Effects of dissolved oxygen stress on larval *Haminoea vesicula* survivorship and growth

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Abstract

Dissolved oxygen in coastal marine systems is projected to become more variable and is on the average declining in coastal systems. As surface temperatures and eutrophication continue to increase with climate change and anthropogenic inputs into marine systems, benthic marine invertebrates are expected to experience delayed development and reduced survival to metamorphosis. This study assesses the effect of dissolved oxygen (DO) stress on recently hatched *Haminoea vesicula* for the first 4 days of development as a planktotrophic veliger. Larvae survivorship and growth were compared between a reduced (~4.8 mg/L) and saturated DO (~8 mg/L) treatment. Dissolved oxygen stress did not significantly affect survivorship or any of the shell metrics measured. There was considerable variation in initial shell size (maximum shell dimension) which alone could not explain variance in growth rates within and among replicates and treatments. Given the small sample size, it is possible that limited statistical power precluded detection of an effect of DO on larval growth and survivorship. However, further research is needed to determine whether *Haminoea vesicula*, which experience DO stress during encapsulated embryonic development, are robust to DO stress. Additionally, studies should investigate if early exposure to DO stress post-hatching could have lasting effects via affecting time to competency, success at settlement, and juvenile performance.

Introduction

Oxygen undersaturated conditions may occur naturally or by anthropogenic causes in marine ecosystems. In shallow coastal waters, thermal stratification and nutrient input have been found to lower dissolved oxygen (Pan, Marcoval, Bazzini, Vallina, & De Marco, 2015). Along coastal environments, there is an increasing trend towards lowered dissolved oxygen (DO) water bodies because of riverine nutrients from human activities and ocean warming (Diaz & Rosenberg, 1995; Levin et al., 2009; Rabalais et al., 2010).

This decrease in DO could impact both benthic organisms, as reviewed by Diaz and Rosenberg (1995), and organisms living in the water column (Gray, Wu & Ying, 2002). Among gastropods, the majority of studies have assessed responses, either behavioral, physiological or morphological, of adults and juveniles (Galic, Hawkins, & Forbes, 2019). Effects of hypoxic or low DO conditions have been measured in larvae of a few marine gastropods, but largely within the context of functional constraints on egg mass size and morphology, and early encapsulated development. A study on morphology and swimming of early stages of *Nassarius festivus* (Chan, Xu, Shin, & Cheung, 2008) describes how oxygen undersaturated treatments resulted in delayed embryonic development and a reduction in the percentage of larvae to successfully metamorphose.

The bubble shell snail *Haminoea vesicula*, a cephalaspidean opisthobranch, is a common subtidal gastropod in the San Juan Archipelago, in the Northern Pacific Coast. It is abundant in mudflats and eelgrasses, where it lays ribbon-shaped egg masses on any available hard substrate. Egg masses are densely packed with small, ~90 μm diameter eggs that undergo encapsulated development for 9 days before hatching as a planktotrophic, veliger larva (Strathmann, 1987; Gibson & Chia, 1989). At the time of hatching, veligers have a small shell with one full or nearly fully-formed whorl (Strathmann, 1987).

Given the importance to understand responses of early developmental stages of marine invertebrates to environmental stressors, we investigated the effects of rearing larvae of *H. vesicula* under low DO conditions on shell growth (as a proxy for metabolism) and survivorship.

Methods

Larval background and rearing

Egg masses from adult *Haminoea vesicula* (collected from False Bay, San Juan Island Marine Reserve) were collected from a flow-through culture at Friday Harbor Laboratories. Egg masses were maintained in 1 liter containers until hatching, aerated gently by paddle and maintained at ambient seawater temperature in a water bath receiving nearshore seawater. Recently released veliger larvae of *H. vesicula* from four egg masses were initially pipetted into ten, one-liter jars at a density of 800 larvae per liter for calcein staining. At two days post-hatching (dph), larvae were reduced to a density of 300 larvae/L and allocated into one of two dissolved oxygen treatments: hypoxic (~4.8 mg/L) and saturated DO (~8 mg/L). Larvae were maintained at treatment levels in a seatable array (Figure 1) until 6 dph.

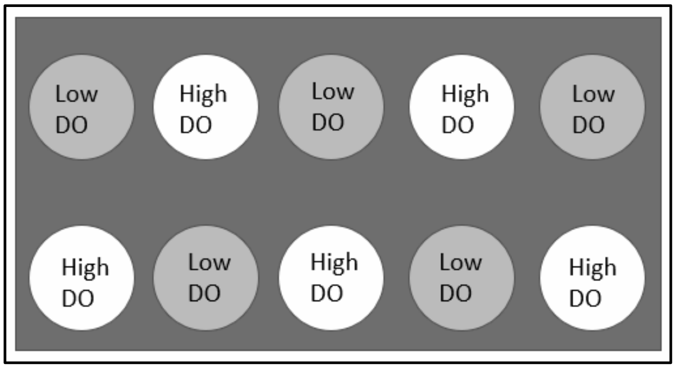

Figure 1. Array of 1L larval cultures (hypoxic/Low DO, n=5; saturated/High DO, n=5) in flow-through seatable at ambient seawater temperature.

Water changes and monitoring

Water changes were performed daily by gently pouring cultures onto a 15 µl mesh filter submerged in filtered seawater. Larvae were transferred into custard dishes using a squirt bottle and then into 1L jars containing either bubbled or deoxygenated 0.45 µl filtered seawater at a total volume of 800mL. Hypoxic treatment water was obtained by deoxygenating 0.45 µl filtered seawater in a side-arm flask and vacuum pump (Welch Director 8915, Welch Vacuum Technology, IL, USA). Dissolved oxygen saturated filtered seawater was obtained by bubbling overnight with ambient air by an airstone and aquarium pump. Temperature and dissolved oxygen were monitored before and after each water change (approximately 2-3 times per day) using a ProODO meter(YSI, OR, USA). Salinity was measured periodically throughout the experiment using a refractometer.

Algal culturing and feeding

Larvae were fed *Isochrysis galbana* daily a concentration of 1x10^4 cells/mL (initial concentration of 2.67x10^4 cells/larva/day). *Isochrysis galbana* was centrifuged and resuspended in 0.45µl filtered seawater before feeding. Algal density was determined by using a hemocytometer.

Calcein staining

Calcein staining was performed at a concentration of 100mg/L, with fed larvae maintained in the stain for the first 24 hours ph. Larvae were thoroughly rinsed with 0.45μl filtered seawater following staining and exposed to treatment conditions at two dph.

Growth

At four days post-hatch, larvae were subsampled from each replicate culture for shell size measurements. Larvae were embedded in modeling clay on microscope slides with the umbilicus positioned down. Images were taken with a fluorescence filter (EX 460-500, DM 505, BA 510-560) to identify individual growth of larvae between day 0 and 6 ph. Measurements were calibrated at 10x and 20x magnification using a stage micrometer. Three measurements were performed for each larva to determine initial shell size, accretion of new shell at the operculum since calcein staining, and maximum shell dimension (Figure 2).

Survivorship

Experimental larval cultures were initiated by manually pipetting individual larva to reach a density of 300 larvae per replicate culture. Larval survivorship was determined at 5 and 7 dph by enumerating surviving larvae prior to water changes.

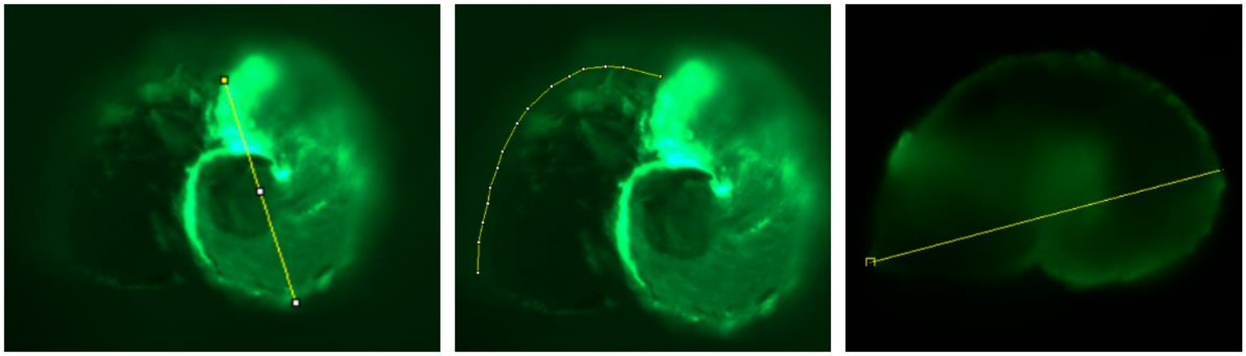

Figure 2. Individual shell measurements: initial maximum shell dimension (left), accretion of new shell at the operculum since calcein staining (middle), and maximum shell dimension (right).

Results

Rearing conditions

Average dissolved oxygen levels of hypoxic and saturated DO treatments ranged between 4.3-5.3 mg/L (Avg. 4.8 mg/L, 56.9%) and 7.9-8.1mg/L (Avg. 8.0 mg/L, 96.9%), respectively (Figure 3). Temperature covaried with external nearshore conditions but covaried between hypoxic (Avg. 14.2°C; 14.1-14.3°C) and saturated (Avg. 15.35°C; 14-16.7°C) DO treatments. Salinity ranged from 25 and 27 psu over the duration of the experiment.

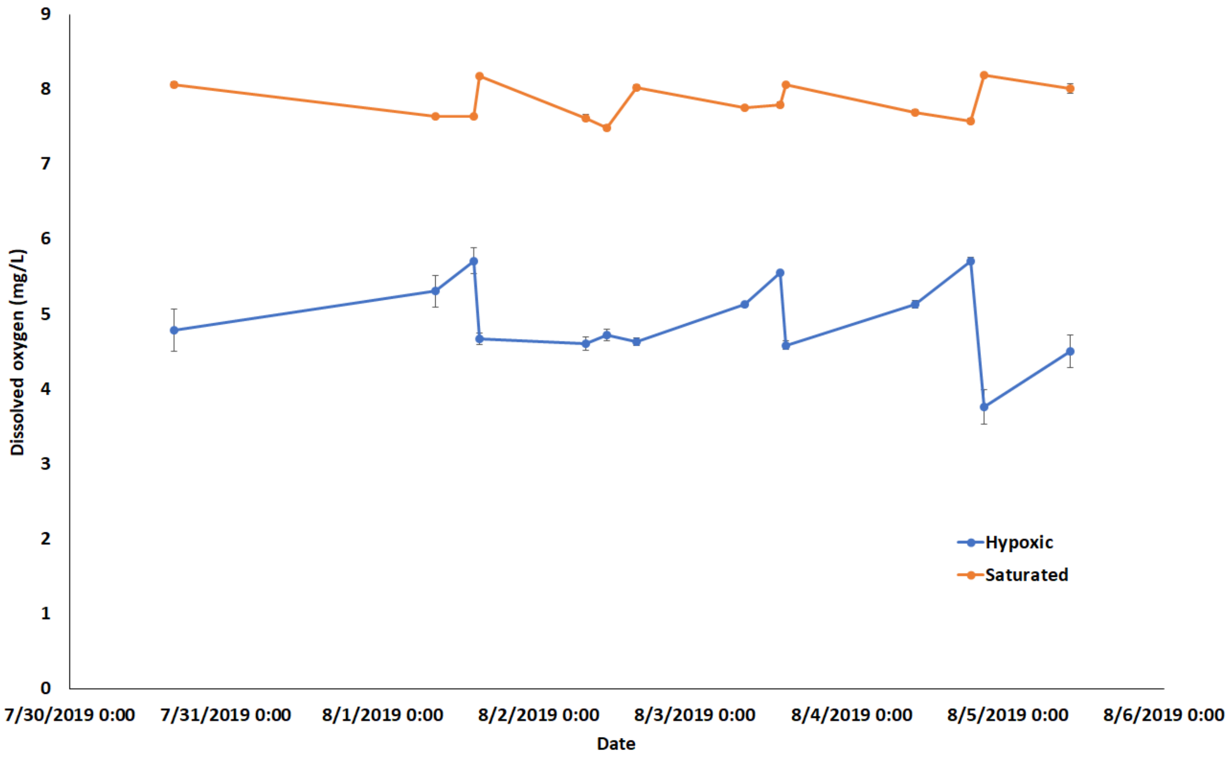

Figure 3. Average dissolved oxygen (mg/L) +/- standard error in hypoxic and saturated treatments for the duration of the experimental exposure (July 30 to August 4, 2019).

Growth

Average initial maximum shell dimension, interpreted as the maximum shell dimension at the time of hatching, was similar between hypoxic and saturated DO treatments overall: 139.57 +/- 1.65 µm standard error (122.8-155.5 µm; n= 27) and 138.09 +/- 1.60 µm (119.6-156.9 µm; n=30), respectively. Average maximum shell distance at 6 dph was also similar among hypoxic (158.12 µm +/- 2.77, n= 27) and saturated (154.44 µm +/- 2.78, n= 30) treatments. Overall, average shell growth at the operculum was also similar at 60.93 +/- 4.28 µm (n=27) and 55.90 +/- 6.23 µm (n=30) for hypoxic and saturated treatments, respectively. Using the linear model function of the MASS R package, a stepwise regression was performed to assess whether treatment, initial maximum length, or maximum length were predictors of larval growth and determine the best fit model (Larval growth ~ initial dimension + treatment + maximum dimension + treatment:maximum dimension). Treatment and initial maximum dimension were not significant predictors of larval growth (p =0.29, df = 1, F = 1.12; p = 0.49, df = 1, F = 0.464).

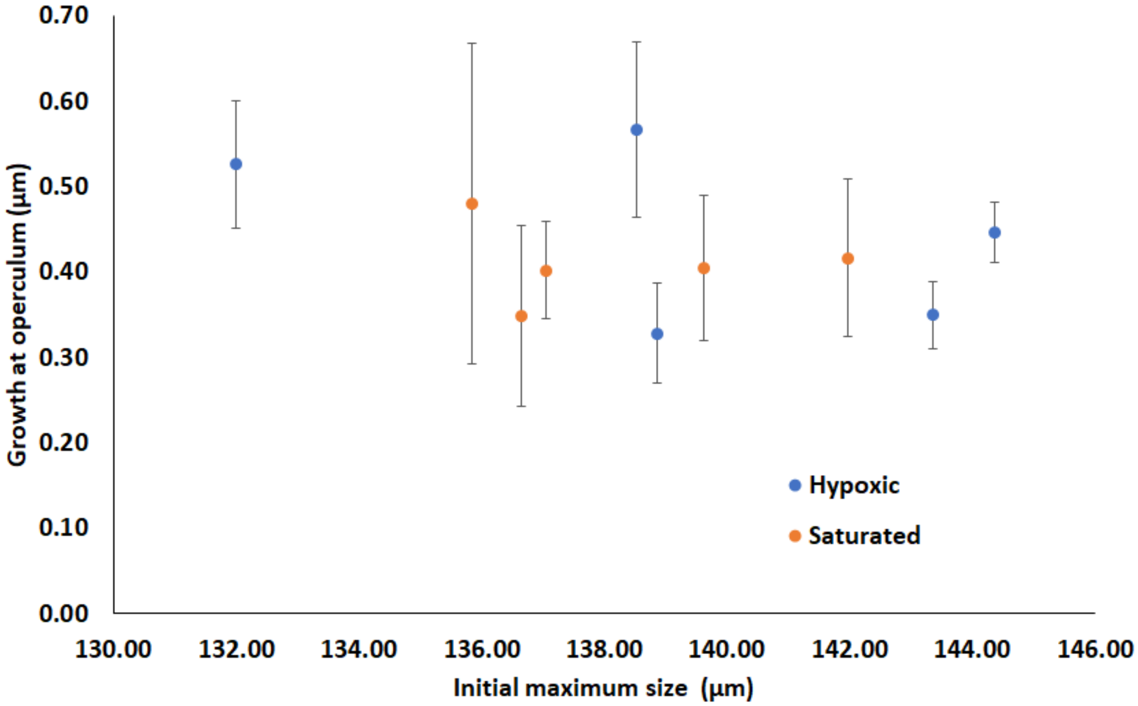

Figure 4. Average growth (µm) as a function of initial size +/- standard error bars of larvae in hypoxic and saturated treatments.

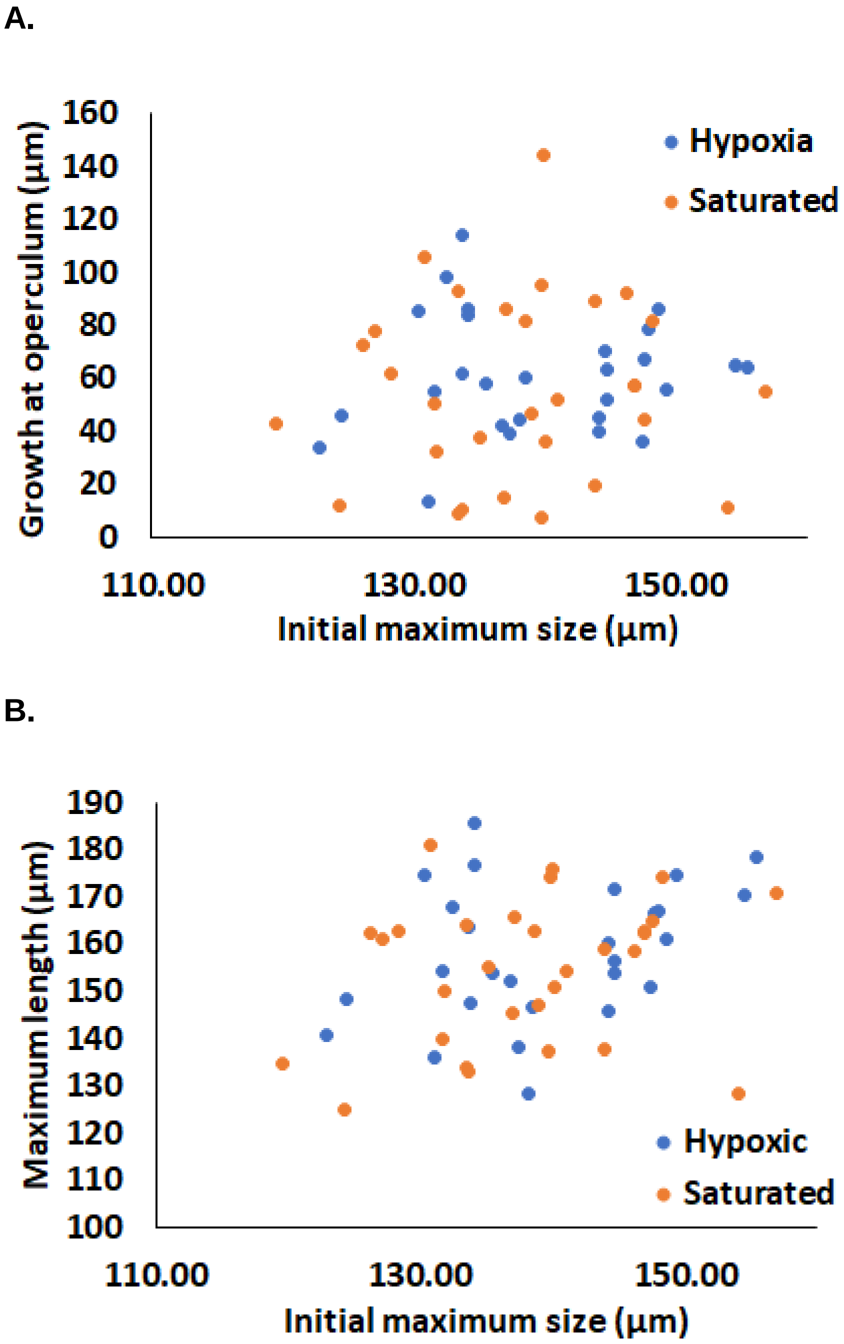

Figure 5. Individual shell dimensions as a function of initial shell size: (A) shell growth between 2 and 6 days post hatching, and (B) maximum shell dimension at 7 dph.

Survivorship

Larval survivorship was assessed at 5 and 7 dph. Larval survivorship was calculated as the proportion of larvae surviving since the start of the experiment unless otherwise noted. Due to an accident during the water change at day 5 post- hatching, there is high uncertainty in the survivorship of larvae between 2 and 5 dph for three of the five hypoxic replicate cultures. Although we report larval survivorship for all replicates for the duration of the experiment (Figure 6), comparisons could only be made between between 5 and 7 dph. Survivorship between 5 and 7 dph was not significantly different between hypoxic and saturated treatments (p = 0.30, n = 5).

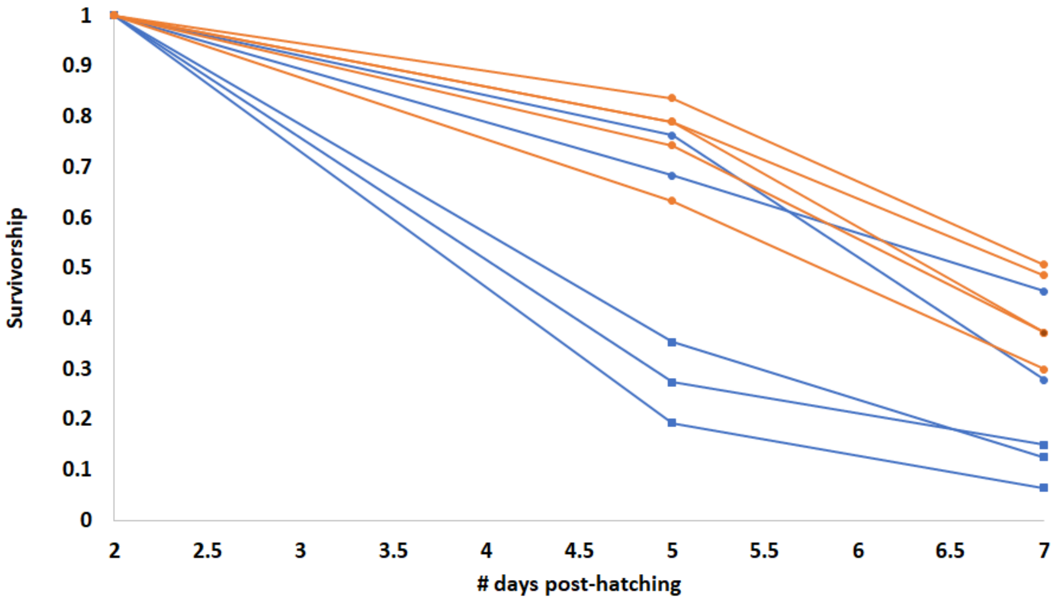

Figure 6. Survivorship (proportion of larvae surviving of replicate cultures from 2 to 7 dph for hypoxic (blue circles) and saturated (orange circles) treatments. Hypoxic r replicates with uncertain decrease in survivorship at 5 and 7 dph have square symbols.

Discussion

In this study, exposure to hypoxia for the first four days post-hatching was not found to affect *H. vesicula* larval growth or survivorship. Errors made during one water change preclude any statistical comparisons of survivorship over the duration of the experiment and among all replicates. Survivorship curves of two hypoxic replicate cultures are nested within those of the five saturated replicate cultures, suggesting that the treatment had no effect on survivorship during the two time points measured post-hatching. Our study cannot extrapolate beyond the 4 dph exposure time or to populations outside of False bay. However, although no response was seen within a short experiment duration immediately after hatching, carry over effects may have been measureable or persistent later in development. Exposure to food limitation, ocean acidification, and thermal stress have been identified to influence larval survivorship and juvenile growth in some bivalve species (e.g. *Mercenaria mercenaria*) through combined larval exposure and juvenile outplanting experiments.

Shell growth was not found to be a function of initial maximum shell, suggesting that larval size alone cannot be used as a predictor of growth rates later in development (i.e. larvae of a similar initial size at the same rearing conditions do not have the same growth trajectories). Plasticity in larval development and growth has been linked to differential maternal investment, genetics, and genetic by environment interactions. It is possible that the genetic background of larvae from the four egg masses and differential maternal investment in larvae within and among egg masses may have contributed to variance in initial shell size. Differences in initial shell size may also be a function of the age of the egg mass (time since deposition) and therefore slight differences in the time spent as encapsulated larvae.

In this study, multiple sources of variance in larval growth cannot be accounted for: (1) *H. vesicula* may have a broad tolerance to DO stress resulting in a wide range of phenotypically plasticity morphologies and responses, (2) the duration or magnitude of DO stress was not severe enough to have a measurable effect, and (3) a small sample size and low statistical power precluded our ability to detect any effect of DO stress on growth and survivorship.

Considering the natural history of *H. vesicula* and the field site where the parental generation of larvae used in this study were sourced from, this species may exhibit a broad DO tolerance that extends beyond reproductive adults and encapsulated larval development. Cohen and Strathmann (1996) describe DO concentrations within egg masses as varying from 85 to 10% saturation over the encapsulated development *H. vesicula* (Cohen and Strathmann 1996). Selective mortality and delayed development of some individuals at the egg mass stage (and therefore biased representation in our experiment which has a hatching time resolution of 1 day) may explain some of the variance we see in the initial size data.

Measurable effects of DO stress on larval *H. vesicula* energy budgeting and metabolism may have been observed using alternative metrics. In this study shell growth, measured as the distance between the fluorescent stain and the aperture, was used a proxy intended to link the cost of maintenance and homeostasis to development. Other studies have measured gastropod shell geometric morphometrics and structure/material properties in the context of plastic responses to predator cues and other environmental stressors. These would provide a more holistic view of how an individual is investing energy, but may only be important under food limitation.

Future studies on *H. vesicula* may benefit by accounting for differences in maternal investment, parental background, and extending low DO stress studies to follow survivorship and growth over ontogeny, from encapsulated development to larval settlement and metamorphosis. Additional studies assessing larval tolerance that compare the effects of site and the interaction between genetics and environment may help parse out whether broad tolerance to low DO is an adaptive trait shared among species with encapsulated larval development.

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