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Comparison of larval development in domesticated and naturalized stocks of the Pacific oyster *Crassostrea gigas* exposed to high pCO_2 conditions

Evan Durland[1,*], George Waldbusser[2], Chris Langdon[1]

[1]Department of Fisheries and Wildlife and Coastal Oregon Marine Experiment Station, Hatfield Marine Science Center, Oregon State University, Newport, Oregon 97365, USA

[2]College of Earth, Ocean, and Atmospheric Sciences, Oregon State University, Corvallis, Oregon 97331, USA

ABSTRACT: Ocean acidification (OA) has had significant negative effects on oyster populations on the west coast of North America over the past decade. Many studies have focused on the physiological challenges experienced by young oyster larvae in high pCO_2/low pH seawater with reduced aragonite saturation state (Ω_{arag}), which is characteristic of OA. Relatively few, by contrast, have evaluated these impacts upon fitness traits across multiple larval stages and between discrete oyster populations. In this study, we conducted 2 replicated experiments, in 2015 and 2016, using larvae from naturalized 'wild' and selectively bred stocks of the Pacific oyster *Crassostrea gigas* from the US Pacific Northwest and reared them in ambient (~400 µatm) or high (~1600 µatm) pCO_2 seawater from fertilization through final metamorphosis to juvenile 'spat.' In each year, high pCO_2 seawater inhibited early larval development and affected the timing, but not the magnitude, of mortality during this stage. The effects of acidified seawater on metamorphosis of pediveligers to spat were variable between years, with no effect of seawater pCO_2 in the first experiment but a ~42% reduction in spat in the second. Despite this variability, larvae from selectively bred oysters produced, on average, more (+ 55 and 37%) and larger (+ 5 and 23%) spat in ambient and high pCO_2 seawater, respectively. These findings highlight the variable and stage-specific sensitivity of larval oysters to acidified seawater and the influence that genetic factors have in determining the larval performance of *C. gigas* exposed to high pCO_2 seawater.

KEY WORDS: Ocean acidification · Pacific oyster · *Crassostrea gigas* · Larval development · Genetics · Domestication

1. INTRODUCTION

1.1. Effects of ocean acidification on bivalve larval fitness

The effects of ocean acidification (OA) upon marine organisms have been widely studied by biologists (Kroeker et al. 2013), ecologists (Miller et al. 2009, Gaylord et al. 2015) and aquaculturists (Barton et al. 2012, 2015) over the past decade. While OA conditions have diverse impacts on marine ecosystems, marine bivalves are particularly vulnerable to shifts in seawater carbonate chemistry, especially during embryogenesis when the initial larval shell is being formed (prodissoconch I or PDI stage) and the sites of calcification are exposed to ambient seawater conditions (Waldbusser et al. 2013). Reduced seawater aragonite saturation (Ω_{arag}), which is a typical consequence of OA conditions, leads to an increase in morphological abnormalities of PDI or 'D-hinge'

*Corresponding author: durlandevan@gmail.com

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veliger larvae at ~24–48 h post fertilization (hpf) as well as smaller, normally developed larvae (Kurihara et al. 2007, Waldbusser et al. 2015a). In addition to impeding calcification, the multi-stressor environment of OA conditions (low pH, high pCO_2, reduced Ω_{arag}) has diverse effects upon larval physiology and metabolism (Dineshram et al. 2012, Waldbusser et al. 2015b), many of which may be difficult to assess (Frieder et al. 2017).

Oyster larval development is also complex; it encompasses 2 distinct metamorphic transitions, is impacted by numerous water quality parameters (Pörtner 2010, Przeslawski et al. 2015), and fitness traits are prone to variation based on maternal/egg quality (Gallager et al. 1986, Myrina et al. 2015) and nutritional aspects of the larval culture (Ben Kheder et al. 2010, Marshall et al. 2010). While much attention has been paid to early development and shell formation in bivalve larvae, especially with regards to bioenergetics, calcification and egg lipids (Waldbusser et al. 2013, Frieder et al. 2017), the impacts of OA conditions across the entire larval period are important to consider in order to expand the scope of inference from discrete physiological impacts to overall population survival and fitness (Gobler & Talmage 2013, Brunner et al. 2016).

1.2. Impacts of OA on US west coast oyster stocks

In the US Pacific Northwest (PNW), Pacific oysters *Crassostrea gigas* were established from numerous importations of Japanese founder stocks in the early 20th century. They have successfully established self-recruiting naturalized populations in several bays of Washington State that are frequently exposed to upwelled seawater during summer months (May–September). These upwelling events bring deep water onto the continental shelf which is naturally low in pH, owing to microbial respiration, and which has become more strongly acidified in recent years due to OA (Feely et al. 2004) and affects seawater chemistry of coastal bays in the PNW (Feely et al. 2008, 2016). It is difficult to establish a correlation between the inherently stochastic spawning and settlement trends of Pacific oysters in these habitats and the dynamic effects of upwelling and OA (Weisberg et al. 2016, Ruesink et al. 2018), but overall recruitment for these stocks has been in decline for the past 30 yr (Dumbauld et al. 2011), and increasingly acidified seawater environments are likely to be one of the factors contributing to this trend (Hales et al. 2017).

Notably, the same upwelling events that affect naturalized Pacific oyster populations have also had severe impacts on shellfish hatcheries operating in this region which have experienced significantly reduced rates of larval growth and survival during upwelling events in the past decade (Barton et al. 2012). In response to these challenges, commercial hatcheries in this region now measure seawater carbonate chemistry, strategically time larval production cycles, chemically buffer incoming seawater in order to maintain optimal carbonate chemistry conditions and are implementing breeding programs to produce shellfish stocks that may have greater resistance to OA effects (Barton et al. 2015). The potential for breeding to improve OA-specific larval fitness traits remains uncertain, but growth and survival of larvae in high pCO_2 seawater appear to be fitness traits which are partially genotype dependent, displaying significant variation within (Sunday et al. 2011, Frieder et al. 2017) and between bivalve populations (Parker et al. 2011, Wright et al. 2014). The variable response of bivalve larvae to acidified conditions is also consistent with broader phenotypic plasticity and adaptive capacity to OA observed in other marine invertebrates (Kelly & Hofmann 2013, Sunday et al. 2014, Thor & Dupont 2015).

1.3. Oyster breeding for improved fitness

Since 1996, the Molluscan Broodstock Program (MBP) at Oregon State University has conducted a selective breeding program for Pacific oysters farmed on the west coast of the USA (De Melo et al. 2016, 2018). The commercial shellfish industry has collaborated with MBP to enhance commercially valuable field traits (growth and survival of juveniles and adults at farm sites) while maintaining genetic diversity and limiting inbreeding accumulation in the stocks through family-based structured mating designs using bi-parental crosses. To date, there has been no selection directed upon any larval performance traits, but 6 generations of rearing larval cohorts (defined here as a group of separate or mixed families reared simultaneously) in hatchery environments may have resulted in unintentional selection that improves mean larval growth and survival under hatchery conditions. Additionally, larvae from most of these MBP cohorts were cultured during summer periods with likely, but unmeasured, upwelling events and may have acquired additional traits which benefitted their survival in high pCO_2 seawater. Interestingly, over the past ~10 yr, commercial shellfish hatcheries

that partner with MBP have reported that during periods of intense upwelling, larvae spawned from MBP broodstock survive and grow better when compared to larvae spawned from naturalized populations (S. Cudd pers. comm.). Little is known about stock-based sensitivity of Pacific oysters in the PNW to the effects of OA, and anecdotal evidence that selected lines exhibit increased larval fitness in acidified conditions is surprising given that any such gain in MBP stocks would be incidental. Furthermore, naturalized oysters, which possess substantially larger populations and greater genetic diversity (Camara 2011, Sun & Hedgecock 2017) and are exposed to upwelled, low Ω_{arag} conditions more frequently (Hales et al. 2017), should be theoretically favored to more rapidly acquire beneficial adaptations to these stressors.

More directly, any realized differences in larval fitness between selectively bred, or 'domesticated,' lines of oysters and naturalized stocks have important implications for the sustainability of the shellfish industry in the PNW along with potential genetic consequences for naturalized stocks of *C. gigas* in the region. Improved resilience of domesticated oyster larvae to acidified conditions would not only be a commercially valuable trait but could also potentially alter population dynamics between domesticated oysters and naturally occurring populations which are located close to aquaculture operations and frequently interbreed (e.g. 'migration' in Sun & Hedgecock 2017). Differences in larval fitness between the stocks, especially for performance in high pCO_2 conditions, could possibly augment the rate of gene flow from hatchery-reared, domesticated lines to established natural stocks and have unknown long-term implications for these populations.

In this study, we aimed to compare the resilience of larvae from selected MBP broodstock with that of oysters collected from Willapa Bay (the most abundant local source of naturalized *C. gigas*) when cultured in both ambient and high pCO_2 seawater. The structured pedigree of MBP oyster families (De Melo et al. 2016) allows for the creation of genetically diverse pools of larvae for experimentation that are not compromised by inbreeding and are similar to those used by commercial hatcheries. Additionally, access to pedigreed oyster families allows the use of similar genetic pools across multiple experiments, enabling a level of comparison unavailable to other studies that use broodstock of unknown genetic composition. Utilizing this resource, we conducted 2 long-term (22–24 d) experiments across 2 years in which we compared the performance of larvae from pools of mixed MBP families to those of larvae from Willapa stocks (of unknown genetic structure) to determine stage-specific sensitivities in ambient (~400 µatm) and high (~1600 µatm) pCO_2 conditions.

2. MATERIALS AND METHODS

2.1. Broodstock selection and conditioning

For the first experiment, conducted in 2015, full sibling oyster families from MBP's fifth-generation cohorts were ranked based on farm yields (De Melo et al. 2016), and the top 20 performing families were selected for spawning. In April 2014, naturalized oysters were obtained from the Naselle region of Willapa Bay, WA (46° 25′ 15.2″ N, 123° 51′ 47.6″ W) and transported to the Hatfield Marine Science Center (HMSC), OR, where all MBP breeding stocks are maintained in Yaquina Bay (44° 37′ 27.6″ N, 124° 02′ 35.2″ W). In late April 2015, ~20 broodstock from each MBP family (~400 total) and Willapa broodstock (~60 total) were transferred from the broodstock repository to conditioning tanks (~50 l, n = 12) to facilitate gonadal development. Oysters in conditioning tanks were provided with seawater (~2 l min^{-1} flow-through, from a common head tank) and fed on a microalgal diet of 50/50 (by cell concentration) of *Isochrysis galbana* (C-iso) and *Chaetoceros gracilis* at approximately 20 000–30 000 cells ml^{-1}. Seawater was filtered to ~10 µm and buffered with sodium carbonate to maintain pH ~8.0 for the duration of the conditioning period. After a 3 d acclimation period in ambient conditions (11°C), water temperature was increased by 1°C d^{-1} until it reached 20°C, where it was maintained for 25 d until broodstock were removed for spawning (see Section 2.2). For the second experiment, in 2016, no Willapa oysters remained from the previous year's supply; therefore, new adult oysters were collected from the intertidal rocks at Stony Point in Willapa Bay (46° 40′ 21.3″ N, 123° 55′ 31.4″ W). Recent analyses by Sun & Hedgecock (2017) suggest that there is little genetic differentiation between naturalized *C. gigas* stocks across a broad range of PNW environments, so we do not expect that these broodstock were substantially genetically distinct from those used in 2015. In April 2016, ~60 adult oysters were collected from this site and acclimated in seawater holding troughs at HMSC for 4 wk. In May 2016, MBP oysters from each of the same families used previously (~20 each, or ~400 in total) were obtained from the broodstock repository and were transferred to con-

ditioning tanks along with acclimated Willapa broodstock. After this point, conditioning protocols for the 2016 experiment were identical to those of the prior year.

2.2. Cross design and spawning

For each broodstock group, in each year, 95 single-pair matings (1 female × 1 male) were created from available parents. For MBP, families were crossed in a semi-factorial fashion: 1 male and 1 female oyster from each family were individually paired with 4–6 individuals (of opposite gender) from other MBP families with a low coefficient of co-ancestry (<10%) with no reciprocation. Crosses were conducted in this way with 19 and 16 MBP families in 2015 and 2016, respectively. Oysters from Willapa Bay in both years exhibited a heavily skewed sex ratio (~10:1 female:male), and thus 95 crosses were made from 19 females and 5 males in each year with a fully crossed mating design (every male paired with every female).

Crosses were performed by stripping ripe gametes from each male and female, and suspending them in beakers filled with seawater. Eggs from each female were enumerated (see Section 2.5) and divided into 5 replicate beakers for fertilization. Each replicate beaker was independently fertilized with appropriate aliquots of sperm suspension from different males. After 1 h, each cross was checked for fertilization by sampling eggs and visually confirming polar bodies or cell division. Eggs were then rinsed of excess sperm on a 25 µm screen and proportionally combined to form 2 composite larval pools (1 each for MBP and Willapa) that contained approximately equal quantities of fertilized eggs from each of the 95 crosses. Pooling larvae in this way, with approximately equal input from each of 95 crosses and ~19 females, spreads the effects of individual parental contributions to larval fitness (maternal, genetic or both) to an aggregate group. This method provides a better representation of 'mean' fitness of these 2 populations than could be practically obtained by rearing a much more limited number of families separately.

2.3. Seawater manipulations and sampling

Ambient seawater (~400 µatm CO_2, pH = 7.9–8.1, Ω_{arag} = 2.3–2.7) was created by filling a 200 l tank with standard hatchery seawater (25°C, 32 ppt, 10 µm filtered) and equilibrating it overnight via vigorous aeration with outside air. High pCO_2 treatment water (~1600 µatm CO_2, pH ~7.5–7.6, Ω_{arag} = 0.9–1.2) was created by filling an identical tank with hatchery seawater and vigorously aerating it for several hours with a gas mixture of CO_2-stripped air and pure CO_2 to result in a final pCO_2 concentration of ~1600 µatm. Gas mixing was controlled by paired mass flow controllers (Alicat): 1 each for air and CO_2. Culture units consisted of 10 l polycarbonate chambers (BearVault) fitted with a sealing lid and rubber ring seal (McMaster-Carr). No supplemental aeration was supplied to the larval rearing units throughout the experimental period.

Seawater conditions of individual culture chambers were monitored by daily measurements of pH (Orion Star A11; Thermo-Fisher) calibrated with NIST buffers (calibrated to a seawater standard: Batch 22, A.G. Dickson, Scripps Institution of Oceanography) and dissolved oxygen (YSI 85). Seawater samples for carbonate analysis were also obtained from seawater used to fill the chambers after 48 h of culture (before each water change) to account for changes in chemistry arising from off-gassing or respiration. These samples were stored in sealed, gas-tight 350 ml amber glass bottles and poisoned with 30 µl of saturated mercuric chloride ($HgCl_2$) solution for later analysis. These water samples were analyzed at the lab of Dr. Burke Hales at Oregon State University, following the procedure outlined by Hales et al. (2005) and Bandstra et al. (2006) to obtain values for sample total dissolved carbon dioxide (TCO_2), pCO_2 and seawater pH, from which Ω_{arag} and Ω_{calc} values were calculated. This method has been shown to be highly accurate, providing TCO_2 and pCO_2 estimates with <0.2 and <5% uncertainty, respectively (Waldbusser et al. 2013). A summary of seawater carbonate chemistries is provided in Table 1, and changes in pH and Ω_{arag} values throughout each experiment are illustrated in Fig. 1.

2.4. Larval culture

The embryo pools of each group (MBP and Willapa) were counted, and aliquots of ~200 000 embryos were distributed among culture units approximately 5 hpf for an effective stocking rate of 20 larvae ml^{-1}. Culture units were filled with either ambient (~400 µatm) or high (~1600 µatm) pCO_2 seawater. In 2015, each treatment level (broodstock × water treatment) was replicated 5 times, resulting in a total of 20 culture units. In 2016, each level was

Table 1. Mean (±SD) temperature, salinity, total alkalinity, total CO_2 (TCO_2), partial pressure CO_2 (pCO_2), bicarbonate (µmol kg^{-1}), carbonate (µmol kg^{-1}), pH (pH_T = pH on the total scale) and saturation states of calcite (Ω_{calc}) and aragonite (Ω_{arag}) for ambient and high pCO_2 seawater treatments in Molluscan Broodstock Program (MBP) and Willapa Pacific oyster larval culture replicates across the 22–24 d culture period

	Temp. (°C)	Salinity	Alkalinity (µeq kg^{-1})	TCO_2 (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	pH_T	Ω_{calc}	Ω_{arag}
2015										
MBP										
Ambient	24.5 ± 0.86	29.8 ± 0.27	2249 ± 34	2069 ± 50	651 ± 215.1	1911 ± 72	139.3 ± 32.1	7.89 ± 0.13	3.43 ± 0.79	2.28 ± 0.53
High pCO_2	24.4 ± 1.02	30.5 ± 0.26	2294 ± 31	2260 ± 18	1791 ± 364.3	2146 ± 17	61.0 ± 10.3	7.48 ± 0.09	1.50 ± 0.25	1.00 ± 0.16
Willapa										
Ambient	24.5 ± 0.90	29.8 ± 2.27	2267 ± 30	2083 ± 56	640 ± 203.1	1922 ± 77	142.0 ± 29.7	7.89 ± 0.12	3.50 ± 0.73	2.33 ± 0.48
High pCO_2	24.3 ± 1.00	30.5 ± 0.36	2304 ± 23	2270 ± 24	1790 ± 326.8	2156 ± 23	61.1 ± 9.2	7.48 ± 0.08	1.50 ± 0.22	1.00 ± 0.15
2016										
MBP										
Ambient	24.6 ± 0.5	30.9 ± 0.2	2246 ± 58	2104 ± 80	862 ± 321.8	1963 ± 96	115.7 ± 31.0	7.78 ± 0.14	2.82 ± 0.76	1.88 ± 0.50
High pCO_2	24.6 ± 0.9	30.8 ± 0.4	2302 ± 50	2296 ± 74	2318 ± 610.7	2177 ± 69	51.4 ± 13.9	7.38 ± 0.12	1.25 ± 0.34	0.83 ± 0.23
Willapa										
Ambient	24.8 ± 0.7	30.8 ± 0.3	2262 ± 59	2112 ± 91	817 ± 289.6	1968 ± 105	120.5 ± 26.3	7.8 ± 0.12	2.93 ± 0.64	1.95 ± 0.42
High pCO_2	24.6 ± 0.9	30.8 ± 0.4	2340 ± 115	2302 ± 72	2126 ± 969.2	2168 ± 67	71.3 ± 56.8	7.46 ± 0.26	1.74 ± 1.4	1.16 ± 0.93

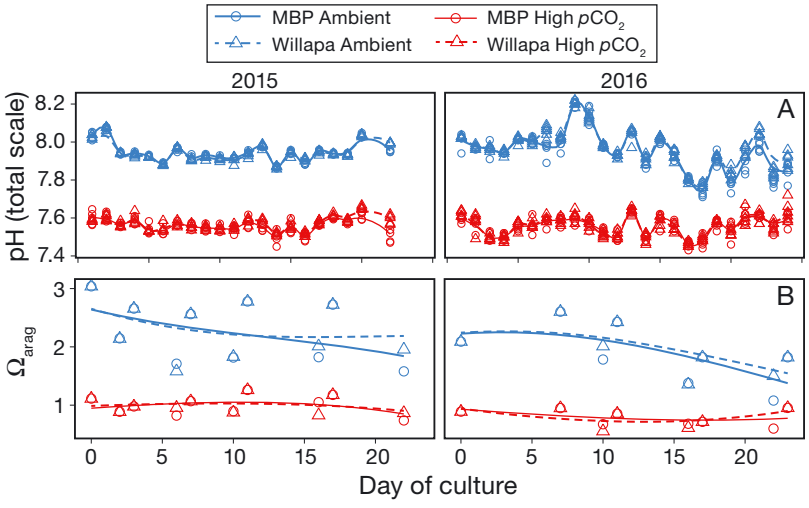

Fig. 1. Seawater carbonate chemistry. (A) Daily pH measurements of Molluscan Broodstock Program (MBP) and Willapa Pacific oyster larvae static cultures reared at ambient and high pCO_2 levels for 0 to 22 (2015) or 24 (2016) d post fertilization. Seawater (prepared at ~400 and ~1600 µatm for ambient and high pCO_2, respectively) was replaced in each culture every 48 h; fluctuations in pH values reflect changes due to respiration between water changes. (B) Aragonite saturation state (Ω_{arag}) in ambient (~400 µatm) and high (~1600 µatm) pCO_2 cultures across the culture period. Individual points represent estimates from discrete samples, alternating between those obtained from newly prepared seawater (equilibrated to ~400 and ~1600 pCO_2) and *in situ* samples from vessels after 48 h of static culture. Lines represent local regression (LOESS) estimates of mean values

replicated 6 times, to improve statistical power, resulting in 24 culture units. Larval culture protocols were similar to those of Langdon et al. (2003); briefly: every 2 d, culture water was exchanged with fresh seawater, equilibrated to treatment pCO_2 levels. The contents of culture units were poured onto a sieve (see screen sizes below) to retain larvae, then washed, re-filled, resupplied with algae and re-stocked with larvae. Antibiotics were added prophylactically in order to reduce bacterial respiration in culture units that would unduly affect seawater carbonate chemistry (Waldbusser et al. 2015b). Antibiotics were alternated at each water change between a chloramphenicol/ampicillin mixture (2 and 10 ppm, respectively) and 20 ppm streptomycin to reduce the risks of development of resistant bacterial strains.

Microalgal diets were supplied once daily, starting 2 d post fertilization (dpf), beginning with C-iso at 20 000 cells ml^{-1}. Rations were increased by 5000 cells ml^{-1} d^{-1}, and the diatom species *C. gracilis* was gradually incorporated into the diet starting at 5% (based on cell concentrations) on Day 4, until it accounted for 50% of the algal diet by Day 11, where it was maintained for the duration of the experiments. Fertilized eggs were stocked at a rate of 20 ml^{-1}, and larval densities were reduced to 10 ml^{-1} on Day 2, 5 ml^{-1} on Day 6 and 1 ml^{-1} at the pediveliger stage, i.e. Day 14 in 2015 or Day 12 in 2016. Larval density was maintained in this fashion to provide equal and optimal

environments for growth and survival and limit respiratory contribution to seawater pCO_2 levels. One ambient pCO_2 culture unit in 2015 suffered complete larval mortality on Day 10, but all prior data points were retained for analysis.

Throughout the experiments, there was no selection for larval size, and larvae were screened on conservative screen sizes to retain slow-growing individuals: 25 µm to Day 4, 37 µm on Day 6, 45 µm on Day 8, 64 µm on Days 10 and 12 and 80 µm for the remainder of the experiments. Reductions in larval density (as above) were carried out in the same way for all larval populations with no selection on size. After the appearance of eyed larvae on Day 16, larvae were additionally screened on a 240 µm sieve to retain pediveliger larvae which were subsequently induced to settle by exposure to 1.8×10^{-4} M epinephrine for 2 h (Coon et al. 1986). After this time, all larvae and newly settled spat were rinsed in seawater and returned to the culture vessel with the remainder of the larval group. Additional experiments were conducted that confirmed that neither seawater pCO_2 nor the antibiotics we used had interactive effects with epinephrine in determining settlement success (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m621p107_supp.pdf).

2.5. Larval sampling and measurement

Larval survival was estimated from counts of larvae in each culture unit on Days 2, 6, 10, 14, 16 and 22 (Day 24 in 2016). Counts were obtained by concentrating larvae from each 10 l culture unit into a 250 ml beaker with 100–150 ml of seawater and removing 5 subsamples (~30–50 µl each) for counts, with additional re-sampling when the coefficient of variation among samples exceeded 10%. Survival estimates accounted for animals removed for sampling and for adjustments in densities by multiplying survivorship between sampling/counting events to obtain a cumulative survival estimate across the entire experimental period. Samples for larval size analysis were preserved by collecting ~200 larvae from each replicate, adding them to 10 ml seawater and fixing them with 200 µl of 10% buffered (pH = 8.1–8.3) formalin. Mean larval size was measured as the maximal anterior–posterior shell width parallel to the hinge for 30–50 larvae from each sample. Developmental stages were assessed as the proportion of 'normal' D-hinge larvae on Day 2, pediveliger or 'eyed' larvae after Day 16 and spat on Day 22 in 2015 and Day 24 in 2016. On Day 2, 'normal' larvae were characterized by a straight hinge, smooth shell along the perimeter of the valve and tissue contained within the translucent shell (ASTM International 2012). Images were analyzed using ImageJ (NIH).

2.6. Lipid analysis

Directly after fertilization, eggs were sub-sampled from each fertilized egg pool, filtered on a pre-ashed glass fiber filter (Whatman GF/A) and stored under chloroform and nitrogen gas at −4°C for later analysis. In 2015, ~20 000 eggs were sampled, in triplicate, from each pool. In 2016, females yielded fewer eggs overall and only ~5000–6000 eggs were available for sampling, in triplicate. An internal standard of the fatty acid 23:0 was added prior to extraction at an amount that was approximately 10% of expected total fatty acid content in order to correct for losses during extraction and analysis. Lipid extraction, derivatization and analysis of fatty acid methyl esters followed the methods detailed by Copeman et al. (2016). Given the disparity in sample size between experiments, additional egg samples were later collected (in 2018) with sample sizes of: 5000, 10 000, 20 000, 50 000 and 100 000, in duplicate, and processed in the same fashion to assess the efficiency of the extraction method. No relationship was observed between sample size (no. of eggs) and estimated total fatty acid egg^{-1} in this range. Analysis of the fatty acid content and profile of Willapa eggs obtained in 2016 (n = 3) indicated that these samples were contaminated during filtration; they were therefore removed from analysis. Lipid composition of each egg pool is summarized in Table S1 in the Supplement.

2.7. Data analysis

We categorized larval oyster development into 3 distinct stages: (1) early larval development (from fertilized egg to D-hinge veliger larvae), (2) mid-veliger stages (from Days 6 to 16) and (3) settlement and metamorphosis (from pediveliger stage on Day 16 to 'spat' on Day 22/24). Each of these developmental stages has distinct patterns of feeding, growth and survival (see Fig. 2). Consequently, in order to appropriately characterize treatment and broodstock effects upon larval performance metrics, the data collected in these trials were first analyzed as a whole and subsequently partitioned by developmental stage for analysis. The whole and stage-separated datasets

for larval performance metrics were analyzed using generalized linear models with binary fixed effects: seawater treatment (low or high pCO_2), broodstock type (MBP or Willapa) and year/cohort (2015 or 2016). Each model was subjected to backward and forward stepwise selection (Venables & Ripley 2002) to resolve a final model with fixed effects and interactions that minimized residuals. Models for spat size data included survival rate as a covariate to test for the influence of density-dependent growth during this stage. All data satisfied normality and homogeneity of variance assumptions as evaluated with Bartlett's and Levene's tests, and significance was determined at $p < 0.05$ on the Type III sums of squares. All statistical analyses were conducted in R version 3.4.1 (R Core Team 2015). All models, summary tables and associated p-values can be found in the Supplement.

3. RESULTS

3.1. Carbonate chemistry

Both ambient (~400 pCO_2, pH ~8.0, Ω_{arag} ~2.7) and high pCO_2 (~1600 pCO_2, pH ~7.6, Ω_{arag} ~1.0) culture replicates showed fluctuations in carbonate chemistry between water changes. For ambient cultures, pH averaged 7.89 ± 0.13 (mean ± SD) and 7.79 ± 0.13 in 2015 and 2016, with an average pCO_2 level of 645 ± 210 and 840 ± 305 µatm, respectively. High pCO_2 cultures had an average pH of 7.48 ± 0.09 and 7.43 ± 0.19 in 2015 and 2016, with average pCO_2 levels of 1790 ± 345 and 2222 ± 788, respectively (Table 1, Fig. 1A). The metabolically produced CO_2 in these static systems contributed to these fluctuations, but Ω_{arag} levels (which are arguably the most biologically relevant parameter; Waldbusser et al. 2015a) remained distinct during these experiments: averaging 2.3 ± 0.5 and 1.95 ± 0.46 in ambient conditions and 1.00 ± 0.16 and 0.995 ± 0.58 in high pCO_2 replicates in 2015 and 2016, respectively ($p < 0.001$; Table 1, Fig. 1B). There were no significant differences in treatment conditions between the broodstock groups in a given year, but overall, the 2016 trial had lower Ω_{arag} for both ambient and high pCO_2 treatments than the 2015 trial ($p < 0.01$). Despite this, Ω_{arag} treatments for both experiments were maintained above and below the Ω_{arag} 'threshold' (~1.5–1.6) for oyster larvae that has been recently suggested as the minimum condition for early development and commercial production of *Crassostrea gigas* (Barton et al. 2012, Gimenez et al. 2018).

3.2. Overall survival and growth

When analyzed over the total period of larval development, larval survival from fertilization (Day 0) through metamorphosis and settlement (Day 22 in 2015 and Day 24 in 2016) was similar between experiments, treatments and broodstock groups. Both experiments had low initial survival for young larvae (~30% surviving on Day 6) followed by stable populations of mid-veliger larvae and additional mortality occurring during the settlement period (Table 2, Fig. 2A). Cumulative survival of all larvae and spat at the termination of the experiments averaged ~11.2 ± 5.0% in 2015, compared to ~7.0 ± 3.5% surviving in 2016. Group-specific survival and size estimates at each stage are given in Table 2. Growth rates of larvae were similarly uniform across groups and experiments: ~10.4 ± 0.5 µm d^{-1} during veliger larval stages and ~28.7 ± 10.4 µm d^{-1} for post-metamorphic spat (Fig. 2B). The overall effects of broodstock type, seawater treatment and experiment iteration upon larval growth and survival during the entire 22 (24) d culture period were not statistically different (Tables S2–S4 in the Supplement). Stage-specific larval performance metrics, however, provide greater insight into how treatment conditions impacted developing oyster larvae.

3.3. Early larval development

Survival from fertilization to shelled D-hinge larvae at 48 h was higher in 2015 than 2016, with an average of 60.0 ± 5.3% and 69.6 ± 12.2% of MBP and Willapa larvae surviving in 2015, compared to 52.0 ± 8.0% and 38.2 ± 5.6%, respectively, in 2016 (Table 2). The difference in survival to 48 hpf between the years, however, was significant only for Willapa larvae in 2016 ($p < 0.001$; Fig. 3A, Table S5 in the Supplement). Survival was slightly greater in high pCO_2 treatments overall (+ ~4.6%), although this effect was statistically marginal ($p = 0.063$; Table S5). The difference in larval survival to 48 hpf between the years did not appear to be a function of lipid content of the eggs: the mean total fatty acid content of eggs from MBP and Willapa groups in 2015 was 3.38 ± 0.31 and 3.66 ± 0.39 ng egg^{-1}, respectively, compared with 4.33 ± 0.35 ng egg^{-1} for the 2016 MBP cohort ($p = 0.0134$, Fig. S2, Table S1 in the Supplement).

Among the surviving larvae at 48 h, an average of 75% were fully shelled 'normal' D-hinge larvae in ambient pCO_2 conditions; ~13% more, on average, than their high pCO_2 counterparts ($p < 0.001$; Fig. 3B,

Table 2. Mean (± SD) cumulative percent survival (%) and shell length (µm) at D-hinge, pediveliger and spat stages for Molluscan Broodstock Program (MBP) and Willapa Pacific oyster larvae reared at ambient and high pCO_2 conditions in replicated experiments in 2015 and 2016. Pediveliger larvae were sampled on Days 14 and 12 in 2015 and 2016, respectively. Spat stage metrics are separated into (1) all larvae and spat (Total), (2) pre-settlement larvae (Pre-set) and (3) settled spat (Spat). (–): not meausured

Larval stage:	D-hinge		Pediveliger		Spat					
					Total		Pre-set		Spat	
	%	µm	%	µm	%	µm	%	µm	%	µm
2015										
MBP										
Ambient	58.8 (6.7)	81.8 (1.2)	17.3 (3)	220 (56.0)	10.1 (3.1)	–	4.3 (1.9)	304.6 (25.9)	5.8 (1.9)	568.2 (18.4)
High pCO_2	61.3 (3.9)	79.6 (3.6)	16.6 (5)	209.2 (47.3)	11.1 (3.4)	–	5.0 (1.6)	302.4 (31.8)	6.1 (1.6)	593.9 (31.5)
Willapa										
Ambient	67.4 (13.9)	78.7 (1.2)	19.6 (8.7)	216.7 (49.2)	12.3 (6.7)	–	8.2 (2.6)	295.4 (28.2)	4.1 (2.6)	509.4 (26.7)
High pCO_2	71.7 (11.3)	78.5 (2.2)	19.2 (7.7)	208.1 (40.6)	11.3 (7.9)	–	7.3 (3.9)	299.0 (32.8)	4.0 (3.9)	492.1 (45.3)
2016										
MBP										
Ambient	44.6 (6.0)	80.7 (0.9)	18 (2.6)	181.2 (5.8)	10.7 (3.4)	–	1.4 (3.4)	248.7 (13.7)	9.3 (3.4)	634.2 (43.1)
High pCO_2	54.2 (5.9)	79.4 (0.8)	21.6 (2.3)	177.4 (8.0)	3.5 (2.4)	–	1.5 (1.0)	240.6 (21.2)	2.0 (1.0)	722.6 (88.4)
Willapa										
Ambient	37.3 (6.2)	81.0 (0.6)	16.7 (3.2)	164.5 (5.2)	8.2 (2.2)	–	2.3 (2.2)	280.1 (15.4)	5.9 (2.2)	632.4 (28.7)
High pCO_2	38.2 (5.2)	79.7 (0.7)	17.8 (2.7)	167.5 (12.2)	5.1 (1.5)	–	4.0 (0.8)	273.7 (14.5)	1.0 (0.8)	616.6 (86.5)

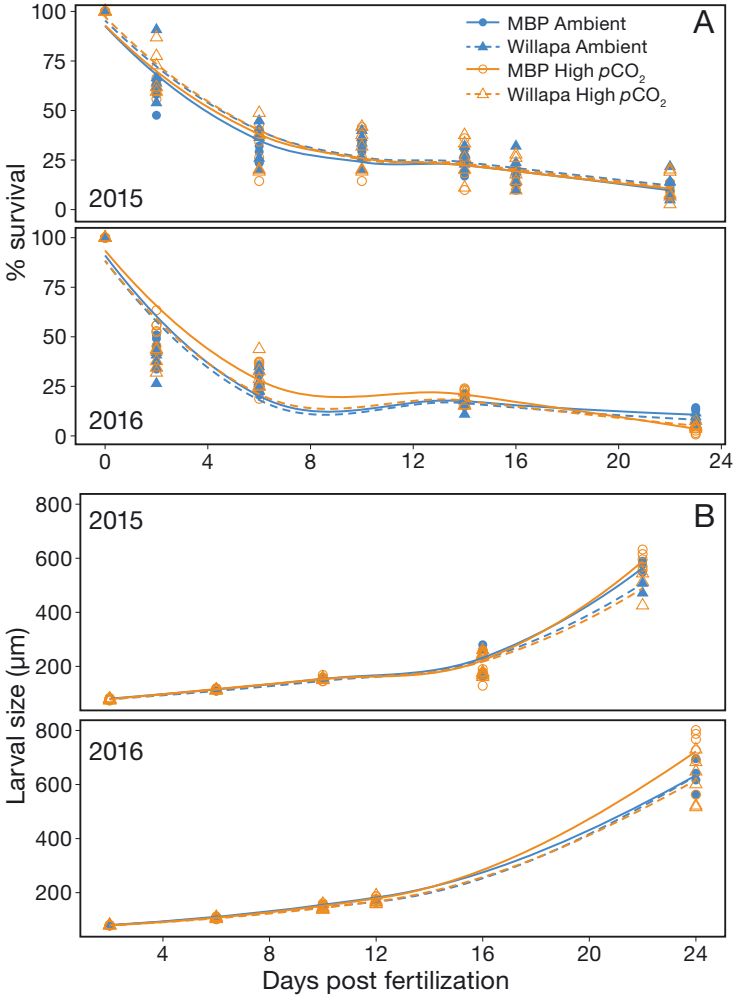

Fig. 2. Cumulative (A) survival and (B) mean size from 0 to 22 (2015) or 24 (2016) d post fertilization for Molluscan Broodstock Program (MBP) and Willapa Pacific oyster larvae reared at ambient and high pCO_2 conditions in replicated experiments in 2015 and 2016. Mean sizes in (B) on Days 22 (2015) and 24 (2016) are representative of metamorphosed spat only. Pre-metamorphic veliger larvae were measured but are not represented in this figure

Table S6 in the Supplement). Normal larvae in ambient replicates were also ~1.3 ± 0.5 µm larger, on average, than those in high pCO_2 conditions (p = 0.011, Fig. 3D, Table S7 in the Supplement). When the total number of D-larvae (no. of all larvae × % normal D-hinge) is estimated, however, there is no significant difference between seawater types in either year (p = 0.37; Table S8 in the Supplement, Fig. 3C), and by Day 6, all groups in both experiments averaged 30.8 ± 1.2% cumulative survival of all larvae with no significant differences between broodstock types, seawater treatment or years (Fig. 2, Table S2). Additionally, the proportion of normal larvae at 48 hpf was correlated with neither subsequent survival (from Day 2 to Day 6) nor mean larval size on Day 6 (Fig. 4A,C). Total larval survival (normal and abnormal) on Day 2, however, displayed a significant neg-

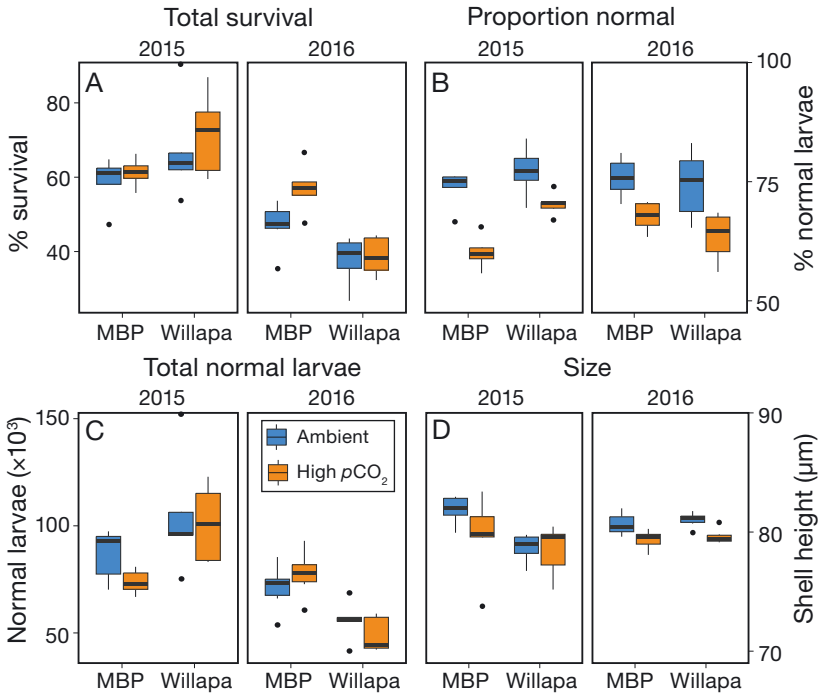

Fig. 3. (A) Total Pacific oyster larval survival, (B) proportion normal, (C) total number of normal larvae present and (D) mean size (shell height; µm) for D-hinge veliger larvae at 48 h post fertilization in Molluscan Broodstock Program (MBP) and Willapa larval groups reared in ambient and high pCO_2 seawater conditions in 2015 and 2016. The horizontal line in each box represents the median, the bottom and top of the boxes represent the inter-quartile range (IQR; at 25th and 75th percentiles, respectively), and error bars define range of the data, up to 1.5 × IQR (data points falling outside these percentiles are marked by dots)

ative correlation with survival from Day 2 to Day 6 (p = 0.027, Fig. 4B, Table S9 in the Supplement) and a weak but positive correlation with larval size on Day 6 (p = 0.08, Fig. 4D, Table S10 in the Supplement). Among all of the early larval development performance metrics, broodstock effects were marginal and inconsistent between years, suggesting little difference overall between MBP and Willapa larvae at this age (Fig. 3).

3.4. Veliger stages

During veliger development, from Days 6–16, survival was high in all cultures, with an average mortality rate of 1.37 ± 0.09 % d^{-1} with no significant effect of broodstock or water treatment (Fig. 2, Table S11 in the Supplement). At the last sampling point prior to metamorphosis, cultures had an average cumulative survival (from fertilization) of 18.3 ± 4.1 %. Similarly, growth rates were relatively uniform during this period, averaging a daily 10.4 ±

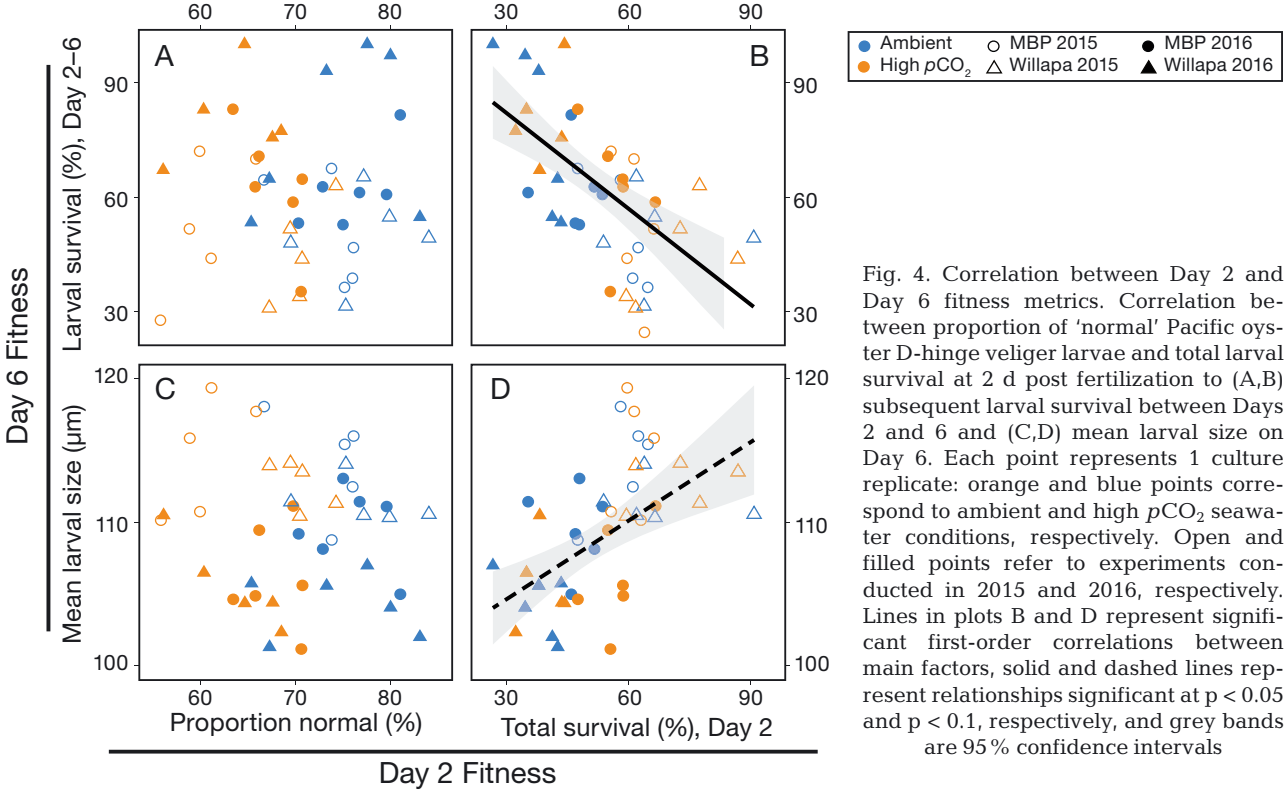

Fig. 4. Correlation between Day 2 and Day 6 fitness metrics. Correlation between proportion of 'normal' Pacific oyster D-hinge veliger larvae and total larval survival at 2 d post fertilization to (A,B) subsequent larval survival between Days 2 and 6 and (C,D) mean larval size on Day 6. Each point represents 1 culture replicate: orange and blue points correspond to ambient and high pCO_2 seawater conditions, respectively. Open and filled points refer to experiments conducted in 2015 and 2016, respectively. Lines in plots B and D represent significant first-order correlations between main factors, solid and dashed lines represent relationships significant at $p < 0.05$ and $p < 0.1$, respectively, and grey bands are 95 % confidence intervals

0.5 µm increase in shell length across all treatments in both experiments (Table S12 in the Supplement). Prior to induction of settlement, pediveliger larvae measured, on average, 213.2 ± 46.4 µm on Day 16 in 2015 and 172.7 ± 10.4 µm on Day 12 in 2016. Although pediveliger larvae (Day 16) were not measured in 2016, the similarity in growth rates between treatments and years over this period resulted in a linear model of best fit with only age (dpf) as an explanatory variable, with a model-predicted mean larval size of 213.5 ± 40.7 µm on Day 16 for all treatments.

3.5. Settlement and metamorphosis

Survival rates for all larvae and spat from the pediveliger stage through the settlement phase (Days 16–22) averaged 59.2 ± 12.8% in 2015, with no significant effect of broodstock or seawater pCO_2 ($p > 0.1$, Fig. 5A, Table S13 in the Supplement). In 2016, survival rates during this stage were similar for ambient conditions: ~66 and ~53% in MBP and Willapa groups, respectively, but high pCO_2 seawater significantly reduced survivorship of larvae: only ~18% in MBP and ~30% in Willapa were surviving on Day 24 in acidified cultures in this year ($p < 0.001$, Fig. 4A). The final larval/spat samples collected at the conclusion of the trial were size separated on a 240 µm screen (see Section 2.4 above). In all replicates (for both experiments), <1% of larvae/spat in the >240 µm size fraction were dead or moribund, indicating that mortality during this phase was almost entirely due to the loss of slow-growing larvae (<240 µm) and not due to spat mortality.

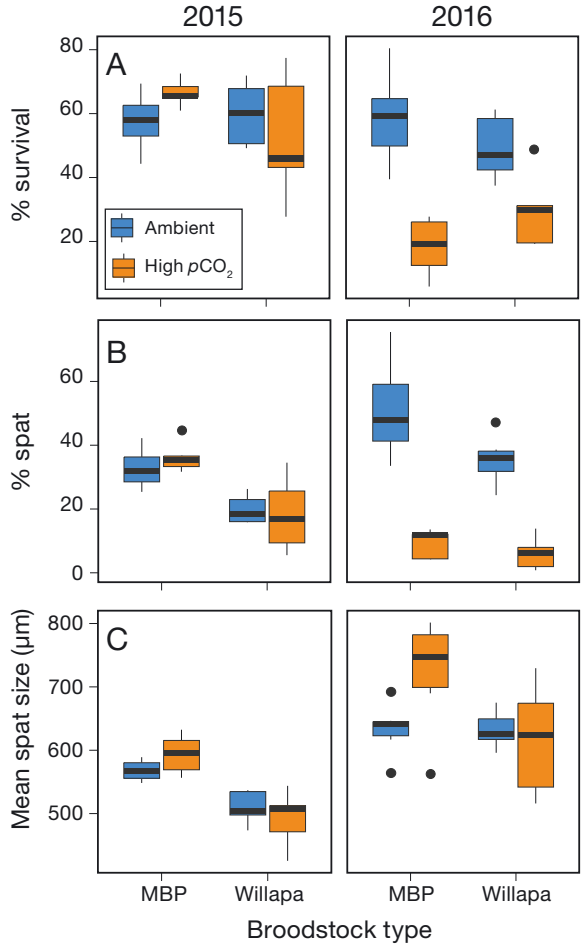

Fig. 5. (A) Percent survival, (B) settlement success (% spat) and (C) mean shell length (µm) at 22 and 24 d post fertilization in Molluscan Broodstock Program (MBP) and Willapa Pacific oyster larval groups in 2015 and 2016. Percentage survival and settlement are calculated from pediveliger stage, not initial stocking density. Box plot parameters as in Fig. 3

The disparate effects of high pCO_2 culture between the years were also reflected in the settlement rates of these groups. In 2015, there was no apparent effect of acidified seawater on settlement, with ~35% of MBP and ~19% of Willapa pediveligers successfully metamorphosing to spat by Day 22, unaffected by seawater treatment ($p = 0.56$). In 2016, by contrast, ~51 vs. 9% of pediveligers in MBP groups and ~36 vs. 6% in Willapa groups settled out in ambient and high pCO_2 conditions, respectively. This represents an average ~42% reduction in settlement success in acidified seawater ($p < 0.001$; Fig. 5B, Table S14 in the Supplement). Overall, broodstock effects upon settlement in both trials were significant (Table S15 in the Supplement). On average, MBP pediveliger larvae resulted in 55 and 37% more spat in ambient and acidified conditions overall, respectively, when compared to Willapa groups ($p = 0.036$, Fig. 5B, Table S14).

Larvae from MBP stocks also resulted in larger spat, on average, at the conclusion of both experiments; averaging a ~28.0 µm (5.1%) increase in ambient and ~123.1 µm (22.5%) increase in high pCO_2 conditions across both experiments ($p = 0.042$ and $p = 0.027$, respectively, Fig. 5C; Table S16 in the Supplement). The mean size of spat was, on average, significantly greater in all groups in 2016 (+ ~255.5 µm), likely owing to the additional 2 d of growth in this trial (24 vs. 22 dpf). Survival rates of all larvae during settlement (from Day 16) were modeled as a covariate in the analysis of spat size to account for possible density-dependent growth. In 2016, where survival differences were more pronounced, survival was significantly negatively correlated with spat size: an estimated ~2.2 µm reduction in mean spat size for every percent increase in total survival ($p = 0.005$, Table S16). This effect was not significant, however, in the 2015 experiment ($p = 0.296$) where survival

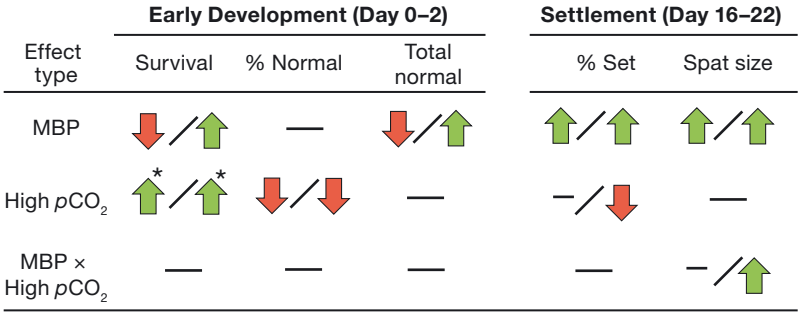

Fig. 6. Summary of significant net effects of Pacific oyster broodstock type (Molluscan Broodstock Program, MBP), seawater treatment (High pCO_2), and their interaction (MBP × High pCO_2) on larval performance. Early larval development metrics (0–2 d post fertilization) include: survival, proportion normal (%) and total normal D-larvae. Metrics for performance over the settlement stage from pediveliger to spat (16–22 d) include: percent metamorphosed (% set) and size of spat (spat size). Green and red arrows indicate a positive or negative effect, respectively (–: no significant effect), with year-specific effects separated by a '/' (i.e.+. '2015/2016'). The positive effect of High pCO_2 culture on larval survival was marginally significant (p = 0.063)

rates were relatively uniform. A summary of the most significant overall effects of broodstock type, seawater treatment and their interaction is depicted in Fig. 6.

4. DISCUSSION

These experiments contribute to a relatively sparse body of work concerning the long-term effects of low Ω_{arag} seawater upon Pacific oyster larvae and build on this information by comparing the relative sensitivity of domesticated and naturalized stocks to these conditions. The stage-specific analysis of the response of oyster larvae to acidified seawater provides greater insight into the physiological effects of low Ω_{arag} seawater than analyses based on overall impacts or those upon single larval stage alone. These experiments also uniquely re-create both the larval rearing environment and genetic composition of MBP larval pools in 2 experiments, allowing us to examine inherent differences between experiments that arise from factors not incorporated into typical designs of OA experiments (e.g. Gimenez et al. 2018).

4.1. Early larval development

Shell morphology is a useful and sensitive metric to assess the fitness of 'D-larvae' at 48 hpf and their sensitivity to low Ω_{arag} seawater during formation of the PDI and early larval development (Kurihara et al. 2007, Parker et al. 2011, Waldbusser et al. 2015a). In these experiments, we observed a similar reduction in the proportion (%) and size (µm) of normal larvae in both MBP and Willapa larval groups reared in high pCO_2 seawater (Fig. 3B,D). In addition to % normal D-larvae (which is the metric most frequently used in OA studies on bivalve larvae), here we also report the total percent survival of all larvae at 48 hpf (abnormal and normal alike) relative to the stocking density of eggs following fertilization (20 ml^{-1}; ~200 000 $replicate^{-1}$; Fig. 3A). Although this distinction may seem to be a minor detail, there was an overall, if subtle, higher total survival rate of all larvae in high pCO_2 seawater treatments compared with ambient treatments ($\bar{x}$ = +4.6 %, p = 0.063). Interestingly, the average difference in total normal larvae (% survival × % normal) in these experiments is statistically indistinguishable between low (~1) and high (>2) Ω_{arag} seawater conditions within each experiment (p = 0.37; Table S8, Fig. 3C). This difference of effect between proportion-normal and total-normal is due to the greater abundance of abnormal larvae surviving to this age (48 hpf) in low Ω_{arag} replicates. While low Ω_{arag} is likely to impede precipitation of the PDI, it is unlikely that the physiochemical barrier to calcification presented by these conditions (Lannig et al. 2010, Waldbusser et al. 2013, 2015a) should also lead to increased total survival of abnormal larvae to this age. This finding may instead result from an overlap of 2 other coinciding biological processes: developmental delays incurred by low Ω_{arag} (Timmins-Schiffman et al. 2013, De Wit et al. 2018) and stage-specific genetic inviability (Plough et al. 2016).

4.2. Genetic load and larval mortality

The broadly adapted and highly polymorphic genome of the Pacific oyster (Zhang et al. 2012) contains an abundance of negative or deleterious alleles, referred to as a high 'genetic load' (Launey & Hedgecock 2001). Many of these alleles appear to be involved with developmental transitions (Plough 2011, 2018), resulting in stage-specific mortality patterns that render a large majority of *Crassostrea gigas* larvae genetically inviable (Plough et al. 2016). Larval mortality owing to these genetic impediments is manifest as a function of developmental progression

rather than absolute time (Plough 2018). In the context of our experiments, under ambient seawater conditions, early larval development and associated genetic mortality proceed at a 'normal' rate, and samples taken at 48 hpf cumulatively represent both processes. In low Ω_{arag} seawater conditions, in contrast, developmental processes are delayed and protracted across a longer time frame (Timmins-Schiffman et al. 2013, De Wit et al. 2018), which not only results in smaller fully-formed surviving larvae (Fig. 3D; also observed by Waldbusser et al. 2015a) but also an increased total abundance of 'abnormal' larvae, many of which may have genetic impediments to proper development (Fig. 3A,B). The hypothesis that low Ω_{arag} seawater delays both embryogenesis and the timing of genetically mediated mortality during this stage is supported by the comparison of larval performance metrics at 48 hpf with that of subsequent larval growth and survival to 6 dpf. Total survival rate on Day 2 has a surprisingly robust negative correlation with subsequent survival between Days 2 and 6 (Fig. 4B), indicating that seawater pCO_2 (and correlated carbonate parameters) appears to have had an effect on the timing, but not the magnitude, of early larval mortality to Day 6 in our experiments. These data also suggest that total survival and proportional normality at 48 h in these experiments are ephemeral performance metrics; by Day 6, cumulative larval survival was similar among all groups (~30%), and we found no correlation between proportion normal at 48 hpf and subsequent survival or larval size on Day 6 (Fig. 4A,C). A general (albeit ambiguous) positive correlation between survival on Day 2 and mean larval size on Day 6 (Fig. 4D) suggests that culture conditions in both seawater pCO_2 treatments were adequate to maintain healthy growing larvae, and survival to 6 dpf was not negatively affected by larval density or food availability.

4.3. Veliger growth and survival

During mid-veliger stages, from 6 to 16 dpf, we observed no discernable differences in survival or growth rate of veliger larvae in either water treatment, broodstock group or experiment. Shelled veliger larvae are somewhat more resilient to elevated seawater pCO_2 and low Ω_{arag} (Ramesh et al. 2017), but these conditions have nevertheless been shown to exhibit distinct effects on the physiology of larval oysters. Timmins-Schiffman et al. (2013) and Frieder et al. (2017) indicated that acidified seawater reduces net calcification rates of early larvae, and Pan et al. (2015) and Frieder et al. (2018) suggested that acidification stress alters the allocation of metabolic energy within larvae. Dineshram et al. (2012, 2013, 2016) demonstrated similar impacts on global proteomic expression. Despite the acknowledged significance of bioenergetics in larval physiology under acidification stress (Waldbusser et al. 2013, 2015a, Frieder et al. 2018), many studies which report reduced larval growth rates in high pCO_2 conditions frequently overlook the quality of microalgal diets, and research is undertaken with larvae fed on a mono-specific diet of *Isochrysis galbana* (e.g. Miller et al. 2009, Talmage & Gobler 2009, 2011, 2012, Thiyagarajan & Ginger 2012, Gobler & Talmage 2014, Clark & Gobler 2016, Frieder et al. 2017, 2018). This species of microalga is widely used in bivalve culture but is nutritionally sub-optimal for larval oysters, leading to slower growth, poorer survival and reduced settlement when compared to mixed-species diets that include diatoms, such as those in the genus *Chaetoceros* (Langdon & Robinson 1996, Rico-Villa et al. 2006, Marshall et al. 2010). Sub-optimal algal diets may result in nutritional stress of larvae, affecting their response to the additional stress of acidified seawater. The more complete diets used in our study may have improved mid-veliger performance in high pCO_2 seawater, but null effects of acidified conditions on the growth of veliger larvae are not without precedent; for example, Miller et al. (2009) saw no reduced growth of *Crassostrea ariakensis* larvae when reared in seawater with Ω_{arag} as low as 0.6, and others (Thiyagarajan & Ginger 2012, Hettinger et al. 2013, Ko et al. 2014) have suggested that the negative effects of acidified seawater on growth and survival may be negated by increased culture temperature or food availability. The lack of measurable effects of acidified seawater we report during mid-veliger stages does not suggest that oyster larvae were not impacted by the high pCO_2 experimental conditions, but rather that in our culture environment their effects were not discernable during this stage from gross performance metrics such as size and survival, as suggested by Brunner et al. (2016).

4.4. Settlement and metamorphosis

In contrast to similar growth and survival rates between pCO_2 treatments observed during the veliger stages, the metamorphic period, from 16 to 22 dpf (2015) or to 24 dpf (2016), displayed distinct yet variable effects of high pCO_2 seawater. During metamor-

phosis, pediveliger larvae settled with relatively similar success in ambient conditions in 2015 and 2016, resulting in ~43 and ~28% settlement success, on average, in MBP and Willapa groups, respectively (Fig. 5B). Larvae undergoing metamorphosis in high pCO_2/low Ω_{arag} replicates in the first experiment (2015) appeared unaffected by acidification, but in the second experiment (2016) high pCO_2/low Ω_{arag} conditions resulted in ~40% greater mortality during metamorphosis and ~42% fewer spat, on average, when compared to ambient conditions (Fig. 5A,B). In both of these experiments, however, ~99% of the observed mortality occurred with small, underdeveloped larvae that passed through a 240 µm screen, and we observed almost no mortality of size-competent pediveliger larvae (>240 µm; Coon et al. 1990) or settled spat. This distinction is a useful detail because it indicates that the increased mortality observed during settlement in high pCO_2 replicates in 2016 was a function of development to pediveliger competency and not due to an effect of acidified seawater on the metamorphic transition from pediveliger to spat. It appears that the chronic stress of high pCO_2 seawater in the 2016 experiment was masked in growth and survival measurements through much of the veliger stage (6–16 dpf). The cumulative physiological impacts of this stress, nevertheless, inhibited development to pediveliger competency for a large portion of the larval population in these cultures, resulting in death. In 2015, larval oysters likely experienced similar physiological challenges in high pCO_2 cultures, but it appears that some unknown additional set of factors (discussed in Section 4.6) helped ameliorate these effects enough to permit comparable rates of survival and settlement in ambient and high pCO_2 treatments.

It is worth acknowledging that the environmental and behavioral factors contributing to the stochastic nature of oyster settlement (Fitt et al. 1990, Tamburri et al. 1992, Turner et al. 1994) were circumvented in these experiments by the use of epinephrine to chemically induce metamorphosis (Coon et al. 1986, Bonar et al. 1990). We adopted this technique in order to standardize the culture period between replicates and treatments and facilitate counting of spat. Haws et al. (1993) demonstrated that this method increases the rate but not the magnitude of larval settlement, relative to 'natural' controls, and we conducted additional experiments that confirmed that neither seawater pCO_2 nor the antibiotics we used had interactive effects with epinephrine in determining settlement success (Fig. S1). Nevertheless, this methodology is an artificial one, and the results should be interpreted with caution in other contexts where environmental cues, such as seawater pCO_2, may have behavioral effects upon pediveliger larvae and influence settlement timing and success (e.g. Pechenik 1990, Pechenik et al. 1990).

4.5. Comparison with previous work

Several studies have documented the physiological effects of acidified seawater environments on larval bivalves over multiple developmental stages (e.g. Miller et al. 2009, Talmage & Gobler 2011, Frieder et al. 2017). Among these, however, many initiated experimental exposure likely after PDI development (~16 hpf; Miller et al. 2009, Talmage & Gobler 2009, Thiyagarajan & Ginger 2012, Ko et al. 2013, Gobler & Talmage 2014, Clark & Gobler 2016), provided potentially inadequate microalgal diets (mentioned previously), and based their estimates of size and developmental progression on a limited number of remaining individuals (Talmage & Gobler 2009, 2012, Gobler & Talmage 2014), all of which can skew results and weaken conclusions with regards to treatment effects. Additionally, few studies have examined larval performance over multiple cohorts in the same analysis. The reported variability between experiments evaluating the long-term response of oyster larvae to OA conditions is large; for example, some researchers (Talmage & Gobler 2009, 2012, Gobler & Talmage 2014, Clark & Gobler 2016) reported that average larval survival to the pediveliger stage of *C. virginica* under ambient conditions (~400 pCO_2) ranged from ~15–50% (from initial stocking at ~6–24 hpf), and the final percentage of larvae that settled to produce spat ranged from ~25–45% (for those experiments which included metamorphosis). Low Ω_{arag} seawater in these studies (0.39–1.52) had generally negative impacts on survival and metamorphosis of *C. virginica* but also produced variable results, relative to the control. In 2 of the 4 studies, moderate Ω_{arag} levels (1.83–1.91) resulted in positive effects on larval performance, compared to high Ω_{arag} controls (2.91–3.68; Talmage & Gobler 2009, Gobler & Talmage 2014). This substantial variation in overall and relative larval performance among these studies (and between the 2 experiments reported here) demonstrate the inherent variability between larval oyster cohorts and the importance of replicated experiments and extensive sampling regimes to better understand larval responses to OA conditions.

In this case, the differential effect of seawater pCO_2 upon spat production between the 2015 and 2016 experiments similarly highlights the sensitivity of larval fitness to a multitude of factors. High pCO_2 cultures in 2016 had lower mean Ω_{arag} values overall (0.995 ± 0.58 compared to 1.00 ± 0.16 in 2015), but this slight difference is unlikely to be the sole explanatory variable for the substantially disparate effects of acidified seawater on larval fitness between experiments. Underlying variation in water quality parameters that traditionally go unmeasured, such as algal blooms and marine toxins (such as those observed earlier in 2015; McCabe et al. 2016), may also potentially affect larval fitness and relative sensitivity to stressors like high pCO_2 (e.g. Gimenez et al. 2018). In our experiments, we were unable to discern any substantial difference in larval performance in control (ambient) conditions that suggest the presence of any such confounding factors, but it remains a possibility that we cannot entirely reject.

Differences in the quality of broodstock available for each of the experiments is another plausible explanation for the observed difference in larval sensitivity to high pCO_2 seawater. Larval growth and survival is strongly influenced by broodstock egg quality (Gallager et al. 1986, Kennedy 1996, Bochenek et al. 2001), and although broodstock in both years were conditioned similarly for ~4 wk prior to spawning, it has been reported that gametes are formed from reserves that are accumulated ~6 mo prior to conditioning (Berthelin et al. 2000). A strong El Niño event during the 2015–2016 winter (NOAA 2018) produced both warmer seawater temperatures and increased precipitation at the broodstock holding site (HMSC; Fig. S3 in the Supplement), potentially disrupting natural patterns of gonad re-absorption and gametogenesis (Dutertre et al. 2009) and reducing feeding rates (Gray & Langdon 2018). Sub-optimal overwintering conditions likely contributed to the observed poor condition of broodstock in 2016 and significantly fewer eggs obtained per female (Fig. S4 in the Supplement) despite having significantly greater estimates of total lipid per egg (Fig. S2). Differences in overwintering location for 2016 broodstock (Yaquina vs. Willapa Bay) could have also impacted egg quality, resulting in early larval survival differences between the broodstock types (Fig. 3), but uniform performance over the veliger period and consistent broodstock-level effects on settlement metrics suggest that season, more than overwintering location, was a stronger source of variation. Gametes physically removed from underdeveloped broodstock (i.e. strip spawning) may remove 'unripe' gametes that are compromised in other ways that are difficult to assess (Pauletto et al. 2017), and, as noted by Myrina et al. (2015), total lipid content may not be a reliable predictor of oocyte quality. Natural spawning is likely a preferred method for producing robust larvae for experimentation, but the extensive cross design adopted in these experiments made this approach unfeasible.

4.6. Effect of broodstock type

Despite substantial inter-experimental variation in the effect of seawater treatment upon settlement results, pediveliger larvae from selected MBP broodstock, when evaluated across both experiments and seawater types, resulted in significantly more and larger spat, on average, when compared to their naturalized counterparts (Table 2, Fig. 5B,C, Tables S14 & S16). The increased settlement rate of MBP groups in ambient seawater appeared to be somewhat muted by high pCO_2 conditions (+55 vs. +37%, respectively, overall) but differences in mean spat size were magnified by acidified environments: MBP spat were, on average, ~5 and ~23% larger in ambient and high pCO_2 conditions, respectively, than Willapa spat (Fig. 5C, Table S16). The overall trends for increased size of MBP spat are somewhat obscured by inter-annual variation and interactive effects: in the 2015 experiment, MBP spat were larger overall (+76 µm, on average, calculated from model-predicted estimates) with little effect of treatment, and in 2016 there was a stronger interactive effect between seawater treatment and broodstock type, which resulted in larger spat for MBP groups specifically in high pCO_2 treatments (+130 µm, on average, calculated as above), but less significant size differences in ambient cultures (Tables S16 & S17 in the Supplement). This multi-level variation in relative performance may be due to variability in culture conditions between the experiments (discussed previously) or minor differences in genetic composition of Willapa larval pools of unknown background (discussed below). While it is tempting to interpret size differences as evidence for an improved growth rate of spat, the sampling design in these experiments makes that conclusion tenuous. Metamorphosis and settlement are dynamic and sporadic processes in larval oyster populations, and with a single sampling point, we cannot distinguish from these data whether differences in spat size between broodstock groups or treatments are due to altered timing of settlement or differences in

spat growth rates. Further studies are warranted to investigate the specific effects of acidified seawater on bivalve settlement behavior and physiology to better parse these 2 distinct potential effects.

MBP Pacific oyster families have been selectively bred over 5 generations for improved yields on farms (De Melo et al. 2016, 2018), but improved larval performance has not been intentionally selected, so differences of this magnitude in the performance of larvae and juveniles of MBP and Willapa stocks are surprising. Larval performance traits have been found to be heritable (Ernande et al. 2003), and 5 generations of larval rearing in hatchery systems is likely to have resulted in unintentional selection for faster growth and higher survival in these conditions (stable 25°C seawater together with abundant, high-quality algal diets). Although the genetic background of Willapa broodstock potentially varied between the 2 sites from which they were collected for 2015 and 2016, the difference is expected to be small (Sun & Hedgecock 2017). A reduced sire input from Willapa groups in each year (n = 5 males, compared to 19 for MBP) likely reduces the overall genetic diversity for Willapa larval pools to a degree, but the large number of females used in each year (n = 19 each) still results in 95 individual crosses that represent a broad combination of genotypes for experimentation. Moreover, the general comparisons between MBP larval pools (which had a high degree of genetic similarity) and Willapa counterparts were highly consistent between years, with only minor significant differences observed in early larval stages (Fig. 3). Epinephrine-induced settlement could also be a selective pressure that favors MBP groups in these experiments (resulting in increased settlement) but we did not see a higher proportion of 'unresponsive' pediveliger larvae at the final sampling point in Willapa groups to support this hypothesis (Fig. S5 in the Supplement). In 2016, high pCO_2 seawater negatively impacted both MBP and Willapa larvae, but MBP pediveligers still produced ~50% more spat than Willapa groups in these adverse conditions (Tables 2 & S15). It is possible that one or more previous generations in the MBP breeding program were spawned during a period of coastal upwelling, thus inadvertently exerting selection pressure for genotypes that are resilient to high pCO_2 conditions. More broadly, improvements in general larval performance traits such as metabolic efficiency, growth and survival could also possibly reduce the negative effects of acidified seawater upon larval physiology. This latter explanation is perhaps more plausible, as we see no apparent performance advantage in MBP stocks for initial shell formation (assessed at 48 hpf), a stage when the impacts of acidified seawater are most pronounced and consistent. Increased spat production from MBP groups in all treatment combinations, however, suggests that incidental selection for larval performance has unintentionally improved early life fitness traits in stable hatchery conditions for these selected lines.

4.7. Implications for *C. gigas* populations in the PNW

The effect of larval fitness differences between domesticated and naturalized oyster stocks on population dynamics in natural environments in the PNW is difficult to predict. In theory, improved larval fitness represents a competitive reproductive advantage for domesticated oysters, but it is uncertain whether these advantages are maintained in the highly variable and dynamic environments that naturalized Pacific oyster larvae experience (Hales et al. 2017). Furthermore, reproductive success of *C. gigas* in the PNW is highly stochastic, temporally variable and vulnerable to the effects of 'sweepstakes recruitment' which severely limits the effective population size (N_e) and increases the rate of inbreeding accumulation (Hedgecock et al. 1992). All of these factors increase the relative strength of genetic drift over directive selection as a mechanism of genetic change and may reduce or overwhelm any more subtle effects that the introduction of domesticated genotypes has on current populations of naturalized stocks. The continuing effects of climate change on atmospheric and oceanic processes, however, make for an uncertain future for *C. gigas* populations in the PNW (Barton et al. 2015, Lemasson et al. 2018). If the Pacific oyster aquaculture industry in the region becomes increasingly reliant on hatchery reared, selectively bred oysters for continued production, the genetic impacts of domesticated stocks on naturalized 'wild' populations may also increase accordingly.

5. CONCLUSIONS

We have demonstrated that Pacific oyster larvae display sensitivities to high pCO_2 culture conditions that differed across larval developmental periods; furthermore, we found that larval settlement and survival in simulated OA conditions are highly variable between spawning cohorts. The negative effects of low Ω_{arag} seawater upon shell formation and initial

larval development were consistent with the reported literature (e.g. Waldbusser et al. 2015a). However, these impacts did not result in a significant decrease of the total number of 'normal' D-stage larvae at 48 hpf, nor did they display any persistently negative effects on later veliger larval stages. High pCO_2 seawater had variable effects on total metamorphic success in these experiments but, importantly, did not appear to directly impede the physiological process of metamorphosis from pediveliger larvae to juvenile spat. The negative overall impacts of high pCO_2 seawater we observed in the 2016 experiment, but the lack of effect of similar conditions in the experiment conducted in 2015, suggest that stress responses due to acidification are influenced by other biotic and abiotic parameters, e.g. broodstock quality, food availability and culture conditions, as suggested by Hettinger et al. (2013), Thomsen et al. (2013) and Cole et al. (2016). The consistently improved settlement results of MBP stocks relative to larvae from Willapa oysters, in both ambient and high pCO_2 seawater (Fig. 6), supports previous findings which indicate a genetic effect on larval resilience to OA (Parker et al. 2011, Sunday et al. 2011, Goncalves et al. 2018) and a potential for Pacific oysters to adapt to acidified environments.

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