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## LARVAL RESPONSE TO PARENTAL LOW pH EXPOSURE IN THE PACIFIC OYSTER *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 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 ( $7.31 \pm 0.02$ ) or ambient pH ( $7.82 \pm 0.02$ ) conditions for 7 wk. Oysters were then held for 8 wk in ambient conditions, and subsequently reproductively conditioned for 4 wk at ambient pH. After conditioning, the oysters were strip-spawned to create four families based on maternal and paternal ocean acidification exposure. The number of D-hinge larvae was counted 18 h postfertilization. A sex-specific brood stock 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 organismal ability to persist in the face of environmental change.

**KEY WORDS:** 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 the Sydney rock oyster *Saccostrea glomerata* to high pCO<sub>2</sub> conditions (856  $\mu$ atm, pH<sub>NBS</sub> 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 than those from parents reared in ambient pH conditions (Parker et al. 2012, 2015, 2017). Conversely, similar experiments conducted with the northern quahog *Mercenaria mercenaria* (also called hard clam) and the bay scallop *Argopecten irradians* demonstrated negative larval carryover effects (Griffith & Gobler 2017). Larvae from adult *A. irradians* and *M. mercenaria* exposed to low pH were more sensitive to acidified conditions than those spawned from parents exposed to ambient pH during reproductive conditioning (Griffith & 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. Although fertilization still occurs under near-future ocean acidification conditions (Kurihara et al. 2007, Havenhand & 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 (Dineshran et al. 2012). During metamorphosis, oyster larvae experience down-regulation of proteins related to energy production, metabolism, and protein synthesis (Dineshran et al. 2016). Adult *C. gigas* calcification rates decrease as seawater pCO<sub>2</sub> increases (Gazeau et al. 2007), with oysters grown at 2,800  $\mu$ atm displaying significantly lower fracture toughness than oyster shells from ambient conditions (Timmins-Schiffman et al. 2014). Exposure to ocean acidification also affects adult antioxidant responses, carbohydrate metabolism, transcription, and translation protein pathways (Timmins-Schiffman et al. 2014). Predator-prey interactions can also change under experimental ocean acidification (Wright et al. 2018). There is limited evidence, however, of how ocean acidification influences Pacific oysters across multiple generations.

The current study is the first to discern how exposure to experimental ocean acidification before reproductive conditioning affects larval abundance in *Crassostrea 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. In addition, the study demonstrates how environmental perturbation experienced before reproductive maturity affects the subsequent generation, even if the stressor is long-removed.

### MATERIALS AND 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 Manchester Field

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Station (47° 34' 09.1" N 122° 33' 19.0" W, Manchester, WA) in 2017. Adult hatchery-raised *Crassostrea gigas* (average shell length = 117.46 ± 19.16 cm) were acclimated in the facility for 10 days and then exposed to either low or ambient pH conditions for 48 days (Fig. 1). After pH exposure, oysters were held at ambient pH and water temperature conditions for 90 days. Oysters underwent reproductive conditioning for 22 days and were then strip-spawned. D-hinge larvae were counted 18 h after fertilization occurred.

#### Experimental pH Exposure

The experimental system consisted of a 1,610-L storage tank that fed two 757-L header tanks. Water from Clam Bay, WA, was pumped through a sand filter and then UV-treated. The UV-treated water passed through a set of three sock filters (100, 50, and 25 µm) and a degassing column. Once degassed, water passed through three more sock filters (25, then 10, 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<sub>2</sub> in the water could be equilibrated with atmospheric CO<sub>2</sub>. Equilibrated water flowed into the two header tanks, each of which fed three 50-L flow-through (1.2 L/min) experimental tanks (six experimental tanks in 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. The addition of CO<sub>2</sub> 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<sub>2</sub> airline with a back pressure of 15 psi, controlled with a regulator, injected CO<sub>2</sub> into the low pH header tank every 180 sec with an injection duration of 0.4 sec. 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<sub>2</sub>-rich water to lower pH. There were no CO<sub>2</sub> injections in the ambient header tank.

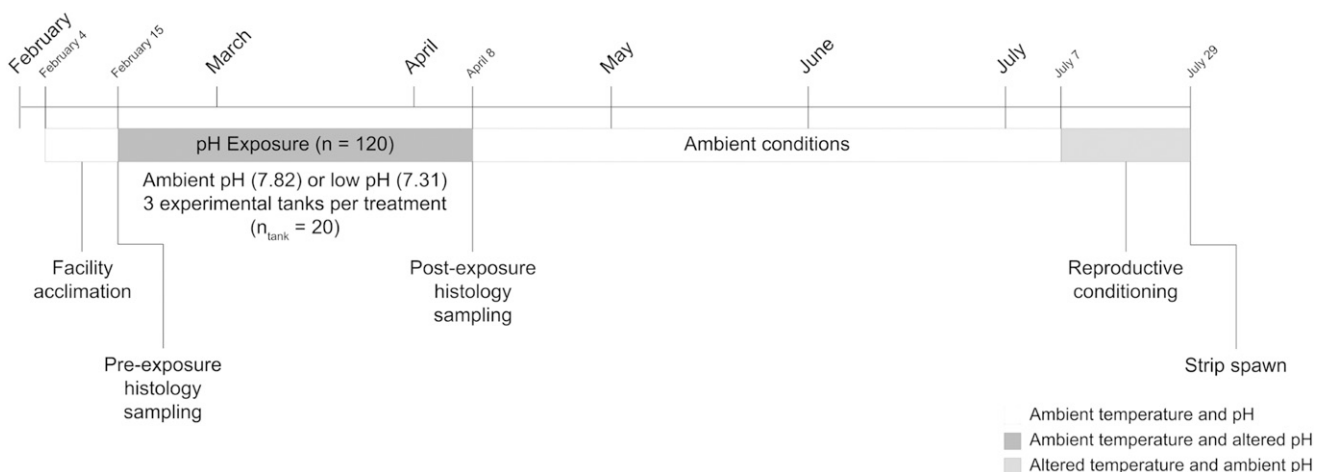
Before the pH exposure trial, 20 randomly selected *Crassostrea gigas* were lethally sampled to assess gonadal status (see

Histological Analysis). Oysters were placed in each flow-through experimental tank in ambient water conditions and exposed to ambient or low pH conditions for 7 wk. Each treatment consisted of three 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 200 L of ambient pH seawater (Helm et al. 2004). Algae were 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 (1 L) were collected from each header and oyster experimental tank. For each sample, salinity 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 (°C) was measured using a Traceable digital thermometer (Model 15-077, Fisher). To calibrate the pH probe, Tris buffer (0.08 M, 28.0 salinity) was prepared using 0.3603 mol of NaCl (J. T. Baker), 0.0106 mol of KCl (Fisher Scientific), 0.0293 mol MgSO<sub>4</sub>·(H<sub>2</sub>O)<sub>7</sub> (Fisher Scientific), 0.0107 mol of CaCl<sub>2</sub>·2(H<sub>2</sub>O) (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 1 L. 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 from discrete samples was used to calculate total alkalinity, using the *seacarb* library in R (Gattuso et al. 2018). Calculated pH, total alkalinity,



**Figure 1.** Experimental timeline. Pacific oysters ( $n = 140$ ) were acclimated for 15 days, then 20 were randomly sampled for histological analyses. Remaining oysters were divided into ambient pH or low pH treatments for 7 wk. Three experimental tanks for each treatment were used with 20 oysters per tank for a total of 60 oysters per treatment. At the end of the pH exposure, a total of 10 oysters were randomly selected from each treatment and sampled for histological analyses. All remaining oysters were then held in ambient pH conditions for 3 mo. Finally, the oysters were reproductively conditioned and strip-spawned. Larvae were counted 18 h postfertilization.

temperature, and salinity were also used in *seacarb* to calculate *in situ* pH, pCO<sub>2</sub>, dissolved inorganic carbon (DIC), calcite saturation ( $\Omega_{\text{calcite}}$ ), and aragonite saturation ( $\Omega_{\text{aragonite}}$ ) 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 *Crassostrea gigas* were lethally sampled before pH exposure for histological analyses. On the last day of low pH exposure, 10 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 40 $\times$  magnification (Fabioux et al. 2005, Enríquez-Díaz et al. 2008).

### Reproductive Conditioning

Following 7 weeks of low pH exposure, oysters were returned to a common garden and maintained at ambient pH conditions for 8 wk. Afterward, oysters were reproductively conditioned. Water temperatures and food quantity are known to regulate the timing, speed, and intensity of gametogenesis in *Crassostrea gigas* (Enríquez-Díaz et al. 2008). Conditioning protocol was modeled after standard hatchery practices (Molly Jackson, brood stock manager at Taylor Shellfish, personal communication, June 2017). Water temperature was raised from ambient conditions (13°C) to 23°C over 3 wk (1°C/2 days) because optimal temperature for *C. gigas* gametogenesis is between 18°C and 26°C (Parker et al. 2010). Conditions were maintained at 23°C for one more week before spawning. During conditioning, *C. gigas* were fed 700–800 mL of Shellfish Diet 1800 daily (Helm et al. 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 10 $\times$  magnification. Only *Crassostrea gigas* with active sperm or eggs were used for crosses ( $n_{\text{male, low}} = 6$ ,  $n_{\text{female, low}} = 22$ ,  $n_{\text{male, ambient}} = 6$ , and  $n_{\text{female, ambient}} = 26$ ). 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), 1 g of mature gonad from each ripe female was pooled. The number of eggs in both the ambient and low pH pools was 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  $\mu$ L) from individual males.

Four half-sibling families were created based on parental pH exposure: low pH female (pool)  $\times$  low pH male, low pH female (pool)  $\times$  ambient pH male, ambient pH female (pool)  $\times$  low pH male, and ambient pH (pool) female  $\times$  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  $\times$  low pH male-01, low pH female pool  $\times$  low pH male-02,... low pH female pool  $\times$  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 (1 L) for 20 min with static 23°C filtered seawater (1  $\mu$ m) in ambient pH conditions. After confirming polar body formation, beaker contents were transferred to larger plastic tanks (19 L) with aerated, static 23°C filtered seawater (1  $\mu$ m) for 18 h of incubation. Duplicate containers were combined 18 h post-fertilization, and D-hinge larvae were counted for each cross ( $n = 24$ ).

### Statistical Analyses

Differences in *in situ* pH, total alkalinity, pCO<sub>2</sub>, DIC,  $\Omega_{\text{calcite}}$ , and  $\Omega_{\text{aragonite}}$  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-square 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, was 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

Pacific oysters 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;  $F_{1, 16} = 5,838.7810$ ,  $P = 6.1165e-22$ ), pCO<sub>2</sub> (one-way ANOVA;  $F_{1, 16} = 235.4018$ ,  $P = 5.4421e-11$ ), DIC (one-way ANOVA;  $F_{1, 16} = 7.1222$ ,  $P = 0.0168$ ),  $\Omega_{\text{calcite}}$  (one-way ANOVA;  $F_{1, 16} = 528.9468$ ,  $P = 1.0989e-13$ ), and  $\Omega_{\text{aragonite}}$  (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 *Crassostrea gigas* sampled before and after pH treatment (binomial GLM;  $F_{2, 37} = 0.7973$ ,  $P = 0.3442$ ). In addition, 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 (Venkataraman et al. 2018). Sex ratios were also similar between low and



TABLE 1.  
Average ( $\pm$ SE) pH, total alkalinity ( $\mu\text{mol}/\text{kg}$ ),  $\text{pCO}_2$  ( $\mu\text{atm}$ ), dissolved inorganic carbon (DIC;  $\mu\text{mol}/\text{kg}$ ), calcite saturation state ( $\Omega_{\text{calcite}}$ ), and aragonite saturation state ( $\Omega_{\text{aragonite}}$ ) for three time points during low pH exposure (day).

Day	pH		Total alkalinity		$\text{pCO}_2$ ( $\mu\text{atm}$ )		DIC ( $\mu\text{mol}/\text{kg}$ )		$\Omega_{\text{calcite}}$		$\Omega_{\text{aragonite}}$	
	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment
5	7.82 $\pm$ 0.004	7.33 $\pm$ 0.002	2,307.41 $\pm$ 25.45	2,332.36 $\pm$ 31.05	747.51 $\pm$ 13.94	2,481.23 $\pm$ 29.83	2,233.41 $\pm$ 25.29	2,408.51 $\pm$ 31.76	1.86 $\pm$ 0.02	0.62 $\pm$ 0.01	1.16 $\pm$ 0.012	0.58 $\pm$ 0.007
33	7.81 $\pm$ 0.005	7.31 $\pm$ 0.004	2,747.00 $\pm$ 21.13	2,917.60 $\pm$ 18.36	912.22 $\pm$ 12.69	3,309.52 $\pm$ 7.22	2,664.57 $\pm$ 19.99	3,020.99 $\pm$ 17.99	2.23 $\pm$ 0.03	0.77 $\pm$ 0.02	1.40 $\pm$ 0.020	0.48 $\pm$ 0.014
48	7.82 $\pm$ 0.015	7.29 $\pm$ 0.004	2,611.40 $\pm$ 31.01	2,808.39 $\pm$ 12.24	863.47 $\pm$ 42.42	3,343.89 $\pm$ 49.49	2,533.28 $\pm$ 35.45	2,920.52 $\pm$ 15.11	2.13 $\pm$ 0.06	0.68 $\pm$ 0.01	1.32 $\pm$ 0.035	0.42 $\pm$ 0.004

The *seacarb* library in R was used to calculate total alkalinity, and *in situ*  $\text{pCO}_2$ , dissolved inorganic carbon (DIC), calcite saturation ( $\Omega_{\text{calcite}}$ ), and aragonite saturation ( $\Omega_{\text{aragonite}}$ ) for each oyster tank. Averages for both control (ambient pH) and experimental (low pH) values were calculated from three replicate tanks each. Between all 3 days, pH (one-way ANOVA;  $F_{1,16} = 5.838, 7810, P = 6.1165e-22$ ),  $\text{pCO}_2$  (one-way ANOVA;  $F_{1,16} = 235.4018, P = 5.4421e-11$ ), DIC (one-way ANOVA;  $F_{1,16} = 7.1222, P = 0.0168$ ),  $\Omega_{\text{calcite}}$  (one-way ANOVA;  $F_{1,16} = 528.9468, P = 1.0989e-13$ ), and  $\Omega_{\text{aragonite}}$  (one-way ANOVA;  $F_{1,16} = 526.5207, P = 1.1389e-13$ ) were significantly lower in the experimental treatment. Total alkalinity, however, was not significantly different between treatments (one-way ANOVA;  $F_{1,16} = 1.382, P = 0.2570$ ).

ambient pH treatments (chi-squared test for homogeneity;  $\chi^2_2 = 3.2279, P = 0.1942$ ).

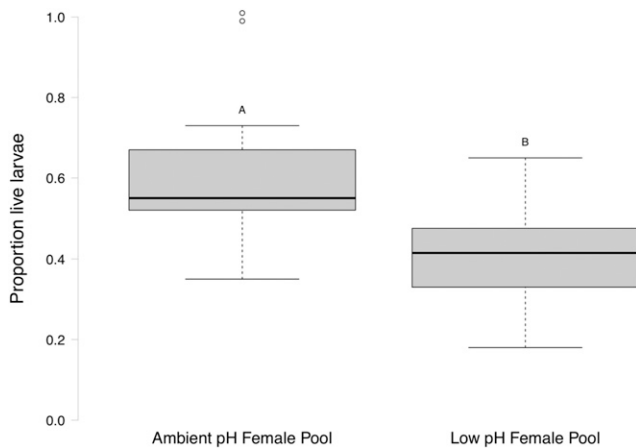
#### Larval Survival

A linear mixed effect model, with female pool and sire as random effects, demonstrated no significant difference in the number of D-hinge larvae counted 18 h postfertilization between all four parental families (Linear mixed effect model;  $\chi^2_3 = 3.1325, P = 0.1066$ ). 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 (Fig. 2; linear mixed effect model;  $\chi^2_1 = 8.1781, P = 0.0042$ ), with sire accounting for 0.3116053% of total variance.

#### DISCUSSION

The present study is the first to document the transgenerational influence of ocean acidification on Pacific oysters. Larval *Crassostrea gigas* were negatively impacted when maternal brood stock 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 3 mo before reproductive conditioning, and then were kept solely in ambient pH conditions through strip-spawning and larval rearing. Because environmental perturbation experienced before Pacific oysters were mature still affected larval oysters, the results indicate a role for environmental memory in *C. gigas* response to ocean acidification. Mechanisms for transgenerational environmental memory have been explored in response to acute stressors in other species. For example, *Daphnia magna* exposed to high salinity conditions had altered DNA methylation patterns, and these patterns were inherited by the following three nonexposed 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.

Although it is evident that acute exposure to low pH experienced by adult *Crassostrea 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. The tanner crab *Chionoecetes bairdi* were 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, yet substantial effects on physiology were observed when larvae had a history of maternal exposure during oogenesis (Long et al. 2016). Larvae from the adult northern quahog *Mercenaria mercenaria* (also known as hard clam) and the bay scallop *Argopecten irradians* developed slower when parents were reproductively conditioned in low pH conditions (pH<sub>T</sub> = 7.4) (Griffith & Gobler 2017). In addition, larvae with a history of parental low pH exposure were more vulnerable to additional stressors such as thermal stress, limited food, and harmful algae exposure (Griffith & Gobler 2017). Although *C.*



**Figure 2.** Proportion of live D-hinge larvae 18 h postfertilization 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 indicating 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 a letter.

*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 4 mo 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 offspring demonstrate transgenerational acclimatization to stressors. For example, the negative carryover effect observed in *Crassostrea gigas* is different from the positive carryover effects observed in ocean acidification experiments conducted with Sydney rock oysters. When adult *Saccostrea glomerata* were exposed to acidified seawater ( $p\text{CO}_2 = 856 \mu\text{atm}$ ;  $\text{pH}_{\text{NBS}} = 7.89\text{--}7.90$ ) during reproductive conditioning, the resultant larvae were larger and developed faster in acidified conditions than larvae from parents exposed to ambient conditions (Parker et al. 2012). This positive carryover effect was found to persist in the  $F_2$  generation. In acidified conditions,  $F_2$  offspring with a history of transgenerational ( $F_0$  and  $F_1$ )  $p\text{CO}_2$  exposure grew faster and demonstrated fewer shell abnormalities (Parker et al. 2015). Although 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  $p\text{CO}_2$  treatment of  $856 \mu\text{atm}$  ( $\text{pH} = 7.89\text{--}7.90$ ), with a control of  $380\text{--}385 \mu\text{atm}$  ( $\text{pH} = 8.19\text{--}8.20$ ). Therefore, the elevated  $p\text{CO}_2$  treatment used in Parker et al. (2012, 2015, 2017) is similar to the ambient pH treatment ( $7.82$ ;  $p\text{CO}_2 = 747.51\text{--}912.22$ ) 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 than larvae with no transgenerational exposure history (Parker et al. 2012). With a relatively smaller effect size and a milder treatment than that 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 & Gobler 2017:  $\text{pH}_T = 7.4$ ; this study:  $\text{pH} = 7.31$ ). Both of these studies used treatment levels more extreme than the International Panel on Climate Change projections for open ocean acidification, but consistent with coastal and estuarine acidification scenarios experienced at study locations (Feely et al. 2010, Griffith & 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 3 mo before 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 the eastern oyster *Crassostrea virginica* that experienced severe ocean acidification conditions during reproductive conditioning ( $\text{pH} = 7.71$ ,  $5,584 \mu\text{atm}$ ) (Boulais et al. 2017). The green sea urchin *Strongylocentrotus droebachiensis* exposed to high  $p\text{CO}_2$  ( $1,200 \mu\text{atm}$ ) conditions for 4 mo demonstrated low fecundity (Dupont et al. 2013), and *Saccostrea glomerata* conditioned in high  $p\text{CO}_2$  ( $856 \mu\text{atm}$ ) conditions exhibited reduced rates of gametogenesis, smaller gonad area, and reduced fecundity (Parker et al. 2018). Gonad histology from *Crassostrea 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 3 mo may have reversed any detrimental effects.

Reduced *Crassostrea 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, such as lipids or proteins, into eggs—or decrease provisioning because of energetic constraints (Liu et al. 2010, Uthicke et al. 2013). For example, changes in fatty acid provisioning from maternal exposure to high  $p\text{CO}_2$  conditions ( $2,300 \mu\text{atm}$ ) in the 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; although elevated  $p\text{CO}_2$  conditions ( $856 \mu\text{atm}$ ) reduced the amount of energy invested in maternal gonads, these conditions did not impact *Saccostrea glomerata* egg size or total lipid content (Parker et al. 2018). Because 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 3 mo before reproductive conditioning.

The documented effect on Pacific oyster larval abundance 4 mo after low pH exposure indicates an important role for environmental memory in *Crassostrea gigas* response to ocean

acidification. Low pH exposure may have induced epigenetic modifications (e.g., 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 & Roberts 2017). One notable study on *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. Several experimental ocean acidification studies, however, hint at the role of epigenetic memory. The Olympia oyster *Ostrea lurida* exposed to high pCO<sub>2</sub> (1,000 μatm) conditions still grew less in the juvenile life stage than the counterparts reared in ambient pCO<sub>2</sub>, even after the stressor had been removed (Hettinger et al. 2013). Similarly, transgenerational acclimation of *Saccostrea glomerata* larvae with a history of exposure to acidified conditions could be explained by changes in epigenome that affect organismal performance (Parker et al. 2012, 2015, 2017). 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 4 mo 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 4 mo after the exposure ended. Stressor timing and duration can impact transgenerational responses between mature parents and offspring (Donelson et al. 2018). Although experimental ocean acidification (pH 7.7; pCO<sub>2</sub> = 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 (17°C or 21°C) in the three-spined stickleback *Gasterosteus aculeatus* differed based on exposure duration (Shama & Wegner 2014). Grandparents ( $F_0$ ) were only exposed to treatment temperatures during reproductive conditioning, whereas 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 & Wegner 2014). 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 the

marine polychaete *Ophryotrocha 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 led 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 *Ostrea lurida* when they were co-occurring stressors, but 2–4 wk 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 4 mo after brood stock was exposed to acidified seawater have implications for both aquaculture and natural *Crassostrea 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 afterward. Hatchery-reared *C. gigas* larvae can also experience different conditions than brood stock. 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 & 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 *Crassostrea gigas* experienced experimental ocean acidification, larval abundance of female oysters exposed to low pH was significantly lower than those exposed to ambient pH 18 h postfertilization. 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 before reproductive conditioning, larval abundance was still significantly affected. Therefore, conditions experienced by aquaculture brood stock 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 multi-generational 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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