.g.*, Pechenik and Tyrell, 2015). T-ISO was concentrated by centrifugation and resuspended in filtered sea-

Table 1

Summary of experiments on Crepidula fornicata

Experiment no.	Population	Larval pH and nutrition	Larval measurements	Juvenile pH and nutrition	Age at metamorphosis (d post-hatch)	Shell length at metamorphosis (μm ± SD)	Juvenile measurement
1	Totten Inlet, WA	8.0 HF 7.6 HF 7.5 HF	Shell growth Survivorship	–	–	–	–
2	Totten Inlet, WA	8.0 HF 7.5 HF	Shell growth Survivorship	8.0 HF 7.5 HF	12	834 ± 104	Shell growth
3	Buzzards Bay, MA	8.0 HF 8.0 LF 7.6 HF 7.6 LF	Shell growth	8.0 HF 8.0 LF 7.6 HF 7.6 LF	19	1221 ± 87	Shell growth
4	Buzzards Bay, MA	8.0 HF 8.0 LF 7.6 HF 7.6 LF	Shell growth Metamorphosis	8.0 HF	14 (8.0 HF) 18 (7.6 HF) 22 (7.6LF, 8.0LF)	1097 ± 111	Shell growth

The four experiments performed during this study are described according to the geographic populations of *C. fornicata* used, the life-history stages examined, the culturing conditions applied to these stages, and what organismal processes were examined during each stage. HF, high food; LF, low food. Each juvenile growth condition included animals derived from all larval conditions in that experiment, except that in Experiment 3, only HF larvae were carried forward as juveniles.

water before addition to larval cultures, to minimize the transfer of algal growth medium. Food and culture seawater were changed every two days in Experiment 1 and daily in Experiment 2.

Two experiments (Experiments 3 and 4) were carried out at Dickinson College in Carlisle, Pennsylvania. For these experiments, adult *C. fornicata* were collected by the Marine Biological Laboratory Marine Resources Center in Woods Hole, Massachusetts, and shipped to Dickinson College during late winter. Adults were fed Shellfish Diet 1800 (Reed Mariculture, Campbell, CA) twice daily and maintained on a 14h:10h light:dark photoperiod in seawater that was bubbled with ambient air and replaced every other day. Adult incubation temperature began at 7 °C and was increased 1 °C d^{-1} until it reached 20 °C, in order to stimulate oviposition (Pires, 2014). Upon release from brooding adults, larvae were collected as described above and distributed across larval pH and nutrition treatments, each represented by 4 replicate 800-mL culture jars each containing 150 larvae. Larval culture jars were emptied every 2 days and refilled with seawater of pH 8.0 or 7.6. High or low concentrations of T-ISO algae were added to cultures at each water change. High food (HF) cultures contained 15×10^4 cells mL^{-1}. Low food (LF) cultures contained 1×10^4 cells mL^{-1} (*e.g.*, Pechenik and Tyrell, 2015). Both of these experiments included all four combinations of larval pH and nutrition (8.0HF, 8.0LF, 7.6HF, and 7.6LF treatments) to enable the observation of interactive effects between larval pH and nutrition on larval and juvenile development (Table 1). Larval cultures were maintained at 20 °C on a 14h:10h photoperiod. Seawater conditions were recorded as during Experiments 1 and 2.

Seawater pH manipulation and carbonate chemistry

In two experiments (Experiments 1 and 2), seawater conditioning and carbonate chemical analyses were carried out in the Ocean Acidification Environmental Laboratory at Friday Harbor Laboratories. For Experiment 1, incoming seawater was filtered at 1 μm and equilibrated overnight at 20 °C by bubbling with ambient air (for pH 8.0) or with mixtures of CO_2 and CO_2-free air delivered by Aalborg GFC17 mass-flow controllers (Orangeburg, NY; for pH 7.5 and pH 7.6). For Experiment 2, seawater pH was dynamically conditioned at 20 °C by a feedback system that utilized a Honeywell Durafet pH electrode and Honeywell UDA2182 process controller (Charlotte, NC) to regulate injection of CO_2 and CO_2-free air to stabilize seawater pH at set points of 7.5 or 8.0 (O'Donnell *et al.*, 2013). In both Experiments 1 and 2, seawater pH was measured immediately before loading into culture jars with a Honeywell Durafet pH electrode calibrated to the total scale by the cresol purple spectrophotometric method described by Dickson *et al.*, (2007). The pH in culture jars was also measured when they were opened for water changes. In Experiment 1, headspaces of culture jars were continuously ventilated with the same gas mixtures used to condition the seawater pH treatments during the two-day intervals between culture water changes. In Experiment 2, culture jars were sealed without ventilation, and water was changed daily. Temperature and salinity

were measured with a YSI Pro Series 1030 meter (Yellow Springs, OH).

Additional studies (Experiments 3 and 4) were conducted at Dickinson College. For these experiments, seawater was collected at Buzzards Bay, Massachusetts, and filtered at 1 μm. Seawater pH was conditioned by overnight equilibration with ambient air (for pH 8.0) or with a mixture of ambient air and CO_2 delivered by Aalborg GFC17 mass-flow controllers (for pH 7.6). These gas mixtures were used to ventilate culture jar headspaces as in Experiment 1. Seawater pH was measured before and after culture water changes at two-day intervals as described for Experiment 1, but with a YSI Pro Series 1030 meter calibrated to National Institute of Standards and Technology standards. The pH measurements were converted to total scale by recalibration of the YSI glass electrode with the cresol purple method by Dickson *et al.*, (2007). Temperature and salinity were measured as in Experiments 1 and 2.

In all experiments, seawater samples representing each treatment were fixed with mercuric chloride and were titrated to determine total alkalinity (TA) using a Mettler-Toledo DL15 automated titrator (Columbus, OH) calibrated to certified reference materials (Dickson laboratory, Scripps Institution of Oceanography). In Experiments 1–3, pCO_2 and Ω_{Ar} (saturation state of aragonite) were estimated based on empirical measurements of pH and TA by using CO2Sys 2.1 (Pierrot *et al.*, 2006). In Experiment 4, pCO_2 was also measured empirically by using an infrared CO_2 meter (CO2Meter CM-0001, Ormond Beach, FL) calibrated to an ISO-certified 1000 ppm CO_2 standard

Table 2

Physical seawater data recorded during Experiments 1–4 throughout larval and juvenile rearing of Crepidula fornicata

Experiment no.	Nominal pH (total)	Value	Actual pH (total)	Salinity (ppt)	Temperature (°C)	TA (μmol kg^{-1})	Ω_{Ar}	pCO_2 (μatm)	HF pH at change (total)	LF pH at change (total)
1	8.0	Mean	7.95	29.64	19.68	2097.3	1.98	490.1	7.92	–
		SD	0.02	0.15	0.66	4.3	0.10	38.3	0.03	–
		n	5	5	5	3	3	3	4	–
	7.6	Mean	7.63	29.86	19.88	2105.0	1.06	1103.5	7.61	–
		SD	0.03	0.15	0.13	7.7	0.10	100.3	0.03	–
		n	5	5	5	3	3	3	4	–
	7.5	Mean	7.54	30.00	20.06	2115.3	0.89	1393.1	7.54	–
		SD	0.02	0.20	0.30	14.6	0.07	77.7	0.03	–
		n	5	5	5	3	3	3	4	–
2	8.0	Mean	7.95	30.11	20.04	2104.6	2.04	524.2	7.88	–
		SD	0.03	0.25	0.39	6.5	0.22	69.5	0.03	–
		n	22	22	22	6	6	6	18	–
	7.5	Mean	7.50	30.09	20.14	2108.4	0.85	1527.3	7.51	–
		SD	0.04	0.26	0.51	12.0	0.07	87.5	0.07	–
		n	22	22	22	6	6	6	18	–
3	8.0	Mean	8.07	30.97	19.12	2263.7	2.63	402.9	7.98	8.03
		SD	0.04	0.19	0.57	110.7	0.16	56.7	0.04	0.05
		n	16	16	16	8	8	8	13	13
	7.6	Mean	7.63	30.89	19.45	2255.7	1.13	1196.9	7.55	7.58
		SD	0.03	0.35	0.75	125.8	0.06	129.2	0.04	0.05
		n	15	15	15	7	7	7	12	12
4	8.0	Mean	7.99	31.36	19.81	2180.8	2.29	435.3	8.15	8.05
		SD	0.02	0.49	0.27	68.8	0.21	23.3	0.10	0.09
		n	11	12	12	3	11	10	10	10
	7.6	Mean	7.59	31.48	20.06	2210.3	1.13	1425.2	7.78	7.59
		SD	0.03	0.26	0.39	17.5	0.13	36.4	0.09	0.05
		n	10	11	11	2	10	10	10	10

Values of pH are reported (from left) as the nominal treatment target values, the actual values for new seawater added to cultures, and the values recorded in high food (HF) and low food (LF) cultures immediately before regular seawater changes. Ω_{Ar}, saturation state of aragonite; TA, total alkalinity.

(Gasco, Oldsmar, FL). The chemical and physical properties of seawater used in all experiments are given in Table 2.

Induction of metamorphosis

In Experiments 2–4, larval competence for metamorphosis was assayed twice per replicate before whole cultures were exposed to a metamorphic inducer. In each assay, 10 larvae per replicate were transferred to 8 mL of seawater conditioned to the larval rearing pH containing 20 mmol L^{-1} (Experiments 2 and 3) or 15 mmol L^{-1} (Experiment 4) elevated KCl (Pechenik and Gee, 1993). Larvae were incubated at 20 °C in 6-well plates inside plastic boxes ventilated with the same gas mixtures used to condition seawater pH. In Experiment 2, larvae used to measure competence for metamorphosis were taken from auxiliary cultures of pH 8.0 and pH 7.6 containing 150 larvae each, so as not to deplete the sample size of larval cultures used for measuring larval growth rates. These auxiliary cultures were reared in tandem with replicates in which larval growth was measured, and they were treated with the same conditioned seawater. The frequency of metamorphosis per replicate was measured by counting the proportion of larvae that had shed their velum after 6 and 24 h of exposure to elevated KCl. Larvae tested for competence were not returned to culture. When larvae in each treatment group achieved ≥80% metamorphosis after 24 h of exposure to KCl, the remaining larvae in that treatment were exposed to KCl to produce juveniles. Metamorphosis assays performed in Experiments 2 and 3 functioned solely to determine appropriate times to produce juveniles rather than to assess the effect of larval pH or nutrition on competence, as was done in Experiment 4 by using more frequent assays.

Competence for metamorphosis was analyzed in Experiment 4 by using a full-factorial general linear model in which larval pH, larval nutrition, age at induction, and time post-induction were categorized as nominal predictor variables (Table A1). These variables were deemed nominal because of the binary nature of their treatment levels. *P*-values corresponding to individual tests within the whole-model ANOVA were adjusted to eliminate false discoveries, using Benjamini-Hochberg false discovery rate (FDR) *P*-value adjustments. Significant differences in frequencies of metamorphosis between treatment levels were evaluated using Student's *t* tests.

Juvenile culture

During Experiment 2, newly metamorphosed juveniles derived from pH 8.0 and pH 7.5 larval cultures were evenly distributed across pH 8.0 and pH 7.5 juvenile culture treatments. Juvenile treatment groups included 12 individuals, with each individual serving as a replicate. Juveniles were individually housed in 50-mL glass jars with transparent lids containing 50 mL of pH-conditioned seawater. Seawater inside juvenile jars was replaced each day with seawater of the appropriate pH and with 15×10^4 cells T-ISO mL^{-1}. Seawater conditions were recorded as they were during larval culture. Juvenile cultures were incubated in a 20 °C bath and were exposed to a 12h:12h photoperiod. Juvenile growth extended for eight days.

In Experiment 3, juveniles derived from 8.0HF and 7.6HF larval treatments were evenly distributed across 4 juvenile treatments: 8.0HF, 8.0LF, 7.6HF, and 7.6LF. Juveniles derived from 8.0LF and 7.6LF larval treatments were not included because few of those larvae became competent for metamorphosis in the time available for this experiment. Experiment 4 included juveniles derived from all 4 larval treatments, but these juveniles were all cultured at pH 8.0 with HF (Table 1). Each juvenile treatment included 12 (Experiment 3) or 10 (Experiment 4) replicate individuals. These animals were individually housed in 50-mL plastic cups containing 40 mL of pH-conditioned seawater. Juvenile culture dishes of the same pH were grouped in plastic boxes ventilated with the appropriate gas mixture and were maintained at 20 °C on a 14h:10h photoperiod. Seawater and food were replaced every two days. Juvenile HF and LF concentrations were the same as those used in larval cultures. Juvenile growth extended for 8 d in Experiment 2, 14 d in Experiment 3, and 12 d in Experiment 4.

Determination of growth rates and survivorship

Growth rates of larvae and juveniles of *C. fornicata* were measured as the change of shell length over time. Individuals were nondestructively imaged using a Motic camera (Kowloon, Hong Kong) fitted to a Leica Wild M3C dissecting microscope (Leica Microsystems, Buffalo Grove, IL) and then returned to culture. Shell lengths were measured offline in ImageJ (Schneider *et al.*, 2012). Larvae were measured on their hatching day and then at four-day intervals. All individuals ($n = 20$) in each replicate culture were measured in Experiment 2. Larvae were blindly subsampled for measurement from the larger cultures used in Experiments 1, 3, and 4 ($n = 20$, 15, and 10 individuals per replicate culture, respectively). Each juvenile treatment group in Experiments 2–4 consisted of individually cultured animals, all of which were measured on the day of metamorphosis and again at three- to four-day intervals until the experiments concluded.

The survivorship of larvae was measured in Experiments 1 and 2 by counting the numbers of living larvae and empty shells within replicate cultures over time. Larval survivorship was analyzed using one-way ANOVAs with measurements that were made 10 d post-hatch. Student's *t* tests were used to perform *post hoc* comparisons of mean survivorship between treatments. All juveniles survived the duration of their experimental growth periods (Experiments 2–4); and, therefore, juvenile survivorship data are not presented. However, some juveniles developed significant epibiont growth on top of their shells during Experiment 3. This epibiont growth had the potential to obstruct movement and feeding. Four of 96 ju-

veniles were removed from culture before the completion of Experiment 3. No more than one juvenile was removed from any given treatment group.

The effects of pH and nutrition on larval shell growth were assessed throughout larval development using one-way or two-way ANOVAs to analyze shell lengths recorded at each date of measurement. Significant differences in mean shell length across treatment groups were evaluated using Student's *t* tests (Tables A2–A4).

The effects of pH and nutrition on juvenile shell growth were examined using repeated-measures analyses derived from linear mixed models. These models included the identity of each juvenile replicate as a random effect in order to account for temporal pseudoreplication. In all experiments, covariance of the "replicate identity" effect was equal to zero (Wald *P*-value = 0.0001–0.0059). Unlike larval growth data, juvenile growth measurements included sufficient replication for including a random effect within the model. For this reason, a linear mixed model was selected to analyze juvenile growth but not larval growth. Linear mixed-model parameters and results can be found in Tables A5–A7.

Results

Effects of pH and nutrition on larval performance

After 8 days of growth, larvae reared at pH 7.5 under HF began to show reduced shell lengths relative to larvae reared at pH 7.6 or pH 8.0. The shells of pH 7.5 larvae were 17% and 14% shorter than those from pH 7.6 and pH 8.0 cultures, respectively, in Experiment 1 ($P = 0.001$; Fig. 1A; Table A2) and 11% smaller than those of pH 8.0 larvae in Experiment 2 ($P = 0.015$; Fig. 1B).

Larval shell growth was less affected by rearing at pH 7.6. In Experiment 1, larvae from pH 7.6 and pH 8.0 cultures grew at the same rate during the first 8 d post-hatch (Fig. 1A). In Experiment 3, larvae reared at pH 7.6 and pH 8.0 grew at equal rates when fed LF (Fig. 1C; Table A3). When fed HF, however, larvae cultured at pH 7.6 were smaller than those cultured at pH 8.0 at 4 d ($P < 0.0001$) and 8 d ($P = 0.0064$) after hatching. By 12 d post-hatch, however, pH 7.6 larvae recovered their growth such that their shell lengths were equivalent to those cultured at pH 8.0 (Fig. 1C). Similarly, pH 8.0 and pH 7.6 larvae in Experiment 4 that were fed HF achieved equivalent shell lengths at 20 d post-hatch (Fig. 1D; Table A4). Larvae fed HF consistently grew faster than larvae fed LF at each pH level in both Experiment 3 (Table A3) and Experiment 4 (Table A3). Nutritional stress and pH stress did not appear to interactively influence the larval shell growth of *Crepidula fornicata* (Fig. 1C, D).

By 10 d post-hatch, larvae reared during Experiment 1 achieved a mean survivorship of 85.4%, and larvae reared during Experiment 2 achieved a mean survivorship of 83.6%. There was a high degree of variability in survivorship across pH treatments and experiments. Unexpectedly, in Experiment 1, larvae reared at pH 8.0 achieved 77% survivorship by 10 d post-hatch, while those reared at pH 7.6 achieved 91% and those reared at pH 7.5 achieved 88% ($F_{2, 11} = 8.28$, $P = 0.0091$). In Experiment 2, 93% of pH 8.0 larvae survived by 10 d post-hatch while 72% of pH 7.5 larvae survived ($F_{1, 5} = 6.87$, $P = 0.0471$), demonstrating a coupling of decreased growth rate and survivorship at pH 7.5.

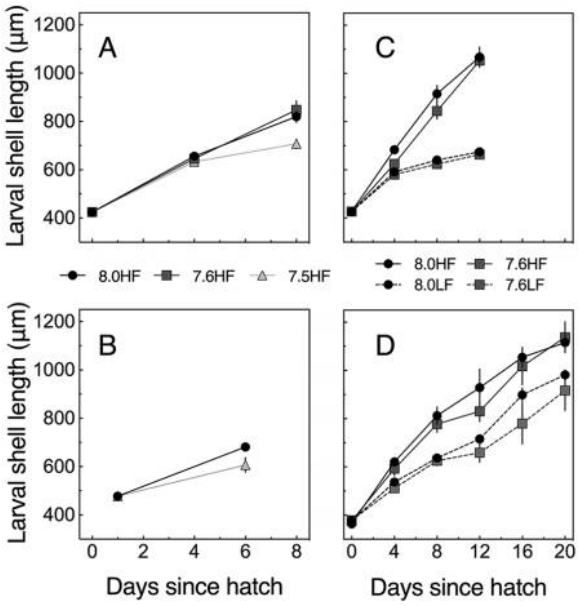

Figure 1. Effects of seawater pH and nutrition on larval shell growth in *Crepidula fornicata*. All experiments were performed with independent broods of larvae derived from different adult broodstock. (A) Experiment 1: effects of seawater pH (7.5, 7.6, 8.0). (B) Experiment 2: effects of seawater pH (7.5, 8.0). (C) Experiment 3: combined effects of seawater pH (7.6, 8.0) and nutrition (HF, high food; LF, low food). (D) Experiment 4: combined effects of seawater pH (7.6, 8.0) and nutrition (HF, LF). In all experiments, mean shell lengths were derived from four replicate cultures per treatment. Error bars represent ±SD.

Since fewer pH 8.0 larvae survived culture relative to pH 7.6 and pH 7.5 treatments during Experiment 1, it is possible that pH 8.0 larvae experienced an unknown stressor in that experiment. Such a stressor could have decreased pH 8.0 growth rates and contributed to the similarity in growth between pH 8.0 and pH 7.6 cultures during Experiment 1. However, 15% more pH 8.0 larvae survived by 10 d post-hatch in Experiment 2 compared to Experiment 1 while pH 8.0 larvae from Experiment 1 grew 18% faster than pH 8.0 larvae reared in Experiment 2. This demonstrated that survivorship and growth rate were not coupled among pH 8.0 experimental treatments.

Synergistic effects of pH and nutrition on competence for metamorphosis

Nutrition, pH, and larval age interactively affected the acquisition of competence for metamorphosis by larvae of *C. fornicata* ($F_{1, 49} = 19.9$, FDR-adjusted $P = 0.0001$). At

12 d post-hatch, 55% of 8.0HF larvae metamorphosed 6 h after induction, and 95% metamorphosed after 24 h. The 7.6HF cultures were less competent by 12 d post-hatch, with only 23% of larvae metamorphosing within 6 h after induction and only 68% after 24 h. The 12-d-old LF cultures did not exhibit any metamorphosis 6 h after induction and metamorphosed at frequencies of 10% in 8.0LF and 18% in 7.6LF after 24 h (Fig. 2).

By 16 days of age, 8.0HF, 7.6HF, and 8.0LF larval cultures showed comparable frequencies of metamorphosis 6 and 24 h after they were induced to metamorphose. However, few larvae experiencing both low pH and low nutrition became competent by this age. Larvae reared at 7.6LF metamorphosed at a lower frequency than those reared at 8.0HF, 7.6HF, or 8.0LF after both 6 and 24 h of KCl exposure ($P < 0.0001$; Fig. 2).

Consequences of larval pH and nutritional stress for juvenile growth

Larval exposure to low pH influenced juvenile growth rates in three experiments (Experiments 2, 3, and 4; Fig. 3). In Experiment 2, juveniles derived from larvae that had been reared at pH 7.5 grew 33% more slowly than those derived from larvae reared at pH 8.0 ($F = 6.07$, $P = 0.0166$) when juveniles were cultured at pH 8.0 (Fig. 3A; Table A5). Thus, these juveniles incurred a carryover effect of larval acidification experience. Larval carryover effects were not apparent in juvenile *C. fornicata* reared at pH 7.5 because these cultures stopped growing 3 d post-metamorphosis regardless of their larval pH condition. Juvenile growth rates were also diminished by a main effect of juvenile pH ($F = 126.72$, $P < 0.0001$). *Crepidula fornicata* reared at pH 7.5 as juveniles grew 82% more slowly than pH 8.0 juveniles when individuals were derived from pH 8.0 larval cultures. Among juveniles derived from pH 7.5 larval cultures, juveniles reared at pH 7.5 grew 63% more slowly than pH 8.0 juveniles (Fig. 3A).

In Experiment 3, *C. fornicata* reared at pH 7.6 as juveniles grew at a similar rate to pH 8.0 juveniles. However, larval

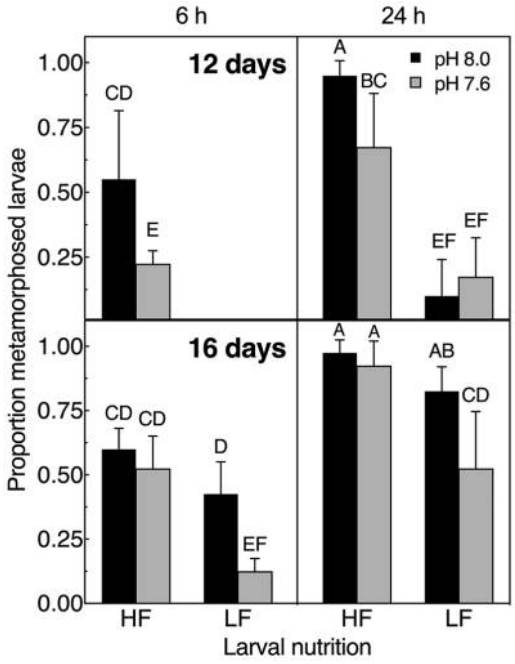

Figure 2. Experiment 4: interactive effects of pH (7.6, 8.0) and nutrition (HF, high food; LF, low food) on competence for metamorphosis in *Crepidula fornicata*. Means are derived from 4 replicate cultures per treatment; $n = 10$ larvae per replicate. Error bars represent ±SD. Values corresponding to different letters have significantly different means as determined by Student's *t* tests. Rows are separated by larval age. Columns are separated by time post-induction.

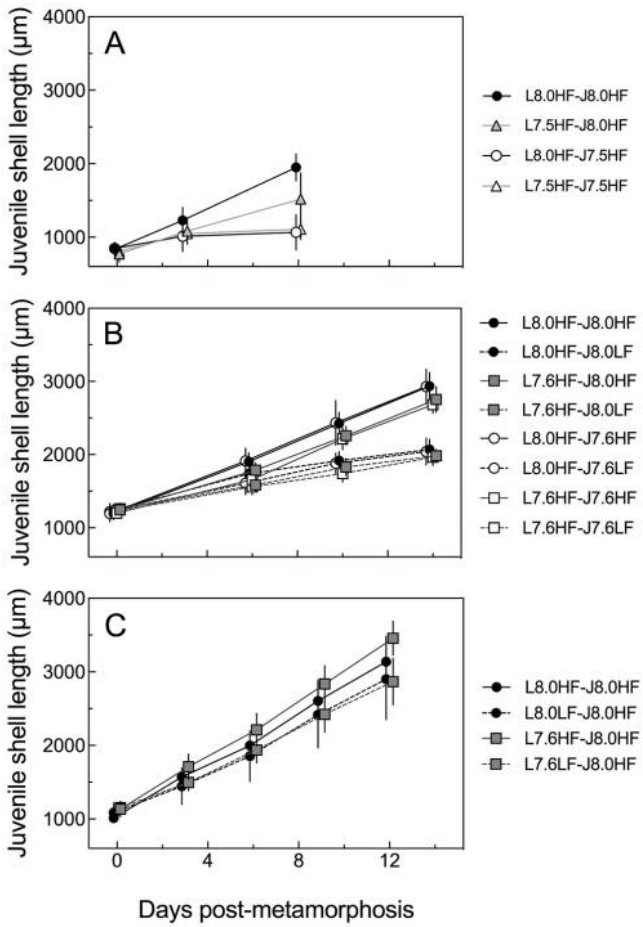

Figure 3. Effects of nutrition and pH conditioning during larval and juvenile stages on the juvenile shell growth of *Crepidula fornicata*. All experiments were performed with independent broods of larvae derived from different adult broodstock. Error bars represent ±SD. Values measured on the same day have been minimally pushed left or right of their correct position to reduce overlap of point symbols. (A) Experiment 2: effects of larval pH (L7.5, L8.0) and juvenile pH (J7.5, J8.0) on juvenile shell growth. (B) Experiment 3: combined effects of larval pH (L7.6, L8.0), juvenile pH (J7.6, J8.0), and juvenile nutrition (HF, high food; LF, low food) on juvenile shell growth. (C) Experiment 4: combined effects of larval pH (L7.6, L8.0) and larval nutrition (HF, LF) on juvenile shell growth.

culture at pH 7.6 exerted a latent effect on juvenile development that reduced post-metamorphic growth rates by 14% in juveniles fed HF and 12% in LF juveniles ($F = 29.11$, $P < 0.0001$; Fig. 3B; Table A6). Larval exposure to pH 7.6 influenced juvenile growth *via* a latent effect rather than a carryover effect, because the larvae cultured at pH 7.6 had achieved growth equivalent to their siblings cultured at pH 8.0 before they were induced to metamorphose (Fig. 1C). Moreover, the effect of larval pH on juvenile growth was influenced by juvenile diet ($F = 5.05$, $P = 0.0255$). The latent effect of larval pH 7.6 exposure was more evident among juvenile *C. fornicata* fed HF than among those fed LF (Fig. 3B).

In Experiment 4, juvenile *C. fornicata* that were fed LF as larvae grew more slowly than juveniles derived from HF larval cultures ($F = 43.86$, $P < 0.0001$; Fig. 3C; Table A7), reflecting a carryover effect similar to that caused by larval conditioning to pH 7.5. However, larval nutritional stress caused a greater carryover effect among juveniles derived from pH 7.6 larval cultures than among those derived from pH 8.0 larval cultures ($F = 5.40$, $P = 0.0214$). When larvae were reared at pH 7.6, juveniles from LF larval cultures grew 25% slower than their HF counterparts. However, when larvae were reared at pH 8.0, juveniles from LF larval cultures grew only 13% slower than their HF counterparts (Fig. 3C). Thus, the carryover effect of poor larval diet on juvenile growth was exacerbated by larval pH stress, but the direct effects of these two stressors did not interactively influence larval development. In Experiment 4, unlike in Experiments 2 and 3, larval exposure to low pH alone did not exert a negative effect on juvenile shell growth.

Discussion

Larval and juvenile growth of Crepidula fornicata *only declined past a pH tipping point*

The larval shell growth of *Crepidula fornicata* responded non-linearly to decreases in pH. Only after culture conditions crossed a pH threshold between 7.6 and 7.5 did mean larval growth rates significantly decrease; larval shell growth was unaffected by low-pH conditioning at pH 7.6 (Fig. 1A, C). However, larvae reared at pH 7.5 grew significantly slower than those maintained at pH 8.0 or pH 7.6 (Fig. 1A, B). Our observations on the growth of *C. fornicata* under pH 8.0, pH 7.6, and pH 7.5 are consistent with those of Kriefall *et al.* (2018), who also demonstrated subtle yet divergent transcriptomic responses of *C. fornicata* larvae to pH 7.6 and pH 7.5 conditioning, and greater transcriptional variation between pH 8.0 larvae and those in both low pH treatments. A pH tipping point was also apparent during juvenile culture. *Crepidula fornicata* did not experience reduced growth at pH 7.6, relative to pH 8.0, during its juvenile stage (Fig. 3B). Like larvae, juveniles reared at pH 7.5 grew significantly slower than those reared at pH 8.0 (Fig. 3A). Multiple experiments have thus demonstrated that *C. fornicata* is resilient to decreased pH, incurring only minor mortality and sublethal growth effects at pH 7.5 during its larval and juvenile stages.

Non-linear calcification responses to increased pCO_2 or decreased pH are common among marine calcifiers (Doney *et al.*, 2009; Ries *et al.*, 2009, 2010; Waldbusser *et al.*, 2015a). Such thresholds are functions of calcium carbonate undersaturation (Doney *et al.*, 2009) or proton flux limitation (Jokiel *et al.*, 2011b), because saturation states of calcium carbonate and $[H^+]$ are tightly coupled (Cyronak *et al.*, 2015). Indeed, total pH and estimated Ω_{Ar} were correlated across experiments. The results of Experiment 1 show that larval growth began to decline between a mean pH of 7.63–7.54 and a Ω_{Ar} range of 1.06–0.89, within which aragonite began to undersaturate (Table 2). The parameter Ω_{Ar} is of interest here because aragonite is the major form of $CaCO_3$ that precipitates during calcification in larval and juvenile marine molluscs, including *C. fornicata* (Eyster, 1986; Falini *et al.*, 1996; Weiss *et al.*, 2002; Ries *et al.*, 2009). The decline of larval shell growth below pH 7.6 may be partially attributed to accelerated shell dissolution caused by an undersaturation of aragonite in pH 7.5 culture conditions. Likewise, Noisette *et al.* (2016) reported that net calcification rates in adult *C. fornicata* became negative at a mean pH near 7.5 in a temperature-dependent manner that was linked to Ω_{Ar}.

Larval pH stress persisted after metamorphosis as a latent or carryover effect

Rearing larvae at pH 7.5 or pH 7.6 resulted in markedly reduced rates of juvenile growth even when juveniles were reared at pH 8.0. Larval conditioning at pH 7.5 directly reduced larval growth and indirectly depressed juvenile growth after metamorphosis, thus persisting as a carryover effect (Figs. 1B, 3A). It is possible that the strength of the carryover effect of pH 7.5 conditioning was reduced by the increased mortality incurred by pH 7.5 larvae. Larvae that died during pH 7.5 exposure would likely have shown even poorer performance as juveniles had they survived through metamorphosis. Our finding that the effect of pH on growth persists across metamorphosis in *C. fornicata* as a larval carryover effect is consistent with past reports on larva-to-juvenile transitions in bivalves (Hettinger *et al.*, 2012, 2013) and echinoids (Dupont *et al.*, 2013), as well as embryo-to-larva transitions in crustaceans (Long *et al.*, 2016).

Acidification stress endured by one life-history stage has not previously been shown to affect the performance of a later life-history stage (i) when direct effects of pH are absent in both stages, or (ii) when the first stage is able to recover from acidification stress before transition to the next, as we have shown in the present work. Larvae reared at pH 7.6 recovered to pH 8.0-equivalent shell lengths before metamorphosis (Fig. 1C, D); yet juveniles that experienced pH 7.6 as larvae grew significantly more slowly than juveniles reared at

pH 8.0 as larvae, regardless of juvenile pH conditions in Experiment 3 (Fig. 3B). A latent effect caused by rearing at pH 7.6 was absent among Experiment 4 juveniles, demonstrating that trans-life-cycle effects of pH conditioning can vary across broods of *C. fornicata*. Because larval mortality was not measured in Experiments 3 and 4, it is also possible that the absence of a pH latent effect in Experiment 4 was the result of positive selection rather than inter-brood plasticity. Many taxa have been deemed resilient to OA after showing no declines in calcification or shell growth during examinations of single life-history stages (Miller *et al.*, 2009; Gazeau *et al.*, 2011; Talmage and Gobler, 2011). *Crepidula fornicata* has shown resilience to direct effects of OA during single life-history stages (Noisette *et al.*, 2015, 2016; Kriefall *et al.*, 2018), but latent pH effects from larval exposure to OA can result in additional reductions in performance.

Interactive effects of larval nutrition and larval pH before and after metamorphosis

The potential for adequate nutrition to offset physiological effects of OA has been demonstrated in multiple marine calcifiers, diet types, and life-history stages (Hettinger *et al.*, 2013b; Sanders *et al.*, 2013; Thomsen *et al.*, 2013; Pansch *et al.*, 2014; Ramajo *et al.*, 2015, 2016). Optimal nutrition did not lessen the direct effect of low pH on larval shell development in *C. fornicata* (Fig. 1C, D). Rather, low larval pH amplified negative effects of larval nutritional stress on competence for metamorphosis and juvenile shell growth.

The development and shell structure of larvae of the brooding oyster *Ostrea angasi* responded similarly to interactions between pCO_2 and nutrition; delayed eye development and increases in shell abnormalities in larvae with a low food supply only arose under high pCO_2 (Cole *et al.*, 2016). There are numerous explanations for this interaction. Mechanistically, pH stress likely impairs response pathways to nutritional deprivation that would otherwise maintain performance (Sokolova, 2013). In an energy budgeting framework, *C. fornicata* and *O. angasi* may be able to invest optimally in shell development despite nutritional stress, but that budgeted investment becomes insufficient when low pH increases energetic demands for shell development. Additionally, OA can delay the initiation of feeding in planktotrophic molluscan larvae (Gray *et al.*, 2017) and can decrease larval clearance rates (Cole *et al.*, 2016), further decreasing access to nutrition.

For juvenile *C. fornicata*, investment in shell development during low nutrition and low pH arises after the additional energetic cost of metamorphosis. A similar phenomenon (*i.e.*, interactions among larval stressors arising only after metamorphosis) was found in the congeneric species *Crepidula onyx* following larval exposure to low nutrition and hypoxic stress: larval exposure to hypoxia reduced juvenile growth, but only when larvae had poor access to food (Chiu *et al.*, 2007). In cases such as this, multiple stressors appear to dynamically interact across development. This interaction is a crucial yet overlooked element of the net effect of OA on the performance and fitness of calcifying invertebrates.

The responses of growth and metamorphosis by *C. fornicata* to low pH and nutrition contrast with responses by its congener, *C. onyx*, to similar stressors (Maboloc and Chan, 2017). Larvae of *C. onyx* exhibited only minor reductions in shell length at the time of competence when reared at pH 7.38. Furthermore, *C. onyx* settled earlier, rather than later, when it was reared at low pH and fed a nutritionally poor diet (Maboloc and Chan, 2017). The differing responses between *Crepidula* species suggest differences in physiology and developmental strategy during exposure to singular and multiple stressors of OA and nutritional stress. Both species have become globally distributed across diverse environments (Blanchard, 1997; Xu, 2006) but are native to regions where low-pH events occur at distinctly different frequencies. *Crepidula onyx* is native to the Pacific Coast between Southern California and Baja, Mexico (Coe, 1935), where upwelling events reduce pH at a greater frequency and severity than in the North Atlantic, the native habitat of *C. fornicata* (Zaytsev *et al.*, 2003; Feely *et al.*, 2008). Reduction of the pre-competent phase by *C. onyx* incurring low pH and poor nutritional quality may be an adaptive response derived from its native upwelling zones. *Crepidula fornicata* has been shown to extend its pre-competent phase in response to some environmental stressors (Pechenik *et al.*, 1996b), and the delay of metamorphosis by *C. fornicata* incurring low pH and low food access indicates a developmental response to reduced pH that is distinct from that of *C. onyx*. The costs and advantages of delaying or accelerating competence in response to low pH can be comparatively examined among *Crepidula* species to better understand interactions between developmental timing and OA as they relate to performance.

Our findings suggest that the nature of interactions between multiple stressors can vary across life history and persist as trans-life-cycle effects as they are punctuated by developmental events such as metamorphosis. To accurately assess the performance of calcifying invertebrates under predicted future pCO_2 scenarios, a comprehensive framework that examines the relationship between multiple stressors and the legacy of those stressors should be adopted. Expanding multiple stressor studies toward *in situ* observations of environmental mosaics has proven to be a crucial step in documenting population-level consequences of OA in marine organisms (Kroeker *et al.*, 2013, 2016), but incorporating the legacy of stressors in field experiments is a challenging undertaking. Fortunately, trans-life-cycle effects of environmental stress have been successfully recorded *in situ* (Marshall *et al.*, 2003; Ng and Keough, 2003; Emlet and Sadro, 2006; Chiu *et al.*, 2007; Thiyagarajan *et al.*, 2007), including carryover effects of low pH (Hettinger *et al.*, 2013). There is a growing understanding of how biotic and abiotic factors interact across life history and how they may thus impact marine calcifiers in present and future environments.

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Appendix

Table A1

Experiment 4: general linear model parameters and results for mean frequencies of larval metamorphosis of Crepidula fornicata

Fixed effect	df (test, error)	*F*-ratio	FDR *P*-value	Sum of squares
Whole model	14, 49	29.78	<**0.0001**	6.94
Lack of fit	1, 48	0.04	0.8487	0.01
pH	1, 49	23.01	<**0.0001**	0.39
Nutrition	1, 49	155.52	<**0.0001**	2.64
Time post-induction	1, 49	107.34	<**0.0001**	1.82
Age	1, 49	74.53	<**0.0001**	1.27
pH × nutrition	1, 49	0.59	0.5625	0.01
pH × time post-induction	1, 49	0.33	0.6576	0.01
pH × age	1, 49	0.59	0.5625	0.01
Nutrition × time post-induction	1, 49	4.45	0.0667	0.08
Nutrition × age	1, 49	14.72	**0.0007**	0.25
Time post-induction × age	1, 49	2.98	0.1360	0.05
pH × nutrition × age	1, 49	19.47	**0.0001**	0.33
pH × nutrition × time post-induction	1, 49	0.00	1	0
pH × age × time post-induction	1, 49	0.14	0.7539	0.01
Nutrition × age × time post-induction	1, 49	5.30	**0.0485**	0.09

Significant effects (Benjamini-Hochberg false discovery rate [FDR]-adjusted $P < 0.05$) are indicated in bold.

Table A2

Experiment 1: multiple one-way ANOVA results assessing the effect of pH on larval shell length of Crepidula fornicata *at different times post-hatch*

			P-value		
Larval age (d)	*F*-ratio	*P*-value	8.0 *vs.* 7.6	8.0 *vs.* 7.5	7.6 *vs.* 7.5
4	4.08	0.0548	na	na	na
8	26.77	**0.0002**	0.1968	**0.0004**	<**0.0001**

Significant differences ($P < 0.05$) demonstrated by Student's *t* tests are indicated in bold. "na" indicates *post hoc* comparisons that could not be made because of lack of a significant corresponding effect.

Table A3

Experiment 3: multiple two-way ANOVA results assessing the effect of pH and nutrition on larval shell length of Crepidula fornicata *at different times post-hatch*

				P-value					
Larval age (d)	Fixed effect	*F*-ratio	*P*-value	8.0HF *vs.* 8.0LF	7.6HF *vs.* 7.6LF	8.0HF *vs.* 7.6LF	8.0LF *vs.* 7.6HF	8.0HF *vs.* 7.6HF	8.0LF *vs.* 7.6LF
	Nutrition	223.71	**<0.0001**		**<0.0001**			–	–
4	pH	59.07	**<0.0001**	–	–		**<0.0001**		
	Interaction	26.61	**0.0002**	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**	**<0.0001**	**0.0992**
	Nutrition	860.40	**<0.0001**		**<0.0001**			–	–
8	pH	0.94	0.0037	–	–		**0.0037**		
	Interaction	0.02	0.0419	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0014**	**0.3717**
	Nutrition	860.40	**<0.0001**		**<0.0001**			–	–
12	pH	0.94	0.3522	–	–		na		
	Interaction	0.02	0.8821	na	na	na	na	na	na

Significant differences ($P < 0.05$) demonstrated by Student's *t* tests are indicated in bold. *P*-values in merged cells that combine high food (HF) *vs.* low food (LF) or pH 8.0 *vs.* pH 7.6 columns correspond to *post hoc* comparisons of means for all HF or LF or pH 8.0 or pH 7.6 data. "na" indicates *post hoc* comparisons that could not be made because of lack of a significant corresponding effect.

Table A4

Experiment 4: multiple two-way ANOVA results assessing the effect of pH and nutrition on larval shell length of Crepidula fornicata *at different times post-hatch*

				P-value					
Larval age (d)	Fixed effect	*F*-ratio	*P*-value	8.0HF *vs.* 8.0LF	7.6HF *vs.* 7.6LF	8.0HF *vs.* 7.6LF	8.0LF *vs.* 7.6HF	8.0HF *vs.* 7.6HF	8.0LF *vs.* 7.6LF
	Nutrition	71.08	**<0.0001**		**<0.0001**			–	–
4	pH	7.51	**0.0179**	–	–		**0.0179**		
	Interaction	0.03	0.8633	na	na	na	na	na	na
	Nutrition	134.3	**<0.0001**		**< 0.0001**			–	–
8	pH	2.7	0.1263	–	–		na		
	Interaction	0.73	0.4101	na	na	na	na	na	na
	Nutrition	61.69	**<0.0001**		**<0.0001**			–	–
12	pH	10.04	**0.0081**	–	–		**0.0081**		
	Interaction	0.72	0.4137	na	na	na	na	na	na
	Nutrition	39.43	**<0.0001**		**<0.0001**			0.8375	0.0816
16	pH	6.19	**0.0285**	–	–		**0.0285**		
	Interaction	1.7	0.2162	na	na	na	na	na	na
	Nutrition	39.91	**<0.0001**		**<0.0001**			–	–
20	pH	0.56	0.4652	–	–		na		
	Interaction	2.40	0.1472	na	na	na	na	na	na

Significant differences ($P < 0.05$) demonstrated by Student's *t* tests are indicated in bold. *P*-values in merged cells that combine high food (HF) *vs.* low food (LF) or pH 8.0 *vs.* pH 7.6 columns correspond to *post hoc* comparisons of means for all HF or LF or pH 8.0 or pH 7.6 data. "na" indicates *post hoc* comparisons that could not be made because of lack of a significant corresponding effect.

Table A5

Experiment 2: linear mixed model assessing juvenile shell length of Crepidula fornicata *as a function of time post-metamorphosis, larval pH, and juvenile pH*

Fixed effect	df (test, error)	*F*-ratio	*P*-value
Days post-metamorphosis	1, 60	368.14	<**0.0001**
Larval pH	1, 28	3.65	0.0665
Days post-metamorphosis × larval pH	1, 60	6.07	**0.0166**
Juvenile pH	1, 28	23.32	<**0.0001**
Days post-metamorphosis × juvenile pH	1, 60	126.72	<**0.0001**
Larval pH × juvenile pH	1, 28	4.80	**0.0369**
Days post-metamorphosis × larval pH × juvenile pH	1, 60	13.47	**0.0005**

The identity of each juvenile culture was included within the model as a random effect. Significant effects ($P < 0.05$) are indicated in bold.

Table A6

Experiment 3: linear mixed model assessing juvenile shell length of Crepidula fornicata *as a function of time post-metamorphosis, larval pH, juvenile nutrition, and juvenile pH*

Fixed effect	df (test, error)	*F*-ratio	*P*-value
Days post-metamorphosis	1, 275	5881.56	<**0.0001**
Larval pH	1, 86	23.37	<**0.0001**
Days post-metamorphosis × larval pH	1, 275	29.11	<**0.0001**
Juvenile food	1, 86	277.74	<**0.0001**
Days post-metamorphosis × juvenile food	1, 275	713.80	<**0.0001**
Larval pH × juvenile food	1, 86	2.60	0.1113
Days post-metamorphosis × larval pH × juvenile food	1, 275	5.05	**0.0255**
Juvenile pH	1, 86	3.57	0.0621
Days post-metamorphosis × juvenile pH	1, 275	0.01	0.9531
Larval pH × juvenile pH	1, 86	0.51	0.4758
Days post-metamorphosis × larval pH × juvenile pH	1, 275	0.03	0.8723
Juvenile food × juvenile pH	1, 86	0.08	0.7725
Days post-metamorphosis × juvenile food × juvenile pH	1, 275	0.01	0.9499
Larval pH × juvenile food × juvenile pH	1, 86	0.99	0.3220

The identity of each juvenile culture was included within the model as a random effect. Significant effects ($P < 0.05$) are indicated in bold.

Table A7

Experiment 4: linear mixed model assessing juvenile shell length of Crepidula fornicata *as a function of time post-metamorphosis, larval pH, and larval nutrition*

Fixed effect	df (test, error)	*F*-ratio	*P*-value
Days post-metamorphosis	1, 156	3949.55	<**0.0001**
Larval food	1, 36	8.98	**0.0049**
Days post-metamorphosis × larval food	1, 156	43.86	<**0.0001**
Larval pH	1, 36	2.94	0.0950
Days post-metamorphosis × larval pH	1, 156	0.51	0.4765
Larval food × larval pH	1, 36	1.49	0.2304
Larval pH × larval food × days post-metamorphosis	1, 156	5.40	**0.0214**

The identity of each juvenile culture was included within the model as a random effect. Significant effects ($P < 0.05$) are indicated in bold.