

Mothers might matter: effects of dissolved oxygen and food stress on larval *Mytilus trossulus* growth and lipid accumulation

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Abstract

With continued climate change, coastal regimes in dissolved oxygen are expected to become more variable and severe. Larval marine invertebrates, particularly calcifiers like bivalves, have been identified as being sensitive to changing conditions. This study focuses on the effect of low dissolved oxygen (DO) and food stress on larval growth, lipid content, and survivorship following 6-day exposure to treatment conditions from 2 to 8 days post-fertilization. Using selected crosses between four maternal and 1 paternal lines, this study determined the effect of maternal background on larval response variables. Overall, reduced food and oxygen negatively affected growth but had no effect on lipid contents. Cohorts from different maternal lines responded differently within a DO x Food treatment combination, and highest performance did not always occur in the high DO x high food treatment. Counter intuitive differences in growth rates suggest plasticity in maternal investment or possible genetic by environment interactions. High variance in lipid content estimates existed within and among treatment combinations, and may require per larva measurements to account for inter-individual variation extrinsic to treatment level effects. Survivorship was >80% overall and did not significantly differ for any maternal lines or treatment combinations. In this experiment, parental background explained 46% of the variance in larval growth, but only 2% of variance in lipid content. This suggests that larval *M. trossulus* are highly plastic in their energy budgeting during periods of stress, but alternative metrics to assess differences in lipids are needed.

Introduction

The average prevalence of hypoxia has substantially increased globally in coastal systems over the past few decades (Gilbert et al. 2010). With continued climate changes like increasing sea surface temperature and ocean acidification exacerbating local processes, variance in dissolved oxygen (DO) is expected to increase due to diel fluctuations photosynthesis and respiration that will be linked with average decreases in DO as a function of increasing sea surface temperature and anthropogenic eutrophication.

Of the life stages and taxa most likely impacted by changing environmental regimes in coastal marine ecosystems, larval marine invertebrates are expected to be the most vulnerable. Specifically in the Bivalvia, larval stages have been assessed for sensitivity to thermal, food, hypoxia and acidification stress in isolation and in combination, with response metrics generally consisting of growth and survivorship (e.g. Gobler et al. 2014).

Integrating growth and survivorship response metrics with the theory underlying energy budgeting in organisms suggests that larvae may be limited by available energy, but that energy may be supplemented by feeding. This finite quantity may be differentially allocated to somatic maintenance and growth when responding to an environmental stressor. Relative estimates of available energy in bivalve larvae can be made by quantifying neutral lipids with Nile Red staining and then comparing the total

area of lipids to shell area in a 2D image. Quantification of neutral lipid reserves in larvae by this method can be used as a predictor of performance, growth and survivorship, due to rearing conditions (Gallager et al. 1986). Additionally, this method can permit comparisons in metabolic demand of cohorts of larvae exposed to different environmental stressors if deficits in food availability or increased metabolic rates required to maintain homeostasis (e.g. during exposure to reduced pH or elevated temperature) are correlated with changes in total neutral lipid energy reserves. Gallager et al. (1986) measured lipid reserves over ontogeny in three bivalve species (*Crassostrea virginica*, *Mercenaria mercenaria*, and *Ostrea edulis*) as a proxy for larval condition and identified that maternally-provisioned endogenous lipids decreased substantially by the veliger stage. At the veliger stage, neutral lipids began accumulating and overall total accumulation differed by species, temperature, and food availability. Following larvae through metamorphosis, Gallager et al. (1986) determined that lipid content was generally correlated with survival.

Most larval experiments that assess the effects of environmental stressors on response metrics maximize parental genetic diversity by pooling gametes, without accounting for the contribution of maternal provisioning and genetic background (ocean acidification; Waldbusser et al. 2015). The influence of parental background has been studied in the context of ocean acidification tolerance in selected lines for aquaculture of *Crassostrea gigas* (Frieder et al. 2017).

The aim of this experiment was to measure the scale variation in maternal provisioning (inferred from initial size 2 days post fertilization ~ maternal line) on tolerance to reduced DO. As a second factor, the experiment assessed the potential for

food to ameliorate DO on increased metabolic demand. This study focuses on early larval development in the intertidal bivalve *Mytilus trossulus*, native to the northeastern Pacific coastline, which is widely distributed across the Northern Atlantic in Maine and Canada, Northern Europe, and in the Baltic Sea. Using selected crosses between four maternal and one paternal line, this study assessed the impacts of combined dissolved oxygen and food stress on growth, lipid reserves and survivorship. The amount of variance in larval response metrics explained by maternal line and treatment levels were quantified in order to infer the importance of genetic background or differential maternal provisioning on performance.

Methods

Adult collection and spawning

Adult *Mytilus trossulus* were collected from a subtidal region of the docks at Friday Harbor Laboratories (48.545002, -123.012576) on August 8, 2019. Adults were cleaned of biofouling organisms and placed in 1 μ m filtered sea water at nearshore temperature (~12°C). Adults were injected with 1mL of 0.56 mM KCl solution to induce spawning. After 1.5 hours, four females and two males successfully spawned and gametes were cross-fertilized between the four females and one of the males in 0.45 μ m filtered sea water (hereafter FSW) to produce four parental combinations. Embryos from each of the four parental lines were placed into 1L jars with light bubbling of ambient air for the first 48 hours post-fertilization. Each jar was reduced in volume using a 15 μ m filter and poured into a custard dish. A subset of swimming D-stage

veligers were haphazardly allocated to experimental 12 treatments containers per maternal line.

Larval background and rearing

Larvae from each parental line were pipetted into 120 mL culture containers (total volume of 100 mL) at 48 hours post-fertilization (August 10, 2019), at a density of 2 larvae/mL. All larvae were fed and stained with calcein for 24 hours and then exposed to one of four treatments: (1) low dissolved oxygen DO (L; ~45% saturation, ~4 mg/L) and full food ration (F; 1×10^4 cells/mL/day *Isochrysis galbana*), (2) low DO and quarter food ration (Q; 2.5×10^3 cells/mL/day), (3) high DO (H; ~95% saturation, ~8 mg/L) and full ration, (4) high DO and quarter ration. Replicate cultures were organized in a semi-randomized design by DO (H/L) x Food (H/Q) x Parental line treatment (A-D) for a level of replication of $n = 2 \times 2 \times 3$. High food rations (10^4 cells/mL) were determined from other studies on *Mytilus* sp. that result in high survivorship (Clark and Gobler 2016), while low food concentrations were expected to be above lethal conditions. Larvae were fed daily with algae that was centrifuged, resuspended in FSW, and density determined with a hemocytometer.

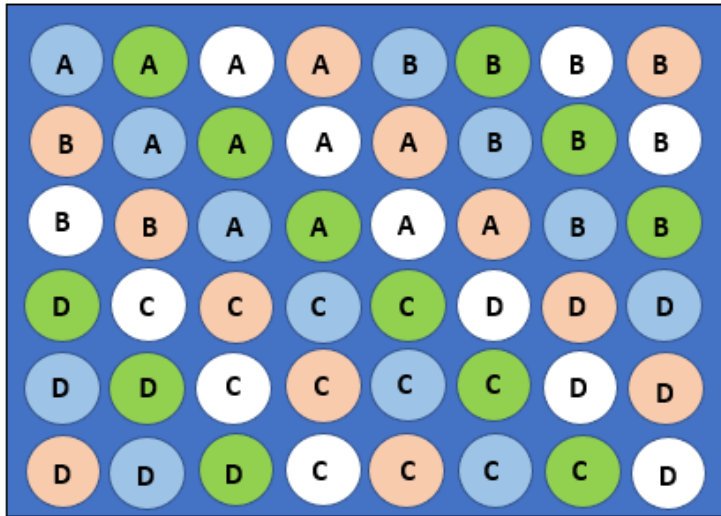


Figure 1. Array of larval cultures in sea table. Treatments include high DO/full ration (light blue circles), high DO/quarter ration (light orange), low DO/full ration (green), low DO/quarter ration (white). Letter (A-D) denote the parental lineage.

Water changes and monitoring

Water changes were performed daily by gently pouring cultures onto a 15µl mesh filter submerged half-way in FSW. Larvae were backwashed into custard dishes using a squirt bottle and then into 120 mL jars containing to a volume of 20mL. Cultures were then fed centrifuged and resuspended algae. Bubbled or deoxygenated FSW was added to reach a total volume of 100mL per culture for high and low DO treatments, respectively. To minimize gas exchange, the headspace of low DO treatment containers were filled with N₂ gas and then tightly capped. Hypoxic treatment water was obtained by bubbling FSW with N₂ gas. Dissolved oxygen saturated FSW was obtained by bubbling overnight with ambient air by an airstone and aquarium pump. High DO containers were lightly capped to minimize contamination but allow for partial gas exchange. Temperature and dissolved oxygen were monitored before and after each water change (1-2 times per day) using a ProODO meter (YSI, OR, USA). Salinity was measured periodically throughout the experiment using a refractometer.

Calcein staining and growth analysis

Two-day old larvae were stained for the first 24 hours of the experiment at a concentration of 100mg/L (Fitzpatrick et al. 2013). Larvae were thoroughly rinsed with FSW following staining and exposed to treatment conditions for 6 days.

At 8 dpf, 10 larvae were subsampled from each replicate culture for shell size measurements. Images were taken with a fluorescence filter (EX 460-500, DM 505, BA 510-560) to identify individual growth of larvae between 2 and 8 dpf. Measurements were calibrated at 10x magnification using a stage micrometer. Two measurements were performed for each larva: (1) shell height at 2pdf and (2) shell height at 8 dpf. These measurements were used to calculate daily growth rates as $(\text{size at 8dpf} - \text{size at 2dpf})/6 \text{ days}$.

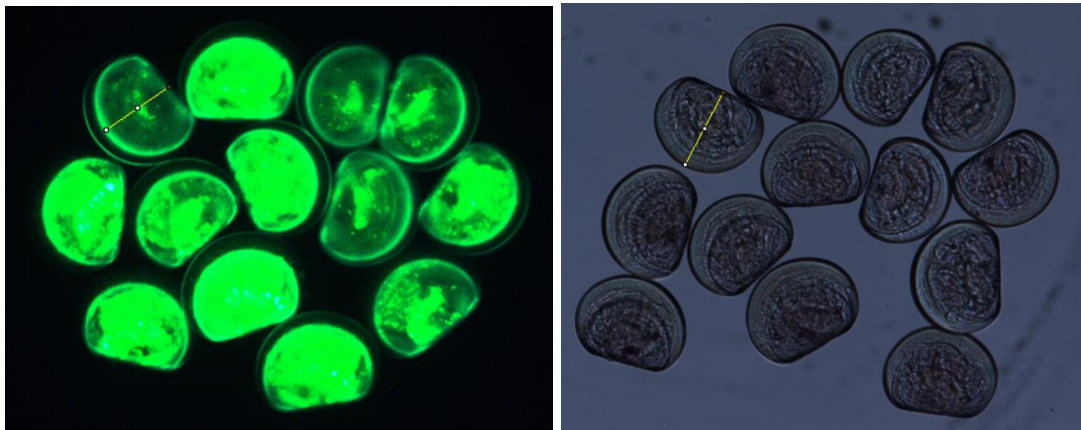


Figure 2. Individual shell measurements: shell height at 2pdf (yellow line; left), (2) shell height at 8 dpf (yellow line; middle). Measurements calibrated with a stage micrometer at 10x magnification.

Lipid staining and analysis

Water changes were started early on the 6th day of the experiment (8dpf) to allow for larvae to depurate for 6 hours prior to staining. The same larvae imaged for growth were also photographed for lipid content. Staining with Nile red was performed at a concentration of 1.25 $\mu\text{g}/\text{mL}$ for 1.5 hours in 12-well plates (Castell and Mann, 1994). Photos were taking within 5 hours of staining. At that time, larvae were rinsed with FSW and transferred to a depression slide for imaging under a fluorescence filter (EX 528-553, DM 565, BA 600-660). Lipids were quantified using the CountColors image analysis R package, whereby a global threshold in the RGB spectrum was set for fluorescing neutral lipids. The number of pixels identified as neutral lipids were enumerated using the `img.fraction` summary statistic which calculates the proportion of the image within a specified color range. The same statistic was used to estimate the total shell area per image using a separate color range. Since multiple larvae were in each image (within a replicate larval culture), a unitless estimate of lipid content was calculated as $(\text{img.fraction}_{\text{lipids}} / \text{larva}) / (\text{img.fraction}_{\text{total area}} / \text{larva})$ for each of the 3 replicate cultures.

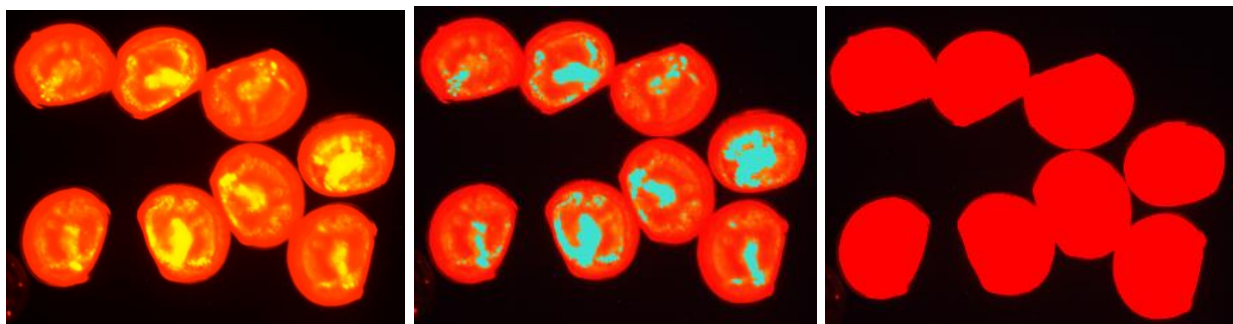


Figure 3. Image processing for lipid analysis: (1) identifying threshold range for differentiating polar (red) and neutral (yellow) lipids (left), (2) quantifying turquoise area of neutral lipids (middle), and total shell area (right).

Results

Rearing conditions

Average dissolved oxygen levels of low and high treatments ranged between 50.55-52.15% saturation and 81.05-93.55%, respectively (Table 1). Temperature varied by between 13 and 14.8 °C during the experiment, but was similar across food treatments and parental lineages. Average salinity was 24 ± 2 PSU and similar across all treatment levels.

Table 1. Summary of average temperature and dissolved oxygen (\pm SEM) maintained during larval experiment.

DO Treat	Food Treatment	Parental Lineage	Average temperature (°C)		Average DO (%)	
High	Full	A	14.08	± 0.29	93.55	± 0.90
High	Full	B	14.14	± 0.30	91.77	± 1.55
High	Full	C	13.89	± 0.25	91.41	± 1.49
High	Full	D	14.05	± 0.35	91.27	± 1.50
High	Quarter	A	14.09	± 0.34	92.90	± 1.04
High	Quarter	B	14.06	± 0.30	87.40	± 4.93
High	Quarter	C	13.82	± 0.21	92.35	± 1.55
High	Quarter	D	13.96	± 0.29	91.49	± 1.50
Low	Full	A	14.38	± 0.32	52.15	± 4.87
Low	Full	C	14.15	± 0.47	51.71	± 4.17
Low	Full	D	14.16	± 0.47	51.48	± 3.83
Low	Quarter	A	14.57	± 0.53	50.87	± 3.65
Low	Quarter	B	14.13	± 0.45	55.23	± 4.25
Low	Quarter	C	13.97	± 0.40	55.15	± 5.81
Low	Quarter	D	14.20	± 0.39	50.55	± 3.22

Growth

Growth measurements at 2dpf and 8 dpf were taken for 477 individuals, including 9-10 pairwise measurements per replicate culture. Shell height at 2 dpf varied greatly

among and within treatment levels, but all individuals grew over the duration of the experiment (Figure 4).

A linear model was used to test the effect of DO, food, and maternal lineage on growth rate of individual larva (growth rate ~ DO + Food + Parent). Growth rate was best predicted by parental line, accounting for 46% of the variance (Table 2). However, parental line was only a significant predictor of growth rate for lines A, B, and D ($p < 0.005$), but not for C ($p = 0.081$). There was a significant effect of DO treatment on growth rate ($p = 0.0503$).

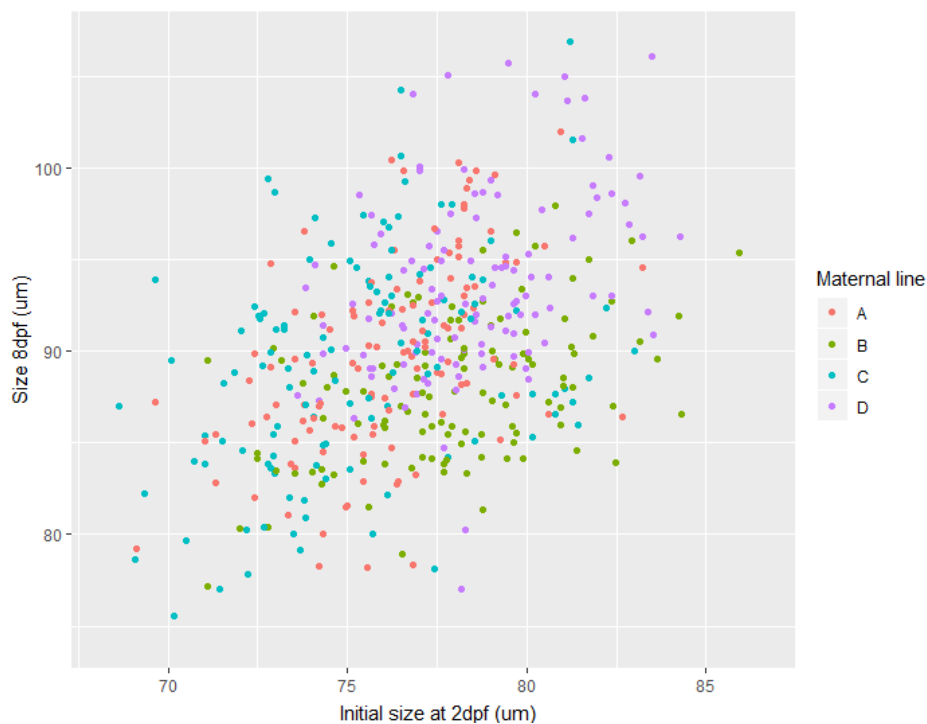


Figure 4. Size at the end of the experiment (8dpf) as a function of initial size at 2 dpf for each parental line.

Table 2. Variance explained by treatment conditions and parental line calculated from ANOVA sum of squares for growth and Lipid.

Response metric	DO	Food	Parental line	Residual variance
Growth	0.05	0.0002	0.46	0.50
Lipids	0.001	0.01	0.02	0.97

Trends suggest that growth rate was reduced under low dissolved oxygen for some parental lines (A, C, D), but that this was not mediated by having higher food concentrations. Parental line A had the highest growth rates under high DO for both food treatments. Parental line B had similar growth rates across all treatment levels, with the highest growth rate under low DO and low food. Parental line C had the highest growth rate under high DO and food, but similar growth rates at other treatment levels. Growth rates for line D were significantly lower for H x Q, L X H, and L X Q treatments relative to H x H. Within a treatment level, growth rates among parental lines significantly differed under H X H ($B < D < A < C$). In all other treatments, parental lines A and C had larger growth rates than D.

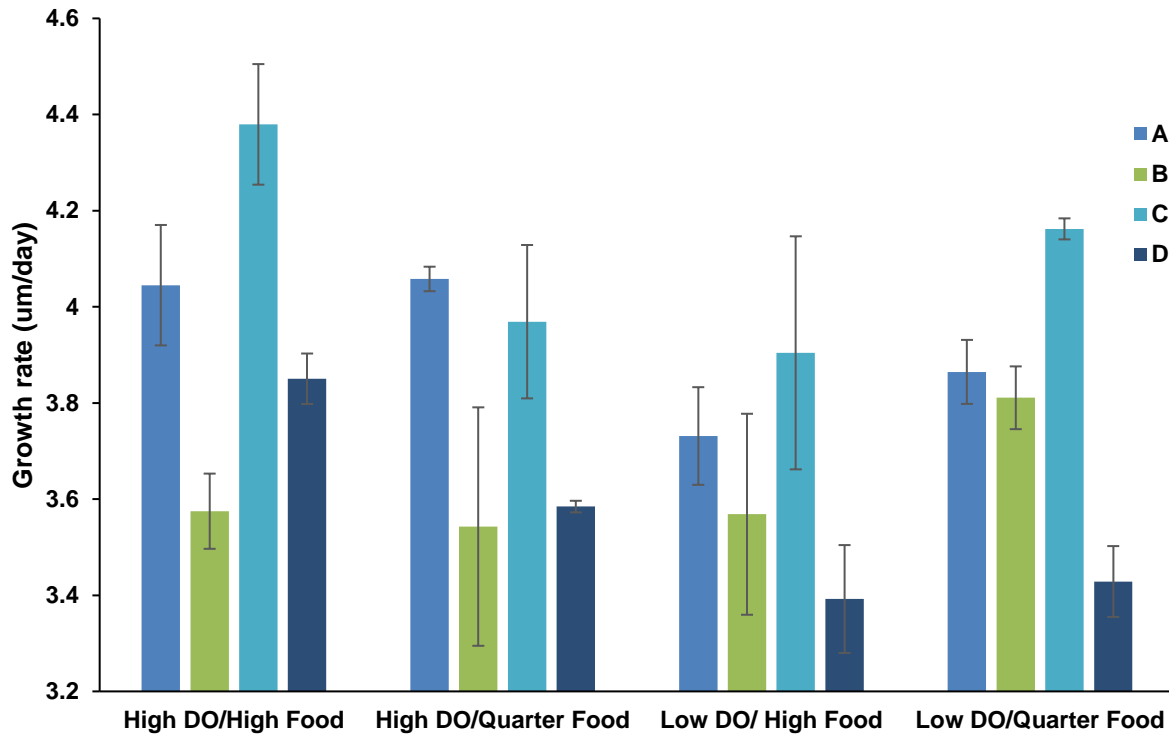


Figure 5. Individual growth rate ($\mu\text{m}/\text{day} \pm \text{SEM}$) by treatment level ($n=3$). Treatment notation follows that stated in Figure 4.

Lipid analysis

Lipids were quantified for 651 larvae, with 6-20 larvae per DO x Food x parental treatment combination. A linear regression was used to test the effect of DO, food, and maternal lineage on lipid content of individual larva (lipid area \sim DO + Food + Parent). There was no effect of any treatment level on lipid content for the full model ($p = 0.93$).

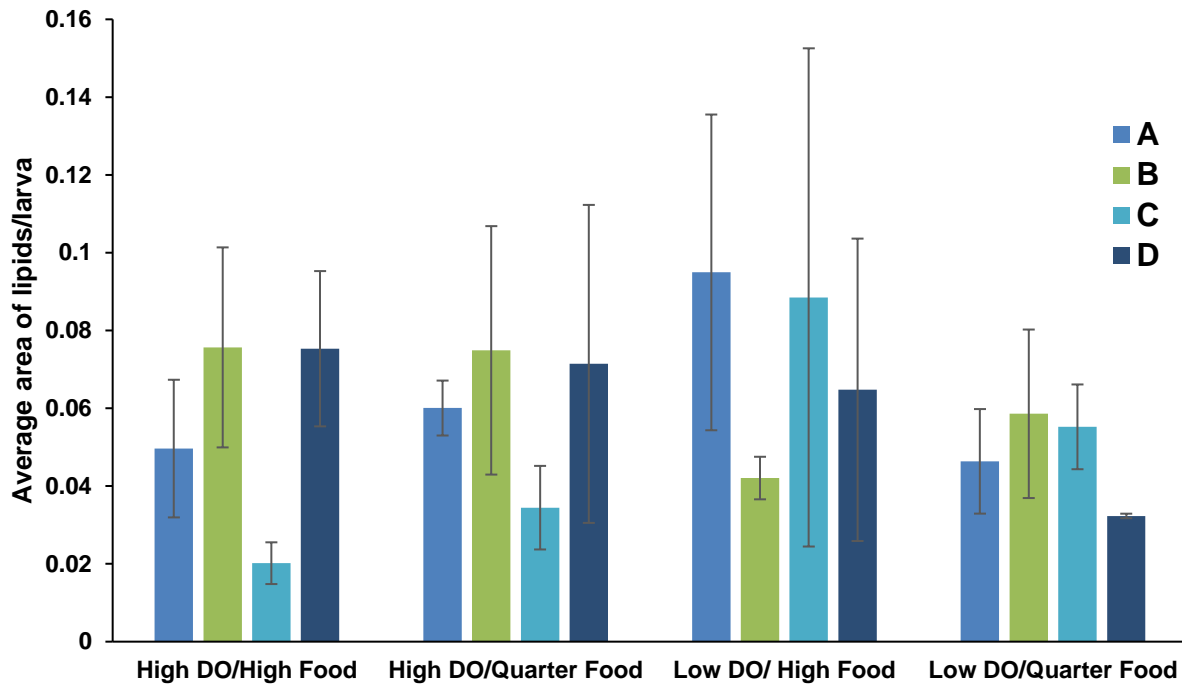


Figure 6. Average area of lipids shell per larva after controlling for shell area by treatment level. Treatment notation follows that stated in Figure 4 (n=3).

Discussion

Larval survivorship by the end of the experiment was >80% for all cultures. This is suggestive of *M. trossulus* larva being robust to low DO and food stress and that there are multiple solutions to tolerating environmental stressors by augmenting energy budgeting. In this study, variance in growth and lipid accumulation may have been an emergent property of the tradeoff between accumulating neutral lipids accumulation and accreting shell or new soft tissue. However, it may also be the case that all treatment levels were equally as stressful (or not stressful at all), with variance in growth and lipid contents being due to differences in genetic and plasticity in maternal provisioning. Overall, reduced DO was found to reduce growth, but differences across treatment

conditions were not consistent among maternal lines. Alternative metrics, such as comparing changes in total surface area between day 2 and 8 post-fertilization may better capture the influence of treatment levels on growth.

However, in this experiment, maternal line was found to explain a large proportion of the variance in growth, but negligible amounts of variance in lipid contents. More than 95% of the variance was unexplained by the linear model, suggesting that the metric used was not sufficient to parse out differences in lipid content due to genetics, plasticity, or environmental conditions. It is possible that the metrics used to quantify lipids (relative area of lipid contents to shell), which were averaged across full-siblings within a replicate culture, was too confounded by high variance to detect a treatment effect. High variance in lipid contents was observed most maternal lines at high and low DO and high food. By comparing the lipid/shell area per larva and making pairwise comparisons with growth, differences as a function of DO x food treatment and parental background might be detectable.

Relative to other studies, low DO exposure was on the average approximately 30% saturation higher than hypoxic levels used to assess larval performance in other bivalve species (e.g. *Crassostrea virginica* pediveligers; Baker and Mann, 1994). However, variance in DO was likely greater than that captured by measurements taken before and after daily water changes. Low DO treatments were observed to be as low as 26% saturation, a greater than 50% change in DO over the course of 24 hours. This is likely due to cumulative decreases in DO from respiration (and shifts in photosynthesis: respiration from algae at night) from larval cultures being in a closed container. High DO treatments varied substantially less (~10-16%).

General trends in growth and lipids suggest that combined DO and food stress has differential effects on larval performance (with growth and lipid availability as proxy measures) depending on maternal lineage. In some lineages, growth differences are counterintuitive, with higher growth at low DO treatments. Additionally, increased food was found to ameliorate the negative effects of reduced DO in some lineages (B, C, D), but not all (A). The effect of DO x food was statistically detectable, albeit high overall variance in individual differences in growth, by partitioning variance due to maternal line. This suggests that differences in larval performance may be a function of genetic background or maternal investment. High variance in lipid accumulation estimates is likely due to averaging across groups of full sibling larvae with different initial sizes/growth rates and likely differences in maternal investment.

Mytilus species have been assessed for tolerance to low DO during larval development, with responses potentially differing as a function of duration of exposure, DO treatment levels, and high genetic diversity within a cohort of larvae. Frieder et al. 2014 demonstrated some bivalve species may be robust to low DO and pH (separately and in combination). After exposure to variable pH and DO for the first 8 days of development post-fertilization, survivorship and larval development were not significantly affected in *Mytilus galloprovincialis* and *M. californianus*. Wang and Widdows (1991) found that *Mytilus edulis* exposed to hypoxia and allowed to recover at normoxic levels as early prodissochonch larvae did not exhibit compensatory growth, although feeding rates were found to increase under hypoxic conditions.

In another bivalve species, Baker and Mann (1994) found effects of hypoxia on *Crassostrea virginica*, found effects on development, growth and survivorship when

exposure began at the pediveliger stage. Tolerance to environmental stressors, specifically hypoxia, is expected to increase over development. This has been demonstrated in *C. virginica* larvae reared under low DO conditions for short-term exposure times at multiple stages in development, from prodissochonch to pediveliger (Widdows et al. 1989). *Argopecten irradians* and *Mercenaria mercenaria* have been observed to have reduced growth and increased mortality, respectively, under hypoxic conditions (Gobler et al. 2014).

Responses to hypoxia appear in bivalve larvae appear to vary greatly by species and larval developmental stage as a function of treatment conditions. Differences observed within a cohort, especially under hypoxic conditions may be heavily influenced by genetics and lesser so by maternal investment, as larvae cannot use lipid reserves to undergo anerobic catabolism.

This study investigated the cumulative effects of exposure to low DO and food stress on larval growth and lipid availability during early development. Although differences in growth were not followed through to the juvenile stage, it is important to assess for the potential of legacy effects later in development in order to have any predictive ability of population level responses to environmental stressors.

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