Adult low pH exposure influences larval abundance in Pacific oysters (*Crassostrea gigas*)

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Abstract

As negative effects of ocean acidification are experienced by coastal ecosystems, there is a growing trend to investigate the effect ocean acidification has on multiple generations. Parental exposure to ocean acidification has been shown to induce larval carryover effects, but whether or not an acute exposure to a stressor as an adult can influence the larval generation long after the stress has been removed has yet to be tested. To assess how a temporary exposure to experimental ocean acidification affects the ecologically and commercially relevant Pacific oyster (*Crassostrea gigas*), adult oysters were exposed to either low pH (7.31 ± 0.02) or ambient pH (7.82 ± 0.02) conditions for seven weeks. Oysters were then held for eight weeks in ambient conditions, and subsequently reproductively conditioned for four weeks at ambient pH. After conditioning, oysters were strip-spawned to create four families based on maternal and paternal ocean acidification exposure. The number of D-hinge larvae were counted eighteen hours post fertilization. A sex-specific broodstock response was observed, where female exposure to low pH conditions resulted in fewer D-hinge larvae. This study demonstrates that the effects of ocean acidification can last beyond the time from when the environmental perturbation is experienced. Broadening the understanding of environmental memory will be valuable when considering an organism's ability to persist in the face of environmental change.

Keywords: ocean acidification, maternal effect, carryover effect, Pacific oyster, Crassostrea gigas, D-hinge larvae, response timing

Introduction

Determining how parental exposure to ocean acidification carries over into early larval stages is important for understanding cumulative effects of climate-related environmental change. Gametogenesis is a key period during which parental exposure to ocean acidification can influence offspring (Donelson et al. 2018). Several studies exposing Sydney rock oysters (*Saccostrea glomerata*) to high pCO_2 conditions (856 µatm, pH_{NBS} 7.89-7.90) during reproductive conditioning identified positive larval carryover effects (Parker et al. 2012, 2015, 2017). Specifically, larvae from parents exposed to low pH conditions were larger and developed faster in acidified conditions compared to those from parents reared in ambient pH conditions (Parker et al. 2012, 2015, 2017). Conversely, similar experiments conducted with hard clams (*Mercenaria mercenaria*) and bay scallops (*Argopecten irradians*) demonstrated negative larval carryover effects (Griffith and Gobler 2017). *A. irradians* and *M. mercenaria* larvae from parents exposed to low pH were more sensitive to acidified conditions than those spawned from parents exposed to ambient pH during reproductive conditioning (Griffith and Gobler 2017). These studies demonstrate the importance of parental exposure during reproductive conditioning (late stage gametogenesis) on offspring.

As the Pacific oyster (*Crassostrea gigas*; Thunberg, 1793) is a commercially and ecologically relevant species in much of the world, several research efforts have identified consequences of ocean acidification for distinct *C. gigas* life stages. While fertilization still occurs under near-future ocean acidification conditions (Kurihara et al. 2007; Havenhand and Schlegel 2009; Boulais et al. 2018), fertilization success in acidified conditions is variable between *C. gigas* populations (Parker et al. 2010; Barros et al. 2013). Researchers have found that larvae experience developmental delays and reduced shell growth when exposed to experimental ocean acidification conditions (Kurihara et al. 2007; Gazeau et al. 2011; Timmins-Schiffman et al. 2013; Waldbusser et al. 2014). Natural upwelling-induced ocean acidification conditions also reduced larval production and growth in a hatchery setting (Barton et al. 2012). Ocean acidification hampers protein expression in larvae, especially for proteins related to calcification and cytoskeleton production (Dineshram et al. 2012). During metamorphosis, oyster larvae experience down-regulation of proteins related to energy production, metabolism, and protein synthesis (Dineshram et al. 2016). Adult *C. gigas* calcification rates decrease as seawater pCO_2 increases (Gazeau et al. 2007), with oysters grown at 2800 µatm displaying significantly lower fracture toughness than oyster shells from ambient conditions (Timmins-Schiffman et al. 2014). Exposure to ocean acidification also affects adults' antioxidant response, carbohydrate metabolism, transcription, and translation protein pathways (Timmins-Schiffman et al. 2014). There is also evidence of predator-prey interactions changing under experimental ocean acidification (Wright et al. 2018). However, there is limited evidence of how ocean acidification influences Pacific oysters across multiple generations.

The current study is the first to discern how exposure to experimental ocean acidification prior to reproductive conditioning affects larval abundance in *C. gigas*. This experiment not only describes how isolated exposure to low pH during early gametogenesis influences larvae, but also provides information on the effects of acute pH exposure on adult gonad morphology. Additionally, the study demonstrates how environmental perturbation experienced before reproductive maturity affects the subsequent generation, even if the stressor is long-removed.

Methods

Experimental overview

Experimental trials were conducted at the Kenneth K. Chew Center for Shellfish Research and Restoration at the National Oceanic and Atmospheric Administration (NOAA) Manchester Field Station (47°34'09.1"N 122°33'19.0"W, Manchester, Washington, USA) in 2017. Adult hatchery-raised *C. gigas* (average shell length = 117.46 ± 19.16 cm) were acclimated in the facility for 10 days, then exposed to either low or ambient pH conditions for 48 days (Figure 1). After pH exposure, oysters were held at ambient pH and water temperature conditions for 90 days. Oysters underwent reproductive conditioning for 22 days, then strip-spawned. D-hinge larvae were counted eighteen hours after fertilization occurred.

Experimental pH exposure

The experimental system consisted of a 1,610 liter storage tank that fed two 757 liter header tanks. Water from Clam Bay, WA was pumped through a sand filter, then UV-treated. The UV-treated water passed through a set of three sock filters (100 µm, 50 µm, and 25 µm) and a degassing column. Once degassed, water passed through three more sock filters (25 µm, then 10 µm, and 5 µm) before entering the storage tank. The storage tank was outfitted with an off-gas vent and pump to recirculate water such that CO_2 in the water could be equilibrated with atmospheric CO_2. Equilibrated water flowed into the two header tanks, each of which fed three 50L flow-through (1.2 L/min) experimental tanks (six experimental tanks total). For all header and experimental tanks, pH in header and experimental tanks was continuously monitored using Durafet pH probes (Honeywell Model 51453503-505) and an AVTECH system. Addition of CO_2 in the low pH header tank was controlled using a solenoid valve. A Dual Input Analytical Analyzer (Honeywell Model 50003691-501) automatically mediated solenoid injections. A CO_2 air line with a back pressure of 15 psi, controlled with a regulator, injected CO_2 into the low pH header tank every 180 seconds with an injection duration of 0.4 seconds. Injections only occurred if real-time pH from the Durafet was above pH 7.22. A venturi injector connected to the ambient water line mixed ambient pH water with CO_2-rich water to lower pH. There were no CO_2 injections in the ambient header tank.

Prior to the pH exposure trial, twenty randomly selected *C. gigas* were lethally sampled to assess gonadal status (see Histological analysis). *C. gigas* were placed in each flow-through experimental tank in ambient water conditions and exposed to ambient or low pH conditions for seven weeks. Each treatment consisted of 3 tanks, each with 20 oysters. All experimental tanks received algae from a common reservoir. The algal tank contained 300-500 mL of Shellfish Diet 1800® (Reed Mariculture) diluted in 200L of ambient pH seawater (Helm and Bourne 2004). Algae was continuously dosed to oyster experimental tanks using an Iwaki Metering Pump. Algal lines were cleaned twice weekly, and experimental tanks were fully drained and cleaned once a week.

Seawater chemistry analysis

Twice a week, water samples (1L) were collected from each header and oyster experimental tank. For each sample, salinity (PSU) was measured with a Bench/Portable Conductivity Meter (Model 23226-505, VWR), pH (mV) was measured with a Combination pH Electrode (Model 11278-220, Mettler Toledo), and temperature (ºC) was measured using a Traceable Digital Thermometer (Model 15-077, Fisher). To calibrate the pH probe, a Tris buffer (0.08 M, 28.0 PSU) was prepared using 0.3603 mol of NaCl (J.T. Baker), 0.0106 mol of KCl (Fisher Scientific), 0.0293 mol MgSO4-$(H_2O)_7$ (Fisher Scientific), 0.0107 mol of $CaCl_2$-2(H_2O) (MP Biomedicals), 0.0401 HCl (J.T. Baker), and 0.0799 mol of Tris base (Fisher Scientific). Deionized water was added for a final volume of 1L. Salinity, temperature, and pH measurements for the Tris buffer were obtained at five temperatures before measuring samples to generate a standard curve. This standard curve was used to calibrate the pH electrode and convert measured millivolts to pH units.

For total alkalinity measurements, duplicate seawater samples (250 mL) were collected from experimental tanks twice weekly and dosed with mercuric chloride (50 µL of 0.18 M solution) to preserve samples (Bandstra et al. 2006). Samples from days 5, 33, and 48 were run on a T5 Excellence titrator (Mettler Toledo) to determine alkalinity. Salinity (PSU) from discrete samples was used to calculate total alkalinity, using the *seacarb* library in R (Gattuso et al. 2018). Calculated pH, total alkalinity, temperature, and salinity were also used in *seacarb* to calculate in situ pH, pCO_2, dissolved organic carbon (DIC), calcite saturation ($\Omega_{calcite}$), and aragonite saturation ($\Omega_{aragonite}$) for days 5, 33, and 48. R code used to calculate water chemistry parameters is available (Venkataraman et al. 2018).

Histological analysis

Twenty randomly selected *C. gigas* were lethally sampled before pH exposure for histological analyses. On the last day of low pH exposure, ten oysters from each treatment — randomly selected from each tank — were also lethally sampled to assess gonadal status. For each sampled oyster, a piece of gonad tissue was cut and placed in a histology cassette. Gonad tissue in cassettes was fixed for histology using PAXgene Tissue FIX and STABILIZER and sent to Diagnostic Pathology Medical Group, Inc. (Sacramento, CA) for staining with hematoxylin and eosin and slide preparation. Tissues exposed to ambient pH were confounded during processing, preventing any tank identification. Maturation state and organism sex was evaluated histologically at 40x magnification (Fabioux et al. 2005; Enríquez-Díaz et al. 2008).

Reproductive conditioning

Following seven weeks of low pH exposure, oysters were returned to a common garden and maintained at ambient pH conditions for eight weeks. Afterwards, oysters were reproductively conditioned. Water temperatures and food quantity are known to regulate the timing, speed, and intensity of gametogenesis in *C. gigas* (Enríquez-Díaz et al. 2008). Conditioning protocol was modeled after standard hatchery practices (Molly Jackson, Broodstock Manager at Taylor Shellfish, pers. comm., June 2017). Water temperature was raised from ambient conditions (13ºC) to 23ºC over three weeks (1ºC/2 days), since optimal temperature for *C. gigas* gametogenesis is between 18ºC and 26ºC (Parker et al. 2010). Conditions were maintained at 23ºC for one more week prior to spawning. During conditioning, *C. gigas* were fed 700-800 mL of Shellfish Diet 1800® daily (Helm and Bourne 2004).

Strip spawning and larval rearing

After reproductive conditioning, all surviving oysters were prepared for strip spawning. A sample of gonad from each individual was assessed for presence of active sperm or eggs using a microscope at 10x magnification. Only *C. gigas* with active sperm or eggs were used for crosses ($n_{male,\ low}$ = 6, $n_{female,\ low}$ = 22, $n_{male,\ ambient}$ = 6, $n_{female,\ ambient}$ = 26). Presence of mature gametes and ripe oysters indicated that oysters were in good condition and not affected by use of Shellfish Diet 1800® instead of live algae during reproductive conditioning. For each treatment (low pH and ambient conditions), one gram of mature gonad from each ripe female was pooled. The number of eggs in both the ambient and low pH pools were counted to determine the number of eggs used for parental crosses. Parental crosses were created using 210,000 eggs from the female egg pools and sperm (200 µL) from individual males.

Four half-sibling families were created based on parental pH exposure: low pH female (pool) x low pH male, low pH female (pool) x ambient pH male, ambient pH female (pool) x low pH male, and ambient pH (pool) female x ambient pH male. These crosses were conducted using pooled eggs from either low pH or ambient pH females, and sperm from one of six males within each pH treatment (e.g. low pH female pool x low pH male-01, low pH female pool x low pH male-02, ... low pH female pool x low pH male-06), totaling 24 crosses. All crosses were performed in duplicate, resulting in 48 separate fertilization events.

Fertilization was carried out in plastic beakers (1L) for 20 minutes with static 23ºC filtered seawater (1 µm) in ambient pH conditions. After confirming polar body formation, beaker contents were transferred to larger plastic tanks (19L) with aerated, static 23ºC filtered seawater (1 µm) for eighteen hours of incubation. Duplicate containers were combined eighteen hours post-fertilization, and D-hinge larvae were counted for each cross (n= 24).

Statistical analyses

Differences in *in situ* pH, total alkalinity, pCO_2, DIC, $\Omega_{calcite}$, and $\Omega_{aragonite}$ between pH treatments were evaluated with a one-way ANOVA. Because tissue samples were confounded during histological processing, a binomial GLM model was used to compare gonad maturation between pH treatments. Differences in sex ratios between pH treatments were evaluated using a chi-squared test of homogeneity. To identify differences in D-hinge larval counts, a linear mixed model was used, with sire and female egg pool as random effects. Differences in D-hinge larval counts by female treatment were assessed using a similar linear mixed model, with only sire as a random effect. Normality of data, as well as independence and homoscedasticity, were verified visually. All statistical analyses were carried out in R (Version 3.4.0). R Scripts are available in the supplementary Github repository (Venkataraman et al. 2018).

Results

Water chemistry

C. gigas exposed to low pH experienced different water chemistry parameters than those in the ambient pH treatment (Table 1). Using water samples from days 5, 33, and 48, pH (One-way ANOVA; $F_{1, 16}$ = 5838.7810, p = 6.1165e-22), pCO_2 (One-way ANOVA; $F_{1, 16}$ = 235.4018, p = 5.4421e-11), DIC (One-way ANOVA; $F_{1, 16}$ = 7.1222, p = 0.0168), $\Omega_{calcite}$ (One-way ANOVA; $F_{1, 16}$ = 528.9468, p = 1.0989e-13), $\Omega_{aragonite}$ (One-way ANOVA; $F_{1, 16}$ = 526.5207, p =1.1389e-13) were significantly lower in the low pH treatment. Total alkalinity, however, was not significantly different between pH treatments (One-way ANOVA; $F_{1, 16}$ = F = 1.382, p = 0.2570).

Gonad maturation

A binomial GLM was used to compare gonad maturation of individuals sampled before and immediately after pH exposure, but before reproductive conditioning. The most parsimonious model included only sampling time (before or after pH treatment). Gonad maturation status was not significantly different between *C. gigas* sampled before and after pH treatment (binomial GLM; $F_{2, 37} = 0.7973$, $p = 0.3442$). Additionally, maturation status was not different between pH treatments (binomial GLM; $F_{3, 36} = 2.2675$, $p = 0.1408$). No sampled oysters possessed fully mature gametes, but some males sampled appeared to be undergoing resorption (Table S1; Figure S1). Sex ratios were also similar between low and ambient pH treatments (Chi-squared test for homogeneity; $X^2_2 = 3.2279$; $p = 0.1942$).

Larval Survival

A linear mixed effect model, with female pool and sire as a random effects, demonstrated no significant difference in the number of D-hinge larvae counted eighteen hours post-fertilization between all four parental families (Linear mixed effect model; $X^2_3 = 3.1325$; $p = 0.1066$). Sire and female egg pools accounted for 0.8530% and 3.1623% of total variance, respectively. Significantly fewer D-hinge larvae were present in half-sibling families where females were exposed to low pH conditions (Figure 2; Linear mixed effect model; $t = -2.999$; $p = 0.0119$), with sire accounting for 0.3116% of total variance.

Discussion

The present study is the first to document the transgenerational influence of ocean acidification on Pacific oysters. Larval *C. gigas* was negatively impacted when maternal broodstock were exposed to low pH (pH = 7.31), suggesting a maternal carryover effect. The experimental design of this study is also unique — adult *C. gigas* experienced low pH conditions three months prior to reproductive conditioning, then were kept solely in ambient pH conditions through strip spawning and larval rearing. Since environmental perturbation experienced before *C. gigas* were mature still affected larval oysters, the results indicate a role for environmental memory in the Pacific oyster's response to ocean acidification. Mechanisms for transgenerational environmental memory have been explored in response to acute stressors in other species. *Daphnia magna* exposed to high salinity conditions had altered DNA methylation patterns, and these patterns were inherited by the following three non-exposed generations (Jeremias et al. 2018). Significant carryover effects observed in *C. gigas* — solely exposed to low pH when immature — broaden the current understanding of stressor timing and its effect on organismal physiology.

While it is evident that acute exposure to low pH experienced by adult *C. gigas* resulted in detrimental effects for larvae, the fact that larvae were not reared in acidified conditions makes cross-study comparison difficult. If *C. gigas* larvae were also reared in acidified conditions, it is possible that larvae with a history of parental exposure to experimental ocean acidification may have exhibited a negative carryover effect on larval growth and performance. Negative carryover effects have been found in other marine invertebrate taxa, but all studies involved exposure to experimental ocean acidification during reproductive conditioning and larval rearing in acidified conditions. Tanner crabs (*Chionoecetes bairdi*) solely exposed to acidified water (pH 7.5 or 7.8) as larvae did not exhibit significant changes in morphology, size, Ca/Mg content, or metabolic rate (Long et al. 2016). However, substantial effects on physiology was observed when larvae had a history of maternal exposure during oogenesis (Long et al. 2016). Larvae from adult Atlantic hard clams (*Mercenaria mercenaria*) and bay scallops (*Argopecten irradians*) developed slower when parents were reproductively conditioned in low pH conditions (pH_T = 7.4) (Griffith and Gobler 2017). Additionally, larvae with a history of parental low pH exposure were more vulnerable to additional stressors like thermal stress, limited food, and harmful algae exposure (Griffith and Gobler 2017). Although *C. gigas* were not reproductively conditioned in acidified water, and the present study cannot distinguish between hatching success and early mortality, identifying a similar negative larval carryover effect four months after an acute environmental perturbation is arguably more surprising and significant, particularly in terms of efforts to understand the mechanism of environmental memory.

The severity of conditions experienced by organisms may also explain whether or not offspring demonstrate transgenerational acclimatization to stressors. For example, the negative carryover effect observed in *C. gigas* is different from the positive carryover effects observed in ocean acidification experiments conducted with Sydney rock oysters. When adult *S. glomerata* were exposed to acidified seawater (pCO_2 = 856 µatm; pH_{NBS} = 7.89-7.90) during reproductive conditioning, resultant larvae were larger and developed faster in acidified conditions when compared to larvae from parents exposed to ambient conditions (Parker et al. 2012). This positive carryover effect was found to persist in the F2 generation. In acidified conditions, F_2 offspring with a history of transgenerational (F_0 and F_1) pCO_2 exposure grew faster and demonstrated fewer shell abnormalities (Parker et al. 2015). While species-specific responses can certainly explain the observed differences in larval phenotypes, it is also likely that inconsistencies in treatment conditions between experiments resulted in dose-dependent effects. Parker et al. (2012, 2015, 2017) used a high pCO_2 treatment of 856 µatm (pH = 7.89-7.90), with a control of 380-385 µatm (pH = 8.19-8.20). Therefore, the elevated pCO_2 treatment used in Parker et al. (2012, 2015, 2017) is similar to the ambient pH treatment (7.82; pCO_2 = 747.51-912.22) in the present study. Sydney rock oyster larvae with a history of transgenerational exposure exhibited faster development, but exhibited similar survival and were only 10% larger in acidified conditions when compared to larvae with no transgenerational exposure history (Parker et al. 2012). With a relatively smaller effect size and a milder treatment than used in this study, it is possible these studies are not at odds, but reflect dose-dependent effects on larval phenotypes. Negative carryover effects demonstrated in this study and in Griffith and Gobler (2017) can also be attributed to similar treatment pH levels (Griffith and

Gobler 2017: pH_T = 7.4, this study: pH = 7.31). Both of these studies used treatment levels more extreme than International Panel of Climate Change projections for open ocean acidification, but consistent with coastal and estuarine acidification scenarios experienced at study locations (Feely et al. 2010; Griffith and Gobler 2017; Pelletier et al. 2018). More research is required to understand how location-specific conditions will affect multiple generations in a single species.

Although the effect of water chemistry on gametogenesis has been recorded in other taxa, it is unlikely that a low pH exposure occurring three months prior to reproductive conditioning could have affected gonad maturation. Studies in which reproductive conditioning and experimental ocean acidification occur concurrently have demonstrated negative effects on maturation and fecundity. Gametogenesis, especially oogenesis, was disrupted in Eastern oysters (*Crassostrea virginica*) that experienced severe ocean acidification conditions during reproductive conditioning (pH = 7.71, 5584 µatm) (Boulais et al. 2017). Green sea urchins (*Stronglyocentrotus droebachiensis*) exposed to high pCO_2 (1200 µatm) conditions for four months demonstrated low fecundity (Dupont et al. 2013), and *S. glomerata* conditioned in high pCO_2 (856 µatm) conditions exhibited reduced rates of gametogenesis, smaller gonad area, and reduced fecundity (Parker et al. 2018). Gonad histology from *C. gigas* taken immediately after low or ambient pH exposure did not indicate any differences in maturation state, or interaction between sex and maturation state, between treatments. Even if fecundity or rates of gametogenesis differed between treatments, a return to ambient conditions for three months may have reversed any detrimental effects.

Reduced *C. gigas* larval abundance could have been a result of altered maternal provisioning in female oysters exposed to low pH conditions. In the face of stressors, females can either increase maternal provisioning (Allen et al. 2008; Sunday et al. 2011) — diverting more resources, like lipids or proteins, into eggs — or decrease provisioning due to energetic constraints (Liu et al. 2010; Uthicke et al. 2013). For example, changes in fatty acid provisioning from maternal exposure to high pCO_2 conditions (2300 µatm) in Atlantic silverside (*Menidia*

menidia) resulted in lower embryo survival when eggs lacked certain fatty acids (Snyder et al. 2018). This phenomenon, however, was not documented in the Sydney rock oyster: while elevated pCO_2 conditions (856 µatm) reduced the amount of energy invested in maternal gonads, these conditions did not impact *S. glomerata* egg size or total lipid content (Parker et al. 2018). Since adult *C. gigas* did not experience environmental perturbation after low pH exposure, and received enough food to spawn well, any impact on maternal provisioning and subsequent larval abundance was likely a result of low pH three months prior to reproductive conditioning.

The documented effect on Pacific oyster larval abundance four months after low pH exposure indicates an important a role for environmental memory in *C. gigas* response to ocean acidification. Low pH exposure may have induced epigenetic modifications (eg. changes in DNA methylation) in adult *C. gigas*. Studies of finfish and shellfish aquaculture species have demonstrated environmentally-induced epigenetic modifications that modify phenotypic responses in organisms (Gavery and Roberts 2017). One notable study in *C. gigas* examined parental effects of adult pollutant exposure on offspring (Rondon et al. 2017). Spat from parents exposed to the herbicide diuron had differential methylation in coding regions, with some changes leading to differential gene expression (Rondon et al. 2017). This research indicates that a mechanism crucial for phenotypic plasticity and acclimation across generations exists, and this knowledge can be analyzed in the context of climate-related environmental stressors. Epigenetic modifications in response to ocean acidification have been documented in coral species (Putnam et al. 2016), but not in molluscs. However, several experimental ocean acidification studies hint at the role of epigenetic memory. Hettinger et al.'s (2013) finding that Olympia oyster (*Ostrea lurida*) exposed to high pCO_2 (1000 µatm) conditions still grew less in the juvenile life stage than counterparts reared in ambient pCO_2, even after the stressor had been removed, and Parker et al.'s (2012, 2015, 2017) documentation of transgenerational acclimation in *S. glomerata* larvae with a history of exposure to acidified conditions could be explained by changes in the epigenome that affect organismal performance. Methylation levels are known to increase over the course of gametogenesis, with male and female *C. gigas* exhibiting significantly different methylation patterns (Zhang et al. 2018). If epigenetic modifications were acquired by female oysters during low pH exposure, it could explain why a significant effect on larval abundance was detected four months after the exposure ended. Epigenetic mechanisms and altered maternal provisioning are not necessarily mutually exclusive — changes in the methylome could influence maternal provisioning — and both could contribute to the results observed in this study.

The results of this study emphasize the need to broaden the scope of when environmental perturbation experienced by an organism is considered stressful, and when an effect can be detected. Although there was no observable effect on adult gonad maturation right after low pH exposure, significant differences in larval abundance were detected four months after the exposure ended. Stressor timing and duration can impact transgenerational responses between mature parents and offspring (Donelson et al. 2018). While experimental ocean acidification (pH 7.7; pCO_2 = 800 µatm) increased female investment in amphipods (*Gammarus locusta*), the subsequent generation exhibited fewer eggs and lower fecundity in the same conditions (Borges et al. 2018). Transgenerational benefits of maternal exposure to different temperatures (17ºC or 21ºC) in threespine stickleback (*Gasterosteus aculeatus*) differed based on exposure duration (Shama and Wegner 2014). Grandparents (F_0) were only exposed to treatment temperatures during reproductive conditioning, while parents (F_1) experienced either temperature over the course of development. The F_1 generation exhibited temperature tolerances similar to the F_0 maternal rearing environment, but the F_2 generation tolerance was more similar to the F_0 generation than the F_1 generation (Shama and Wegner 2014). However, the present study demonstrates that length and timing of environmental perturbation experienced by immature individuals can still affect offspring. Massamba-N’Siala et al. (2014) elucidated a similar phenomenon with marine polychaetes (*Ophryotocha labronica*): offspring experienced positive carryover effects of female exposure to temperature conditioning only when mothers were exposed to these conditions during late oogenesis; exposure during early oogenesis lead to negative carryover effects. More research should be conducted to understand how stressor timing, specifically before reproductive maturity, can impact carryover effects.

Most other experiments investigating stressor timing are conducted in a multiple stressor framework (Gunderson et al. 2016). For example, elevated temperatures and low salinity had synergistic effects on *O. lurida* when they were co-occurring stressors, but two to four weeks of recovery in between stressors negated these effects (Bible et al. 2017). Incorporating recovery time in a single-stressor experimental design is also crucial for accurately understanding how environmental perturbation impacts organism physiology. Exposure at one point in time may elicit a response much later in time, in a different environmental setting, or in a different generation, as evidenced by the present study and Hettinger et al. (2013). The experimental design in the present study is unique, featuring a significant recovery time between low pH exposure and spawning. More single-stressor experiments should incorporate lag times between exposure to stress and measuring response variables to understand if these responses change over time. Adding a multigenerational component to such experiments can elucidate if acute exposures generate carryover effects.

Significant decreases in larval abundance four months after broodstock were exposed to acidified seawater has implications for both aquaculture and natural *C. gigas* populations. Parents and offspring — or even different offspring life stages — may not experience the same environmental chemistry. For example, upwelling conditions affecting adult *C. gigas* may subside once spawning occurs. Long-term monitoring of wild Pacific oyster populations, with detailed environmental chemistry reporting, will be crucial for understanding how brief exposures to adverse conditions affect reproductive success and larval abundance in the field. Responses to stressors should not only be documented during and after the perturbation occurs, but also for an extended time afterwards. Hatchery-reared *C. gigas* larvae can also experience different conditions than broodstock. Facilities unable to control water chemistry conditions may be exposing immature individuals to environmental perturbations that could affect larvae once spawned. The success of “priming” — exposing *C. gigas* to stressful conditions to induce environmental memory and increase fitness — hinges on the identification of “programming windows” (Gavery and Roberts 2017). The present study shows that the period of time before reproductive conditioning can be important for transferring environmental memory, although only negative carryover effects have been demonstrated in *C. gigas.*

Conclusion

Four months after adult *C. gigas* experienced experimental ocean acidification, larval abundance of female oysters exposed to low pH was significantly lower than those exposed to ambient pH eighteen hours post-fertilization. Not only did this experiment elucidate intergenerational effects of ocean acidification on the Pacific oyster, but it also demonstrated a need to consider the timing of altered environmental conditions on organismal physiology. Although adult oysters experienced a low pH stressor prior to reproductive conditioning, larval abundance was still significantly affected. Therefore, conditions experienced by aquaculture broodstock before reproductive conditioning should be taken into consideration. Likewise these results should be considered when modeling large-scale ecosystem responses to ocean change. Future work on multigenerational responses to ocean acidification should investigate how exposure to adverse conditions while an organism is immature can affect reproductive success and offspring fitness. The significant lag time between the end of the low pH exposure and spawning possibly indicates some form of epigenetic “memory.” Additional research is needed to investigate the degree of environmental memory that can be maintained and the contributing epigenetic phenomenon.

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Tables and Figures

Table 1. Average (± SE) pH, total alkalinity (µmol/kg), pCO_2 (µatm), dissolved organic carbon (DIC; µmol/kg), calcite saturation state ($\Omega_{calcite}$), and aragonite saturation state ($\Omega_{aragonite}$) for three time points during low pH exposure (Day). The *seacarb* library in R was used to calculate total alkalinity, and in situ pCO_2, Dissolved Inorganic Carbon (DIC), calcite saturation ($\Omega_{calcite}$), and aragonite saturation ($\Omega_{aragonite}$) for each oyster tank. Averages for both control (ambient pH) and experimental (low pH) values were calculated from three replicate tanks each. Between all three days, pH (One-way ANOVA; $F_{1, 16}$ = 5838.7810, p = 6.1165e-22), pCO_2 (One-way ANOVA; $F_{1, 16}$ = 235.4018, p = 5.4421e-11), DIC (One-way ANOVA; $F_{1, 16}$ = 7.1222, p = 0.0168), $\Omega_{calcite}$ (One-way ANOVA; $F_{1, 16}$ = 528.9468, p = 1.0989e-13), $\Omega_{aragonite}$ (One-way ANOVA; $F_{1, 16}$ = 526.5207, p =1.1389e-13) were significantly lower experimental treatment. Total alkalinity, however, was not significantly different between treatments (One-way ANOVA; $F_{1, 16}$ = 1.382, p = 0.2570).

Day	pH		Total Alkalinity (µmol/kg)		pCO_2 (µatm)		DIC (µmol/kg)		$\Omega_{calcite}$		$\Omega_{aragonite}$	
	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment
5	7.82 ± 0.004	7.33 ± 0.002	2307.41 ± 25.45	2332.36 ± 31.05	747.51 ± 13.94	2481.23 ± 29.83	2233.41 ± 25.29	2408.51 ± 31.76	1.86 ± 0.02	0.62 ± 0.01	1.16 ± 0.012	0.58 ± 0.007
33	7.81 ± 0.005	7.31 ± 0.004	2747.00 ± 21.13	2917.60 ± 18.36	912.22 ± 12.69	3309.52 ± 7.22	2664.57 ± 19.99	3020.99 ± 17.99	2.23 ± 0.03	0.77 ± 0.02	1.40 ± 0.020	0.48 ± 0.014
48	7.82 ± 0.015	7.29 ± 0.004	2611.40 ± 31.01	2808.39 ± 12.24	863.47 ± 42.42	3343.89 ± 49.49	2533.28 ± 35.45	2920.52 ± 15.11	2.13 ± 0.06	0.68 ± 0.01	1.32 ± 0.035	0.42 ± 0.004

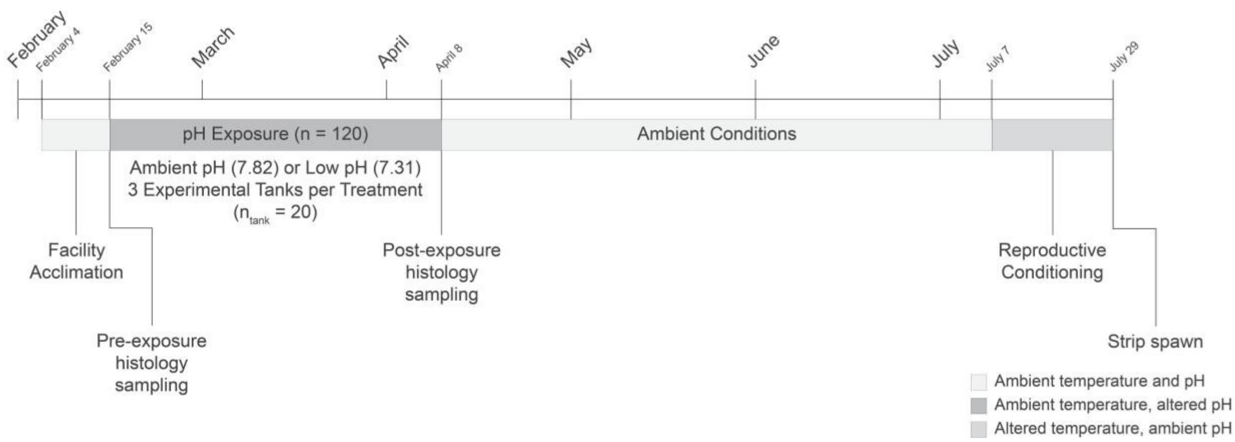

Figure 1. Experimental timeline. Pacific oysters (n = 140) were acclimated for 15 days, then twenty were randomly sampled for histological analyses. Remaining oysters were divided into ambient pH or low pH treatments for seven weeks. Three experimental tanks for each treatment were used with 20 oysters per tank for a total 60 oysters per treatment. At the end of the pH exposure, a total of ten oysters were randomly selected from each treatment and sampled for histological analyses. All remaining oysters were then held in ambient pH conditions for 3 months. Finally, oysters were reproductively conditioned and strip spawned. Larvae were counted eighteen hours post-fertilization.

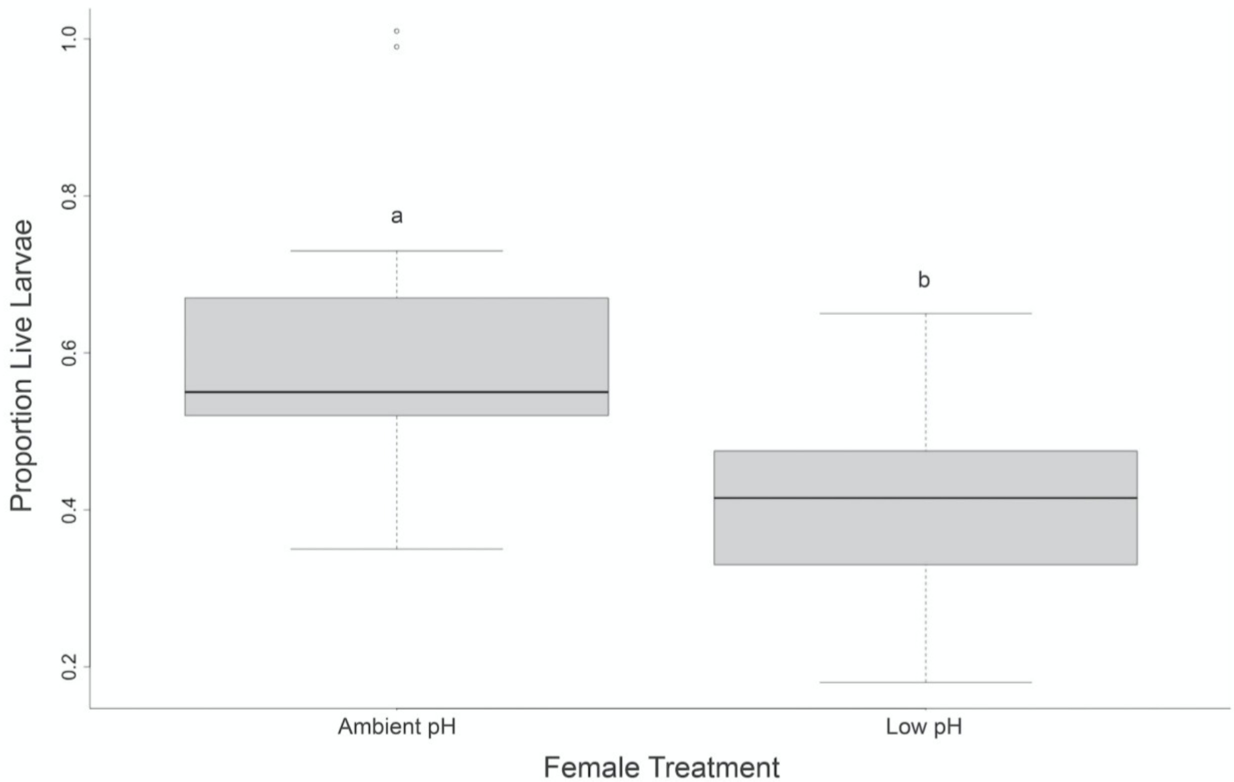

Figure 2. Proportion of live D-hinge larvae eighteen hours post-fertilization by female treatment. Each box represents proportions of live larvae between the first and third quartiles for half-sibling families where the female was exposed to either ambient or low pH conditions. Horizontal lines outside the box indicate the minimum value before the lower fence and the maximum value before the upper fence, with the solid line marks the median. Circles represent outliers. A proportion of 1.0 indicates that all eggs in a cross were successfully fertilized and developed into D-hinge larvae. A linear mixed model, with sire as a random effect, indicated significantly fewer D-hinge larvae were present in half-sibling families where females were exposed to low pH conditions ($t = -2.999$; $p = 0.0119$). Significantly different proportions are indicated by letter.

Supplementary Material

Table S1. Proportion of *C. gigas* sampled at distinct maturation stages before and after a seven week exposure to either ambient (pH = 7.82 ± 0.02) or low (pH = 7.31 ± 0.02) pH conditions. Classifications were adapted from (Fabioux et al. 2005; Enríquez-Díaz et al. 2008). Stage 0 indicates a complete lack of sexuality. Stage 1 gonads feature small follicles and early indications of spermatogonia and oogonia. Primary gametes are apparent in Stage 2, and fully mature gametes are present in Stage 3. Both spawning and resorbing gonads are classified as Stage 4. See Figure S1 for example histology images.

Maturation Stage	Sex	Pre-treatment (n = 20)	Post-treatment: Low pH (n = 10)	Post-treatment: Ambient pH (n = 10)
Stage 0	N/A	0.3	0.5	0.3
Stage 1	Male	0.1	0	0
	Female	0.45	0.2	0.2
Stage 2	Male	0	0	0
	Female	0.05	0.3	0.3
Stage 3	Male	0	0	0
	Female	0	0	0
Stage 4	Male	0.1	0	0.2
	Female	0	0	0

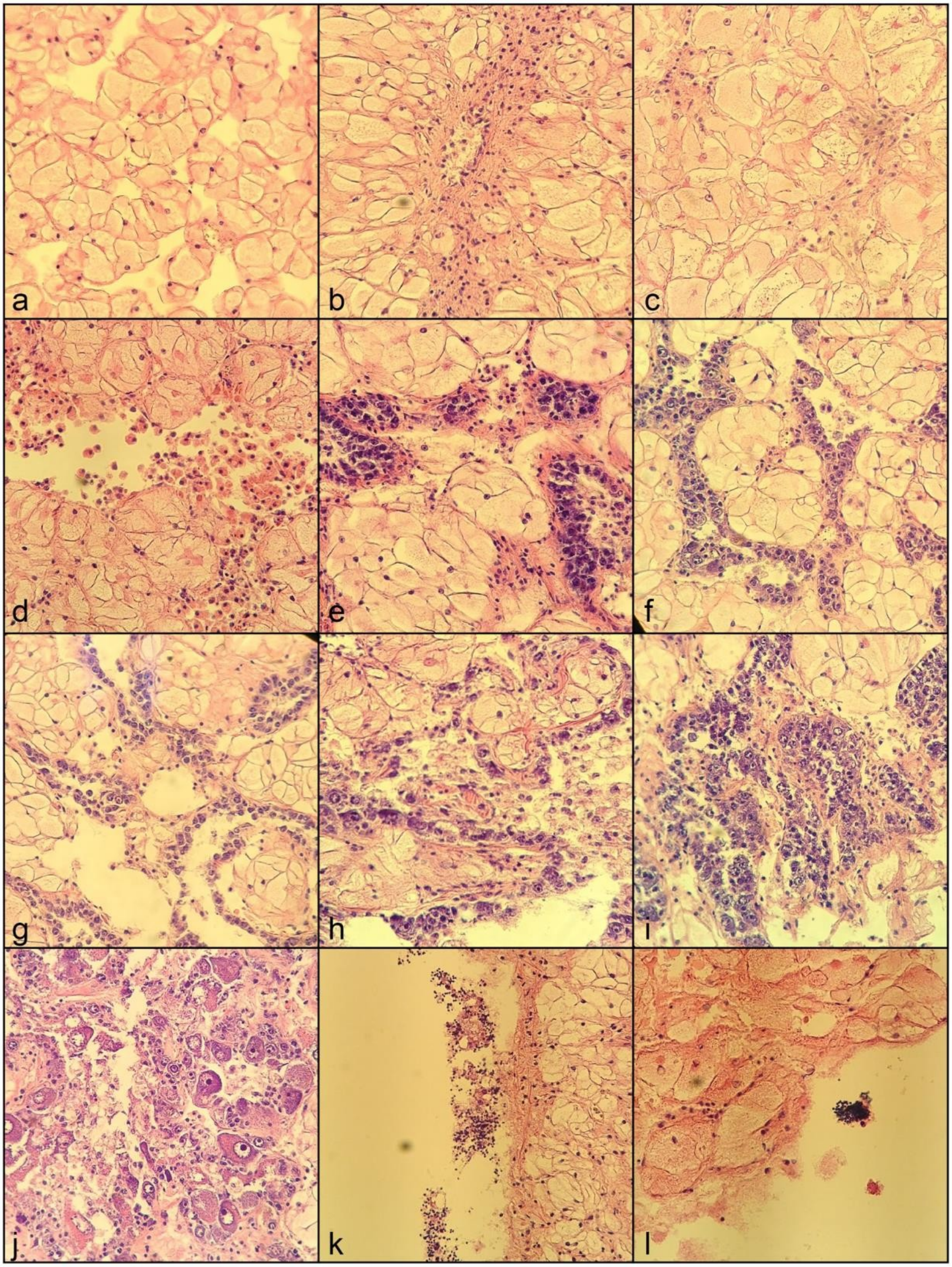
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Figure S1. Example histology images. No Stage 3 individuals of either sex were identified in pre-treatment or post-treatment samples. All images were taken at 40x magnification. All histology images are available in S3.

a. Stage 0 individual from pre-treatment sampling. Individuals at this stage have a complete lack of sexuality.
b. Stage 0 individual from low pH post-treatment sampling. Individuals at this stage have a complete lack of sexuality.
c. Stage 0 individual from ambient pH pre-treatment sampling. Individuals at this stage have a complete lack of sexuality.
d. Stage 1 male from pre-treatment sampling. Gonads feature small follicles and early indications of spermatogonia.
e. Stage 1 female from pre-treatment sampling. Gonads feature small follicles and early indications of oogonia.
f. Stage 1 female from low pH post-treatment sampling. Gonads feature small follicles and early indications of oogonia.
g. Stage 1 female from ambient pH post-treatment sampling. Gonads feature small follicles and early indications of oogonia.
h. Stage 2 female from pre-treatment sampling. Primary gametes are apparent. No Stage 2 males were identified in either pre-treatment or post-treatment samples.
i. Stage 2 female from low pH post-treatment sampling. Primary gametes are apparent. No Stage 2 males were identified in either pre-treatment or post-treatment samples.
j. Stage 2 female from ambient pH post-treatment sampling. Primary gametes are apparent. No Stage 2 males were identified in either pre-treatment or post-treatment samples.
k. Stage 4 male from pre-treatment sampling. Indications of residual spermatozoa. No Stage 4 females were identified in either pre-treatment or post-treatment samples.

I. Stage 4 male from ambient pH post-treatment sampling. Indications of residual spermatozoa. No Stage 4 females were identified in either pre-treatment or post-treatment samples.

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