

Potential Transgenerational Effects of Ocean Acidification on the
Olympia Oyster *Ostrea lurida*: A Three-Part Experimental Study

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Abstract

Potential Transgenerational Effects of Ocean Acidification and Hypoxia on the Olympia Oyster *Ostrea lurida*: A three-part experimental study

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Ocean acidification (OA) is decreasing the pH of surface waters in Puget Sound, Washington, an area already prone to low pH from natural processes such as upwelling, freshwater inputs, and high respiration/decomposition rates. High rates of production and long residence times in Puget Sound can also lead to low dissolved oxygen (DO) levels (hypoxia) in some areas. Studies have shown the negative effects of these stressors on marine organisms, particularly calcifiers. I examined how changes in pH and oxygen in seawater affect adult fecundity and larval survival of the native Olympia oyster (*Ostrea lurida*). Through three discrete trials, I observed the following trends: Adult oysters conditioned at ~400 μatm released significantly more larvae than those conditioned at higher pCO_2 levels ranging from 1000 to 2475 μatm pCO_2 . Larval survival decreased in two multi-stressor treatments when challenged with varying combinations of pCO_2 and DO. Offspring of parents conditioned under high pCO_2 experienced reduced survival when exposed to both high pCO_2 and low DO (14.7% survival). In addition, progeny of adults conditioned under low pCO_2 died when exposed to high pCO_2 and high DO (22% survival). Our results suggest that elevated pCO_2 negatively affects fecundity in *O. lurida* but that the synergistic effects of high CO_2 and low DO on larval survival is more complicated than previously reported. Multigenerational, multi-stressor studies such as this are important in determining how species will respond to an environmental change in the ocean.

Introduction

Ocean acidification (OA) refers to the decreasing pH of the surface oceans resulting from chemical reactions between anthropogenic carbon dioxide (CO₂) and seawater (Feely et al., 2004). The oceans have absorbed roughly one quarter of the anthropogenic CO₂ released into the atmosphere, which in turn has caused the surface ocean pH to decline by approximately 0.1 units, equating to a 30% increase in acidity (Feely et al., 2012). Concomitant with this change is a decrease in the aragonite saturation state of seawater. Aragonite saturation state is a measure of the likelihood that calcium carbonate will form or dissolve (Feely et al., 2004), which is critical to calcifying organisms (shelled species such as bivalves) (Kroeker et al., 2010). Low aragonite saturation state can make it difficult for organisms to build shells and can be fatal for larvae (Kroeker et al., 2010).

OA in Puget Sound, Washington, is exacerbated by natural processes such as upwelling of CO₂ rich water, large freshwater inputs, and high respiration/decomposition rates (Feely et al., 2012). Human activities that produce chemical and nutrient pollution as well as fossil fuel emissions also contribute to OA in Washington waters (Feely et al., 2012). Concern over the effects of OA in Washington state is heightened due to the economic and cultural importance of shellfish in the region. The estimated economic impact of shellfish aquaculture in the state is \$270 million annually (PCSGA). Moreover, many Native American tribes in Washington depend on shellfish for nutritional and cultural purposes. Mabardy et al. (2015) found that roughly half of U.S. West Coast industry members reported being negatively affected by OA but a similar number believed they have the ability to adapt.

In addition to OA, low dissolved oxygen (DO) is an issue of growing concern in the region. Hypoxia (< 2mg/L DO, noaa.gov) can occur when high rates of primary production in surface waters lead to high respiration rates in the waters below, reducing oxygen concentrations (EPA, 2014; Joos et al., 2003). Nutrient pollution from surrounding watersheds can further stimulate primary production leading to eutrophication (Brandenberger et al., 2008). Freshwater inputs, thermal stratification in the summer, along with the presence of glacial sills in Puget Sound result in low mixing and can cause some areas to become hypoxic, especially in Hood Canal (Brandenberger et al., 2008). Hypoxia is expected to increase with climate change as air temperatures rise and stratification increases (Joos et al., 2003). The combination of hypoxia and increasing CO₂ is expected to produce negative synergistic effects on biological processes, but empirical studies are relatively few. Pörtner et al. (2005) suggested that understanding interactions between CO₂ and DO in affected species may aid in forecasting their response to future environmental changes.

Carry-over effects (effects that are expressed within a single generation) and transgenerational effects (those that occur across generations) have been increasingly studied with respect to ocean acidification (Ross et al., 2016). Hettinger et al. (2012) found that juvenile Olympia oysters (*Ostrea lurida*) that were reared (as larvae) at pH 7.8 showed a 41% decrease in shell growth rate compared to those grown at pH 8.0, even when they were moved to ambient seawater after settlement, indicating a negative carry-over effect. Parker et al. (2015) found that the adult Sydney rock oysters (*Saccostrea glomerata*) previously exposed to high pCO₂ (856 µatm) as larvae, were better able to regulate extracellular pH when exposed to OA conditions, indicating a positive carry-over effect. In the same species, Parker et al. (2012) stated that the progeny of adults exposed to high pCO₂ showed increased tolerance to effects of low pH, in terms of larval development rate, shell formation, and abnormalities, an example of

transgenerational effects. Fitzer et al. (2014) discovered that juvenile mussels (*Mytilus edulis*) of parents exposed to 1000 μatm pCO_2 for six months produced only calcite in contrast to control juveniles exposed to ambient conditions and produced both aragonite and calcite, suggesting the species' ability to acclimate to changing seawater chemistry. The potential role of carry-over and transgenerational effects may be an important factor for calcifiers in the face of climate change.

The native Olympia oyster (*O. lurida*) was once an abundant species in many estuaries along the NE Pacific coast and was the basis of a large wild-capture oyster fishery in Willapa Bay, WA (Carpenter, 1864; Stenzel, 1971). Today, the oyster industry is dominated by aquaculture of the non-native Pacific oyster (*Crassostrea gigas*) and produces more oysters than any other estuary in the United States (Ruesink, 2006). Started in the late 1800s, the Olympia oyster fishery had already collapsed by 1920 due to over harvest and poor water quality (Kirby 2004; White et al. 2009). In response to the decline of this native species, the Washington Department of Fish and Wildlife (WDFW) listed *O. lurida* as a Species of Concern - State Candidate (a species with the potential of being sensitive, threatened, or endangered) (WDFW, 2017). Recent analysis of historical *O. lurida* population data has revealed that historical beds in Willapa Bay could have been as expansive as 9,774 hectares (Blake & zu Ermgassen, 2015) but today only 4% of these historic populations remain (Davis & Peabody, 2013).

I hypothesize that in addition to the previously observed negative effects of elevated pCO_2 on larvae and juvenile *O. lurida*, adult fecundity will also be impacted. Further, I hypothesize that the combined effects of DO and OA will result in larval oyster mortality. In this three-part study, I identify the influence of elevated pCO_2 on adult fecundity and larval survival of the Olympia oyster (*O. lurida*). In trials 1 and 2, adult fecundity was quantified in response to varying levels of pCO_2 . Larval survival, growth and shell condition were also examined in trial 1. In the third trial, survival of the larvae was measured in response to reciprocal pCO_2 levels as well as high and low dissolved oxygen. The first two trials aimed to determine if OA can negatively affect the fecundity *O. lurida*. In the third trial, the goal was to detect synergistic effects of ocean acidification and how DO conditions approaching hypoxic levels influence larval survival and shell morphology.

1. Methods

2.1 Experimental System

All trials were conducted at the Ocean Acidification and Environmental Laboratory (OAEL) at the University of Washington's Friday Harbor Laboratories. The OAEL is supplied with running seawater and can accommodate custom blending of pCO_2 to achieve specified treatment conditions. Temperature and pCO_2 were manipulated as described in O'Donnell et al. (2013). Briefly, a DuraFET III pH probe (Honeywell, Morristown, NJ, USA) was used to monitor tank pH and deliver CO_2 gas via solenoid valves and a Honeywell UDA2182 controller to reach experimental set points as outlined in the trials below. DO was similarly controlled via a Honeywell DL5000 sensor (model number is DL5PPM-300-0000-000) and nitrogen (N_2) gas to obtain set points. Tank seawater pH, temperature and dissolved oxygen were monitored continuously and recorded daily. Salinity was recorded daily using a refractometer. I used spectrophotometric pH (see below) and/or calibration using Tris buffer (Dickson et al. 2007) standards to calibrate the Durafet pH probes weekly. Similarly, I performed Winkler titrations weekly to calibrate the DO probes.

2.2 Water Chemistry

Samples were collected, poisoned with 200 µl saturated HgCl₂, and archived for later analysis, seawater samples from each tank weekly. Samples were analyzed for seawater pH, total alkalinity (A_T) and, for trials 1 and 2 only, total dissolved CO₂ (T_{CO2}) were collected according to Dickson et al. (2007) and processed as described below. In trial 3, alkalinity was calculated on a daily basis using measured temperature and salinity values and the LSM model of O'Donnell et al. (2013). This model was generated by OAEL lab personnel and created using measured A_T and salinity (ppt) values of local conditions to create a curve. I measured A_T using an end-point titration (DL15 titrator, Mettler Toledo, Schwerzenbach, Switzerland) and the equation of SMEWW (1998) modified to provide alkalinity in µmol/kg seawater and quality assurance following SOP 3b of Dickson et al. (2007):

$$A_T = [(volume\ titrant)(normality\ of\ titrant)(Mass\ of\ CaCO_3)(10^5)]/(mass\ of\ sample).$$

T_{CO2} was measured using a Licor LI-700 CO₂ detector after acidification as outlined in SOP2 (Dickson et al. 2007). Spectrophotometric pH was measured on weekly samples using SOP 6b of Dickson et al. (2007). To calculate pCO₂, pH, T_{CO2}, and saturation state (Ω) of aragonite (Ω_{ARAG}) and calcite (Ω_{CAL}) I used the Excel version of CO₂ System Calculations (Pierrot, Lewis & Wallace 2006). The known parameters I entered were salinity, temperature, A_T (calculated from OAEL model), and pH. The following constants were used in the CO₂CALC program: CO₂ constants: Lueker et al. (2000); KHSO₄: Dickson (1990); pH scale: total scale (mol/kg SW); and air-sea flux: Wanninkhof (1993). Precision [(standard deviation / mean measured value) x 100] and accuracy [(mean measured value - CRM) / CRM] x 100 value were determined using measurements of certified reference materials (CRMs; supplied by the Dickson laboratory, Scripps Institution of Oceanography, La Jolla, CA). See table 1 for a summary of relevant seawater chemistry (measured and calculated).

2.3 Experimental Design

Cultivated Olympia oysters were obtained from the commercial grower Taylor Shellfish and shipped to the OAEL. All oysters were held in mesh bags in a flow-through tank receiving sand-filtered ambient seawater (9.5°C, 29.7 ppt) until the oysters were measured and tagged. I measured individual length (mm), width (mm), and weight (g) (see table 2 for summary). Adults were batch-fed Reed Shellfish Diet 1800 once per day (~120,000 cells/ml) until being tagged and placed into their assigned treatment systems. Each system received flowing seawater in which water chemistry was maintained in a mixing tank before flowing into experimental chambers as described in O'Donnell et al. (2013). To induce maturation and spawning, immersion heaters (Finnex TH Series) were used to increase seawater temperatures from ambient (~10-12°C) to our target temperature of 20°C by daily increments of 1°C per day. In trial 1, adult and larval oysters were batch fed Reed Shellfish Diet 1800 (Reed Mariculture) at a target of 120,000 cells/ml at a rate of 1.9 L/hr for 36 mins, three times per day using peristaltic pumps (Mityflex). In trials 2 and 3, oysters were fed Shellfish Diet supplemented with 30% live algae. Live microalgae consisting of 60% *Isochrysis galbana* and 40% of either *Pavlova* sp. and *Tetraselmis* sp. was provided by the Taylor Shellfish Farm.

2.3.1 Trial 1 - 2012 Experimental Design

Oysters (n=120) were collected from Totten Inlet, south Puget Sound and shipped in two days to the OEAL where they were randomly placed into experimental chambers (n=11 oysters per chamber) containing ambient seawater (~10°C) for one hour. Three replicated systems were established for each of the four target pCO₂ treatment levels (400, 1000, 1600 or 2200 µatm; see Appendix A for diagram of experimental design). We analyzed adult fecundity by quantifying larval releases over time per chamber. To measure larval survival, approximately 25,000-35,000 larvae were placed into three down-welling silos per CO₂ level. These silos consisted of 7 inch lengths of 4 inch PVC pipe with 100µm mesh screen attached on the bottom and were suspended ~ 1 inch off the bottom of a tank. The silos received the same water from their parent's treatment tank delivered via an irrigation dripper at a flow rate of 60 ml/min. Every other day, we placed an aliquot of larvae (~100-500 µL) in 100% molecular grade ethanol and stored at room temperature for later morphometric and shell analyses. Larval survival was measured over an 8-day period as outlined below.

2.2.2 Trial 2 - 2013 Experimental Design

In trial 2, adult oysters from various sites and of varying ages were conditioned at two pCO₂ levels to measure the effects of pCO₂ on reproduction. Collection sites and spawn years (=origin of oysters) included Hammersley Inlet (2010 spawn), Totten Inlet (2009 spawn), Totten Inlet (2008 spawn), and wild oysters from Burly lagoon. Triplicate groups of 10 adults per origin were placed in 100 µm mesh bags and distributed across treatments (targets of 400 and 1600 µatm pCO₂) and between the duplicated systems for each treatment level. For each oyster origin, two replicate bags were placed in one duplicate system and one bag was placed in the other system (see Appendix B for diagram). We analyzed fecundity through quantifying larval releases over time; larval survival was not tested in this trial.

2.3.2 Trial 3 - 2015 Experimental Design

Wild oysters that originated from South Sound (North Bay), North Sound (Fidalgo Bay), and Hood Canal locations were collected, packed on ice and shipped overnight to the OAEL. Half of the oysters from each origin (FB: n=39; SS: n=39; HC: n=33) were placed into conditioning tanks of either high CO₂ (target =1600 pCO₂) or low CO₂ (target = 400 µatm pCO₂). Only FB and SS released larvae during this trial. Upon release (see section 2.2 for spawning induction), larvae were quantified (see larval sampling for counting method) and placed (n ~10,000 larvae) into duplicate larval silos (as described above). Irrigation drippers delivered seawater at a rate of 42-60 ml/min. I assigned every set of duplicate silos to each of the eight treatment groups of various combinations of high and low CO₂ and high and low dissolved oxygen (Figure 1; high: >8.0 mg/L, low: 3.6 mg/L). The duplicate silos were placed in replicate chambers in the same treatment system (two chambers per treatment tank (see Appendix C). I sampled larvae every other day after following spawning as described below over the 11-day trial.

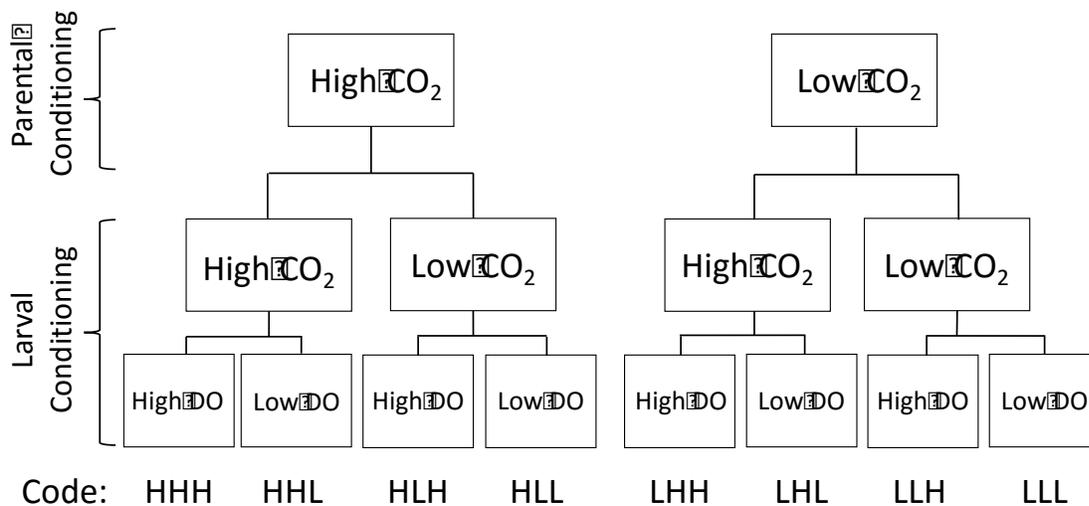


Figure 1. A diagram of experimental treatments for trial 3

2.4 Larval Sampling

The total number of larvae released was estimated by filtering larvae onto a 150µm mesh screen (using seawater to matching chemistry) and finally into a 50ml conical tube from which three 20 µl aliquots were removed after gently mixing. Larvae were counted (total number) and live larvae were differentiated from dead, which had a ‘frosted glass’ appearance or by an empty shell (Appendix B). The proportion of total, live and dead larvae were estimated using the following equation:

$$\text{Larval count} = (\text{Mean counts}/20 \mu\text{l aliquot}) \times (1000 \mu\text{l}/\text{ml}) \times (\text{Volume of conical tube in ml}).$$

For Trial 3, I sampled the larval silos in this manner every other day after larval release.

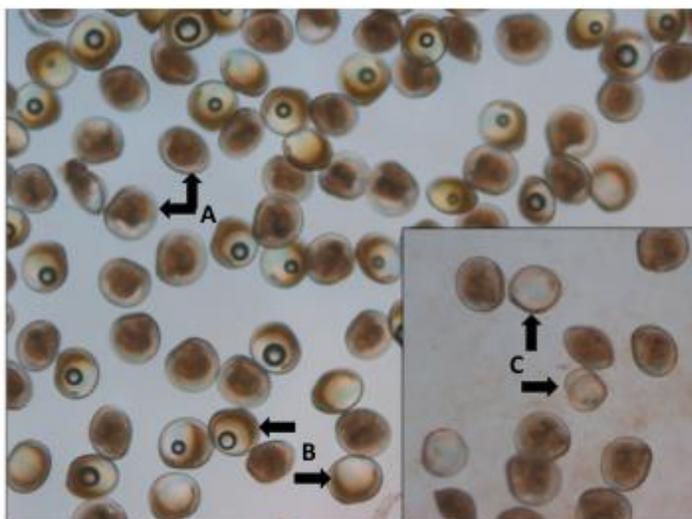


Figure 2. Photograph of larval samplings with examples of (a) live larvae, (b) dead larvae, and (c) empty shells. (date taken, camera info)

During each sampling, an aliquot of larvae (~100-500 μL) was placed in 100% molecular grade ethanol (Fisher Scientific) and stored at room temperature for later length and width measurements. To record images of larvae, we used a Nikon microscope (E600) and camera (Spot); NIS software (Nikon) was used to measure the width, height and hinge length of 30 larvae per replicate per date (trials 1 and 3). We observed shell formation and calcification using double polarized light microscopy (Collin & Voltzow 1998), in which the presence of a Maltese cross indicates birefringence, which confirms calcification (Bielefeld & Becker 1991). To determine any differences in shell morphology among treatments, on day 2 of trial 1 and day 11 of trial 3, larvae were preserved overnight in 4% paraformaldehyde in seawater, rinsed three times in sterile seawater and processed for routine scanning electron microscopy according to Paig-Tran & Summers (2014).

2.5 Data Analysis

Data were entered and organized in Microsoft Excel then transferred to JUMP for statistical analysis. Larval release data were analyzed by individual trial and by pooling all trials together. When pooled, only 400 μatm and 1600 μatm pCO_2 levels were used because these treatments were consistent across all three trials. Larval release by age was only calculated for broodstock for which age was known; this included Totten 2008 & 2009 and Hammersley 2010 each from trial 2. Start day for larval release was counted from the date adults reached ideal reproductive temperature (16°C) to initial date of release. Larval survival counts from the duplicate silos were entered separately and converted to percentage survived. In trial 3, FB released larvae earlier in the experiment but it was not enough to inoculate all silos. On 3/27/15 there was a large release but the trial was ended before we could see true differences in larval survival for that batch of larvae. For these reasons, I utilized the larval release data from FB but not the larval survival. HC adults never released larvae in trial 3.

3 Results:

3.1 Water Chemistry

Table 1. pH (total scale) was measured continuously with a probe during each trial and calibrated weekly with spec pH. Average carbonate chemistry conditions during each trial. Alkalinity was predicted for each day using a LSM model generated from measured alkalinity and salinity values and verified by weekly titrations. pCO₂, TCO₂, Ω Ar, and Ω Ca were calculated using Lueker et al. 2000. ²

Trial	Phase	Treatment	Temp (°C)		Salinity (ppt)		pCO ₂ (µatm)		Alk (µmol/kg)		TCO ₂ (µmol/kg)		pH		Ω Ar		Ω Ca		DO (mg/L)	
			measured	calculated	measured	calculated	measured	calculated	measured	calculated	measured	calculated	measured	calculated	measured	calculated	measured	calculated	measured	calculated
Trial 1 (2012)	Adult Conditioning	400	16.6 ± 0.4	404 ± 138	30.05 ± 0.22	404 ± 138	2079	1882 ± 87	8.06 ± 0.04	2.25 ± 0.90	3.53 ± 1.42	NA	NA							
		1000	17.1 ± 0.2	1088 ± 35	30.03 ± 0.17	1088 ± 35	2084	2035 ± 7	7.64 ± 0.03	0.94 ± 0.03	1.47 ± 0.05	NA	NA							
		1600	17.0 ± 0.1	1707 ± 53	29.98 ± 0.27	1707 ± 53	2079	2083 ± 14	7.46 ± 0.03	0.62 ± 0.02	0.97 ± 0.04	NA	NA							
		2400	17.6 ± 0.1	2475 ± 68	29.99 ± 0.32	2475 ± 68	2084	2129 ± 8	7.30 ± 0.03	0.45 ± 0.01	0.71 ± 0.02	NA	NA							
Trial 2 (2013)	Adult Conditioning	400	18.0 ± 3.5	479 ± 140	30.07 ± 0.12	479 ± 140	2103 ± 22	1927 ± 52	7.97 ± 0.09	2.00 ± 0.48	3.12 ± 0.74	NA	NA							
		1600	18.9 ± 2.96	1475 ± 380	30.06 ± 0.11	1475 ± 380	2105 ± 40	2080 ± 50	7.52 ± 0.10	0.82 ± 0.14	1.28 ± 0.21	NA	NA							
Trial 3 (2015)	Adult Conditioning	LCO ₂	14.24 ± 3.5	409 ± 71	29.44 ± 0.26	409 ± 71	2059 ± 9	1903 ± 27	8.04 ± 0.11	2.81 ± 0.40	1.78 ± 0.26	9.18 ± 0.96								
		HCO ₂	14.39 ± 3.5	1529 ± 1330	29.37 ± 0.31	1529 ± 1330	2038 ± 11	2034 ± 92	7.60 ± 0.2	1.32 ± 0.80	0.83 ± 0.49	8.66 ± 1.19								
	Larval Rearing	HCO ₂ / HDO	19.63 ± 0.4	1557 ± 141	29.78 ± 0.40	1557 ± 141	2069 ± 9	2051 ± 15	7.49 ± 0.04	1.17 ± 0.10	0.75 ± 0.07	8.40 ± 0.39								
		LCO ₂ / LDO	19.81 ± 0.2	1808 ± 75	29.73 ± 0.35	1808 ± 75	2068 ± 5	2068 ± 5	7.43 ± 0.02	1.02 ± 0.04	0.66 ± 0.02	3.63 ± 0.05								
LCO ₂ / HDO	LCO ₂ / HDO	19.67 ± 0.4	515 ± 17	29.88 ± 0.38	515 ± 17	2075 ± 14	1917 ± 13	7.92 ± 0.01	2.91 ± 0.11	1.87 ± 0.07	8.35 ± 0.13									
	LCO ₂ / LDO	19.51 ± 0.8	480 ± 25	29.50 ± 0.20	480 ± 25	2061 ± 0	1895 ± 6	7.96 ± 0.01	3.05 ± 0.09	1.95 ± 0.06	3.77 ± 0.22									

3.2 Larval Release

Adults conditioned at 400 μ atm released significantly more larvae than all other treatment groups (1000, 1600 and 2400 μ atm) which are statistically similar (Tukey HSD, $p < .0001$, $RSq = 0.84$, $\alpha = 0.05$, $Q = 2.59847$, $F = 8.39$). Since it was used in all three trials, our main comparison was between 400 and 1600 μ atm; those conditioned at 400 μ atm released significantly more larvae (all trials combined) (Students t , $\alpha = 0.05$, $t = 1.98217$). In trial 2, a lag in the timing of first larval releases was observed between treatments. Adults conditioned at 1600 μ atm released larvae 8 days later than those conditioned at 400 μ atm (Figure 3). In addition, in this trial, oysters 4 years of age released significantly more than 3 and 5 year old oysters (Tukey HSD, $F = 23.1$, $p < .0001$, $\alpha = 0.05$, $Q = 2.36012$).

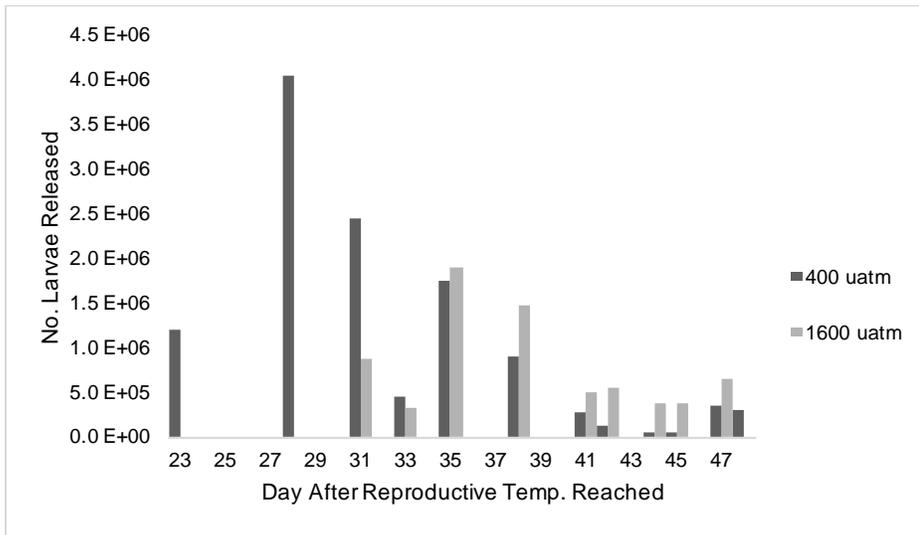


Figure 3. Histogram showing total larval releases for trial 2 for both CO₂ treatments (400 μ atm and 1600 μ atm). The average date of first release (after reproductive temp was reached) was 34 ± 2.9 & 30 ± 6.3 for H & L pCO₂ respectively.

3.3 Larval Survival

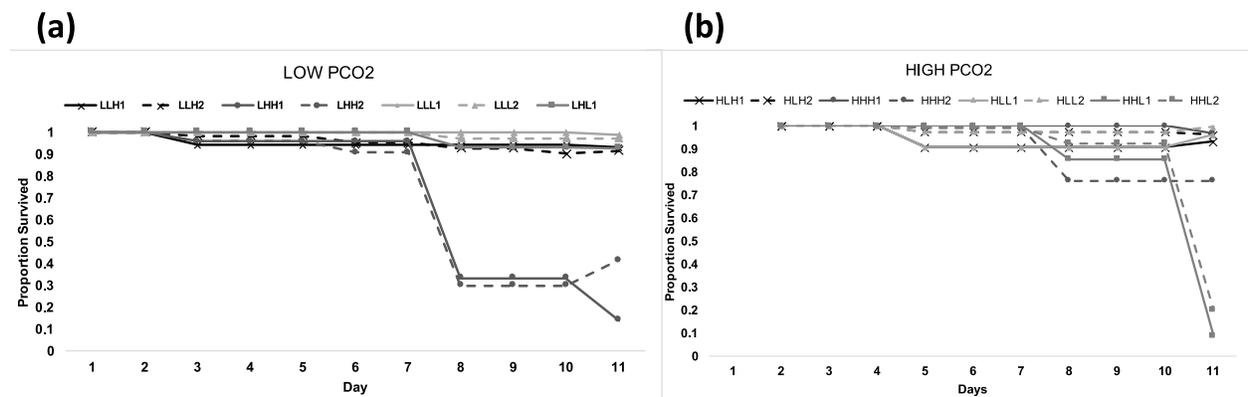


Figure 4. Proportion of larvae surviving per day by treatment for SS broodstock conditioned at (A) 400 μ atm released on 3/23/15 (placed in treatment on 3/24/15) and (B) 1600 μ atm released on 3/25/15. Proportion survived indicates survival for each silo ($n = 10,000$ larvae). Experimental day corresponds to days after the first larval release (3/23/15). See Figure 1 for treatment codes.

Larval survival declined sharply in LHH, between days 7-8, and in HHL treatment, between days 10-11 (Figure 4). In HHL, offspring of parents conditioned at high pCO₂ showed increased mortality when subjected to both high pCO₂ and low dissolved oxygen (14.7% survival on day 11). Larvae of LHH also showed decreased survival (22% on day 11). These are larvae of parents conditioned at low pCO₂ who were placed in a high pCO₂ and high DO larval treatment. All other treatments had greater than 86% survival by day 11.

3.4 Larval Development:

In trial 1, larval size was not influenced by pCO₂ level (K-W ANOVA p>0.05) and was influenced by time (K-W ANOVA p<0.05). Larvae grew an average of 4.8 μm in width (p=0.024) and 5.2 μm in shell height (p=0.021) over the 11 day period of rearing at the four pCO₂ levels (n=3 larval containers per pCO₂ level). No difference in hinge width was observed in relation to pCO₂ level or time (p>0.05). When viewed under SEM, there was no difference in the appearance of larval shells between treatments, and polarized light microscopy revealed the presence of full Maltese crosses indicating full shell development (see appendix D for measurements and images). The same was found in trial 3, that is, no difference in shell morphology between treatments (K-W ANOVA p>0.05) was observed. SEM photos of larval samples taken on the last day of the trial 3 (day 11) for LLH, LLL, LHH, and LHH treatments for comparison are shown in Figure 4.

4 Discussion

Olympia oysters show dual sensitivities to elevated pCO₂ and low DO resulting in lower larval release and decreased larval mortality. Trial 1 demonstrated no difference in larval release between adults conditioned at 1000, 1600, and 2400 μatm but did show that oysters conditioned at 400 μatm released significantly more larvae. When data from all three trials was pooled, oysters conditioned at 400 μatm released significantly more larvae compared to those held at 1600 μatm. These results suggest that the effect of CO₂ on larval release occurs somewhere between 400 and 1000 μatm pCO₂. Four-year-old oysters released greater numbers of larvae than 3 and 5-year-old oysters subjected to the same pCO₂ levels. This may be a result of the hermaphroditic nature of this species whereby the oysters act predominantly as males at the first spawning, and alternate the use of male and female reproductive organs for spawnings thereafter, leading to varying amounts of female and male oysters for each age group (Coe, 1931; Oates et al., 2013). While not significant, a lag was observed in the date of first release (days after reproductive temperature reached) with low pCO₂ conditioned adults releasing larvae an average of 7.8 days earlier than high pCO₂ conditioned adults. We may have missed the actual start of spawning in trials 1 and 3 and suspect that with more trials, this pattern may become significant. Regardless, the pattern is interesting when combined with the difference in the number of larvae released (Figure 3).

Synergistic effects between pCO₂ and DO may play a role in survival of Olympia oyster larvae. Our multi-generational, multi-stressor study looking pCO₂ and DO (trial 3) found that survival decreased in the HHL & LHH treatments. In the first case, only 14.7% of the offspring of parents conditioned at high pCO₂ survived when subjected to high pCO₂ and low DO. Low survival is not surprising given that this treatment was a combination of all stressors tested. Portner et al. (2005) predicted this when they anticipated that exposure to both high pCO₂ and

low DO would have negative synergistic effects, as observed in the HHL treatment. They reasoned that organisms respond to both low oxygen and high pCO₂ through metabolic depression and that the combination of the two stressors will exacerbate this metabolic dampening. The authors go on to explain that under each of these stressors, certain processes are depressed in an attempt to survive the condition, including acid-base regulation, protein synthesis, and aerobic turnover. When combined, the metabolic depression may be too great for the organism to complete the basic life processes needed to survive. While larvae of parents conditioned under high pCO₂ were able to survive one stressor such as low DO (HLL) or high pCO₂ (HHH) alone, the combination of the two stressors may have been too demanding (HHL). Furthermore, Lombardi (2012) reported gaping behaviors of adult *Crassostrea virginica* and *Crassostrea ariakensis* in response to hypoxia. Under hypoxic conditions, the pH around the gaping oysters was decreased significantly (7.06) compared to non-gaping (7.35). This suggests that hypoxia may lead to some kind of behavior by the oyster that decreases the pH in the immediate surrounding waters. Since *O. lurida* is a brooding oyster (Coe, 1931; Oates, 2013), the larvae may be naturally exposed to hypercapnia (high concentrations of CO₂) from their respiring mothers and thus be more resilient to effects of OA.

Larvae in the LHH treatment also showed decreased survival (22% on day 11). These are progeny of parents conditioned at low pCO₂ that were placed in a high pCO₂ and high DO treatment. Based on this result, we would expect decreased larval survival in the presence of high pCO₂ but the high survival of HHH (the same larval treatment but with high pCO₂ conditioned parents) suggests that some kind of transgenerational or carry-over effect may be taking place. Studies have shown that some organisms may possess non-genetic phenotypic plasticity within or across generations that allow for increased success (Ross et al. 2016). Parker et al. (2012) found the progeny of parents exposed to high pCO₂ had better tolerance to the effects of high pCO₂ exposure. In our experiments, larvae from the HHH & HHL treatments could have gained an increased tolerance to high pCO₂ from their parents, but the combination of low oxygen could have led to decreased survival in under HHL conditions.

Since the larvae are brooded, this may be a reflection of a carry-over effect. Since LHH larvae had not yet seen high pCO₂ like HHH larvae, perhaps their metabolism was not yet acclimated to this chemistry. Exposure to high pCO₂ can lead to the perturbation of metabolic processes. The depression of these processes along with hypercapnia (CO₂ retention) can decrease the rate of gas exchange across respiratory epithelia, depleting O₂ stores (Portner et al. 2005). Portner (1990) found that low pH can cause acute effects in the oxygen binding ability in two squid species (*Illex illecebrosus* and *I. pealei*) due to lowered plasma pH, disrupting oxygen transport by pH sensitive hemocyanin. Therefore, it is not without reason to think similar processes may take place in *O. lurida*, another mollusk. Lannig et al. (2010) discovered that under decreased pH seawater (7.68 vs 8.07) the hemolymph of *C. gigas* decreased in both pH (7.1 vs 7.6) and pO₂ (9.43 vs 11.44kPa) further supporting the idea that pCO₂ could have negatively impacted O₂ processes in LHH larvae.

The low survival of larvae exposed to LHH compared with the high larval survival under LHL conditions is more difficult to explain. Three of the four silos in this treatment showed less than 45% survival so experimental error is unlikely, although this treatment had the highest variation among silos (LHH1: 14%, LHH2: 42%, LHH3: 7%, LHH4: 83%). Lei Wei et al. (2015) reported osmotic stress and decreased metabolism of adults exposed to high pCO₂ (2008ppm) based on changes in metabolite composition in the gill tissue of *C. gigas*. This shift in metabolite composition (organic osmolytes, organic & amino acids, and energy storage

compounds) may have led to a shift of O₂ osmotic tolerance (potentially downward). Furthermore, Lei Wei et al. (2015) also found an increase in arginine kinase, a catalyzer of ATP into phosphoarginine. Arginine kinase (AK) has been shown to play a role in pH and oxidative stress response; when expressing high levels of AK, the parasite *Trypanosoma cruzi* exhibited increased survival in response to decreased O₂ and pH stress (Miranda et al. 2006). For larvae exposed to LHL, the exposure to elevated pCO₂ could have led to a shift in metabolites and overexpression of AK, leading to an increased survival response to both the low pH and low DO. Though unlikely, larvae of low pCO₂ parents, could be exhibiting some kind of metabolic preferential shift towards lower DO in response to hypercapnia resulting in high survival of LHL and low survival LHH larvae. This result is the hardest to explain and more research is needed to clarify this finding.

I propose a potential transgenerational or carryover effect exists in progeny of parents conditioned at high pCO₂ that led offspring from the HHH treatment to have greater survival than those in the LHH treatment, potentially decreasing the metabolic stress responses to hypercapnia. When offspring of parents conditioned at high pCO₂ were subjected to high pCO₂ and low DO, however, there were negative synergistic effects on survival were observed. I propose that larvae of parents conditioned under low pCO₂ respond to elevated pCO₂ via a decrease in tolerance to DO levels, potentially through metabolic shifts. Parental/early life history conditioning may play a role, in which parents (and therefore early larvae) conditioned under high pCO₂ prime larvae to deal with low pH but this change may not allow the larvae to respond as well to an additional metabolic depressor like low DO. These results emphasize the importance of multi-generational, multi-stressor studies and the need for more research regarding the interaction of high pCO₂ and hypoxia.

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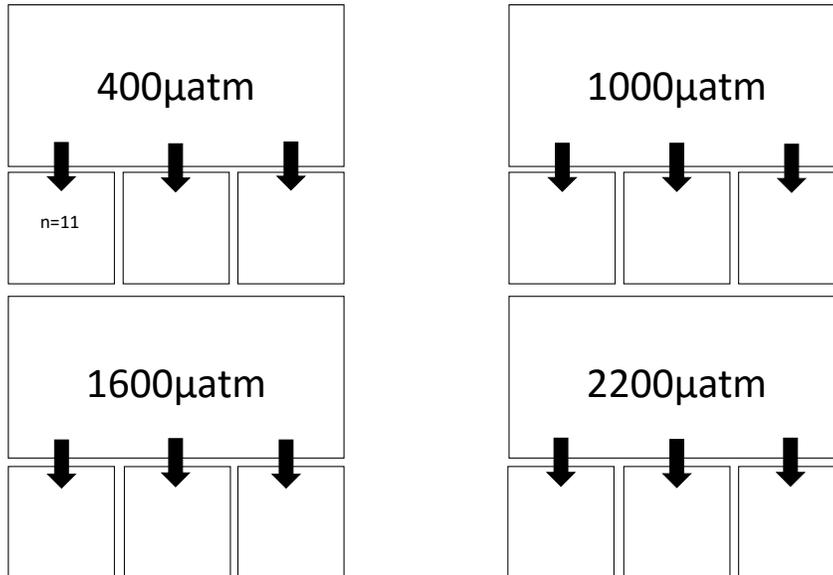
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APPENDIX:

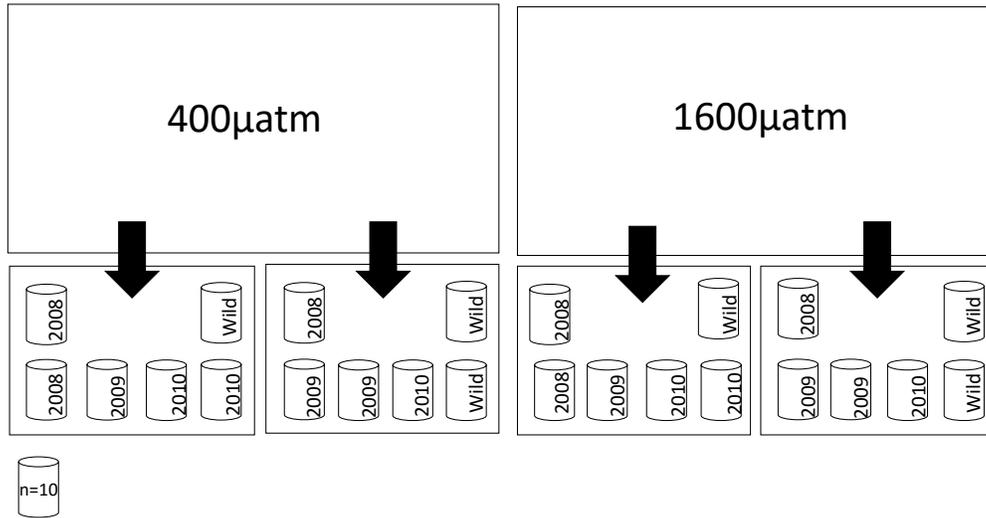
Appendix A. Table of average adult morphometric data for each broodstock group by trial. Measurements taken before adults were placed into treatments.

Trial	Site	Year Spawned	Age	Total Weight (g)	Total Length (mm)
Trial 1	Totten	ukn	ukn	12.7	40.2
Trial 2	Totten	2008	5	16.5	ukn
	Totten	2009	4	13.6	ukn
	Hammersley	2010	3	12.7	ukn
	Wild	ukn	ukn	9.6	ukn
Trial 3	Fidalgo Bay	ukn	ukn	19.4	48.7
	South Sound	ukn	ukn	10.8	38.8
	Hood Canal	ukn	ukn	9	33.6

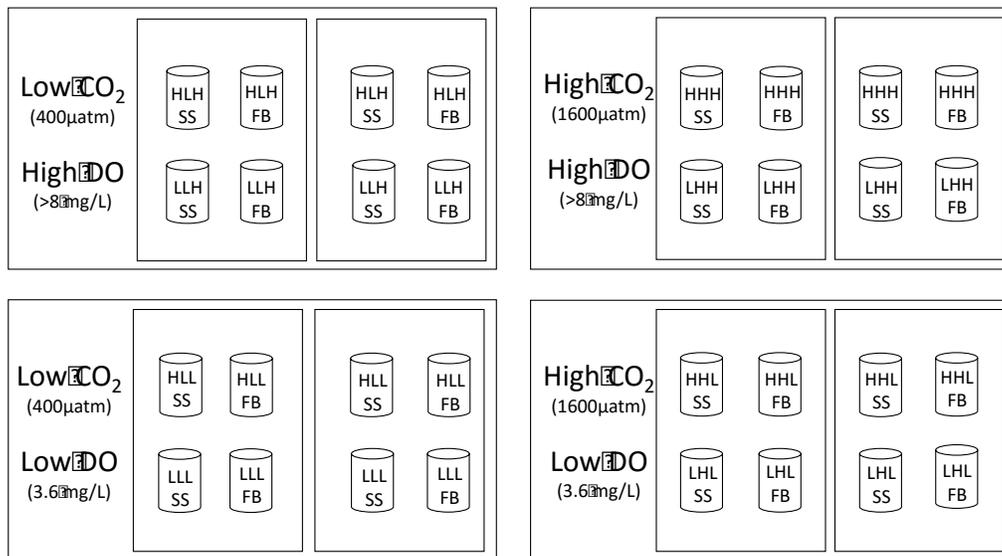
Appendix B. A diagram of experimental set up for Trial 1. Water chemistry for each of the four treatments were obtained in head tanks (coolers) which supplied water to three smaller conditioning tanks (total of 12 conditioning tanks) situated below. Each conditioning tank held n=11 oysters.



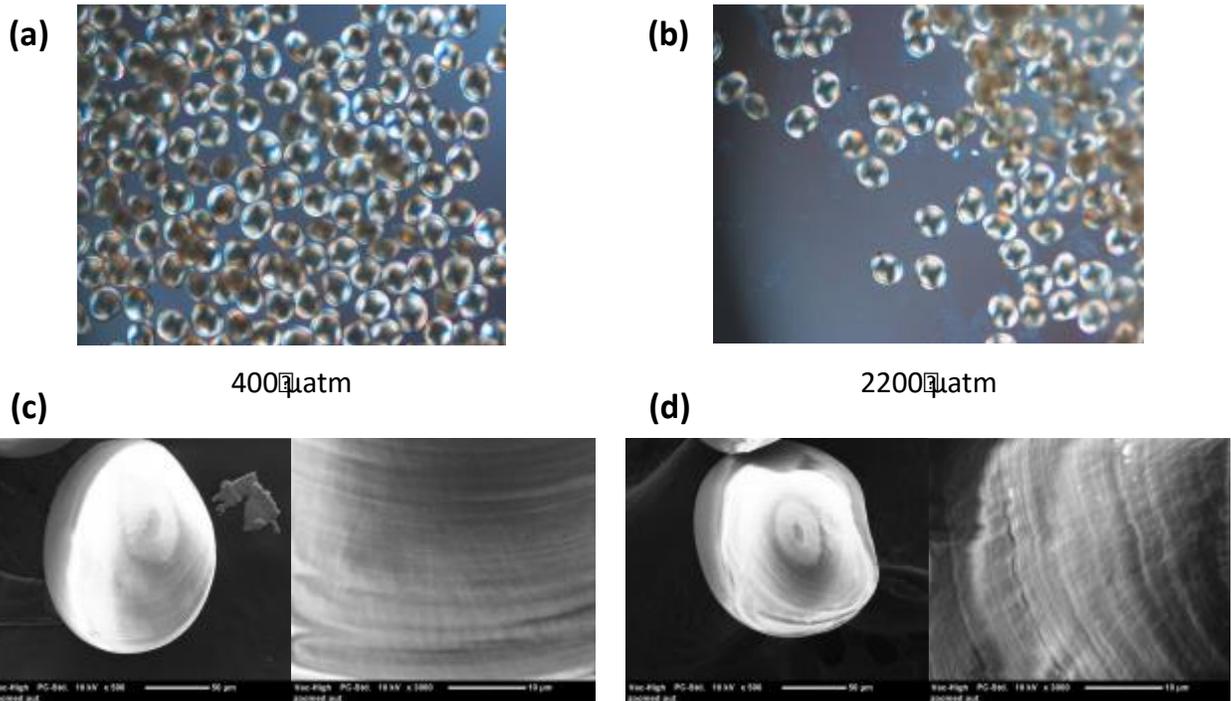
Appendix C. A diagram of experimental set up for trial 2. Water chemistry for each of the four treatments were obtained in head tanks (coolers) which supplied water to two smaller conditioning tanks below (total of four conditioning tanks). Each tank housed six 100 μ m mesh bags containing n=10 adult oysters from various sites and spawning years. This includes Totten inlet (2008 spawn), Totten Inlet (2009 spawn), Hammersley Inlet (2010 spawn), and Wild oysters (spawn year unk). There were three bags of each site per treatment with two bags in one conditioning tank, and one in the other.



Appendix D. A diagram of experimental set up for trial 3. Water chemistry for each of the four treatments were obtained in head tanks (coolers) which supplied water to two smaller conditioning tanks inside (total of four conditioning tanks). Each tank housed eight 100 μ m mesh silos from various spawn dates. Larvae from each spawn were divided into the four larval treatment groups (10,000 larvae/silo) in duplicate with one silo in each tank.



Appendix E. (A&B) are by photographs of larvae from trial 1 sampled on day 11 taken from double polarized light for 404 μ atm and 2475 μ atm treatments (Nikon E600). (C & D) are SEM of larval shells from the same sampling for the same two treatments. (E) table of larval shell width, height, and length (μ m) for trial 1 from samples taken on days 1 and 11 for the four pCO₂ treatments.



e)

pCO ₂ level ¹	Maximum Shell Width (μ m)		Maximum Shell Height (μ m)		Maximum Hinge Length (μ m)	
	Day 1	Day 11	Day 1	Day 11	Day 1	Day 11
400 μ atm	167.8 \pm 8.9 ²	177.8 \pm 10.6	149.1 \pm 7.2	161.5 \pm 10.3	83.7 \pm 9.7	84.2 \pm 8.9
1000 μ atm	171.7 \pm 8.7	174.0 \pm 8.9	154.1 \pm 8.3	157.3 \pm 9.3	86.0 \pm 10.3	92.5 \pm 9.5
1600 μ atm	166.5 \pm 10.6	167.1 \pm 11.5	148.6 \pm 8.1	150.0 \pm 10.3	88.0 \pm 11.0	85.4 \pm 9.9
2200 μ atm	164.0 \pm 8.3	170.4 \pm 8.1	146.4 \pm 7.2	150.4 \pm 7.9	84.5 \pm 11.0	86.1 \pm 10.5

Appendix F. SEM of larvae at the end of trial 3 for four treatment groups. Shell morphology on day 11 was indistinguishable among treatment groups: A = LLH, B = LLL, C = LHH, and D = LHH treatment groups. Bars = 200 μ m. Images were taken with a JOEL CM-5000 NeoScope™.

