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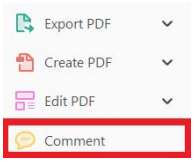

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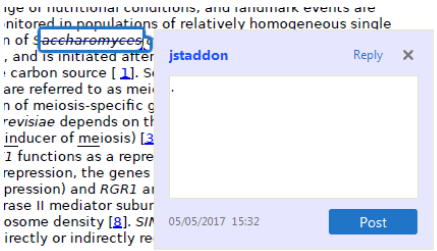

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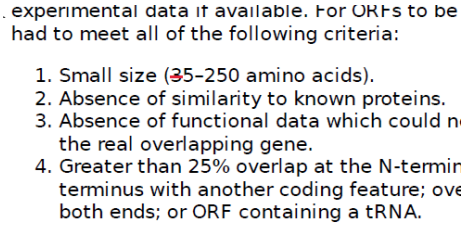

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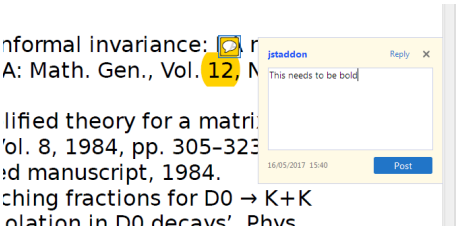

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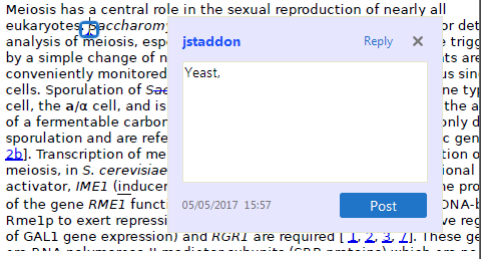

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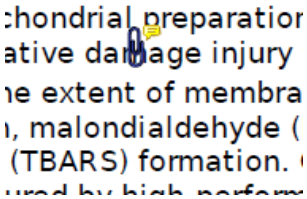

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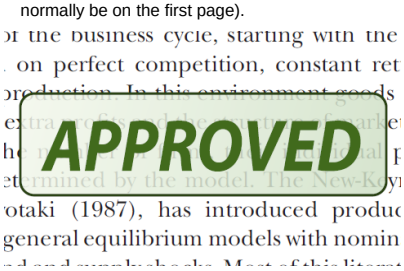

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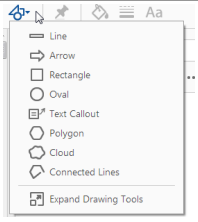

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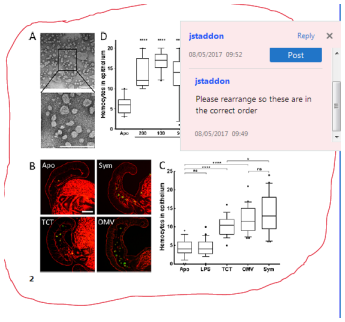

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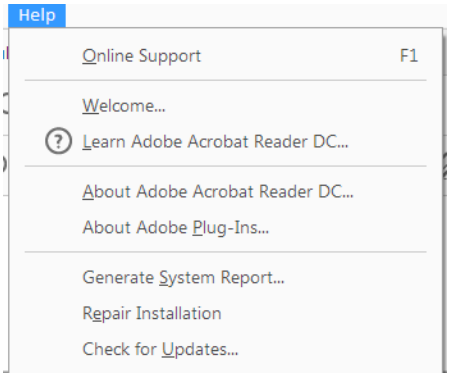

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Carryover effects of temperature and pCO_2 across multiple Olympia oyster populations

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Abstract. Predicting how populations will respond to ocean change across generations is critical to effective conservation of marine species. One emerging factor is the influence of parental exposures on offspring phenotype, known as intergenerational carryover effects. Parental exposure may deliver beneficial or detrimental characteristics to offspring that can influence larval recruitment patterns, thus shaping how populations and community structure respond to ocean change. Impacts of adult exposure to elevated winter temperature and pCO_2 on reproduction and offspring viability were examined in the Olympia oyster (*Ostrea lurida*) using three populations of adult, hatchery-reared *O. lurida,* plus an additional cohort spawned from one of the populations. Oysters were sequentially exposed to elevated temperature (+4°C, at 10°C), followed by elevated pCO_2 (+2,204 μatm, at 3,045 μatm) during winter months. Male gametes were more developed after elevated temperature exposure and less developed after high pCO_2 exposure, but there was no impact on female gametes or sex ratios. Oysters previously exposed to elevated winter temperature released larvae earlier, regardless of pCO_2 exposure. Those exposed to elevated winter temperature as a sole treatment released more larvae on a daily basis but, when also exposed to high pCO_2, there was no effect. These combined results indicate that elevated winter temperature accelerates *O. lurida* spermatogenesis, resulting in earlier larval release and increased production, with elevated pCO_2 exposure negating effects of elevated temperature. Altered recruitment patterns may therefore follow warmer winters due to precocious spawning, but these effects may be masked by coincidental high pCO_2. Offspring were reared in common conditions for ~~1 yr~~, then deployed for ~~3~~ months in four estuarine bays with distinct environmental conditions. Offspring of parents exposed to elevated pCO_2 had higher survival rates in two of the four bays. This carryover effect demonstrates that parental conditions can have substantial ecologically relevant impacts that should be considered when predicting impacts of environmental change. Furthermore, Olympia oysters may be more resilient in certain environments when progenitors are pre-conditioned in stressful conditions. Combined with other recent studies, our work suggests that the Olympia may be more equipped than other oysters for the challenge of a changing ocean.

Key words: *acidification; climate change; intergenerational;* Ostrea lurida; *pH; phenology; reproduction; transgenerational; warming; winter.*

Introduction

The repercussions of ocean warming and acidification on marine invertebrate physiology are complex, but significant recent advances indicate that larval stages of marine taxa are particularly vulnerable (Kurihara 2008, Byrne and Przeslawski 2013, Przeslawski et al. 2015). Understanding how shifting conditions will influence larval recruitment patterns is critical to predicting changing population dynamics, and thus community structure. One emerging consideration is whether larval stages benefit from ancestral exposures, based on evidence that memory of environmental stressors can be transferred between generations through non-genetic inheritance (reviewed in Ross et al. 2016, Donelson et al. 2018, Eirin-Lopez and Putnam 2019). Beneficial, or positive, carryover effects may be important acclimatory mechanisms for marine organisms facing rapid change, particularly those that evolved in dynamic environments like estuaries and the intertidal (Gavery and Roberts 2014, Donelson et al. 2018). These carryover effects are defined as transgenerational when they persist in generations that were never directly exposed.

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Intergenerational, or parental, effects may be due to direct exposure as germ cells (Perez and Lehner 2019). Trans- and intergenerational carryover effects are increasingly reported across marine phyla, including Cnidaria (Putnam and Gates 2015), Echinodermata (Clark et al. 2019), Mollusca (Parker et al. 2015), Arthropoda (Thor and Dupont 2015), and Chordata (for a review, see Munday [2014]).

A foundational series of studies on the Sydney rock oyster (*Saccostrea glomerata*) provide strong evidence for intergenerational carryover effects in bivalves, an ecologically and economically important group of taxa (Dumbauld et al. 2009). Adult *S. glomerata* exposed to high pCO_2 produced larger larvae that were less sensitive to high pCO_2, and the effect persisted in the successive generation (Parker et al. 2012, 2015). In the presence of secondary stressors, however, parental high pCO_2 exposure rendered larvae more sensitive (Parker et al. 2017). Intergenerational carryover effects are increasingly documented in larvae across other bivalve species, and are beneficial in the mussels *Mytilus chilensis* (Diaz et al. 2018) and *Mytilus edulis* (but not juveniles; Thomsen et al. 2017, Kong et al. 2019), and detrimental in the clam *Mercenaria mercenaria*, the scallop *Argopecten irradians* (Griffith and Gobler 2017), and the oyster *Crassostrea gigas* (Venkataraman et al. 2019).

These preliminary studies provide strong evidence for intergenerational carryover effects in bivalves, but the body of work is still narrow in scope. Nearly all studies have exposed parents to stressors during de novo gamete formation (gametogenesis). For many temperate bivalve species, this occurs seasonally in the spring (Bayne 1976). Yet, challenging periods of acidification and warming can occur during other times of the year (Evans et al. 2013, Joesoef et al. 2015, McGrath et al. 2019). The most corrosive carbonate environment in the Puget Sound estuary in Washington State, for example, commonly occurs in the winter when many species are reproductively inactive, while favorable conditions are in the spring when gametogenesis coincides with phytoplankton blooms (Pelletier et al. 2018). Thus, adult exposure to severely corrosive conditions during gametogenesis may not represent the natural estuarine system. To our knowledge, only one study has assessed carryover effects of exposure to acidification before reproductive conditioning in a bivalve, the oyster *C. gigas*, and found negative maternal carryover effects on larval survival (Venkataraman et al. 2019), indicating that pre-gametogenic exposure also matters. No studies have yet attempted to examine intergenerational carryover effects of combined winter warming and acidification in bivalves.

To best predict whether intergenerational carryover effects will be beneficial or detrimental, it is also crucial to understand how warming and acidification will impact fertility and reproductive phenology. Temperature is a major driver of bivalve reproduction, and modulates gametogenesis (Joyce et al. 2013, Oates 2013, Maneiro et al. 2016), influences sex determination (Santerre et al. 2013) and, in many species, triggers spawning (Fabioux et al. 2005) (alongside other factors such as photoperiod, nutrition, lunar/tidal phases). Year-round warming may result in unexpected impacts to larval competency resulting from changes to reproduction. For instance, some temperate bivalve species have a thermal threshold for gametogenesis and enter a period of reproductive inactivity, or "quiescence," which is believed necessary for successive spawning (Hopkins 1937, Loosanoff 1942, Giese 1959). Warmer winters brought on by global climate change (IPCC 2013, 2019) may therefore shift species' reproductive cycles to begin earlier, or eliminate seasonality altogether, resulting in poorly provisioned or ill-timed larvae (Chevillot et al. 2017). Such impacts were clearly demonstrated using a long-term dataset (1973–2001) of estuarine clam *Macoma balthica* reproduction and temperature. Mild winters and earlier springs resulted in low fecundity, earlier spawning, and poor recruitment, which was largely explained by a phenological mismatch between spawning and peak phytoplankton blooms (Philippart et al. 2003). The impacts of winter acidification on estuarine bivalve reproduction are less predictable. The few studies to date show that high pCO_2 delays gametogenesis in the oysters *Crassostrea virginica* and *S. glomerata* (Boulais et al. 2017, Parker et al. 2018), but both studies exposed oysters during gametogenesis. Acidification during the winter months could increase energetic requirements (Sokolova et al. 2012), and deplete glycogen reserves that are later utilized for gametogenesis in the spring (Mathieu and Lubet 1993), but this hypothesis has yet to be tested.

The purpose of this study was to assess whether warmer, less alkaline winters will affect fecundity and offspring viability in the Olympia oyster, *Ostrea lurida*. The Olympia is native to the Pacific coast of North America (McGraw 2009). Overharvest and pollution devastated populations in the early 1900s, and today 2–5% of historic beds remain (Polson and Zacherl 2009, Blake and Bradbury 2012). Restoration efforts are afoot, but *O. lurida* populations continue to struggle, and may be further challenged by changing conditions (Feely et al. 2008, 2012, Barton et al. 2012). For instance, large interannual variability in larval recruitment and frequent recruitment failures were recently reported (Wasson et al. 2016, Kimbro et al. 2019). This variability is presumably related to inconsistent spawning success, larval survival, and retention, and governed predominantly by local conditions (Kimbro et al. 2019). It is unknown how the intensity, timing, and duration of local environmental conditions can predict recruitment failure (Wasson et al. 2016). If winter conditions significantly influence recruitment through direct changes to adult reproductive capacity or timing, or indirect changes through parental carryover effects, population densities, and distributions will inevitably shift with conditions.

Another consideration in this study was the genetic composition of test organisms. *Ostrea lurida* exhibits varying phenotypes among distinct populations (Silliman 2019), which can influence their sensitivity to environmental stressors (Bible and Sanford 2016, Heare et al. 2018, Bible et al. 2019). Indeed, the two groups to measure the response of *O. lurida* larvae to ocean acidification found contrasting results, no effect (Waldbusser et al. 2016) and slower growth (Hettinger et al. 2012, 2013), possibly a result of local adaptation. The source population used for experimental studies may therefore be a critical factor influencing climate-related findings. Furthermore, testing genetically diverse organisms could reveal cryptic genetic variation, alleles that confer stress resilience only under certain settings (Paaby and Rockman 2014, Bitter et al. 2019), which has implications for how wild populations are restored. Therefore, we tested three phenotypically distinct Puget Sound populations (Heare et al. 2017, Silliman et al. 2018), which were hatchery-reared in common conditions to adulthood, to account for intraspecific variation while controlling for within-generation carryover effects (Hettinger et al. 2012, 2013).

Our study is the first to assess the combined effects of elevated winter temperature and pCO_2 on reproduction, and to explore intergenerational carryover in an *Ostrea* ~~spp.~~. We exposed adult *O. lurida* to elevated temperature (+4°C), followed by elevated pCO_2 (+2,204 µatm, −0.51 pH). Gonad development, reproductive timing, and fecundity were assessed for the adults in the laboratory, and offspring performance was assessed in the field. Elevated winter temperature was expected to impede gametogenic quiescence, presumably a critical annual event, subsequently reducing larval production. This prediction was, in part, based on observations of low larval yields in an *O. lurida* restoration hatchery (R. Crim, *unpublished data*) following the winter 2016 marine heat wave in the Northeast Pacific Ocean (Gentemann et al. 2017). Similarly, we predicted that high pCO_2 exposure would result in negative impacts due to increased energy requirements for calcification and cellular maintenance. Finally, we predicted that negative impacts would be amplified upon exposure to both conditions. By assessing the effects of winter warming and acidification on reproduction and offspring viability in multiple Olympia oyster populations, we provide an ecologically relevant picture of how the species will respond to ocean change.

Methods

Adult oyster temperature and pCO_2 exposures

Four cohorts of adult *Ostrea lurida* were used in this study. Three of the cohorts were first-generation hatchery-produced (F1) oysters (32.1 ± 5.0 mm), all hatched in Puget Sound (Port Gamble Bay) in 2013 (Heare et al. 2017). The broodstock used to produce these F1 oysters were wild, harvested from Fidalgo Bay in North Puget Sound (F), Dabob Bay in Hood Canal (D), and Oyster Bay in South Puget Sound (O-1; O in Fig. 1). These populations are considered phenotypically distinct subpopulations (Heare et al. 2017, White et al. 2017). The fourth cohort (O-2, 21.9 ± 3.3 mm) was second-generation, hatchery-produced in 2015 from the aforementioned Oyster Bay F1 cohort, from a single larval release pulse and thus likely one family (Silliman et al. 2018). The O-2 cohort was included to examine whether reproductive and offspring traits were consistent across generations of a population, with the O-2 cohort being closely related to each other (siblings) and 2 ~~yr~~ younger than the other cohorts. Prior to the experiment, all oysters were maintained in pearl nets in Clam Bay (C) for a minimum of 500 d.

Temperature treatment

Oysters were moved from Clam Bay (C) to the Kenneth K. Chew Center for Shellfish Research and

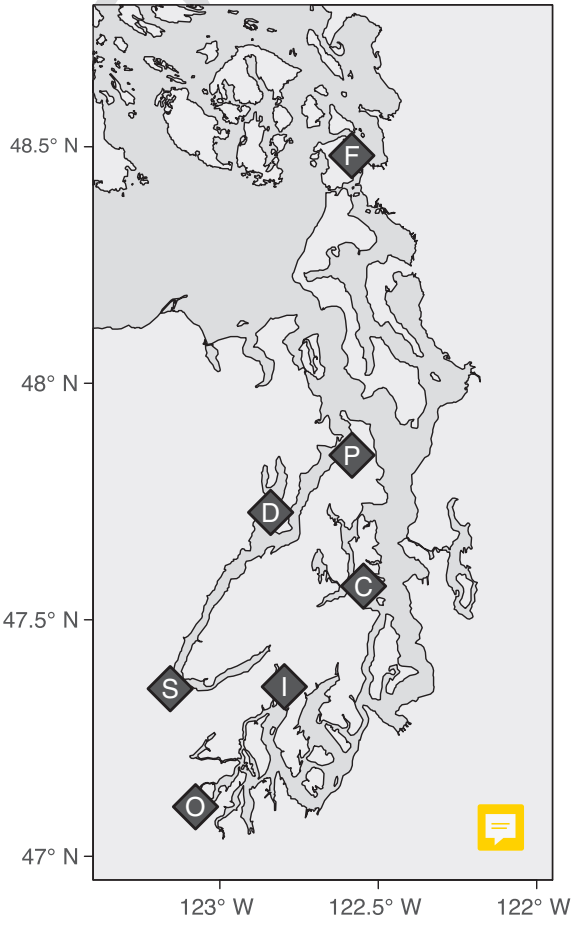

Fig. 1. Locations where *Ostrea lurida* populations' progenitors were collected (F, D, O), where oysters were housed prior to and during the experiment (C), and where offspring were deployed (F, P, S, I). Locations are Fidalgo Bay (F), Port Gamble Bay (P), Dabob Bay (D), Clam Bay (C), Skokomish River Delta (S), Case Inlet (I), and Oyster Bay (O).

Restoration for the temperature and pCO_2 experiments. Oysters were held in one of two temperature regimes (6.1° ± 0.2°C and 10.2° ± 0.5°C) for 60 d beginning 6 December 2016 (Fig. 2). The temperatures correspond to historic local winter temperature (6°C) in Clam Bay, and anomalously warm winter temperature (10°C) as experienced during 2014–2016 (Gentemann et al. 2017). For the temperature exposure, oysters from each cohort (100 for O-1 and F cohorts, 60 for D, and 300 for O-2) were divided into four bags, two bags per temperature, in two flow-through experimental tanks (50 L, 1.2 L/min). Temperature in the 6°C treatment was maintained using an aquarium chiller, and unchilled water was used for the 10°C treatment. Temperatures were recorded continuously with water temperature data loggers.

High pCO_2 treatment

A differential pCO_2 exposure was carried out after the temperature treatment ended. Following a 10-d gradual temperature increase for the 6°C treatment to 10°C, oysters were further divided and held at ambient pCO_2 (841 ± 85 µatm, pH 7.82 ± 0.02) or high pCO_2 (3,045 ± 488 µatm, pH 7.31 ± 0.02) for 52 d (16 February to 8 April 2017, Fig. 2). Animals were housed in six flow-through tanks (50 L, 1.2 L/min), with three replicate tanks per pCO_2 treatment and oyster cohort. High pCO_2 treated water was prepared using CO_2 injection. Filtered seawater (1 µm) first recirculated through a reservoir (1,610 L) with a degassing column to equilibrate with the atmosphere, then flowed into treatment reservoirs (757 L) recirculating through venturi injectors. Durafet pH sensors and a Dual Input Analytical Analyzer monitored pH in treatment reservoirs with readings every 180 s. Using solenoid valves, CO_2 gas was injected through lines at ~~15 psi~~ in 0.4-s pulses if pH exceeded the 7.22 set point. Water pH was continuously monitored in experimental tanks using Durafet pH sensors, and temperature (10.4° ± 0.4°C) was measured using water temperature data loggers. Twice weekly, water samples (1 L) were collected from experimental tanks, and temperature (°C), salinity (PSU), and pH (mV, converted to pH_T) were measured immediately using a digital thermometer, conductivity meter, and pH electrode, respectively. Simultaneously, discrete water samples (120 mL) were collected in duplicate from experimental tanks and preserved with HgCl (50 µL) for later total alkalinity measurements using a titrator. Standard pH curves were generated on each sampling day prior to pH measurements using TRIS buffer prepared in-house at five temperatures (Appendix S1: Section S1). Using the seacarb library in R, pCO_2, dissolved organic carbon (DIC), calcite saturation ($\Omega_{calcite}$), and aragonite saturation ($\Omega_{aragonite}$) were calculated for days 5, 33, and 48 (Appendix S1: Table S1).

During both temperature and pCO_2 treatments, all oysters were fed from a shared algae header tank daily with Shellfish Diet 1800 (300–500-mL; ~~Reed Mariculture~~) diluted in ambient pCO_2 seawater (200 L; Helm and Bourne 2004), dosed continuously with metering pumps. Experimental, reservoir, and algae tanks were drained and cleaned, and oysters were monitored for mortality and rotated within the experimental system twice weekly.

Adult reproductive development

A subset of oysters was sampled for gamete stage and dominant sex immediately before and after pCO_2

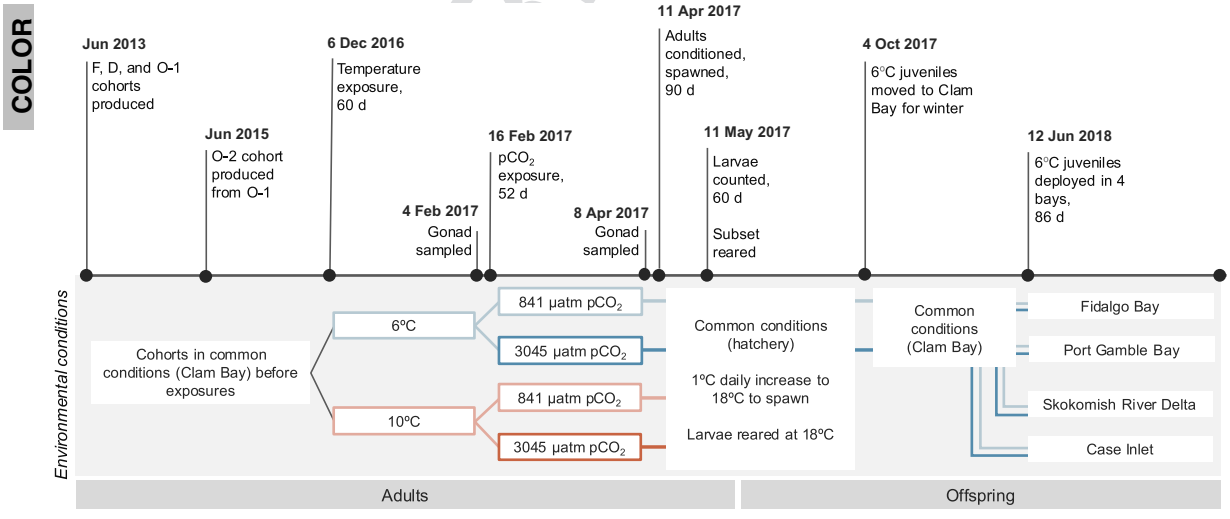

FIG. 2. Experimental timeline. Four cohorts of adult *O. lurida* (F, D, O-1, O-2) were sequentially exposed to two winter temperatures (6.1° ± 0.2°C, 10.2° ± 0.5°C) then two pCO_2 levels (841 ± 85 µatm, 3,045 ± 488 µatm). They were returned to ambient pCO_2 conditions to volitionally spawn. Larvae were collected and reared by cohort × temperature × pCO_2. Juveniles (~1 yr) from 6°C, ambient pCO_2 and 6°C, low pCO_2 adults were deployed in four bays in Puget Sound.

treatments (Fig. 2) to capture developmental differences among treatments. Puget Sound *O. lurida* reportedly enter reproductive quiescence and resorb residual gametes when temperatures are below 12.5°C (Hopkins 1936, 1937), however, recent evidence of low-temperature brooding in Puget Sound (10.5°C; Barber et al. 2016) suggests that reproductive activity may occur during warm winters. Therefore, gonad tissue was sampled to estimate the following: (1) whether residual gametes were resorbed or developed during winter treatments, (2) whether temperature and pCO_2 influenced winter activity, (3) if male and female gametes responded similarly, and (4) if gonad responses correspond with fecundity. Prior to pCO_2 exposure, 15 oysters were sampled from O-1, O-2, and F cohorts, and 9 from the D cohort. After pCO_2 exposure, 9, 6, and 15 oysters were sampled from each treatment for O-1/F, D, and O-2 cohorts, respectively (distributed equally among replicates tanks). Whole visceral mass was excised and preserved in histology cassettes using the PAXgene Tissue FIX System, then processed for gonad analysis by Diagnostic Pathology Medical Group (Sacramento, California, USA).

Adult gonad samples were assigned sex and stage using designations adapted from (da Silva et al. 2009; Appendix S1: Tables S2, S3). Sex was assigned as indeterminate (I), male (M), hermaphroditic primarily male (HPM), hermaphroditic (H), hermaphroditic primarily female (HPF), and female (F). Gonad sex was collapsed into simplified male and female designations for statistical analyses (hermaphroditic-primarily male became male, hermaphroditic-primarily female became female). For stage assignment, male and female gametes were assigned separately due to the high frequency of hermaphroditism (50.8%). Dominant gonad stage was then assigned based on the sex assignment. The da Silva gonad stages were applied for early gametogenesis (stage 1), advanced (stage 2), and ripe (stage 3). Departures from da Silva's stage 0, stage 4 (partially spawned), and stage 5 (fully spawned/resorbing) were as follows: stage 0 in this study represents empty follicles, or no presence of male or female gonad tissue; stage 4 represents both spawned and resorbing gonad; this method did not include a separate stage 5, due to the very high frequency of residual gametes, and no distinct partially spawned oysters (for gonad images, see Appendix S1: Fig. S2; Spencer et al. 2019).

Treatment effects on gonad tissue were assessed for all cohorts combined in four gonad metrics: (1) gonad stage of dominant sex, (2) male gonad tissue when present, (3) female gonad tissue when present, and (4) gonad sex-collapsed (chi-square test of independence). To assess the effects of elevated winter temperature alone, gonad metrics were compared between 6°C and 10°C treatments prior to pCO_2 treatment. To determine the effect of pCO_2 exposure, gonad metrics were compared between ambient and high pCO_2 after 52 d in pCO_2 treatments, including temperature interaction effects. To estimate whether gonad changed during pCO_2 treatment, metrics were compared before and after ambient and high pCO_2 treatments, including temperature interaction effects. *P* values were estimated using Monte-Carlo simulations with 1,000 permutations, and corrected using the Benjamini and Hochberg method and $\alpha = 0.05$ (Benjamini and Hochberg 1995).

Larval production

Following pCO_2 exposure, adult oysters were spawned to assess impacts of winter treatment on larval production timing and magnitude. Beginning on 11 April 2017 (Fig. 2), oysters were reproductively conditioned by raising temperatures gradually (~1°C/d) to 18.1° ± 0.1°C and fed live algae cocktail at 66,000 ± 12,000 cells/mL. Oysters spawned in the hatchery for 90 d volitionally, i.e., naturally releasing gametes without chemical or physical manipulation. Six spawning tanks were used for each temperature × pCO_2 treatment: 6°C, high pCO_2; 6°C, ambient pCO_2; 10°C, high pCO_2; and 10°C, ambient pCO_2. Within the six tanks per treatment, two spawning tanks contained the F cohort (14–17 oysters), two tanks the O-1 cohort (14–17 oysters), one tank the D cohort (9–16 oysters), and one tank the O-2 cohort (111–126 oysters). More O-2 oysters were used due to their small size. Olympia oysters release sperm, but have internal fertilization and release veliger larvae following a ~2 week brooding period (Coe 1931, Hopkins 1937). Therefore, production was assessed by collecting veliger larvae upon maternal release. Spawning tank outflow was collected in 7.5-L buckets using 100-µm screens made from 15.25-cm polyvinyl chloride rings and 100-µm nylon mesh.

Larval collection was assessed for differences in spawn timing and fecundity. Larvae, first observed on 11 May 2017 (Fig. 2), were collected from each spawning tank every 1 or 2 d for 60 d. The number of larvae released was estimated by counting and averaging triplicate subsamples of larvae homogenized in seawater. The following summary statistics were compared between temperature × pCO_2 treatments: total larvae released across the 90-d period, average number of larvae collected on a daily basis (excluding days where no larvae were released), maximum larvae released in 1 d, date of first release, date of maximum release, and number of substantial release days (>10,000 larvae). The total and daily release values were normalized by the number of broodstock × average broodstock height (cm), which can impact fecundity. Distributions were assessed using qqp in the car package for R (Fox and Weisberg 2011), and log-transformed to meet normal distribution assumptions, if necessary. Differences between treatments were assessed using linear regression and three-way ANOVA (cohort was included as a covariate) with backward deletion to determine the most parsimonious models. Tukey honestly significant difference test was calculated using TukeyHSD to assess pairwise comparisons (R Core Team 2016). Dates of peak larval release were also estimated for each pCO_2 × temperature

treatment by smoothing using locally weighted regression, with geom_smooth in the ggplot package (Wickham 2017), with span = 0.3 and degree = 1.

Offspring survival in a natural setting

To assess potential carryover effects of parental pCO_2 exposure, offspring from parents in 6°C, ambient pCO_2 and 6°C, high pCO_2 treatments were reared then deployed in the natural environment. To focus on the effect of parental pCO_2 exposure, only offspring from 6°C parents were tested in the field (Fig. 2). Larvae were collected between 19 May and 22 June 2017, separated by parental pCO_2 exposure and cohort, and reared in common conditions for ~1 yr (Fig. 2; for rearing methods see Appendix S1: Section S6). On 12 June 2018 the juveniles were placed in four bays in Puget Sound, Fidalgo Bay, Port Gamble Bay, Skokomish River Delta, and Case Inlet, with two sites per bay, for a total of eight locations (Fig. 1). Autonomous sensors collected continuous water quality data at each location for pH, salinity (via conductivity), dissolved oxygen, temperature, and chlorophyll. For the F/D and O-1/O-2 cohorts, respectively, 30 and 10 oysters were placed at each location. Initial shell height and group mass were measured, then oysters were enclosed in mesh pouches and affixed inside shellfish bags to exclude predators. At the end of three months, survival, shell height, and group mass were measured for live oysters ~~(Table 1)~~.

Juvenile oyster survival was compared among bays and parental pCO_2 exposure with a binomial generalized linear mixed model (glmm) using glmer from the lme4 package (~~vs. 1.1-19~~). Chi-square tests compared survival differences among factors using the car package ANOVA function (Fox and Weisberg 2011). Mean shell growth ~~was~~ determined by subtracting pre-deployment mean height from post-deployment mean height (not including dead oysters). Both mean shell growth and mass change were compared among factors using ANOVA and *F* statistics to test differences by bay, parental pCO_2, and cohort.

Make and model details for instruments used during treatments and field deployments are available in the Appendix S1: Section S2. All data analysis was performed in R version 3.3.1 using the RStudio interface (R Core Team 2016). Data, gonad histology images, and R code are available on Figshare (Spencer et al. 2019).

Results

Adult reproductive development

After 60 d in temperature treatments (6.1° ± 0.2°C and 10.2° ± 0.5°C), gonad stage of the dominant sex differed significantly between temperatures (Table 2). The 10°C oysters had more instances of advanced gametogenesis (stage 2), and fewer that were resorbing/spawned (stage 4; Fig. 3). This difference was influenced strongly by more advanced male gametes in 10°C oysters, but there were no differences in female gamete stages. No differences in sex ratio were observed between temperature treatments (Fig. 4).

After 52 d in pCO_2 treatments, gonad stage of the dominant sex differed significantly between ambient and high pCO_2 in the oysters previously held in 10°C (Table 2). More mature gametes (stage 3) were found in 10°C, ambient pCO_2 (49%) compared to 10°C, high pCO_2 (33%). This difference was strongly influenced by oysters that were predominantly male, as male gamete stage tended to differ between pCO_2 treatment, but female gamete stage did not (Table 2, Fig. 3). In 6°C treated oysters, there were no pCO_2 effects on gonad stage of the dominant sex, male gamete stage, or female gamete stage. No gonad stage or sex ratio differences were detected among oysters from 10°C, high pCO_2 (combined stressors) and 6°C, ambient pCO_2 (no stressors). Gonad sex did not differ significantly among treatments; however, oysters tended to contain fewer male-only and more female-only gonad tissues in the riper, ambient pCO_2, treated groups ~~than male-only tissues~~ (Fig. 4).

Compared to oysters before pCO_2 exposure, those exposed to high pCO_2 did not differ in gonad sex, stage of the dominant sex, or female gamete stage. Male gametes in the 6°C treated oysters developed while in the high pCO_2 exposure, but there was no change in the 10°C treated oysters. Oysters held in ambient pCO_2 had significantly more advanced gonad compared to before CO_2 exposure regardless of temperature, again influenced strongly by changes in male gamete stage (Table 2).

Table 1. Environmental data during offspring field trial.

Parameter	Fidalgo Bay	Port Gamble Bay	Skokomish River Delta	Case Inlet
Temperature (°C)	15.4 ± 1.5	15.0 ± 1.0	16.2 ± 2.7	16.8 ± 1.7
DO (mg/L)	10.6 ± 2.4	10.5 ± 1.9	10.2 ± 3.9	11.2 ± 2.8
Salinity (PSU)	28.5 ± 3.9	31.9 ± 2.0	29.6 ± 1.3	24.6 ± 1.7
pH_T	8.07 ± 0.15	7.86 ± 0.17	8.01 ± 0.20	8.01 ± 0.16
Chlorophyll (μg/L)	2.27 ± 4.09	2.25 ± 1.45	5.72 ± 15.36	3.31 ± 6.13

Notes: Environmental data was collected from locations where offspring were deployed for 3 months from June through August 2018. Mean ± SD of continuously monitored environmental data are shown for periods of tidal submergence only (tidal height > 0.3 m), collected at two deployment locations within each bay.

TABLE 2. Gonad stage and sex comparisons among treatments.

	6°C			10°C		
Temperature and pCO_2	Pre	Amb	High	Pre	Amb	High
Sex ratio						
6°C						
Pre						
Amb	0.8 (0.93)					
High	4.6 (0.34)	5.4 (0.29)				
10°C						
Pre	5.9 (0.26)					
Amb				6.8 (0.18)		
High		5.3 (0.29)		3.8 (0.46)	0.6 (0.94)	
Stage of the dominant sex						
6°C						
Pre						
Amb	**16.5 (0.013)***					
High	4.6 (0.48)	9.7 (0.090				
10°C						
Pre	**15.8 (0.017)***					
Amb				**12.7 (0.038)***		
High		2.8 (0.78)		5.2 (0.44)	**12.5 (0.038)***	
Male gametes						
6°C						
Pre						
Amb	**24.2 (1.6 × 10^{-3})***					
High	**15.2 (0.013)***	9.0 (0.071)				
10°C						
Pre	**31.1 (1.6 × 10^{-3})***					
Amb				**11.2 (0.038)***		
High		1.7 (0.78)		0.6 (0.95)	9.5 (0.084)	
Percent mature	30	28	15	19	33	21
Female gametes						
6°C						
Pre						
Amb	6.3 (0.18)					
High	3.6 (0.47)	4.4 (0.36)				
10°C						
Pre	2.1 (0.78)					
Amb				4.2 (0.26)		
High		0.8 (0.9)		5.5 (0.17)	0.15 (1.0)	
Percent mature	2	15	8	6	18	21

Notes: Gonad was sampled after temperature treatment but before pCO_2 (6°C Pre and 10°C Pre, n = 54), and after pCO_2 treatment (Amb = 841 ± 85 µatm, n = 39; High = 3045 ± 488 µatm, n = 39). Pearson's chi-square statistics are shown with adjusted P in parentheses for gonad sex, stage of the dominant sex, male gametes when present, and female gametes when present. Cells with * and in boldface type ~~indicate~~ significant differences between ~~comparison;~~ blank cells, not tested; percent mature, percentage of sampled oysters that contained stage 3 gametes in each treatment, for male and female gametes separately.

No sampled oysters contained brooded embryos or larvae. Gonad data and patterns within cohorts are reported in Appendix S1: Figs. S3, S4, and Table S4.

Larval production

Adults exposed to 10°C produced more larvae on a daily basis (excluding days where no larvae were released) than those exposed to 6°C in ambient pCO_2-exposed oysters (P = 0.040), but not in high pCO_2-exposed oysters (P = 0.66; Fig. 6, pCO_2 × temperature interaction, $F_{2,8}$ = 5.1, P = 0.037). Total larvae released over the 90-d spawning period tended to differ by treatment, but not significantly (temperature × pCO_2 interaction, $F_{2,8}$ = 4.0, P = 0.063). Temperature and pCO_2 as single factors did not affect total larvae released or daily averages.

The date of first larval release differed by temperature regardless of pCO_2 (Figs. 5 and 6, $F_{1,8}$ = 11.9, P = 0.0087), and pCO_2 had no effect on timing (not retained in model). Onset was on average 5.2 d earlier in the 10°C treatment. Timing of peak larval release also differed by temperature treatment regardless of pCO_2

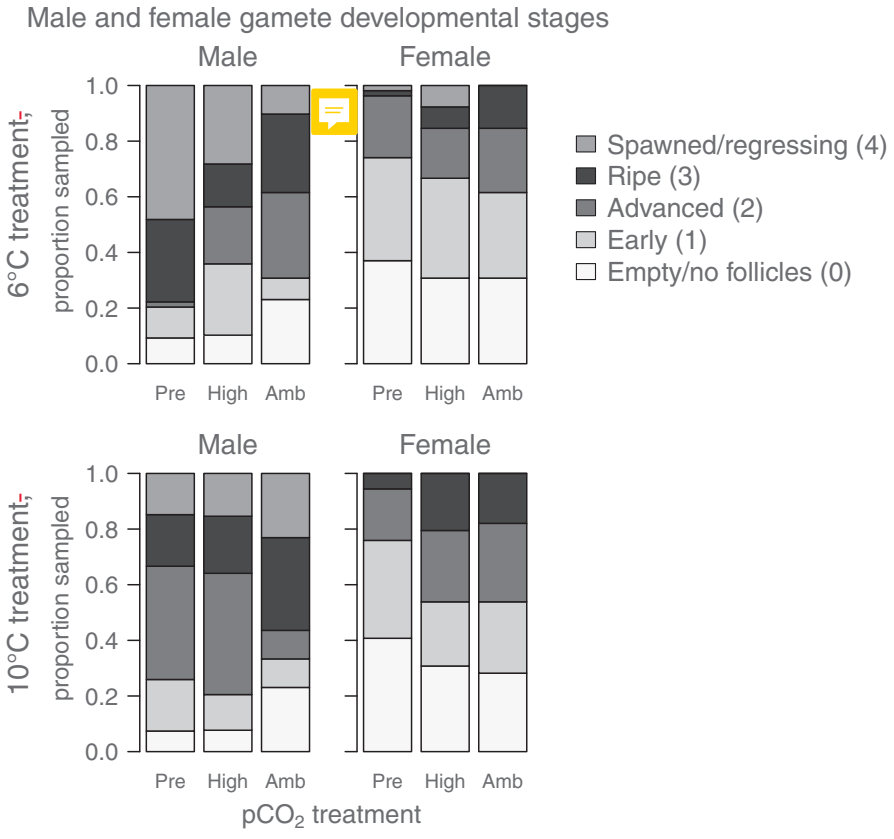

FIG. 3. Gonad developmental stages for male and female gametes after 60 d in temperature treatments but before pCO_2 treatments (Pre, $n = 54$) and after 52 d in high pCO_2 (3,045 ± 488 µatm, $n = 39$) and ambient pCO_2 (Amb, 841 ± 85 µatm, $n = 39$), which indicates that sperm development was influenced by elevated winter temperature (more advanced) and high pCO_2 (less advanced, 10°C treatment only), but oocyte development was not. All oysters were assigned both male and female stages; if no oocytes were present, for example, that oyster was designated as female stage 0.

(Fig. 6, $F_{3,19} = 6.7$, $P = 0.018$), occurring on average 8.3 d earlier in 10°C oysters. The 10°C treated oysters produced more large pulses of larvae, on average 2 additional days, than 6°C ($F_{1,8} = 7.25$, $P = 0.027$).

In total, 18.5 million larvae were collected from 767 oysters. Total larvae produced by each treatment was 3.1, 4.8, 5.9, and 4.5 M for 6°C-ambient pCO_2, 6°C-high pCO_2, 10°C-ambient pCO_2, and 10°C-high pCO_2, respectively. Based on reports of ~215,000 larvae produced per adult *O. lurida* of shell height 35 mm (Hopkins 1936), the number of oysters that spawned as female in this study was ~86, with 14.3, 22.5, 27.6, and 21.0 from the 6°C, ambient pCO_2; 6°C, high pCO_2; 10°C, ambient pCO_2; and 10°C, high pCO_2 treatments, respectively. This estimate is likely low across all treatments, due to the smaller D and O-2 cohorts (mean length in F, D, O-1 and O-2 was 35.7, 29.8, 35.7, and 20.0 mm, respectively); therefore, the total number of oysters that spawned as female and released larvae is likely higher than 86.

Larval production and timing data, including differences among cohorts, are included in Appendix S1: Section S5 and Table S5.

Offspring survival in a natural setting

Juvenile survival after three months in the field was on average 15% higher in cohorts from high-pCO_2-exposed parents than from ambient-pCO_2 parents (44% ± 37% and 29% ± 27%, respectively, $\chi^2 = 10.6$, $P = 0.0011$). The influence of parental pCO_2 on survival varied by bay (bay × parental pCO_2 interaction $\chi^2 = 15.3$, $P = 1.6 \times 10^{-3}$), and by cohort (cohort × parental pCO_2 interaction $\chi^2 = 23.5$, $P = 3.2 \times 10^{-5}$) (Table 3).

Survival in offspring from high pCO_2 parents was higher in the Fidalgo Bay and Port Gamble Bay locations ($\chi^2 = 17.7$, $P = 2.6 \times 10^{-5}$; $\chi^2 = 10.0$, $P = 1.6 \times 10^{-3}$, respectively), but this was not the case in Skokomish River Delta or Case Inlet. Survival in the F cohort was 38% higher in oyster from pCO_2 parents than those from ambient pCO_2 parents across all deployment bays ($\chi^2 = 28.1$, $P = 4.6 \times 10^{-7}$), and within the Fidalgo Bay location ($\chi^2 = 17.6$, adjusted $P = 0.0001$). Survival in the D and O-1 cohorts did not differ significantly between parental pCO_2 across all

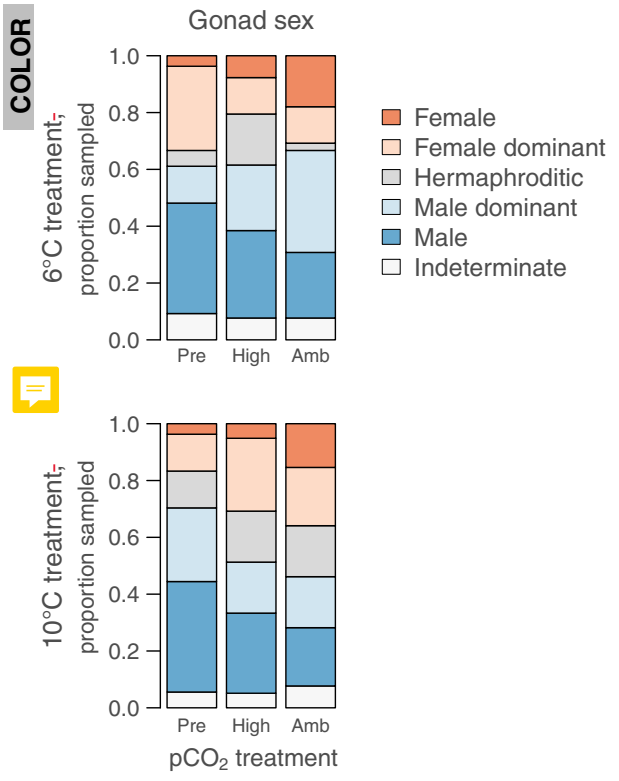

FIG. 4. Gonad sex after 60 d in temperature treatments but before pCO_2 treatments (Pre, $n = 54$) and after 52 d in high pCO_2 (3,045 ± 488 µatm, $n = 39$) and ambient pCO_2 (Amb, 841 ± 85 µatm, $n = 39$). Winter conditions did not significantly influence gonad sex ratios.

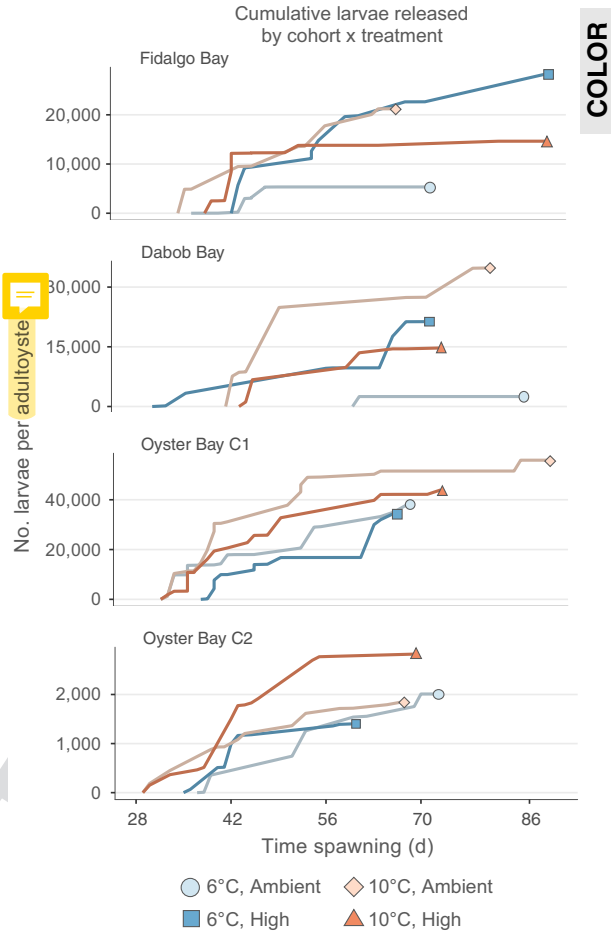

FIG. 5. Cumulative larvae released over 90 d of continuous volitional spawning under hatchery conditions, normalized by the number of adult oysters. Each of the four panels represent a cohort and lines are color coded by winter temperature and pCO_2 treatments, where ambient pCO_2 = 841 µatm (7.8 pH), and high pCO_2 = 3,045 µatm (7.31). Reproductive conditioning and spawning occurred at 18°C, in ambient pCO_2, and with live algae at a density of 66,000 ± 12,000 cells/mL.

bays (D: $\chi^2 = 0.4$, $P = 1$, O-1: $\chi^2 = 2.5$, $P = 0.44$), or within individual bays. More O-2 juveniles with ambient pCO_2 parents survived across all bays ($\chi^2 = 9.1$, $P = 0.010$), and within the Skokomish River Delta ($\chi^2 = 8.9$, $P = 0.011$).

Without considering parental pCO_2, more oysters survived in Port Gamble Bay (49% ± 36%) and Fidalgo Bay (39% ± 36%) than in Case Inlet (29% ± 29%, $P = 0.012$ and $P = 0.037$, respectively; bay factor, $\chi^2 = 18.5$, $P = 3.4 \times 10^{-4}$). Survival at Skokomish River Delta did not differ significantly from other locations (32% ± 27%). No interaction between cohort and bay was detected ($\chi^2 = 9.8$, $P = 0.37$; Fig. 7, Table 3).

Shell length was not affected by bay, cohort or parental pCO_2. The mass per oyster (compared to before deployment) differed by cohort ($F_{3,76} = 15.9$, $P = 4.0 \times 10^{-8}$), due to Dabob Bay cohort growing less than the other three cohorts (Δg/oyster: D = 0.5, F = 1.2, O-1 = 1.6, & O-2 = 1.0). Mass change also differed by bay ($F_{3,76} = 4.8$, $P = 3.9 \times 10^{-3}$) due to less growth in oysters placed at Fidalgo Bay than in Port Gamble Bay and Case Inlet (Δg/oyster: FB = 0.7, PGB = 1.0, CI = 1.1, SK = 0.8; Appendix S1: Fig. S5).

DISCUSSION

Ocean acidification and ocean warming potentially threaten marine organisms, particularly ectothermic calcifiers (Hofmann et al. 2010). An organism's genotype, complete environmental history, and the timing and magnitude of environmental perturbations may all determine its fitness in future ocean conditions. To begin teasing apart these complex factors in the Olympia oyster, this study examined four adult cohorts with distinct genetic structure but known, shared histories. Elevated winter temperature resulted in increased gonad development, which corresponded with earlier and more frequent larval release (on average 5.2 d earlier, two additional days). High pCO_2

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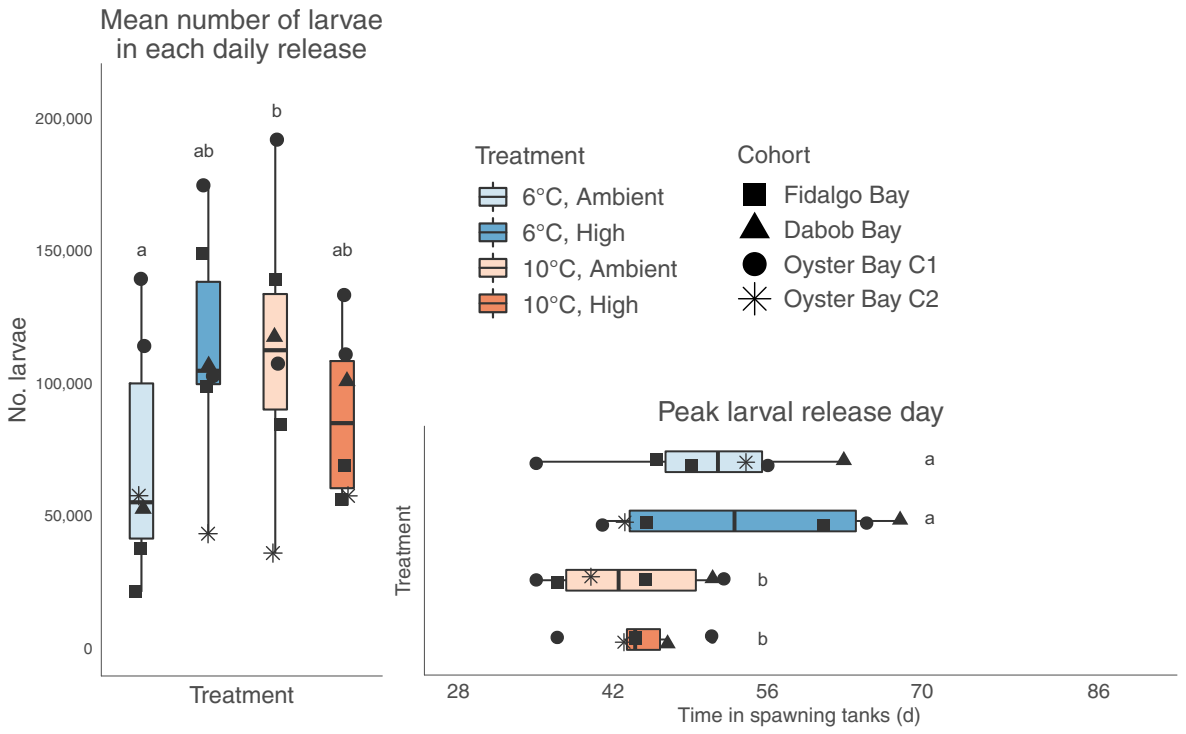

FIG. 6. Left: average number of larvae collected on a daily basis (excluding days where no larvae were released). Daily pulses of larvae were larger ~~in~~ 10°C than 6°C, but only in oysters exposed to ambient pCO_2. For statistical analysis, data were normalized by number of oysters × average oyster height (cm) (data shown is not normalized). Right: number of spawning days until larval release peaked; peak release occurred on average 8.3 d earlier in 10°C treated oysters. Letters (a, ab, b) indicate differences among treatments. Boxes contain values lying within the interquartile range (IQR), with medians indicated by lines in the middle of boxes. Whiskers extend to the largest value not greater than 1.5 × IQR.

10

TABLE 3. Offspring survival in the field.

	Survival (%)									
	Fidalgo Bay, F		Dabob Bay, D		Oyster Bay F1, O-1		Oyster Bay F2, O-2		All cohorts	
Bay	Amb	High	Amb	High	Amb	High	Amb	High	Amb	High
Fidalgo	**20 ± 32***	**85 ± 10***	22 ± 12	38 ± 25	40 ± 46	62 ± 43	11 ± 15	13 ± 23	**25 ± 30$^+$**	**51 ± 37$^+$**
Port Gamble	**33 ± 27***	**74 ± 17***	35 ± 35	63 ± 21	40 ± 47	93 ± 12	21 ± 0	0	**34 ± 33$^+$**	**64 ± 34$^+$**
Skokomish	32 ± 17	51 ± 23	45 ± 11	18 ± 13	20 ± 28	35 ± 41	**33 ± 24***	**0***	32 ± 21	31 ± 33
Case Inlet	20 ± 19	40 ± 30	18 ± 15	15 ± 26	50 ± 26	50 ± 48	14 ± 20	0	27 ± 23	30 ± 35
All bays	**27 ± 22***	**62 ± 29***	30 ± 22	34 ± 28	38 ± 37	58 ± 41	**20 ± 16***	**4 ± 13***	**29 ± 27$^+$**	**44 ± 37$^+$**

Notes: One-year-old juveniles were deployed for three months in four bays in Puget Sound, Washington, USA, in two sites per bay. Survival (mean ± SD) is shown by cohort × bay × parental pCO_2 treatment (Amb = 841 ± 85 µatm, High = 3,045 ± 488 µatm). Only offspring from 6°C-treated adults were deployed. Significant survival differences ($P \leq 0.05$) were detected between parental pCO_2 treatment within the Fidalgo Bay and Oyster Bay F2 cohorts (*), and across all cohorts (+).

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exposure negatively influenced gonad maturation state, but did not affect subsequent fecundity. Offspring from parents exposed to elevated pCO_2 had higher overall survival upon deployment. Differences in juvenile survival among bays and cohorts indicate that carryover effects are dependent upon the environment and genotype, and reinforce the importance of using multiple sources of test organisms in stress-response studies.

Reproduction

We expected elevated winter temperature to reduce fecundity, based on predictions that changes to reproductive quiescence and metabolism would be deleterious to spring reproduction. Counter to this prediction, warm winter temperature positively affected larval production. Oysters in elevated temperature contained more developed male gametes after treatment, and subsequently

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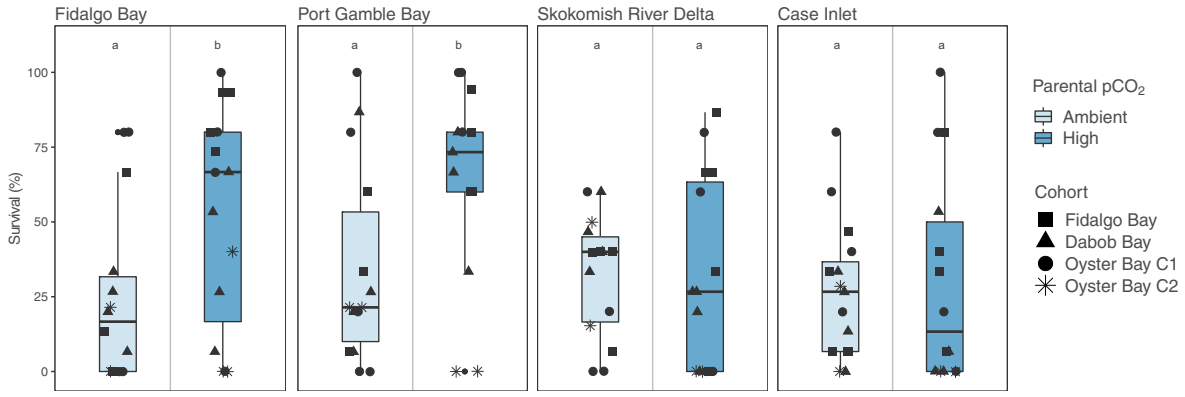

FIG. 7. Percent survival of juvenile offspring in the field. The four panels each represent survival in one bay (Fidalgo Bay, Port Gamble Bay, Skokomish River Delta, Case Inlet). Within each panel, box plots are separated by parental pCO_2 exposure (Ambient = 841 µatm, High = 3,045 µatm). Points indicate percent survival in each deployment pouch, and symbols indicate cohort (Fidalgo Bay, Dabob Bay, Oyster Bay Cohort 1, and Oyster Bay Cohort 2). Letters (a, b) indicate survival differences among parental pCO_2 exposure within each bay. Boxes contain values lying within the interquartile range (IQR), with median survival indicated by lines in the middle of boxes. Whiskers extend to the largest value not greater than 1.5 × IQR.

began releasing larvae earlier and produced more larvae per day compared to cold-treated oysters. We find no evidence that cold winters are critical for spring reproduction, but rather elevated winter temperature may elongate the *O. lurida* spawning season. In comparison, a 29-yr data set of *M. balthica* reproduction showed that as winter temperature increased, spring spawning began earlier and fecundity declined (Philippart et al. 2003). However, the present study was conducted in a hatchery setting, with ample phytoplankton, and did result in a temperature shift during spawning. In the wild, numerous additional abiotic and biotic factors will contribute to *O. lurida* fitness, and warmer winters may result in earlier and longer reproductive seasons only if nutritional requirements are met. Whether larvae released earlier in the spring can survive to recruitment will greatly depend on many factors including food availability and predation. Those modeling larval recruitment (Wasson et al. 2016, Kimbro et al. 2019) should consider including winter temperature as a factor influencing spatiotemporal recruitment patterns.

We predicted that high pCO_2 exposure would redirect energy away from storage to maintenance processes, resulting in delayed gametogenesis and poor fecundity in the spring. After exposure to 3,045 µatm pCO_2 (pH 7.31), fewer oysters contained ripe or advanced male gonad tissue than ~~in~~ ambient pCO_2, signaling reduced spermatogenic activity. Female gonads, sex ratios, and subsequent fecundity were not affected by sole exposure to high pCO_2. Similar impacts on gametogenesis during exposure were observed in the Sydney rock (*S. glomerata*) and Eastern (*C. virginica*) oysters, but with varying pCO_2 thresholds. Parker et al. (2018) found *S. glomerata* gametogenesis to slow in 856 µatm (pH 7.91), and Boulais et al. (2017) found normal rates at 2,260 µatm (pH 7.5), delay at 5,584 µatm (pH 7.1), and full inhibition at 18,480 µatm (pH 6.9) in *C. virginica*. Together, these studies indicate that high pCO_2 slows the rate of gametogenesis, but the level at which pCO_2 affects gametogenesis appears species-specific, and likely reflective of variable physiological mechanisms and reproductive strategies.

The combined effects of sequential elevated temperature and pCO_2 treatments did not act synergistically to delay gonad development, but instead resulted in oysters with gonad stage and fecundity no different from the untreated oysters. Similarly, combined simultaneous temperature and high pCO_2 exposures did not affect *S. glomerata* fecundity (Parker et al. 2018). We did detect a pCO_2 dependent effect of temperature on the average number of larvae released per day. Oysters that had previously been exposed to 10°C produced more larvae than 6°C, but only after ambient pCO_2 exposure, which may reflect a general reproductive arrest that occurs when exposed to high pCO_2. Despite experimental differences (e.g., sequential vs. simultaneous exposures) that can influence outcomes (Bible et al. 2017), both 13
Parker et al. (2018) and the present study indicate that high pCO_2 slows gametogenesis, elevated temperature accelerates it, and these two environmental drivers act antagonistically on gonad development if occurring in the same reproductive season. An important factor not included in either study is ecologically relevant variability. Temperature and pCO_2 oscillations, driven by tides and diurnal photosynthesis, could offer daily refuge or expose oysters to dynamic changes, altering how combined stressors interact (Cheng et al. 2015). 14

In contrast to prior studies, temperature and pCO_2 did not impact *O. lurida* sex ratios, whereas in high pCO_2, *C. virginica* skewed male (Boulais et al. 2017) and *S. glomerata* skewed female (Parker et al. 2018). This observation may be explained by very low incidence of total reproductive inactivity in our *O. lurida* cohorts, only four out of the 108 oysters that were sampled prior

to pCO_2 treatment contained empty follicles, and thus sex ratios may be different if pCO_2 exposure occurs earlier in life during initial sex differentiation. Furthermore, high pCO_2 exposure only occurred in winter, prior to spawning. If high pCO_2 persists during oocyte maturation and spawning, *O. lurida* fecundity may be reduced similar to *C. virginica* and *S. glomerata*. Future research should examine *O. lurida* sexual development during the initial switch from male to female, which can occur the first winter after settlement (Moore et al. 2016), and across a range of pCO_2 to determine conditions in which gametogenesis and sex determination are affected.

Offspring

Abiotic parental stressors can be beneficial, neutral, or detrimental to offspring viability (Donelson et al. 2018). We explored carryover effects of adult exposure to winter pCO_2 on offspring by testing survival in the field. Offspring with high pCO_2 parental histories performed better in two of four locations, Fidalgo Bay and Port Gamble Bay. Carryover effects of parental high pCO_2 exposure may therefore be neutral, or beneficial, to offspring depending on the environmental conditions. Port Gamble Bay and Fidalgo Bay are more influenced by oceanic waters, which could explain cooler observed temperatures. These locations are also typically less stratified than the Skokomish River Delta and Case Inlet. In Port Gamble Bay, where pCO_2 parental history most significantly correlated with offspring survival across cohorts, mean pH was considerably lower than the other deployment locations (−0.17 pH units), and mean salinity was higher (+3.8 PSU). Given the experimental design we are able to clearly demonstrate that manifestation of carryover effects in Olympia oysters is dependent on environmental conditions. Specifically, there is a greater likelihood of beneficial carryover effects when parents are exposed to stressful conditions. Overall, carryover effects of parental pCO_2 treatment were positive; however, negative effects were observed in the O-2 cohort. This discrepancy could relate to unique O-2 juvenile characteristics, as they were bred from siblings, and were third-generation hatchery produced. The complex interactions among parental exposure, bay, and cohort indicate that offspring viability is influenced by ancestral environment history, environmental conditions, and genotype.

Our results contrast with a similar study that exposed *C. gigas* oysters to high pCO_2 during the winter, and found fewer hatched larvae 18 h post-fertilization from exposed females, with no discernable paternal effect (Venkataraman et al. 2019). Hatch rate was not directly measured in this study due to the *O. lurida* brooding behavior; however, no difference in daily and total larvae released suggest that hatch rate was unaffected by pCO_2. The different responses seen in Venkataraman et al. (2019) and the present study may reflect variability among species and spawning method. *C. gigas* gametes were collected artificially by stripping gonads, whereas *O. lurida* late-stage veliger larvae were collected upon release from the brood chamber. For instance, volitionally spawned gamete quality and fertilization rates could vary between the natural vs. artificial settings to influence larval viability. Larval brooding may also be a mechanism by which sensitive larvae are acclimatized to stressors, as the *O. lurida* brood chamber pH and dissolved oxygen can be significantly lower than the environment (Gray et al., *in press*).

Beneficial parental carryover may also be linked to the male-specific gonad effects, and the conditions in which the adult oysters were held. During high pCO_2 exposure, oocyte stage and prevalence did not change, which indicates that oogenesis did not occur. Negative intergenerational carryover effects are commonly linked to variation in oocyte quality, which can be affected by the maternal environment during oogenesis (Utting and Millican 1997). In the Chilean flat oyster (*Ostrea chilensis*), for instance, egg size and lipid content positively correlate with juvenile growth and survival (Wilson et al. 1996). If high pCO_2 exposure were to coincide with oocyte proliferation and growth, *O. lurida* egg quality and larval viability could be compromised. In contrast, male gonad stage advanced significantly during pCO_2 exposure. Intergenerational and transgenerational carryover effects are increasingly linked to the paternal environment in other taxa, such as inheritance of epigenetic changes to the male germ line (Anway et al. 2005, Rodgers et al. 2013, Soubry et al. 2014). Positive carryover effects of environmental stressors observed in this and other marine invertebrate taxa may be due to paternal epigenetic effects, but this link has not yet been observed.

Conclusion

This study clearly demonstrates that exposure to elevated winter temperature and altered carbonate chemistry impacts reproduction and offspring viability in the Olympia oyster. Furthermore, we report the first observations of intergenerational plasticity in an *Ostrea* species, which is dependent on offspring environmental conditions and population. The observed context-dependent carryover effects could have a substantial impact on species resilience. Combined with previous reports of resilience to environmental stressors (Waldbusser et al. 2016, Cheng et al. 2017) and intraspecific variability (Heare et al. 2017, Maynard et al. 2018, Silliman et al. 2018, Bible et al. 2019), the Olympia oyster may be more capable than other marine bivalve species to withstand and adapt to unprecedented ocean change. Furthermore, conserving and restoring *O. lurida* in a variety of settings, including hypoxic, warmer, and less alkaline areas, could increase the probability that future populations are equipped for challenging conditions through selection or intergenerational carryover.

As temperatures rise and ocean acidification progresses, there may be profound and unexpected seasonal changes across marine taxa. Accurate predictions will need to consider parental carryover effects, as they can impart neutral, beneficial, or detrimental characteristics to offspring, which depend on complex interactions among parental exposure timing, reproductive strategies, species plasticity, and standing genetic structure. With these considerations, future biological response studies need to be aware of three possible factors influencing results: (1) source population, (2) environmental history (within-lifetime carryover effects), and (3) ancestral environmental history (inter- and transgenerational carryover effects). Controlling for, or at minimum recognizing and recording these factors, will provide important context for those predicting ecosystem response to environmental change.

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Supporting Information

Additional supporting information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/eap.2060/full

Data Availability

Data are available on Figshare: https://doi.org/10.6084/m9.figshare.8872646