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Original Article

Similar oyster reproduction across estuarine regions differing in carbonate chemistry

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In laboratory studies, shellfish larvae often respond negatively to augmented [CO_2], but no prior tests have related wild bivalve larval performance and carbonate chemistry spatiotemporally. The geography of Willapa Bay (Washington, USA) naturally generates two distinct regions of carbonate chemistry where non-native Pacific oysters (*Crassostrea gigas*) dominate the intertidal fauna and successfully reproduce. On the river-influenced east side, pCO_2 is higher and alkalinity lower, which both contribute to reduced aragonite saturation state ($\Omega_{aragonite}$ 1.3–1.5) relative to the west side receiving low watershed inputs ($\Omega_{aragonite}$ 1.8–1.9). pH_{sws} is also >0.1 lower on the east vs. west sides. Despite this difference in field conditions, no biological signal related to carbonate chemistry was apparent in oyster reproduction based on coupled chemical–biological comparisons over three summers. Instead, survival was equal between the two sides of the bay, and settlement was equal or higher on the low-$\Omega_{aragonite}$, low-pH east side. In a temporal comparison of four larval cohorts, settlement differed by two orders of magnitude and increased with water temperature. These field data on oyster reproduction illustrate that population-level effects may not emerge in higher mean [CO_2] conditions, with possible decoupling due to local adaptation, spatio-temporal heterogeneity, or higher sensitivity to other axes of environmental variability such as temperature.

Keywords: bivalve settlement, estuarine water chemistry, larval mortality, ocean acidification, oyster reproduction.

Introduction

The uptake of anthropogenic carbon into the ocean is projected to change water chemistry in ways that reduce the performance of some marine organisms (Feely *et al.*, 2009; Doney *et al.*, 2012). Most projections derive from controlled laboratory studies where elevated carbon dioxide impairs calcification and other responses (Hendriks *et al.*, 2010; Kroeker *et al.*, 2010). Because of upwelling and river discharge, the northeast Pacific Ocean is one region that naturally exhibits acidified conditions (Ekstrom *et al.*, 2015). Additionally, nearshore waters, in which many benthic organisms spawn and larvae develop, show high spatial and temporal variation in pH (Hofmann *et al.*, 2011; Duarte *et al.*, 2013; Sutton *et al.*, 2016). In this paper, we use natural spatial variability in carbonate chemistry within a northeastern Pacific estuary to test for coincident variation in larval mortality rates and settlement of Pacific oysters, *Crassostrea gigas*.

Reduced growth and higher mortality of bivalve larvae are well-documented to occur under elevated [CO_2], although negative effects are less evident at larger, older life stages (Waldbusser *et al.*, 2010; Talmage and Gobler, 2011; Picard, 2014). Early exposure can influence survival and development, with later exposure primarily reducing growth (Parker *et al.*, 2010). Two primary mechanisms are proposed to link exposure and biological response. First, low pH makes conditions unfavourable for use of bicarbonate as a substrate in calcification (Cyronak *et al.*, 2016). Second, energetic costs of precipitating calcium carbonate shell may increase when the carbonate saturation state (Ω) of the surrounding environment is low (Waldbusser *et al.*, 2013). However,

other work shows that metabolic costs of early shell formation do not vary with carbonate saturation state (Frieder *et al.*, 2017). Highest sensitivity of Pacific oyster larvae occurs within the first hours to days post-spawning, and performance can decline rapidly across threshold levels of carbonate chemistry, even when $\Omega aragonite > 1$ and shell is not expected chemically to dissolve (Waldbusser *et al.*, 2015).

Simple laboratory tests of the effects of elevated pCO_2 compare larval performance between conditions in equilibrium with modern or pre-industrial atmospheric $[CO_2]$ and various future projections (Hendriks *et al.*, 2010; Kroeker *et al.*, 2010). However, these pCO_2 control baselines may be far from what organisms are already experiencing. Coastal temperate waters are frequently supersaturated in pCO_2 relative to the atmosphere, due to upwelling of deep CO_2-rich water, high rates of respiration, and freshwater inflows reducing alkalinity and therefore the CO_2-holding capacity of water (Andersson and Mackenzie, 2012; Duarte *et al.*, 2013). Cultural eutrophication can further acidify coastal waters, associated with hypoxia (Cai *et al.*, 2011; Wallace *et al.*, 2014). Estuaries often release CO_2 to the atmosphere (Frankignoulle *et al.*, 1998; Borges and Abril, 2011; Regnier *et al.*, 2013), in comparison to most other marine environments that have net uptake of atmospheric CO_2 (Cai, 2011). This variability in carbonate chemistry means that stressful conditions for shellfish have likely always occurred at some times and places in estuaries. In the field, organisms may be able to take advantage of spatial or temporal variation in carbonate chemistry to avoid or reduce negative environmental effects, a strategy that is clearly impossible in small containers in the lab (Browman, 2016). Also, field studies can provide evidence of the relative magnitude of environmental variation across multiple potentially limiting factors, enabling an evaluation of what *is* related to performance in the field, rather than what *can* affect performance.

Spatial comparisons of organism performance across gradients of carbonate chemistry illustrate how this stressor manifests in the field. Pteropod calcification has been shown to shift in concert with carbonate chemistry at different locations across an ocean frontal system (Bednarsek and Ohman, 2015). In addition, community structure differs close to and far from carbon dioxide vents (Hall-Spencer *et al.*, 2008; Enochs *et al.*, 2015). In coastal systems, gradients of salinity and ecosystem metabolism establish spatial heterogeneity in carbonate chemistry, with conditions expected to be more challenging for calcification near rivers. However, in studies of juvenile mussels in the Baltic Sea and in Chile, growth actually improved at sites with lower pH (lower $\Omega aragonite$), consistent with food rather than carbonate chemistry as a limiting factor (Thomsen *et al.*, 2013; Perez *et al.*, 2016).

Willapa Bay (Washington, USA) presents an ideal location to study field performance of oysters experiencing natural stressors. The intertidal zone up-estuary contains extensive reefs of naturalized Pacific oysters, a commercially-important and ecologically-influential shellfish species, whose planktonic larvae have a long history of laboratory and hatchery study (Lannan *et al.*, 1980; Chávez-Villalba *et al.*, 2002; Rico-Villa *et al.*, 2006). The unique architecture of the bay provides two regions of similar water residence time and temperature, which together comprise an estuarine nursery for Pacific oysters. The eastern side has lower and more variable pH, associated with higher pCO_2, than the western side (Ruesink *et al.*, 2015). Both sides are equidistant from coastal upwelling, which generates a pH signal at the mouth of the bay but not up-estuary (Ruesink *et al.*, 2015). The surrounding watershed is sparsely populated, thus limiting pollution sources that could harm larvae. We carried out a coupled chemical–biological study of water properties, oyster larval densities, and settlement at stations on both sides of the bay over three summers to test: (i) Oyster reproduction shows a biological signal related to carbonate chemistry in the field, or alternatively is better correlated with temperature conditions or food resources. (ii) Low carbonate saturation state in an estuary in summer derives from net heterotrophy and reduced alkalinity.

Material and methods

Study site

Willapa Bay, Washington, USA (46.6°N 124.0°W) is a shallow (avg. 3.2 m), mesotidal (diurnal range 2.74 m), vertically well-mixed coastal plain estuary with extensive tideflats used for shellfish aquaculture. The southern half of the bay has a month-long water residence time (Banas *et al.*, 2007) and, within a small longitudinal dimension (10 km), is divided into a distinct west and east side by an elongate island (Figure 1). The Naselle River drains into the eastern side, whereas the western side is bordered from the ocean by a narrow sand spit. River discharge peaks in December and is at its lowest in August, therefore salinity throughout the southern half of the bay tends to increase through the summer period of oyster recruitment (Ruesink *et al.* 2015, waterdata.usgs.gov/wa/nwis/current/?type=flow for station 12010000, accessed Mar 14, 2015). Pacific oysters (*C. gigas*) were introduced to Willapa Bay in 1928 to replace native Olympia oysters (*Ostrea lurida*) that were commercially over-exploited in the late 1800s (Kincaid 1968). Pacific oysters typically exhibit a widespread spawn 1–3 times per summer, cued by environmental conditions such as a spike in temperature or as a contagious process when gametes are detected in the water. This coherent spawning simplifies the identification of cohorts in plankton samples because large numbers of larvae appear coherently at an early developmental stage.

The west and east sides of the bay were sampled concurrently for water properties and larvae over three summers. Four cohorts of larvae proceeded to settlement, which was measured on consistent substrates. A nested design was employed with multiple stations within each side to clarify how the sides of the bay differed in these coupled biological–chemical measurements of water properties and oyster reproduction (Figure 1).

Water properties

At each sampling station, concurrent with plankton samples, water was collected for carbonate chemistry and for chlorophyll analyses, with temperature, salinity, and dissolved oxygen measured with a hand-held probe (YSI 85). These collections occurred at 2 m depth from a shallow draft boat in channels during daylight high tides. Samples were collected every 3–5 days in summer for all water properties (3 July–3 September 2010, 30 July–6 September 2011) or measured by hand-held probe only (14 July–24 August 2012). Two to four stations per side were sampled each summer (Table 1).

Discrete water samples for carbonate chemistry were collected in a Niskin bottle and transferred without bubbles to acid- and bleach-washed 350-ml bottles, then poisoned with 0.3 ml saturated $HgCl_2$ and sealed with crimp-seal metal caps. Water samples were analysed for two components of the carbonate chemistry system (dissolved inorganic carbon, C_T, and the partial pressure of carbon dioxide, pCO_2) at Oregon State University

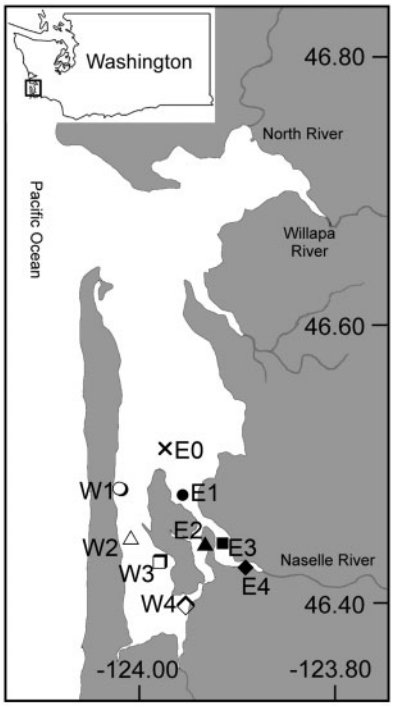

Figure 1. Sampling stations for water properties, larvae, and settlement of Pacific oysters (*Crassostrea gigas*) from 2010 to 2012 in Willapa Bay, Washington. Sampling was concurrent for chemistry and larvae at stations W1–W4 (W1–W2 in 2012) and E1–E3 (plus E4 in 2011). Settlement was measured at W1–W4 and E1–E4 (except E0 instead of E4 in 2010). Settlement of *C. gigas* does not typically occur north of the region spanned by these sampling stations, except for some natural recruitment in the Willapa River.

(Bandstra *et al.*, 2006; Barton *et al.*, 2012). Measurement uncertainty was 0.2% for C_T and 2% for pCO_2 (Hales *et al.*, 2016). To correct for the temperature differential between collection and analysis, a correction was applied by calculating alkalinity from pCO_2 at the analytical temperature, then using this calculated alkalinity (and C_T) to derive carbonate chemistry at field temperatures. Assumptions about non-carbonate components and dissociation constants were included for carbonic acid equilibrium (Millero, 2010), boric acid (Dickson, 1990), dissociation of water (Millero, 1995), and solubility of calcite and aragonite (Mucci, 1983). Calcium concentrations were assumed to scale with salinity. All measured salinities exceeded 20, so equilibrium constants were expected to function similarly to seawater (Dickson and Millero, 1987). From pCO_2 and C_T, the carbonate chemistry system was calculated from standard equations in a program written by the analytical lab, with pH in seawater system units and carbonate saturation state in terms of aragonite ($\Omega aragonite$).

For chlorophyll, 300-ml water samples were collected into Nalgene bottles and kept dark and cool until processing within 3 h. Samples were filtered (GF/F 0.7 µm) and placed immediately in 10 ml of 90% acetone (W/V) for extraction, then kept frozen until the end of each field season for simultaneous analysis. Extracted samples were centrifuged prior to analysis of the supernatant for chlorophyll-*a* via standard acidification procedure on a Turner Designs AU-10 fluorometer (Welschmeyer, 1994).

Data analysis was conducted on the following water properties, by summer: temperature, salinity, dissolved oxygen, chl-*a*, alkalinity, pH, pCO_2, and $\Omega aragonite$. Side of bay, day of year (continuous time), and their two-way interaction were fixed effects, and station was a random effect to account for multiple samples through the summer. This linear mixed effects model structure tested whether water properties differed between the two sides

Table 1. Water properties on the west and east sides of Willapa Bay, Washington, during larval development of four cohorts of Pacific oyster larvae (*Crassostrea gigas*).

		23 Aug–3 Sep 2010	5–19 Aug 2011	24 Aug–6 Sep 2011	13–24 Aug 2012
Number of stations, Times sampled	West	4, 4	4, 7	4, 6	3, 4
	East	3, 3	4, 7	4, 6	2, 4
Temperature °C	West	18.5 (0.5)	18.9 (0.4)	18.8 (0.5)	18.6 (0.3)
	East	18.5 (0.4)	19.3 (0.5)	19.1 (0.8)	19.3 (0.7)
Salinity	West	29.0 (0.3)	25.5 (0.8)	27.3 (1.0)	27.8 (0.4)
	East	28.1 (1.0)	24.4 (2.1)	26.5 (1.4)	26.8 (1.3)
Dissolved oxygen mg l^{-1}	West	7.16 (0.32)	6.50 (0.46)	6.35 (0.09)	6.89 (0.32)
	East	6.61 (0.58)	6.36 (0.29)	6.01 (0.33)	6.42 (0.43)
Chlorophyll-*a* µg l^{-1}	West	3.57 (1.77)	2.25 (0.62)	3.41 (1.12)	
	East	3.50 (1.32)	2.94 (0.62)	3.75 (1.29)	
pH_{sws}	West	8.01 (0.03)	7.98 (0.04)	7.95 (0.03)	
	East	7.89 (0.09)	7.84 (0.12)	7.85 (0.09)	
C_T µmol kg^{-1}	West	1 815 (28)	1 860 (31)	1 944 (17)	
	East	1 812 (35)	1 811 (71)	1 908 (58)	
TA µmol kg^{-1}	West	1 998 (34)	2 017 (41)	2 102 (21)	
	East	1 947 (67)	1 916 (114)	2 026 (84)	
pCO_2 µatm	West	399.0 (23.8)	448.1 (50.9)	494.0 (37.7)	
	East	536.5 (101.1)	630.0 (162.8)	637.0 (131.0)	
$\Omega aragonite$	West	1.89 (0.10)	1.78 (0.18)	1.78 (0.12)	
	East	1.48 (0.31)	1.33 (0.40)	1.42 (0.30)	

Summary statistics are mean (standard deviation) of all samples on each side over the 11–14 day period (total samples therefore is the product of number of stations and times sampled). Note that additional sampling occurred throughout July and August each summer, as presented in Figure 2. TA = total alkalinity, C_T = dissolved inorganic carbon.

and whether they changed through the summer. Any significant interaction would indicate that the seasonal pattern was different on one side than on the other. Significance of main effects and interaction was evaluated by likelihood ratio tests, following fit of different models by maximum likelihood method. We used similar spatio-temporal patterns in Ω*aragonite* and dissolved oxygen for evidence of net heterotrophy as a driver of carbonate saturation state, and in Ω*aragonite* and salinity for evidence of the influence of alkalinity in carbonate chemistry. Linear mixed effects analyses of water properties (as well as biological variables, below) were carried out with the nlme package (Pinheiro *et al.*, 2016) in R (R Core Team, 2015).

Larval sampling

Plankton samples were collected in duplicate at each station in 2010 and 2011, and as single samples in 2012, concurrent with discrete water samples. Cohorts of Pacific oysters were identified by the appearance of straight-hinge or early umbo larvae a few days after spawning and were quantified over about two weeks required to reach setting size (270 µm). A hose extended from an electric pump on the boat to 6 m close to the bottom in channels, a method that accounts for negative phototropism of larvae (Packer 1980). Water (20 gallons = 76 l) was pumped through two Nitex screens, the first 340 µm to remove larger particles, and the second 52–65 µm to retain bivalve larvae. Samples were washed from the bottom screen into small bottles, and the volume determined in a graduated cylinder. Subsamples of 1 ml were placed on a Sedgewick-Rafter cell and examined at 200× with an inverted microscope using cross-polarized light, which produces distinct imagery from larval mollusc shells. Pacific oyster larvae were distinguished from Olympia oyster and clam larvae by size and shape (Loosanoff *et al.*, 1966). Counts were extrapolated to 20 gallons based on the total collected volume, then converted to density per litre. Four cohorts of Pacific oyster larvae were detected, one in 2010, two in 2011, and one in 2012.

We analysed each cohort in two complementary ways: as counts over time between the two sides, and as mortality (=loss) rates between the two sides. Count data were analysed in relation to side, day since spawning (time), and their interaction as fixed effects, and station as a random effect in linear mixed effects models. Count data were natural log-transformed to linearize the exponential declines of cohorts, after assuming that samples with no larvae detected had 0.5 per sample. If significant, a side × time interaction indicated that larval mortality rates differed on the two sides of the bay. Count data from each station were also used to calculate a station-specific mortality rate, based on the effect and standard error from a generalized linear model fit to counts assuming Poisson-distributed error. These mortality rates were then compared between the two sides of the bay using a *t*-test ($n = 2$–4 stations per side) weighted by the inverse of each station's standard error.

Settlement sampling

Each of the four cohorts of larvae tracked in 2010–2012 resulted in a settlement event. Standard settlement substrates were deployed as larvae reached setting size, then collected about a week later when accessible on the next low tide series. Standard substrates consisted of 11 left (flat) valves of Pacific oysters, drilled and stacked smooth-side down on a dowel inserted into the sediment (shellstick). The shellsticks were placed at mean lower low water at four stations on each side of the bay, in close proximity to stations used for water and larval sampling (Figure 1). At each station, two sub-stations were selected at least 100 m distant from each other, with three shellsticks anchored within 10 m at each sub-station. Pacific oysters tend to settle within oyster habitats, rather than bare or vegetated areas (Trimble *et al.*, 2009), therefore placement of shellsticks was within areas of adult oysters that were farmed or naturally-settled. Upon collection, the smooth side of each shell was examined under a dissecting microscope for newly-settled Pacific oysters, which were counted. When settlers exceed three per shell (or half that level per smooth shellface), commercially-viable settlement is considered to have occurred (Kincaid, 1968).

Counts of Pacific oysters per shellstick were modelled as Poisson-distributed and tested separately for each of the four cohorts. Side of bay was a fixed effect in a linear mixed effects model. Station and sub-station (nested in station) were random factors.

In addition to this statistical comparison of settlement between the two sides of the bay, oyster reproduction was compared with water properties during larval development across all four cohorts and two sides. Two response variables—larval mortality rates and settlement—were examined as aspects of oyster reproduction. For predictor variables, water properties were restricted to two east-side stations and three west-side stations and to temperature, salinity and percent dissolved oxygen, which were available for all cohorts. However, carbonate chemistry had proxies in salinity (positive effect on Ω*aragonite* through alkalinity) and dissolved oxygen (positive effect on Ω*aragonite* through autotrophy). Dissolved oxygen was converted from concentration ($mg\,l^{-1}$) to percentage to remove temperature- and salinity-specific oxygen-holding capacity of water (Benson and Krause, 1984; correction tables from http://water.usgs.gov/software/DOTABLES/; last accessed 23 February 2017). Water temperature, salinity, and percent dissolved oxygen were highly correlated (pairwise $|r| > 0.65$, $n = 8$) and therefore unsuitable for inclusion in the same model. As a consequence, we tested the relationship between reproduction and water properties for each predictor separately and applied a model selection approach to compare predictors. All single-factor models and a null model, with Side of bay as a random effect, were included in an evaluation of likelihood by Aikaike's Information Criterion, corrected for small sample size (AICc), which penalizes model complexity in assessing relative model fit [*dredge* function in package MuMin (Barton, 2015) in R (R Core Team, 2015)]. All data are archived at https://doi.pangaea.de/10.1594/PANGAEA.877458.

Results

Water properties—east vs. west side

On the river-influenced east side of Willapa Bay, summer salinity was lower (2010) or started lower and increased faster (2011, 2012) relative to the west side (this and all following results in Figure 2, Supplementary Table S1). Accordingly, alkalinity followed a similar pattern. All carbonate variables differed between the two sides in 2010 and 2011. The east side had lower pH, lower Ω*aragonite*, and higher pCO_2 than the west side. Average Ω*aragonite* for all samples (2010–2011) was 1.37 on the east and 1.79 on the west side. All dissolved oxygen measurements exceeded $5.5\,mg\,l^{-1}$, but concentrations were lower on the east than west side. Chlorophyll concentrations were $\sim 3\,\mu g\,l^{-1}$ in both 2010 and

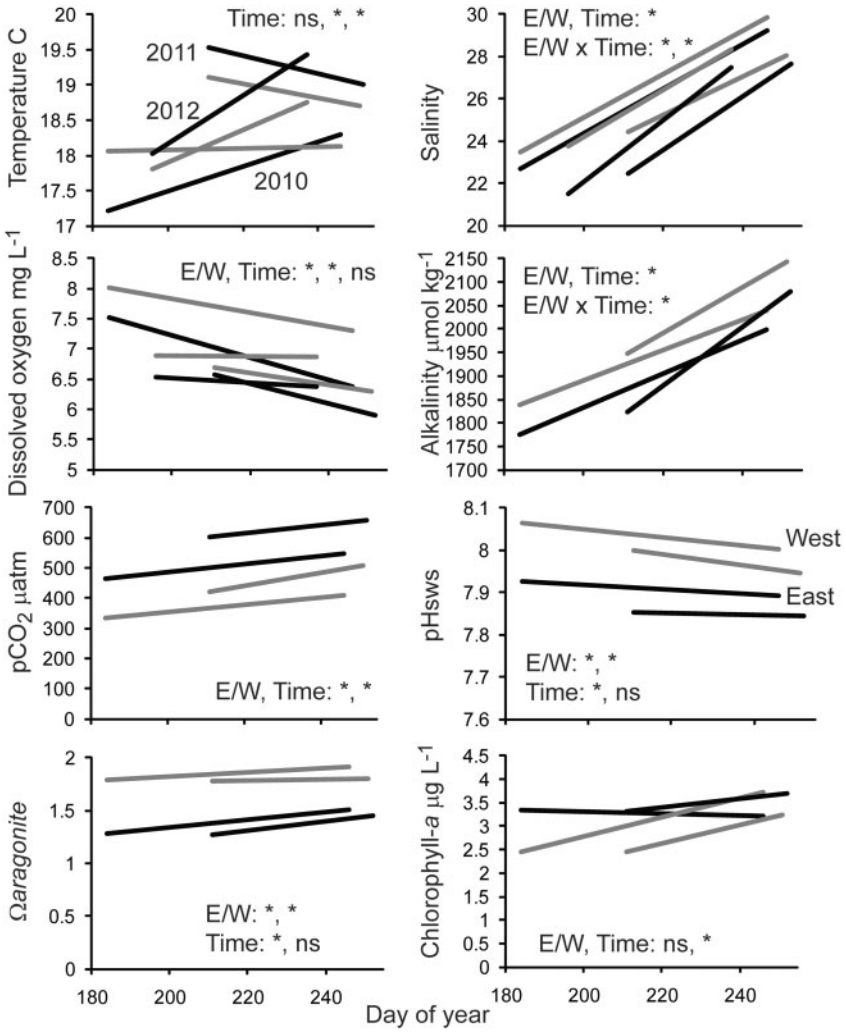

Figure 2. Water properties during daylight high tide on the west and east sides of Willapa Bay in summer 2010–2012. Lines show best fit to all sampling stations on each side. East stations: $n = 3, 4, 3$ across years. West stations: $n = 4, 4, 2$ across years. Sides are distinguished by line colour and years by duration of sampling. Codes on each panel show results of likelihood ratio tests of the linear mixed effects model (random effect of station) testing for a difference between the sides (E/W) and for temporal trends through the summer (Time): $^*p < 0.05$, ns, not significant. Full statistical results are in Supplementary Table S1, and other displays in Supplementary Figures S1–S5.

2011, slightly elevated on the east side in 2011. Water temperatures did not differ significantly between the sides of the bay in any summer. These east vs. west distinctions in water chemistry from linear mixed effects models (Figure 2, Supplementary Table S1) are evident in the mean conditions experienced by each cohort of Pacific oyster larvae, presented in Table 1.

Water properties—change through summer

Salinity increased through summer in all years as river discharge declined, although, as mentioned above, faster on the east than west side in 2011 and 2012 (Figure 2, Supplementary Table S1). Alkalinity followed this temporal trend, which would be expected to raise both pH and Ω*aragonite*, with more buffered water developing through summer. Meanwhile, dissolved oxygen declined while pCO_2 increased over time in 2010 and 2011. This apparent increase in net heterotrophy later in summer led to small overall declining trends in pH in 2010 (while Ω*aragonite* actually increased). No temporal trends in pH or Ω*aragonite* appeared in 2011, with any increase from rising alkalinity apparently cancelled by net heterotrophy. Summer temperatures were variable, with no trend during the measurement period in 2010, a declining trend in 2011, and an increasing trend in 2012 (Figure 2, Supplementary Table S1).

Larval mortality

Initial larval densities of Pacific oysters ranged from 1 to 100 l^{-1} and two weeks later had declined by one to three orders of magnitude (Figure 3). Across the four larval cohorts, mortality rates averaged -0.51, -0.17, -0.21, and -0.44 d^{-1} sequentially (values represent the instantaneous exponential decline in cohort size; Figure 3). Both statistical methods to compare larval mortality between the sides of the bay concurred in showing no difference. Larval density (log-transformed) showed no time × side effect for any cohort, which means that mortality rates did not differ between the two sides of the bay (Figure 3, Table 2). Similarly, direct comparison of mortality rates between the two sides, via

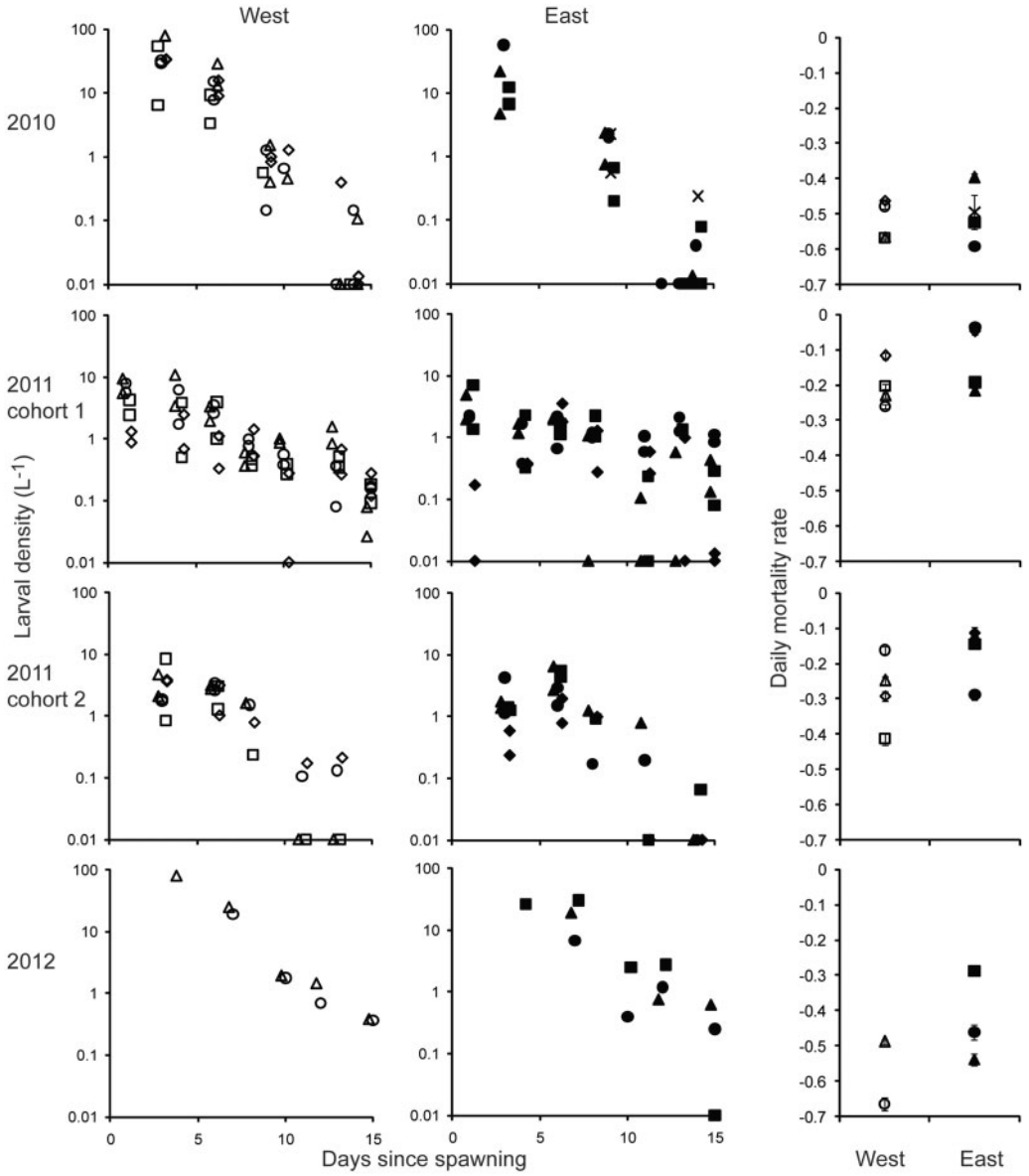

Figure 3. Larval density for four cohorts of larvae of Pacific oysters (*Crassostrea gigas*) on the west and east sides of Willapa Bay. Data are plotted by station: ○W1, ΔW2, □W3, ◊W4, ×E0, ●E1, ▲E2, ■E3, ◆E4. Samples where no larvae were detected are plotted as 0.01 on this log scale. Fits to Poisson-distributed data are shown as daily mortality rates in right-hand column, with error bars from standard error of model fit. Mortality rates that are more negative reflect rapid exponential decline and lower cohort survival.

weighted *t*-test, did not detect a difference between sides of the bay for any of the cohorts (Figure 3): 2010 ($F_{1,5} = 0.03$, $p = 0.87$), 2011 cohort 1 ($F_{1,6} = 2.77$, $p = 0.15$), 2011 cohort 2 ($F_{1,6} = 2.96$, $p = 0.14$), 2012 ($F_{1,3} = 2.33$, $p = 0.23$).

Settlement

Settlement was two orders of magnitude higher in 2012 than in 2010, with 2011 intermediate (Figure 4). Standardized per shellface, settlement met or exceeded commercially-viable levels in 2011, when there were two settlement events summing to 1.5 per shellface, and in 2012 at 11.5 per shellface. No difference in settlement was detectable between the two sides of the bay for three of the four cohorts (Table 3). In 2012, however, Pacific oysters settled at higher density on the river-influenced east than west side of the bay (Figure 4, Table 3). Across cohorts, settlement increased with water temperature (Figure 5), but note that this model had a similar AICc value to the null model (Supplementary Table S3). Predictors of salinity and % dissolved oxygen had AICc values at least 7 higher, therefore poor fit. For larval mortality, none of the three environmental predictors was effective, since each of these models had AICc values >9 higher than the best (null) model (Supplementary Table S3).

Discussion

The primary result of this study was a decoupling of oyster reproduction from carbonate chemistry and pH in the field. We observed statistically-different water properties on the west and east sides of Willapa Bay (Figure 2), which were not reflected in any

Table 2. Results of linear mixed effects models comparing larval densities on the west and east sides of Willapa Bay for four cohorts of Pacific oysters (*Crassostrea gigas*).

	23 Aug–3 Sep 2010	5–19 Aug 2011	24 Aug–6 Sep 2011	13–24 Aug 2012
Total number of samples	62	112	56	21
Time	363.5 (<0.0001)	51.32 (<0.0001)	93.5 (<0.0001)	60.1 (<0.0001)
Side	13.54 (0.01)	1.77 (0.23)	0.57 (0.48)	1.68 (0.29)
Time × Side	0.75 (0.39)	1.19 (0.28)	0.32 (0.58)	0.07 (0.80)

Each column refers to the period when a cohort of larvae was detected in plankton samples. Differential mortality rates between sides would appear as a significant interaction between Time and Side of bay. Results are provided as *F*-value (*p*-value).

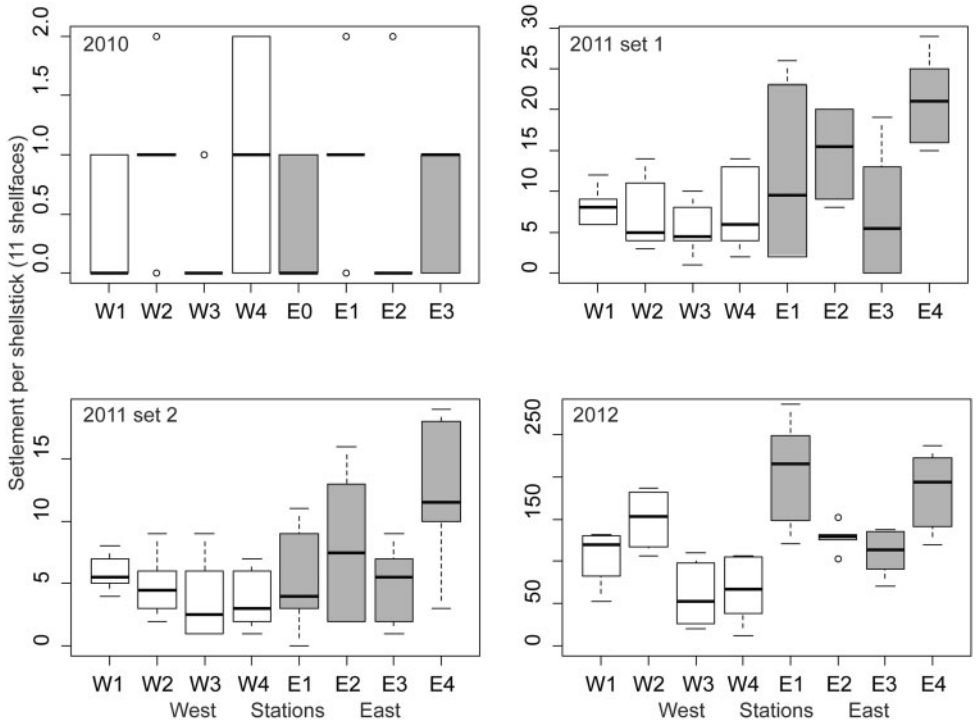

Figure 4. Settlement of four cohorts of Pacific oysters (*Crassostrea gigas*) on the west and east sides of Willapa Bay. Shellsticks were used as a consistent settlement substrate. Boxes show interquartile range and whiskers show range for three shellsticks placed at each of two substations ($n = 6$).

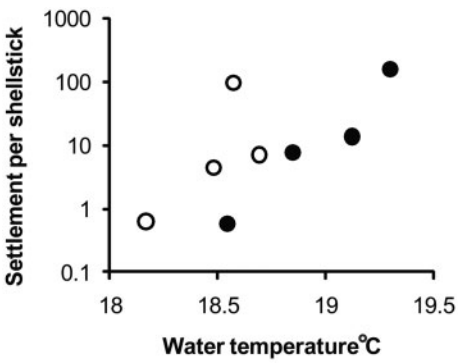

Figure 5. Mean settlement density of Pacific oysters (*Crassostrea gigas*) for each of four cohorts of larvae found on west (○) and east (●) sides of Willapa Bay, 2010–2012. Data are plotted with respect to water temperatures during larval development, based on consistent stations across all years.

biological difference (or in the opposite direction from expected; Figures 3 and 4). This decoupling could occur because water chemistry was not actually differentially stressful between the two sides of the bay, or because other environmental conditions either compensated for carbonate chemistry and pH or had stronger links to biological performance during the study. We address the evidence for these alternative explanations in the following paragraphs.

Were mean conditions of water chemistry differentially stressful on the west and east sides? The differences in carbonate chemistry and pH were statistically-significant but small in magnitude relative to laboratory manipulations that have demonstrated impaired larval performance for Pacific oysters under elevated [CO_2] (Kurihara *et al.*, 2007; Parker *et al.*, 2010; Frieder *et al.*, 2017). The west/east comparison of water chemistry was, however, on the order of change occurring from anthropogenic carbon absorption by the ocean, i.e. $\Delta pH > 0.1$. The challenge for understanding water chemistry as a stressor is whether a biologically-relevant

Table 3. Results of linear mixed effects models comparing settlement of Pacific oysters (*Crassostrea gigas*) on the west and east sides of Willapa Bay.

	5–10 Sep 2010	17–27 Aug 2011	27 Aug–11 Sep 2011	19–30 Aug 2012
Intercept	−0.55 (0.28) $z = -1.97$, $p = 0.049$	2.35 (0.25) $z = 9.39$, $p < 0.0001$	1.83 (0.21) $z = 8.66$, $p < 0.0001$	5.01 (0.17) $z = 29.2$, $p < 0.0001$
Side	0.068 (0.39) $z = 0.17$, $p = 0.86$	−0.48 (0.36) $z = -1.36$, $p = 0.18$	−0.41 (0.30) $z = -1.34$, $p = 0.18$	−0.58 (0.24) $z = -2.39$, $p = 0.017$

Each column refers to the period that shellsticks were deployed to coincide with a cohort of setting-size larvae in plankton samples. Settlement was analysed assuming Poisson-distributed errors, including random effects of station and nested sub-station. $N = 48$ total shellsticks in each analysis. Negative effect sizes indicate lower settlement on west side.

component spanned critical values for larvae, which can only be evaluated through gradients of treatments around critical values. Although the two sides do not constitute a gradient, Ω*aragonite* differed by ∼0.4 (Table 1, Figure 2) and was close to a threshold value reducing larval growth in the lab (Ω*aragonite* < 1.2; Waldbusser *et al.*, 2015).

How does spatio-temporal variability in carbonate chemistry modify any stress related to mean differences? Variability in carbonate chemistry is greater on the east than west side of the bay due to strong salinity gradients that shift their position during tidal exchange. As a consequence, east-side stations exhibited more spatial variability than west-side stations (Table 1, Supplementary Figures S1–S5). Continuous measurements in 2009 and 2010 at one east- and west-side station support that within-station temporal variation was also greater on the east than west side (Ruesink *et al.*, 2015). pH excursions at station E3, where tides rapidly changed the dilution of oceanic salinity by the river, spanned 0.4 pH units (regularly below 7.6), whereas pH was less variable and rarely below 7.9 at station W1 (Ruesink *et al.*, 2015). Because discrete samples were collected at high tide, they are "best case" conditions experienced by larvae, and pH could be much lower on the east side, but only slightly lower on the west side, throughout the remainder of the tidal cycle. For the east side of Willapa Bay, then, variability likely adds to any stress associated with mean conditions. More generally, however, variability in water chemistry has potential to offset problems with mean conditions. For instance, periodic respite from low pH or low Ω*aragonite* may be accompanied by an accumulation of reserves to withstand the next stressful period. The ability of larvae to find and remain in less-stressful water conditions (whether in space or in time) is highly uncertain, although more likely in the field, where such heterogeneity exists, than in a laboratory study. Larvae are known for active behaviours that change their distribution relative to passive particles (Dekshenieks *et al.*, 1996).

Could oyster larvae in Willapa Bay be locally acclimated or adapted to water chemistry? Evidence is accumulating that calcifying species demonstrate population- and family-level differentiation in tolerance of high-CO_2 conditions (Parker *et al.*, 2011; Frieder *et al.*, 2017; Padilla-Gamiño *et al.*, 2016; Thomsen *et al.*, 2017). Non-additive genetic variation and maternal effects can also contribute to tolerance of global change stressors (Chirgwin *et al.*, 2016). The evidence here regarding mortality rates of Pacific oyster larvae in the field provides an opportunity for strong selection, since the daily losses integrated over larval development result in 0.1–8% of the initial cohort surviving to settlement. Unless these oysters were perfectly pre-adapted for the bay's estuarine conditions, evolutionary change since introduction seems likely, but divergence over the small 10-km scale between the two sides of the bay would require that side-specific selection outweighs gene flow.

In addition to the three questions raised above, we have provided evidence that other environmental conditions in Willapa Bay are more consequential than carbonate chemistry and pH for oyster reproduction at present. This conclusion applies especially to the across-cohort differences in oyster settlement, which spanned two orders of magnitude (Figures 4 and 5). Summer water temperatures in Willapa Bay, from 18 to 20 °C, are on the steeply-rising portion of the thermal performance curve for oyster reproduction (Rico-Villa *et al.*, 2010; Figure 2). In other parts of the introduced range of Pacific oysters, settlement clearly improves in years of warmer water temperatures (Diederich *et al.*, 2005; Valdez and Ruesink, 2017). Given covariance of water properties along estuarine gradients, other conditions could improve under more stressful carbonate chemistry. For instance, food resources outweighed carbonate chemistry in two studies comparing juvenile mussel growth across sites (Thomsen *et al.*, 2013; Perez *et al.*, 2016). Well-provisioned larvae often have sufficient energy to overcome poor conditions for shell deposition (Ramajo *et al.* 2016). This point is rather speculative for our study in Willapa Bay, as no evidence exists regarding initial quality of spawned gametes or resource limitation during development (Figure 2). Field concentrations of $\sim 3\ \mu g\ l^{-1}$ chl-*a* were limiting to growth of Dutch mussel larvae (Fotel *et al.*, 1999), and most measurements within the study region of Willapa Bay were similarly low (Figure 2). Nevertheless, it is possible that higher concentrations of chlorophyll on the east vs. west side, as observed in one year of the study, could help offset otherwise stressful conditions.

A second result of this study bears on the drivers of carbonate chemistry and pH in estuaries. The consistently lower aragonite saturation state on the east relative to west side of Willapa Bay reflects both the physics of freshwater mixing with saltwater and the biology of net heterotrophy. First, the east side received more river input in comparison to the west side and was accordingly lower in alkalinity, which reduces Ω*aragonite*, all else being equal. Second, the east side was consistently supersaturated in pCO_2 relative to the west side, in accordance with lower dissolved oxygen, and higher pCO_2 also reduces Ω*aragonite*. In sum, the east side had two causes working in concert that led to lower carbonate saturation state. In contrast, through July and August, these two causes had countervailing temporal trends that served to even out Ω*aragonite* over time. Regionally, summer is a dry season, so as river flow declined and salinity rose, alkalinity also increased through the summer, particularly on the east side. This trend would lead to an increase in Ω*aragonite* through the summer. Yet Willapa Bay also became more super-saturated in pCO_2 through the summer, which would tend to reduce Ω*aragonite*. Because pCO_2 was increasing while dissolved oxygen was declining, a logical explanation is rising net heterotrophy through summer months. High net heterotrophy in late summer has been observed

in other temperate shallow-water coastal environments (Caffrey, 2004; Baumann *et al.*, 2015), although some estuaries can demonstrate highest net production in this season (Maher and Eyre, 2012). The within-season patterns that we report here should be placed in context of other work spanning multiple seasons in Willapa Bay, in which physics (reduced river flow) outdoes biology (net ecosystem metabolism): highest $\Omega_{aragonite}$ occurs in summer, coincident with high salinity and alkalinity (Hales *et al.*, 2016).

Our results highlight some of the difficulties of attributing causality to ocean acidification in the field. These difficulties are also evident in the lack of confirmed population-level consequences of ocean acidification (McElhany, 2017). Overall, such a conclusion would entail a two-step process—first to determine that the cause of high pCO_2 is anthropogenic carbon absorbed from the atmosphere, and second to link reduced population density or dynamics to this elevated pCO_2. Neither step was supported by this study. The first step is particularly challenging in coastal environments, including estuaries, due to high spatiotemporal variability of carbonate chemistry driven by changes in watershed inputs and biological productivity (Duarte *et al.* 2013). The second step of connecting population dynamics to water properties has high value for species in aquaculture (Ellis *et al.*, 2017). However, a focus on larval survival alone could be misleading, as exemplified by seasonal settlement in this study, which was constrained by a range of bottlenecks, including small initial spawning, few cohorts, and high larval mortality during development. The integrated signal of settlement suggests improved population dynamics under warmer temperatures (Figure 5). Therefore, distinct consequences of anthropogenic CO_2 release—rising global temperatures and CO_2 absorption by water bodies—could affect oyster reproduction in opposite ways. Whether or not a more complex framework ultimately aids understanding remains to be seen, as single-factor approaches to biological impacts often serve as well as multiple stressors in field studies of aquatic systems (Nõges *et al.*, 2016).

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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