EFFECTS OF PH, EELGRASS, AND SETTLEMENT SUBSTRATE ON THE GROWTH OF JUVENILE *MAGALLANA* (*CRASSOSTREA*) *GIGAS*, A COMMERCIALLY IMPORTANT OYSTER SPECIES

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ABSTRACT

EFFECTS OF PH, EELGRASS, AND SETTLEMENT SUBSTRATE ON THE GROWTH OF JUVENILE *MAGALLANA* (*CRASSOSTREA*) *GIGAS,* A COMMERCIALLY IMPORTANT OYSTER SPECIES

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Worsening ocean acidification (OA), resulting from ongoing absorption of atmospheric carbon dioxide (CO_2) by the oceans, threatens marine life globally. Calcifying organisms, especially their early life stages, are particularly vulnerable; this includes the economically important Pacific oyster, *Magallana* (*Crassostrea*) *gigas*. Uptake of dissolved CO_2 through photosynthesis by seagrasses, like eelgrass (*Zostera marina*), may benefit calcifying organisms by increasing pH and carbonate availability. I conducted laboratory and field experiments to quantify carbonate chemistry modification by eelgrass and potential mitigation of OA impacts on growth in juvenile Pacific oysters. In the laboratory experiment, daytime net photosynthesis by eelgrass increased seawater pH, while nighttime net respiration reduced pH though to a lesser extent; both effects grew stronger as the pH of incoming seawater decreased. This is consistent with the expectation that eelgrass will benefit from increased aqueous CO_2 levels and suggests that the importance of carbonate chemistry modification by eelgrass and its role as a refugium may increase as OA proceeds. Under the conditions tested, however, eelgrass effects on pH were modest and did not affect oyster growth in the lab or field. In the lab, oysters settled on shell flour grew faster than those on shell chunks, but unlike those on

chunks, the growth rate of oysters on flour decreased significantly in low pH treatments. One hypothesis consistent with these results is that the boundary layer around shell chunks may have slowed oyster growth by limiting food availability but that it also reduced sensitivity to low pH via enhanced carbonate saturation.

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INTRODUCTION

Over the past two centuries, and more clearly over the last four decades, the pH of surface ocean waters has fallen globally by approximately 0.10 units, due primarily to anthropogenic carbon dioxide (CO_2) emissions, showing pH conditions that are uncommon in the last 2 million years, and a rate of change that has not been seen for at least 26,000 years (IPCC 2021). Because the pH scale is logarithmic, this alteration represents an increase in acidity of approximately 30%. The oceans will continue to absorb even more carbon over the coming decades, and under business-as-usual emission scenarios by the end of the century ocean surface waters will be almost 150% more acidic (IPCC 2021). Such a result would yield ocean pH conditions that have not existed in over 20 million years. Worse still, the rapidity of change in pH and ocean carbonate chemistry to which organisms must adapt may be unprecedented over at least the past 300 million years, which spans both the Permian-Triassic and Cretaceous-Tertiary mass extinction events (Hönisch et al. 2012). Deep ocean circulation means that ocean acidification (OA) on the West Coast will continue to intensify for at least 30-50 years; headlands and other parts of the coast that experience stronger and more persistent upwelling are likely to face even greater impacts (Feely et al. 2009). This rapid, ongoing change in ocean chemistry poses a serious threat to marine ecosystems, especially calcifying organisms that depend on carbonate saturation, which decreases along with the pH of seawater (Fabry et al. 2008; Gazeau et al. 2013; Kroeker et al. 2010, 2013; Bednaršek et al. 2019, 2020, 2021a, 2021b).

A major consequence of OA is the reduction of carbonate ion availability and carbonate saturation states, which make it more energetically costly for organisms to build and maintain calcium carbonate shells and other structures (Waldbusser et al. 2013). Ocean acidification has been found to be particularly harmful to the early life history of many calcifiers including numerous bivalve species (e.g., bay scallop [*Argopecten irradians*], Pacific oyster [*Magallana* (*Crassostrea*) *gigas*], hard clam [*Mercenaria mercenaria*], Olympia oyster [*Ostrea lurida*], and Sydney rock oyster [*Saccostrea glomerata*] [Rumrill 1990; Dove and Sammut 2007; Green et al. 2009; Sanford et al. 2014; Waldbusser et al. 2010, 2013, 2014; Barton et al. 2012; Gobler et al. 2014]), suggesting that this is a broad concern.

Recent failures of larval oyster crops in commercial hatcheries are correlated with anomalously intense and persistent upwelling that brings seawater with low pH and low carbonate saturation to the surface and upper water column (Barton et al. 2012). Thresholds of pH with effects on shell-builders are known. These pH thresholds of 8.03 and 7.75 equate to aragonite (a form of carbonate) saturation (Ω_{arag}) values of approximately 1.7 (above which conditions are favorable for shell-building organisms, especially juvenile oysters) and 1.0 (below which seawater is corrosive to aragonite shells including juvenile oysters) respectively (Feely et al. 2004, 2008; Newton et al. 2015; Waldbusser et al. 2015). Because of the ecological and economic importance of oysters, there is great interest in understanding how they will be impacted by OA and potential means of mitigating these effects. As atmospheric CO_2 continues to rise and intensify

OA, it is crucial to investigate impacts on sensitive species, especially commercially important ones, as well as potential means of mitigating these impacts.

Because they consume dissolved aqueous CO_2 (dissolved inorganic carbon [DIC]) during photosynthesis, seagrasses in the family Zosteraceae (*Phyllospadix* spp.; *Zostera* spp.) may benefit from the increasing carbonation of seawater (Beer and Rehnberg 1997; Zimmerman et al. 1997; Koch et al. 2013; Palacios and Zimmerman 2007; Hendriks et al. 2014). Eelgrass is expected to benefit from increasing CO_2 levels as OA progresses because it is carbon limited and readily takes up dissolved CO_2; thus, as seawater grows more acidic due to OA, photosynthesis by eelgrass should increase seawater pH and carbonate saturation more than that of many other photoautotrophs (Palacios and Zimmerman 2007). Their uptake of dissolved carbon for photosynthesis may have a positive indirect effect on nearby calcifying organisms by increasing the pH and carbonate saturation of water, creating a refugium by locally mitigating OA (Jones et al. 1997; Bos et al. 2007; Garrard et al. 2014; Hendriks et al. 2014; Smith 2016; Groner et al. 2018; Koweek et al. 2018; Ricart et al. 2021a). The already broad and important ecological roles of seagrasses including foundation species and ecosystem engineers (Beck et al. 2001; Duffy 2006; Bos et al. 2007; Hughes et al. 2009), nursery (Heck Jr et al. 2003; Bertelli and Unsworth 2014), and general habitat (Moore et al. 2006; Bos et al. 2007), may be further enhanced to the extent that they also provide OA refugia. However, any overall positive effect may depend on the variance in diel physiological processes that seagrasses experience (Pacella et al. 2018). More specifically, OA mitigation and benefits to calcifiers may depend on the balance between net

photosynthesis during the day and respiration during the night (releasing CO_2 with accompanying reduction in pH) from all organisms within seagrass beds; the released CO_2 and accompanying reduction in pH and carbonate saturation may reduce or negate the positive effect of daytime photosynthesis (Waldbusser et al. 2013, 2014, 2015; Pacella et al. 2018; Ricart et al. 2021a, 2021b; etc.). Increasing seagrass density (and photosynthetic biomass) is expected to increase the strength of effects on carbonate chemistry, including total and net photosynthesis but also respiration. Photosynthesis likely increases asymptotically with seagrass density due to increasing self-shading at high density, while respiration increases linearly with density (Koweek et al. 2018; Pacella et al. 2018).

Native and cultivated oysters generally grow in protected bays, sometimes sharing habitat with eelgrass, *Zostera marina* (Linnaeus, 1753). Understanding the interactions between these species with contrasting responses to OA can help us to better understand the implications of OA for oysters and inform aquaculture and habitat management in changing oceanic conditions. A potential strategy for mitigating OA impacts on bivalve farms and restoration efforts is to grow calcifiers where they can benefit from chemistry modification by seagrass and/or other photoautotrophs; this approach may be most feasible in areas where these species co-occur. Given the inherent complexity of these systems, more investigation is necessary to help clarify key relationships and the mechanisms by which these organisms interact.

Bivalve mariculture is an important and growing industry that currently drives $270 million in economic activity and supports approximately 3,000 family wage jobs on

the West Coast (Barton et al. 2015). It is also among the most sustainable sources of animal protein in terms of carbon footprint and other environmental impacts. Humboldt Bay (HB) supports substantial mariculture production of the Pacific oyster, *Magallana* (*Crassostrea*) *gigas* (Thunberg, 1793), which is the most common oyster aquaculture species on the U.S. West Coast (> 90%). California is second only to Washington in U.S. oyster production with the HB oyster industry producing approximately 70% of the oysters sold in California and contributing significantly to the local economy (Richmond et al. 2018). HB also supports extensive eelgrass beds and is subject to natural upwelling-driven OA. Thus, HB provides a model system for researching the potential for eelgrass to reduce the OA impacts on juvenile oysters.

Though multiple studies have investigated OA impacts on juvenile *M. gigas* and other similar species (Gazeau et al. 2007; Kurihara et al. 2007; Miller et al. 2009; Barton et al. 2012; Lemasson and Knights 2021), most have used individuals settled on larger shell chunks/chips, complete valves (shell halves from individual bivalves), or a combination of these and various hard, artificial materials (Gibbons et al. 1989; Nestlerode et al. 2007; Vasquez et al. 2013; Dunn et al. 2014; Smyth et al. 2018; Poirier et al. 2019); fewer examples have excluded shell-based substrates altogether (Hidu et al. 1975; Sonait and Burton 2005). This contrasts with the practice of most commercial operations on the U.S. West Coast that settle their juveniles on finely ground shell flour. The practical significance of this distinction is that shell chunks might be more effective than shell flour in creating a thin boundary layer with significantly elevated carbonate saturation that may protect juvenile oysters from OA impacts (Kervella et al. 2009;

Reidenbach et al. 2013; Cornwall et al. 2014). However, the growth of juvenile oysters on shell chunks may be slower due to reduced flow and food availability in the boundary layer around the chunks as well as potential competition for space and food (Sebens et al. 2017). My project addresses this disconnect by examining the effect of substrate (shell chunks vs. shell flour) on juvenile oyster growth in response to different levels of OA and the presence/absence of eelgrass.

This study will provide important insight into how much eelgrass modifies carbonate chemistry and how much this mitigates OA impacts on cultivated oysters. Studies of similar systems with both seagrasses and bivalves (often focused on larval or adult bivalves in controlled laboratory conditions) have found complex and varied interactions (Peterson et al. 1984; Ruesch 1998; Waldbusser et al. 2010; Saderne et al. 2015; Smith 2016; Valdez et al. 2017; Groner et al. 2018; Koweek et al. 2018; Nielsen et al. 2018; Lowe et al. 2019; Spencer et al. 2019; Ricart et al. 2021b; Abe et al. 2022). Some studies have found that proximity to seagrasses benefit bivalves (Peterson et al. 1984; Ricart et al. 2021b) though these benefits were often context-specific and varied with seagrass species (Smith 2016) and bivalve species (Waldbusser et al. 2010; WDNR 2016; Nielsen et al. 2018; Spencer et al. 2019). Responses were sometimes modest (Groner et al. 2018; Koweek et al. 2018) or dependent on response variable (Reusch 1998). Other studies found little or no benefit to calcifiers from association with seagrasses (Saderne et al. 2015; Greiner 2017; Valdez et al. 2017; Lowe et al. 2019).

Few studies have focused directly on the relationship between the juvenile stages of oysters and eelgrass (Kurihara et al. 2007; Smith 2016; Valdez et al. 2017; Abe et al.

2022), so this work provides valuable information with practical scientific and industrial applications. By providing insight into the efficacy of potential OA adaptation strategies, including different settlement substrates and modification of carbonate chemistry via photosynthesis, this study has relevance for commercial mariculture and conservation and restoration of native bivalves. Results from this study provide data on the effects of OA on juvenile stages of oysters grown in the laboratory and *in situ* – an area of study that is relatively sparse. This information will enhance our understanding of the role that important marine macrophyte foundation species (e.g. kelp and seagrasses) may play in ecosystems as OA progresses.

Objectives and Primary Hypotheses

I devised and conducted laboratory and field experiments to explore the potential for eelgrass to increase pH and carbonate saturation and counteract, to some degree, the negative impacts of OA on the growth of juvenile oysters. The questions I addressed in the laboratory experiment and the hypotheses I formulated about them are:

1. To what extent does eelgrass modify pH, and how does this vary with the pH of incoming seawater?

   Hypothesis: net photosynthesis by eelgrass during the day will increase pH, net respiration during the night will reduce pH, and each of these effects will increase with decreasing pH levels.

2. How much does pH (and carbonate saturation) affect the growth of juvenile oysters?

Hypothesis: juvenile oyster growth will increase with increasing pH and carbonate saturation.

3. How does oyster substrate (shell flour vs. shell chunks) affect the growth of juvenile oysters and alter the effects of pH on their growth?

Hypothesis: The growth of juvenile oysters on shell chunks may be slower due to reduced flow and food availability in the boundary layer around the chunks (as well as potential competition for food and space). However, the boundary layer around chunks likely enhances carbonate saturation and may reduce the impact of low pH on oyster growth.

I conducted a field experiment designed to answer these questions:

4. How much does eelgrass modify pH *in situ*, and how does this vary within an eelgrass bed?

Hypothesis: as compared to outside the eelgrass bed, net photosynthesis by eelgrass during the day will increase pH, net respiration during the night will reduce pH. The effects of both photosynthesis and respiration will be stronger deep within the eelgrass bed than at its edge.

5. How much does position relative to *in situ* eelgrass beds affect the growth of juvenile oysters?

Hypothesis: juvenile oyster growth will increase with pH and carbonate saturation.

6. How does oyster substrate (shell flour vs. shell chunks) affect the growth of juvenile oysters and alter the effects of pH on their growth?

Hypothesis: The boundary layer (as well as potential competition for food and space) around shell chunks may slow the growth of juvenile oysters by reducing flow and reducing food availability. However, the boundary layer around chunks likely possesses elevated carbonate saturation and may reduce the impact of low pH on oyster growth.

METHODS

Laboratory Growth Experiments

Pilot work and preliminary trials

My general approach for laboratory experiments was to supply seawater from pH-controlled header tanks (pH treatment) to replicate aquaria with and without eelgrass (eelgrass treatment) with the drain from the aquaria flowing into tubs containing juvenile oysters settled on different substrates (substrate treatment). I conducted pilot work and two preliminary laboratory trials to test, refine, and troubleshoot methods, including calcein staining protocol; aquarium flow rate (to ensure adequate residence time for eelgrass to modify seawater chemistry); header tank pH control system; addition of natural bay mud to aquaria; methods for measuring eelgrass growth; oyster feed concentration, frequency, and duration; and the inclusion of the symbiotic eelgrass sea hare (*Phyllaplysia taylori*) in hopes that it would graze and naturally clean epiphyte growth from eelgrass leaves. The initial CO_2 dosing system I used was the Digital Aquatics Reef Keeper Elite V2 system, as described by Wilcox-Freeburg et al. (2013); however, due to problems with this system and the simultaneous closing of this company including all customer support, I replaced it with a more reliable dosing system from Neptune Systems (Neptune Systems Apex©).

I ran three trials of a fully factorial lab experiment with three pH treatment levels, two eelgrass treatments (present and absent), and two oyster settlement substrates (ground oyster shell “flour” and larger shell chunks), to test the effects of OA stress and

the presence of eelgrass on oyster growth and condition. The first two trials were preliminary work described here, while the third is described in the following subsection ("Principal experimentation"). Each trial was run for a total of 21 days (Table 1). For each of the three pH treatment levels, I set up two replicate header tanks; the seawater from each header tank flowed into two aquaria, one with and one without eelgrass. Each aquarium fed into two tubs, one with juvenile oysters settled on shell flour the other with juvenile oysters on large chunks of shell. Within each tub were three replicate sieves that held the oysters. Treatment levels and replication were: 3 pH levels × 2 replicate header tanks × 2 eelgrass levels (present/absent) × 2 oyster shell substrates × 3 replicate sieves; thus there were 6 header tanks (2 replicates for each of 3 pH levels), 12 aquaria (one with eelgrass, one without for each header tank), 24 oyster tubs (one with oysters on shell flour, one with oysters on shell chunks for each aquarium), and 72 sieves (three in each oyster tub; Figure 1). The pH of the six large header tanks was regulated using a precise dosing system (Neptune Systems Apex©) that bubbled CO_2 as needed to maintain pH treatment levels. I made the sieves that held the oysters using rings of PVC pipe and 300µm Nitex nylon mesh. These sieves were elevated slightly within the tubs using miniature platforms I made from acrylic egg crate lighting panels; these platforms allowed water to pass freely around the oyster sieves.

I continuously monitored header tank pH using laboratory-grade probes and measured pH in individual aquaria (eelgrass present/absent) and replicate oyster tubs twice daily with a Hach HQ40d hand-held pH meter. The pH meter was calibrated every 2 days using NIST-traceable pH buffers (with pH values of 4.00, 7.00, and 10.00).

During the first two lab trials, the pH treatments were “extra low” (7.65), “low” (7.75), and “ambient” (~7.85) which was unmodified water from the marine lab’s recirculating seawater system (Table 1, L1 and L2). I changed these treatment levels for the third and final trial, as described in the next subsection.

Principal experimentation

For the third lab trial, I followed the methods described above, however, to ensure greater contrast among treatment levels despite pH fluctuations in the lab’s seawater system, as well as some variation in pH maintained by the dosing systems, I decreased the pH of the extra low treatment by 0.10 units to 7.55 and replaced the ambient treatment with a high pH treatment of 8.05 (Table 1, L3). This increased pH level was achieved via precise dosing of a concentrated sodium carbonate (Na_2CO_3) solution using the same dosing system mentioned above. To prevent atmospheric exchange that would alter carbonate chemistry including pH, I covered all aquaria with translucent polyethylene sheeting, sealed oyster tubs with snap sealing lids, and measured the pH in tubs via small semi-enclosed outflow reservoirs, thereby allowing a semi-closed circulation system. Another modification from the preliminary trials was a reduction in outflow rate from each aquarium from ~22ml/s to ~12 ml/s, to increase the time for total water exchange in all aquaria (from ~83 min to ~153min), thereby increasing water residence time and allowing for stronger modification by eelgrass. Each of my experiments included a new cohort of oysters and a fresh set of eelgrass plants. Epibiont growth (primarily diatoms) in all water vessels was monitored and cleaning was done on a regular basis.

As done in the first two trials, I continuously monitored header tank pH using the same laboratory-grade probes and measured pH in individual aquaria (eelgrass present/absent) and replicate oyster tubs twice daily with a hand-held pH meter.

Eelgrass collection and lighting system

I collected nonreproductive/nonflowering eelgrass plants from HB, California, all with intact rhizomes and associated roots. I gently cleaned the shoots with a soft dish sponge, trimmed leaves to a length of 40cm, trimmed rhizomes to length of 8cm, weighed each for wet mass, then attached them to a submersible acrylic panel, and immediately submerged them in 110-liter (76.2 x 30.5 x 45.7 cm) aquaria to create artificial eelgrass beds. Each of these artificial beds included 44 shoots in total, contained within an area of 0.232 m^2. This density is equivalent to approximately 190 shoots/m^2 and lies within the range of average shoot densities (50-230 shoots/ m^2) measured in natural eelgrass beds around Humboldt Bay during the summers of 2017-2019 (Abell et al. *in prep.*). The inflow of header tank water entered at one end and was pumped out at the opposite end for all aquaria to ensure a consistent directional flow and prevent stagnation. The outflow of each aquarium was maintained at approximately 12 ml/s (720 ml/min), therefore it took ~153 minutes for complete water exchange. Each of the twelve aquaria contained a small 910-liter-per-hour water pump to increase circulation and mixing. Eelgrass was allowed a 48-hour acclimation period, before any experimentation began. At the end of each trial, I measured length and wet mass of a subset of eelgrass (25%) plants from each aquarium to quantify growth. Each eelgrass shoot gained an average of 8.48 g in wet mass during the experiment.

I constructed a specialized lighting system to simulate natural light. Fixtures with eight T5-HO full-spectrum aquarium bulbs were suspended above the aquaria. The size of the light fixtures allowed each one to nearly completely cover the top of three aquaria, so a total of four fixtures (32 total T5-HO bulbs) were used to ensure all aquaria received an equal amount of simulated natural light. I arranged the aquaria to alternate between eelgrass present and eelgrass absent (Figure 1). I used digital timers to cycle this lighting system with light/dark periods to mimic natural day/night cycles; which came on at 10:00AM and turned off at 10:00PM, thus allowing 12 hours on and 12 hours off. To ensure darkness similar to natural night conditions, I used internally reflective (white inside/black outside) curtains to hang from a PVC frame surrounding sea tables containing the aquaria. These curtains were also used in similar fashion to cover and surround the smaller subtending sea tables, which contained all tubs with oysters. The use of these curtains around my experimental facilities allowed for a reduction in diatom growth on the walls of the oyster tubs as well as all other nearby/surrounding experimental setups within the marine laboratory.

Oyster acquisition, prep, and measurements

I obtained juvenile oysters from collaborators at Hog Island Oyster Company's oyster hatchery on Humboldt Bay (Samoa, California), and from Pacific Seafood's oyster hatchery in Quilcene, Washington. Juvenile oysters were roughly 40-120μm^2 in shell area size and approximately 1-week post-settlement in age. Half were settled on finely-ground oyster shell flour and the other half on larger chunks of oyster shell roughly 2-2.5cm across. Then I stained juvenile oysters with calcein. I conducted trials using a

concentration of calcein at different incubation times of 4, 6, and 8 hours, ultimately adopting methods similar to those of Moran and Marko (2005) and hatchery staining protocols provided by George Waldbusser (pers. comm.). I found that 8-hour incubation with 10 ml of calcein solution (6.25 g calcein in 1L of deionized water) per 500ml of seawater effectively stained juvenile oyster shells without causing noticeable mortality. After 4 hours, oysters were transferred into a fresh batch of stain-inoculated seawater and incubated for an additional 4 hours. Then I placed 100-200 individuals in each sieve and moved the sieves into their respective tubs. Oysters were dosed every two hours with commercial microalgae concentrate (Shellfish Diet 1800®, Instant Algae, Reed Mariculture) to achieve a chlorophyll concentration of 6µg/L of seawater. This algae food level was based on average fluorometer-based estimates of chlorophyll concentration observed in HB (CeNCOOS).

At the end of each experiment, I measured shell growth using fluorescent microscopy and digital image analysis. First, I photographed individual or small groups of oysters with the Olympus SZX16 stereomicroscope with trinocular tube and Olympus DP series digital camera. Then for each individual oyster, I used Image-J image analysis software (Fiji/Image-J v2.1, NIH; Schindelin et al. 2012) to trace the perimeter of its top shell (final perimeter); as well as the perimeter of the retained fluorescent stain on the top shell (initial perimeter). I then used Fiji/Image-J to calculate initial and final shell area (μm^2) for each oyster. The resulting data was analyzed to compare within and across all treatment combinations. These data were compared within and among lab treatments, as well as to those from the complementary field study.

In Situ Growth Experiment

To investigate the effects of eelgrass on seawater pH and the growth of oysters in the field, I deployed instrument arrays with bags of juvenile oysters along two replicate 50 m transects, each with three evenly spaced positions: an area with no eelgrass (0m), within the edge of the eelgrass bed (25 m), and deep within eelgrass bed (50 m) (Figure 2). All transects and positions were at roughly the same tidal height in the low intertidal. The positions without eelgrass ("Loss") were within areas of eelgrass loss whose cause is still under investigation (W. Gilkerson, pers. comm.). Within the eelgrass bed but 6-7 m from its edge was the "Edge" position; the "Deep" position was 31-32 m from the edge of the eelgrass bed (Figure 2). I obtained and stained juvenile oysters as described for the laboratory trials, but I only used oysters settled on shell chunks. I fashioned small bags of 9 x 9 mm black plastic mesh (L33 Vexar) to contain shell chunks with oysters in the field; I selected the largest mesh size possible that would reliably contain the shell chunks to minimize fouling and resistance to flow through the mesh. In each bag, I placed 3-5 large oyster shell chunks (2-3cm across), each settled with 100-200 calcein-stained juvenile oysters. Bag type and deployment technique closely followed methods described by Smith (2016). At each position on both transects, I attached two replicate oyster bags to the instrument array, which included an Onset HOBO pH logger. This process was repeated for each of two separate two-week deployments, at a field site within the South Humboldt Bay State Marine Recreational Management Area (Table 1). At the end of the experiment, I measured the oysters shell growth via fluorescence microscopy and computer image analysis as described above.

Statistical Analyses

I evaluated the effect of eelgrass on seawater pH in the laboratory experiment by calculating the difference in pH (ΔpH) between each pair of replicate aquaria fed by the same header tank (one with eelgrass and the other without). I used a linear mixed effects (LME) model to test the effects of day vs. night (whether each pH value was measured in the evening after daytime net photosynthesis or in the morning after nightly net respiration) and header tank pH on the response variable ΔpH, with header tank as a random nesting factor.

To analyze oyster growth in the laboratory experiment, I first plotted regressions of log final size (total shell area) versus initial size (calcein-stained shell area). Because initial calcein-stained shell area clearly differed among treatments and settlement substrates, I plotted histograms to visually assess the size distributions of each group. I then calculated relative shell growth ([final shell area – initial shell area] / initial shell area) for each individual oyster and used this the metric of growth in all subsequent analyses. I subsequently ran LME analysis starting with a full model that included all terms: relative shell growth predicted by pH, settlement substrate, eelgrass, and all possible interactions, (with header tank, oyster containment tub, and sieve as hierarchically nested random factors). Then I used stepwise subtractive model selection and the Akaike Information Criterion (AIC) to select the best final model. To explore the significant interaction between pH treatment and oyster substrate, I divided the data into two subsets by substrate (one for shell chunks, one for shell flour), reran the same best fit LME model on each subset, and calculated the effect size for each predictor using

Cohen's *d*. Finally, I created a series of box and whisker plots with relative shell growth by settlement substrate, pH treatment, and eelgrass presence/absence.

Similarly, I analyzed juvenile oyster growth in the field studies using LME with relative shell growth as the metric of growth and stepwise subtractive model selection to choose the best model. I created one set of models using position relative to eelgrass beds (deep in the bed, within the edge of the bed, in an area without eelgrass) as the main effect, with transect and bag of oysters as random nested factors. I also analyzed the second trial using a variety of different pH summary statistics (mean, median, total time while pH > 8.03, total time while pH < 7.75) as predictors. These pH thresholds were selected because while seawater pH is not the only determinant of aragonite (a form of carbonate) saturation (Ω_{arag}), a pH of 8.03 corresponds to Ω_{arag} of approximately 1.7 (Newton et al. 2015), above which conditions are less stressful for shell-building organisms (Waldbusser et al. 2015); a pH of 7.75 corresponds to a Ω_{arag} of roughly 1.0, below which seawater is corrosive to aragonite shell (Feely et al. 2004; Feely et al. 2008) and acutely stressful to oyster larvae (Waldbusser et al. 2015). Because pH data from field trial 1 was unusable (due to failure of the glass electrodes in the HOBO pH loggers), I could only perform this analysis on field trial 2 and even in that trial the logger at the Edge position on the north transect failed. For this reason, I ran a second set of LME analyses for field trial 2 using only pH summary statistics as predictors but without position as a predictor, and a third with pH summary statistics and position as predictors but excluding the Edge position which lacked pH data for the north transect. In these analyses transect and oyster bag were modeled as random nested factors.

All analyses were completed using R (R Core Team 2018) with RStudio (RStudio Team 2020). For LME analyses I used the R package ‘nlme’ (Pinheiro, Bates, and R Core Team 2023). Effect sizes (Cohen’s *d*) were calculated using the ‘effectsize’ package (Ben-Shachar et al. 2020).

Permitting

This work was an extension of a larger project and was conducted in collaboration with California Department of Fish and Wildlife (CDFW) biologist James Ray. Scientific collection of eelgrass was achieved through this collaboration with CDFW as well as the scientific collecting permit held by Dr. Paul Bourdeau. With the help of James Ray (CDFW), I obtained the CDFW permit required to import oyster seed from the oyster hatcheries as well as the Letter of Authorization needed for outplanting the oysters in HB (within California State waters).

Table 1. Breakdown of lab and field experiments, their timeframes, durations, treatment levels and related details. All lab experimentation took place at Cal Poly Humboldt's Telonicher Marine Laboratory in Trinidad, CA. All field experimentation took place within Humboldt Bay (HB), California.

	ID			
LAB	L1	7.85 (Ambient); 7.75 (Low); 7.65 (Extra low)	9/6/2018 – 9/28/2018	21 days
	L2	7.85 (Ambient); 7.75 (Low); 7.65 (Extra low)	10/30/2018 – 11/21/2018	21 days
	L3	8.05 (High); 7.75 (Low); 7.55 (Extra low)	3/3/2020 – 3/25/2020	21 days
FIELD	F1	South HB, North + South Transect	North transect: 40.710413 N, 124.259162 W South transect: 40.709863 N, 124.259304 W	7/24/2021 – 8/7/2021; 14 days
	F2	South HB, North + South Transect	North transect: 40.710413 N, 124.259162 W South transect: 40.709863 N, 124.259304 W	8/22/2021 – 9/5/2021; 14 days

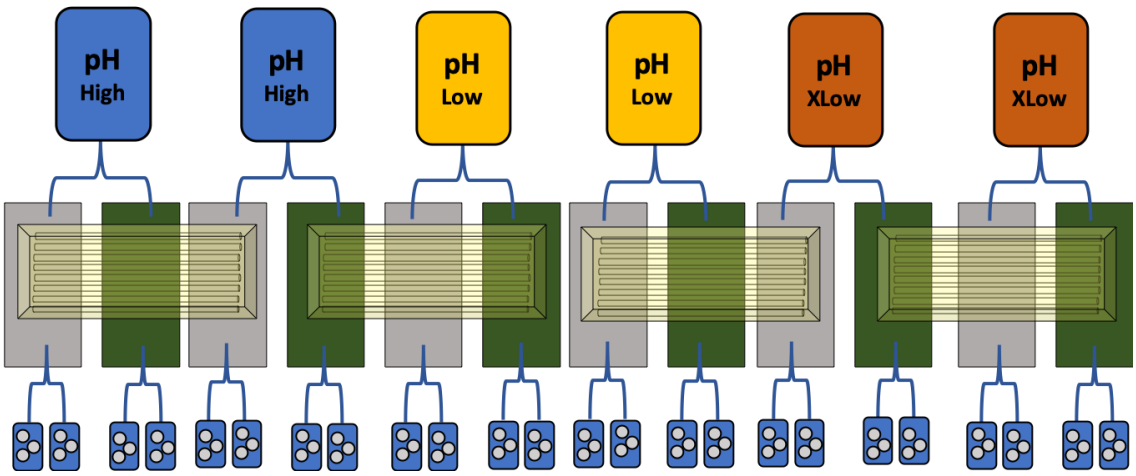

Figure 1. Schematic illustrating the concept of the lab experiment facilities setup.

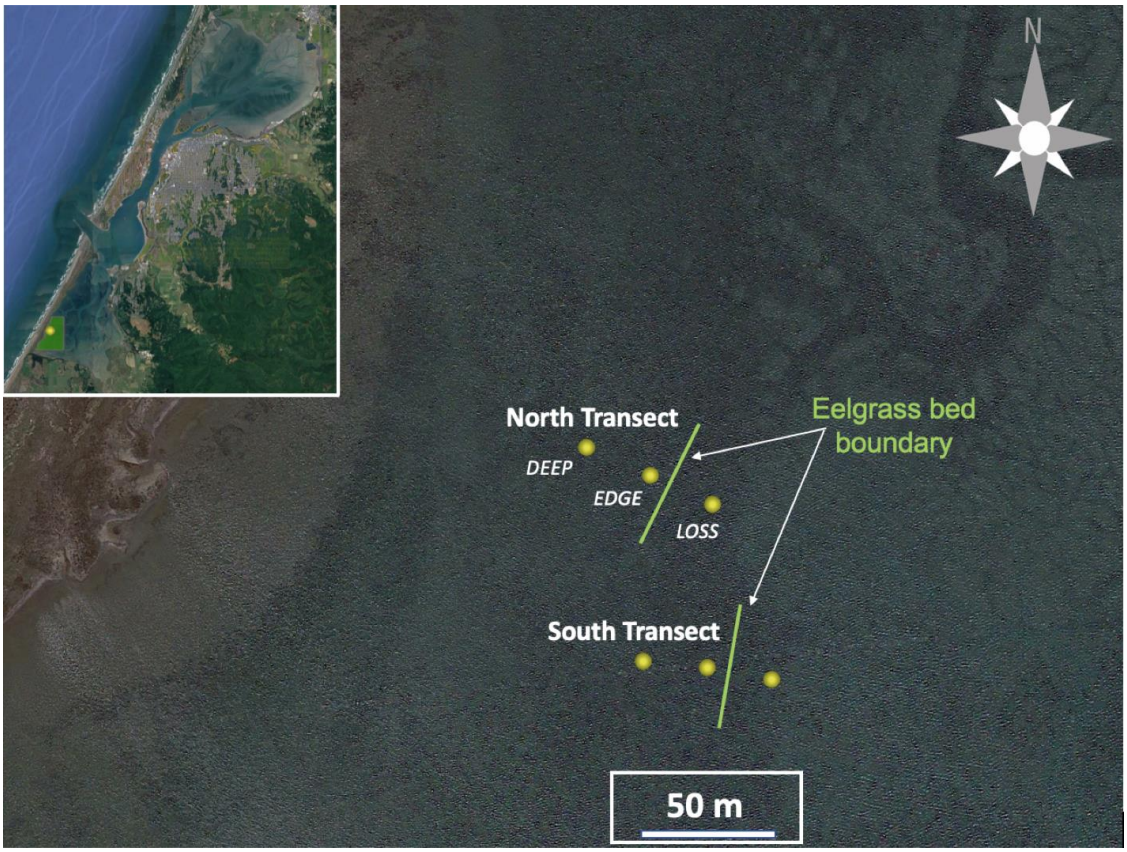

Figure 2. Map of field experiment site in the South Humboldt Bay State Marine Recreational Management Area, California.

RESULTS

Lab Experiment

Eelgrass chemistry modification

The mean header tank pH achieved for each treatment was close to but slightly lower than the nominal targets with values of 7.51, 7.64, and 7.78 for the Extra Low, Low, and High pH treatments, respectively (Table 2). Daytime irradiance provided to eelgrass (measured at the top of aquaria by HOBO Pendant loggers) averaged 4,146 Lux which equates to an average photosynthetically active radiation (PAR) intensity of 124 $\mu E/m^2s$ based on a conversion factor of 0.030 for fluorescent lights with wavelengths optimized for plant growth (Sager and McFarlane 1997); this is roughly 6% of the PAR for clear, midday sun in this region of ~2,000 $\mu E/m^2s$. In all treatments, average pH in aquaria without eelgrass was higher than that of the header tanks (potentially due to a small amount of photosynthesis by fouling algae despite efforts to keep aquaria clean), and pH in the aquaria with eelgrass was higher still (Table 2). In the Extra Low pH treatment, average pH in the eelgrass aquaria was 0.13 units higher than in the aquaria without eelgrass; in the Low pH treatment, the eelgrass aquaria had an average pH only 0.02 units higher, and in the High pH treatments average pH in the eelgrass aquaria was just 0.01 units above the no eelgrass control. The change in aquarium pH driven by eelgrass (measured as ΔpH) was significantly affected by day versus night as well as its interaction with header tank pH (Figures 3, 4, 5 and 6; Table 3). To better visualize the effect of day versus night and its interaction with header tank pH, in addition to the

timeseries of daytime and nighttime ΔpH (Figure 5), I plotted the partial residuals from the LME model (Figure 6). These plots show eelgrass boosting pH in the evening (after daytime net photosynthesis) sometimes by more than 0.2 units; reducing pH in the morning (following nighttime net respiration) to a smaller extent, generally less than 0.10 units; and that both effects increased with decreasing header tank pH (Figure 6).

Table 2. Data of lab pH and eelgrass treatment combinations.

pH		target	mean	SD	min	max
Extra low	Header	7.55	7.51	0.19	7.27	8.26
	No eelgrass	–	7.63	0.09	7.50	7.84
	Eelgrass	–	7.76	0.10	7.57	7.94
Low	Header	7.75	7.64	0.10	7.26	7.76
	No eelgrass	–	7.86*	0.10*	7.69*	8.06*
	Eelgrass	–	7.88	0.14	7.59	8.11
High	Header	8.05	7.78	0.24	7.44	8.45
	No eelgrass	–	7.88	0.31	7.36	8.52
	Eelgrass	–	7.89	0.34	7.33	8.55

**Average excludes results from the no eelgrass aquarium in the 2nd low pH treatment.*

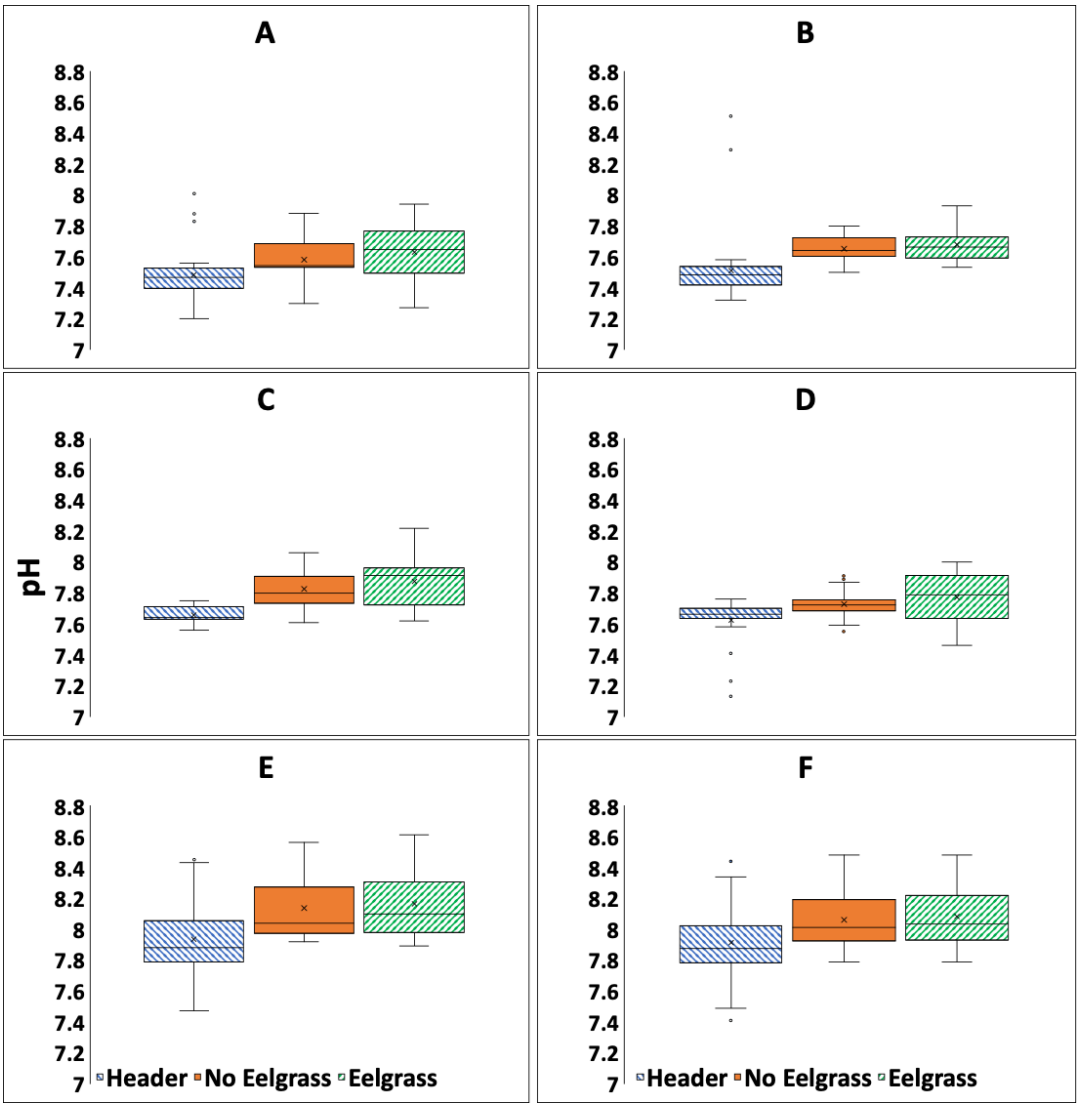

*Figure **3.** Box and whisker plots of pH in replicate header tanks and aquaria by pH treatment: Extra Low pH (A & B); Low pH (C & D), and High pH (E & F). Header tanks are shown in blue, aquaria with eelgrass in green, and aquaria without eelgrass in orange. Boxes span the interquartile range (IQR); horizontal lines within boxes represent medians, and means are represented with an 'X.' Whiskers extend to minimum and maximum values within 1.5 times the IQR of the box; points beyond the whiskers are outliers. The data plotted span the three-week duration of the laboratory experiment from 3 March 2020 to 24 March 2020.*

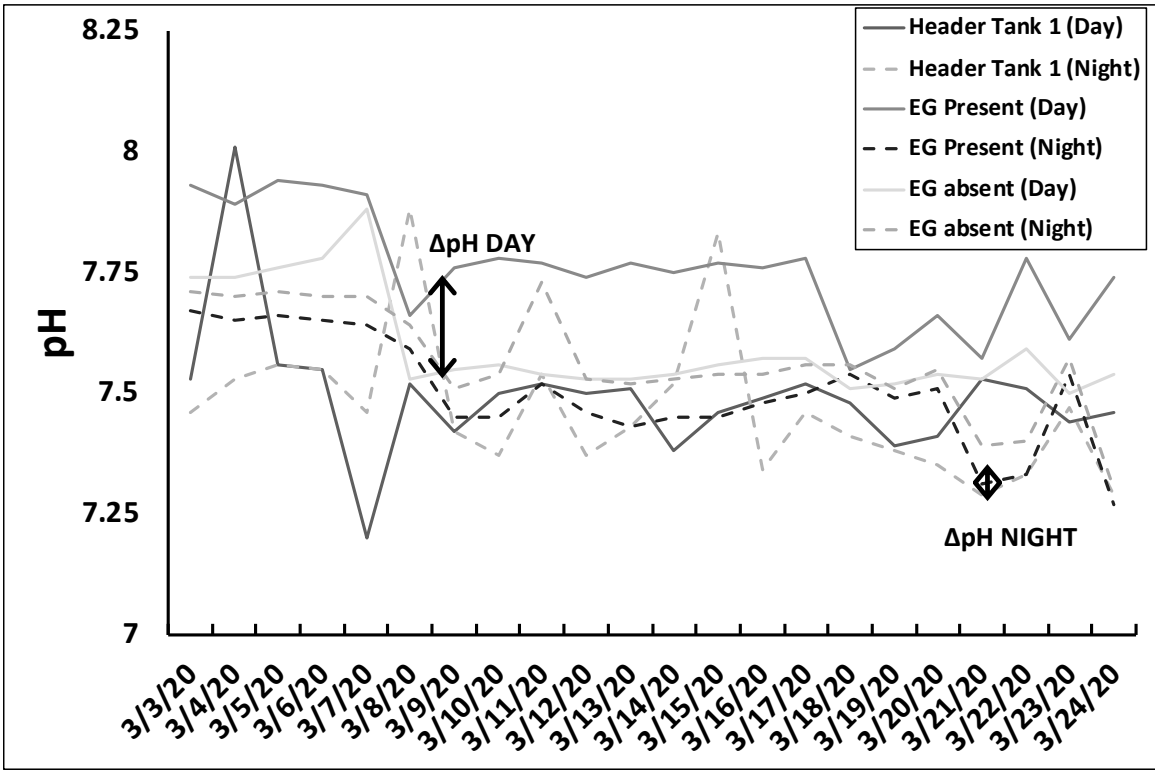

Figure 4. Timeseries from one of the extra low pH treatment header tanks to illustrate the concept of ΔpH (difference in pH between aquaria with and aquaria without eelgrass).

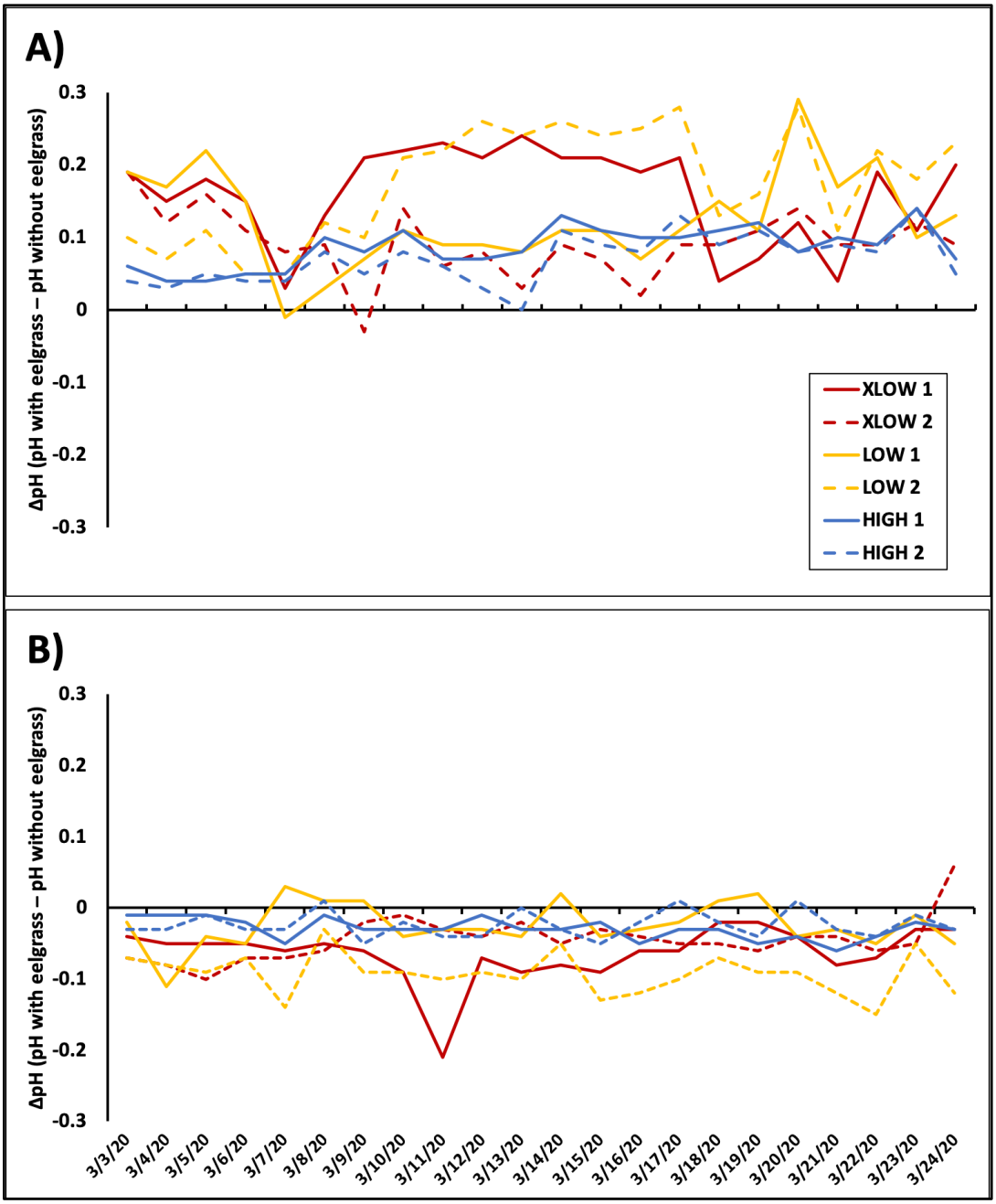

Figure 5. Timeseries of (A) daytime and (B) nighttime ΔpH (difference in pH between aquaria with and aquaria without eelgrass) for each pH treatment and replicate header tank. This timeseries is from 3 March 2020 to 24 March 2020.

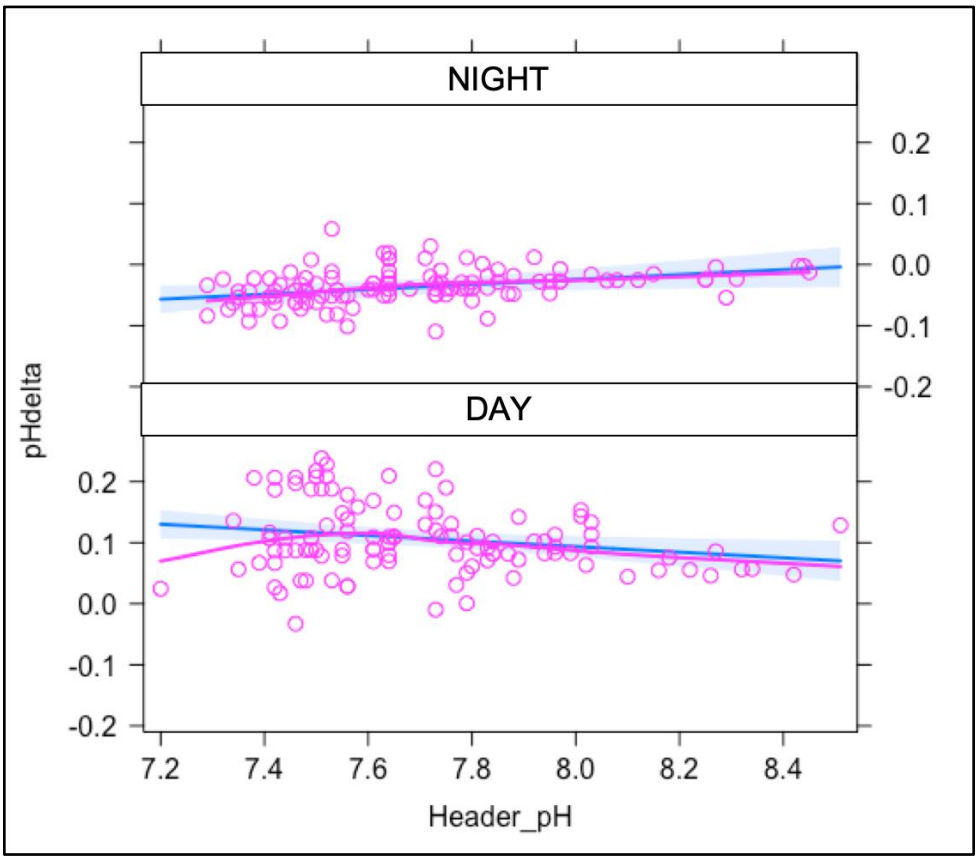

Figure 6. Partial residual plot of ΔpH (difference in pH between aquaria with and aquaria without eelgrass) by header tank pH, for daytime and nighttime. Blue lines represent least squares fit with shaded 95% confidence interval; pink lines are a LOWESS (locally weighted scatterplot smoothing) curve.

Table 3. Linear mixed effects model of eelgrass and header tank effects on ΔpH (the difference in pH between aquaria with and aquaria without eelgrass).

	numDF	denDF	*F*-value	*p*-value
(Intercept)	1	212	33.443	< 0.001*
Header Tank pH	1	212	0.929	0.336
Day or Night	1	212	550.348	**< 0.001***
Header Tank pH:Day or Night	1	212	13.796	**< 0.003***

Effects of pH and substrate on oyster growth

I discovered a methodological issue early in analysis: initial shell area, as estimated by calcein stain, varied substantially with pH treatment particularly on shell flour substrate (Figures 7 and 8). This strongly suggests incidental size-selective sorting occurred when oysters were being divided into pH treatments.

The average relative shell growth of oysters in each pair of aquaria with and without eelgrass was very similar; oysters settled on shell flour grew more overall (Figures 9 and 10; Table 4). Average relative shell growth of oysters decreased with decreasing pH; this trend was much stronger in oysters on shell flour than those on chunks (Figures 9 and 10).

Using LME analysis, I started with a full model (relative shell growth predicted by pH, settlement substrate, eelgrass, and all possible interactions with header tank and oyster containment tub as random nested factors) and conducted stepwise subtraction to select the best model (Table 5). Eelgrass presence was not a significant predictor of juvenile oyster growth, however the effect of pH was marginally significant ($p < 0.07$), substrate was highly significant, as was the interaction between pH and substrate (Table 5). Subsequent investigation of the substrate:pH interaction with the dataset divided by substrate type, analysis with the same best fit LME model, and then calculation of effect size (measured as Cohen's *d*) revealed that this interaction was driven by significantly reduced growth of oysters settled on shell flour in the Low and Extra Low pH treatments (Table 6).

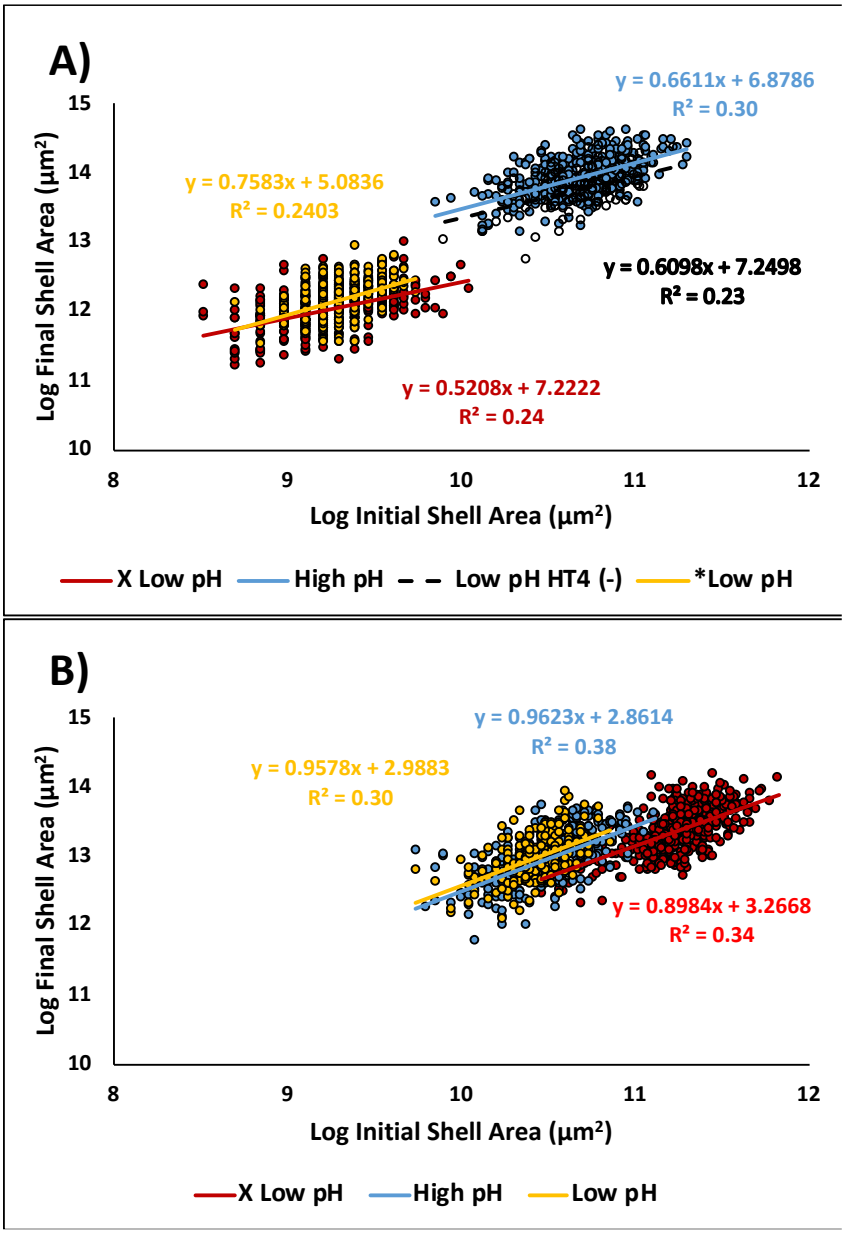

Figure 7. Regression plots of log transformed initial and final oyster shell area, by settlement substrate; Flour (A) and Chunks (B).

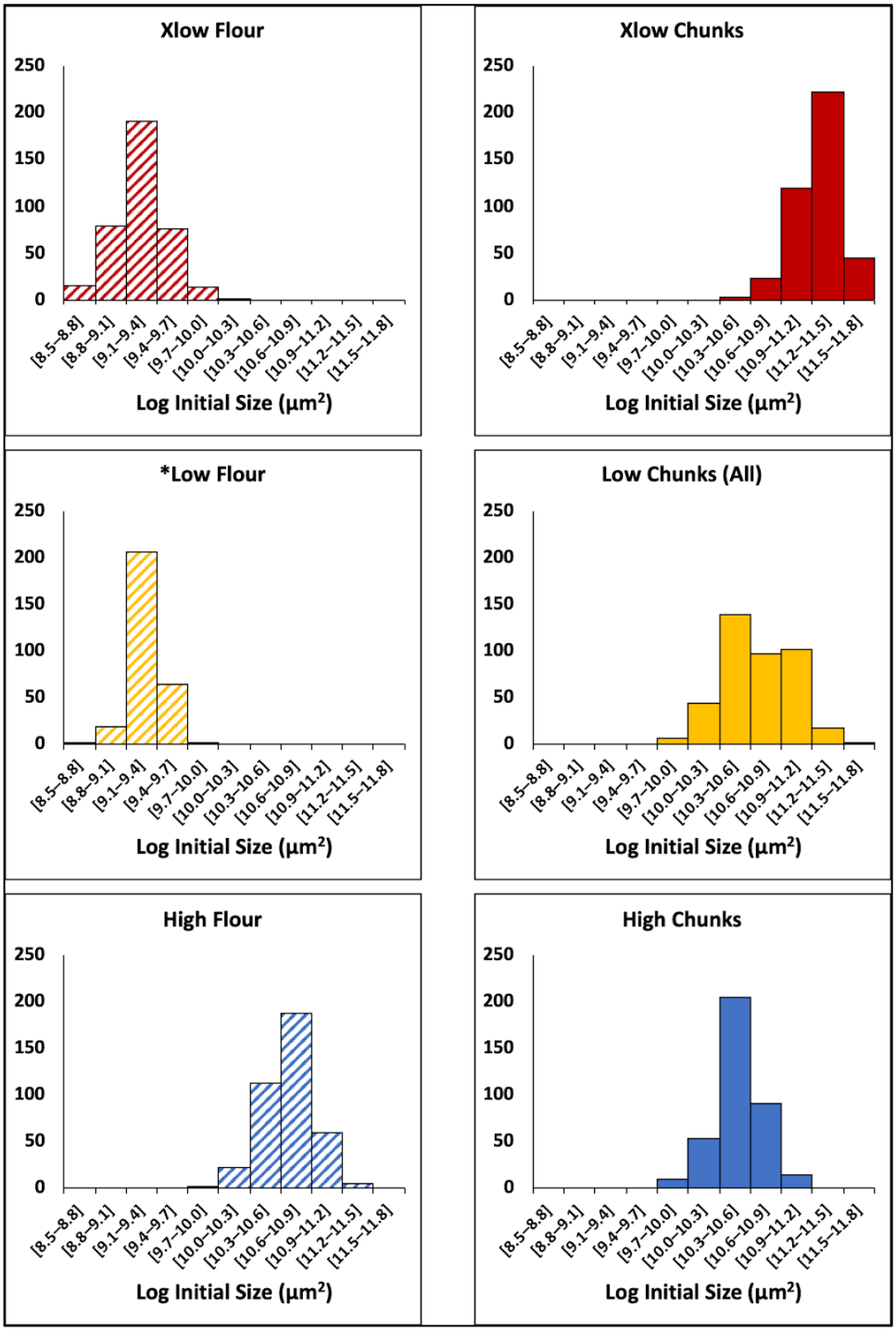

Figure 8. Histograms of log transformed initial oyster shell area by pH treatment and substrate type. Outliers removed.

Table 4. Relative shell growth (sieve means) by settlement substrate and pH and eelgrass treatment from laboratory experiment.

Substrate	pH		mean	SD	min	max
FLOUR	Extra Low	No eelgrass	14.39	0.67	13.64	14.95
		Eelgrass	16.92	2.72	14.41	19.81
		Combined	15.65	2.28	13.53	19.81
	Low	No eelgrass	18.64	1.12	17.40	19.45
		Eelgrass	17.16	1.33	15.89	18.27
		Combined	17.90	1.68	15.89	19.77
	High	No eelgrass	25.96	2.12	24.05	28.18
		Eelgrass	24.67	1.61	23.26	26.42
		Combined	25.31	2.11	17.77	28.18
CHUNKS	Extra Low	No eelgrass	7.27	0.88	6.31	8.04
		Eelgrass	8.00	0.86	7.07	8.70
		Combined	7.64	0.91	6.31	8.73
	Low	No eelgrass	9.67	1.21	8.67	11.02
		Eelgrass	9.63	0.76	8.91	10.39
		Combined	9.65	0.98	8.67	11.20
	High	No eelgrass	11.19	1.73	9.76	13.05
		Eelgrass	11.20	0.57	10.63	11.77
		Combined	11.19	1.61	9.14	13.65

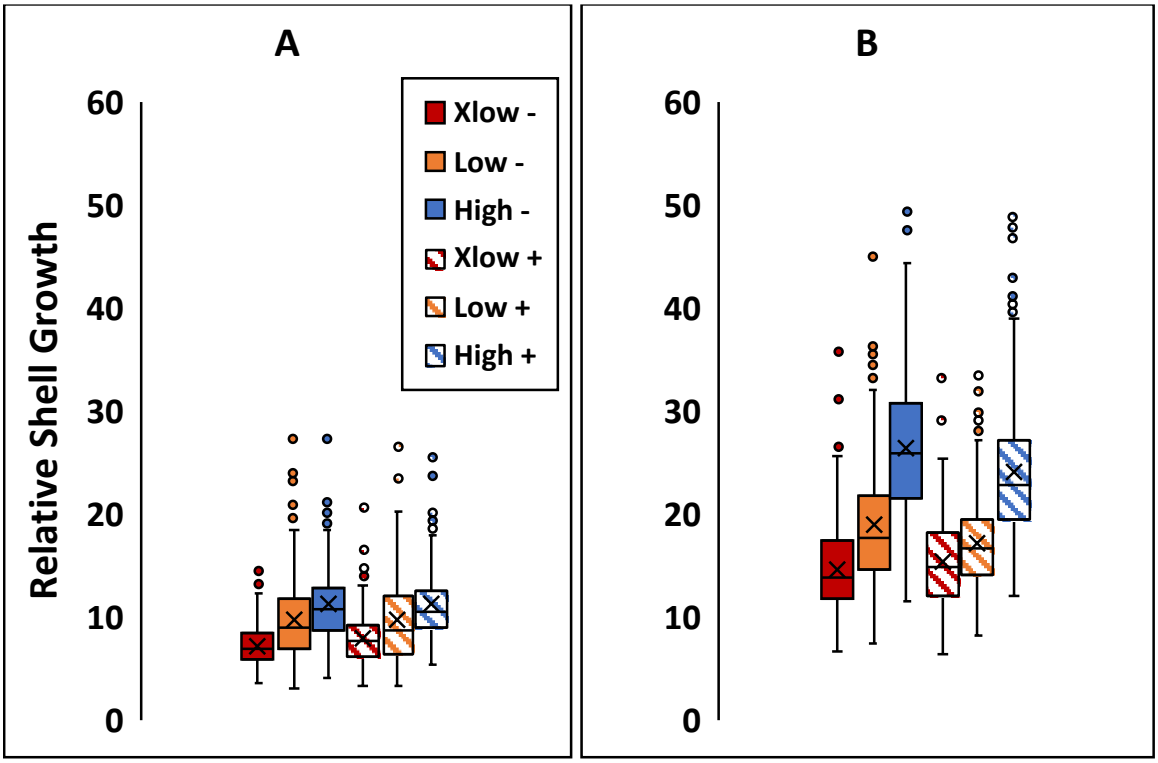

Figure 9. Box and whisker plots of relative shell growth across pH treatments with (+) and without (-) eelgrass, between settlement substrates, Chunks(A) and Flour (B). Boxes span the interquartile range (IQR); horizontal lines within boxes represent medians, and means are represented with an 'X.' Whiskers extend to minimum and maximum values within 1.5 times the IQR of the box; points beyond the whiskers are outliers.

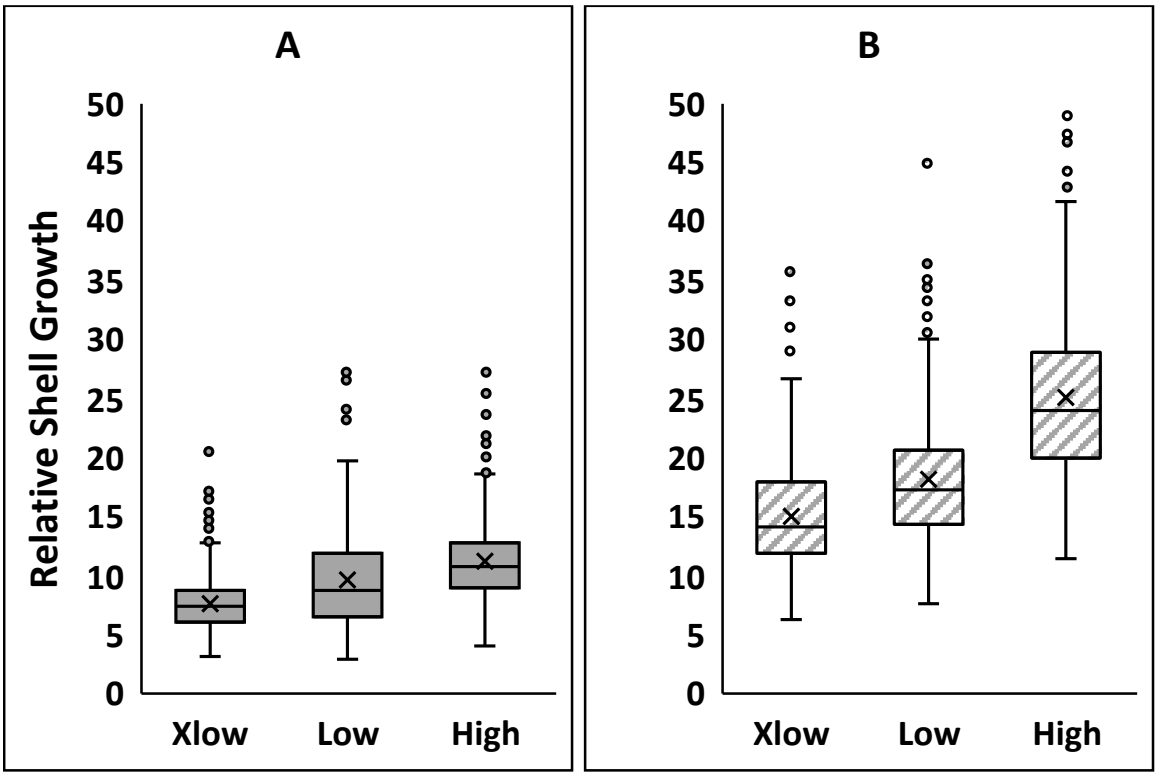

Figure 10. Box and whisker plots of relative shell growth between settlement substrates, chunks (A) and flour (B). Boxes span the interquartile range (IQR); horizontal lines within boxes represent medians, and means are represented with an 'X.' Whiskers extend to minimum and maximum values within 1.5 times the IQR of the box; points beyond the whiskers are outliers.

Table 5. Analysis of treatment effects on oyster relative shell growth using linear mixed effects (LME) modeling.

	numDF	denDF	*F*-value	*p*-value
(Intercept)	1	2265	405.779	<0.0001*
pH	2	3	7.430	0.069
Eelgrass	1	12	0.003	0.957
Substrate	1	12	318.783	**<0.0001***
pH:Eelgrass	2	12	1.877	0.195
pH:Substrate	2	12	12.166	**0.001***

Table 6. Best fit LME model of treatment effects on oyster growth by substrate with effect sizes measured as Cohen's d.

Substrate		Value	SE	DF	*t*-value	*p*-value	Cohen's *d*	Cohen's *d* 95% C.I.
CHUNKS	(Intercept)	11.325	1.650	1151	6.862	< 0.0001	0.40	0.29, 0.52
	Low pH	-1.648	2.332	3	-0.706	0.531	-0.82	-3.10, 1.59
	Xlow pH	-4.045	2.333	3	-1.734	0.181	-2.00	-4.63, 0.83
	EelgrassP.	-0.126	1.242	3	-0.101	0.926	-0.12	-2.37, 2.16
	Low pH: EelgrassP.	0.106	1.754	3	0.060	0.956	0.07	-2.20, 2.33
	Xlow pH: EelgrassP.	0.818	1.754	3	0.466	0.673	0.54	-1.80, 2.80
FLOUR	(Intercept)	26.415	1.493	1114	17.692	< 0.0001	1.06	0.93, 1.19
	Low pH	-7.495	2.112	3	-3.549	**0.038***	-4.10	-7.87, -0.18
	Xlow pH	-11.704	2.113	3	-5.540	**0.012***	-6.40	-11.71, -1.06
	EelgrassP.	-1.261	2.045	3	-0.616	0.581	-0.71	-2.99, 1.67
	Low pH: EelgrassP.	-0.380	2.893	3	-0.131	0.904	-0.15	-2.41, 2.13
	Xlow pH: EelgrassP.	3.841	2.894	3	1.327	0.276	1.53	-1.11, 3.99

Field Experiment

In contrast to the laboratory experiment, initial sizes of oysters used in both trials of the field experiment were relatively uniform (Figures 11 and 12).

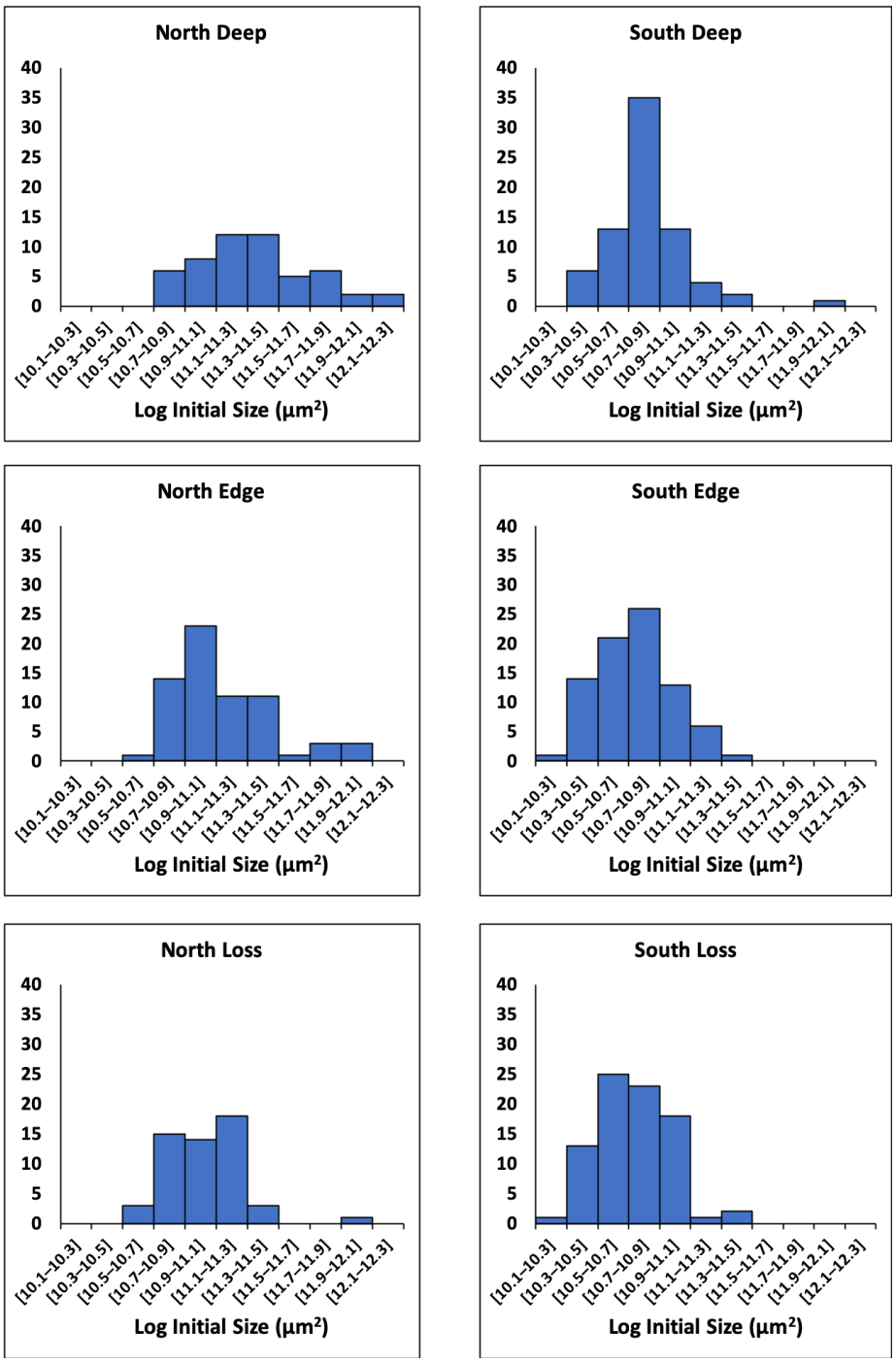

Figure 11. Histograms of log transformed field oysters initial shell area, for field trial 1 of summer 2021, by transect (north is left column, south is right column) and position along transect (first row is deep in the eelgrass bed, second row is in the edge of the bed, third row is in an area where all eelgrass had been lost).

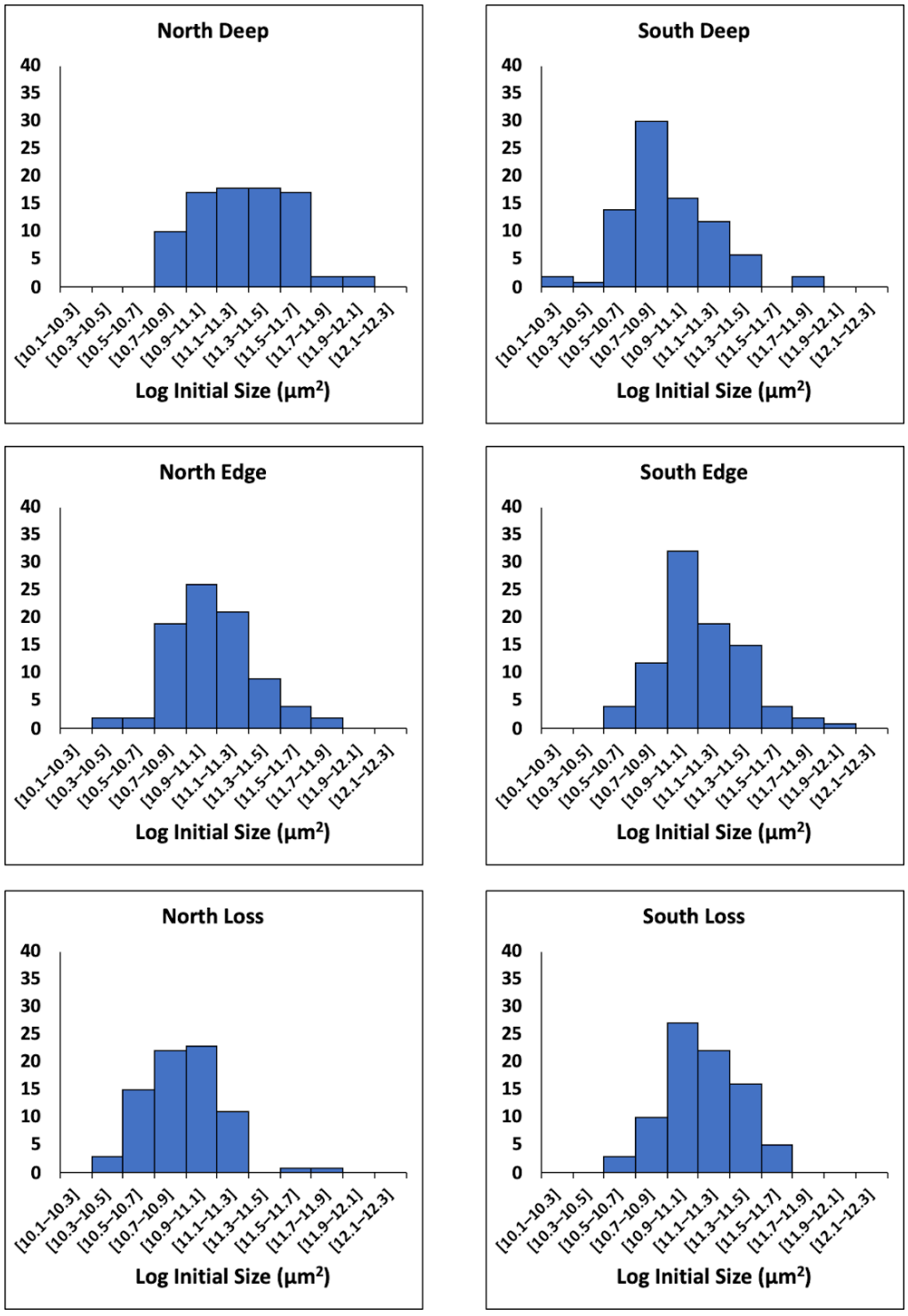

Figure 12. Histograms of log transformed field oysters initial shell area, for field trial 2 of summer 2021 by transect (north is left column, south is right column) and position along transect (first row is deep in the eelgrass bed, second row is in the edge of the bed, third row is in an area where all eelgrass had been lost).

Though data from the pH instruments in the first field trial were unusable due to instrument failure, data from the second field trial showed no obvious difference in pH or temperature by transect location or position relative to eelgrass beds (Figure 13). Data were again analyzed with linear mixed effects models and stepwise subtractive model selection. The first LME analysis of both field trials used position as predictor of relative shell growth and found a marginally significant effect of position in both trials (Table 7; Figure 14).

The LME models of pH effects on relative shell growth for field trial 2 using multiple pH summary statistics (mean, median, total time while pH > 8.03, total time while pH < 7.75) as predictors (with transect and oyster bag as random nested factors) indicated no significant effects.

Table 7. Linear mixed effects analysis results for field experiment trials modeling relative shell growth predicted by position with transect and bag as random nested factors.

Field Trial		numDF	denDF	F-value	*p*-value
1	(Intercept)	1	401	32.884	<0.0001*
	Position	2	6	2.978	0.108
2	(Intercept)	1	489	37.728	<0.0001*
	Position	2	6	3.394	0.059

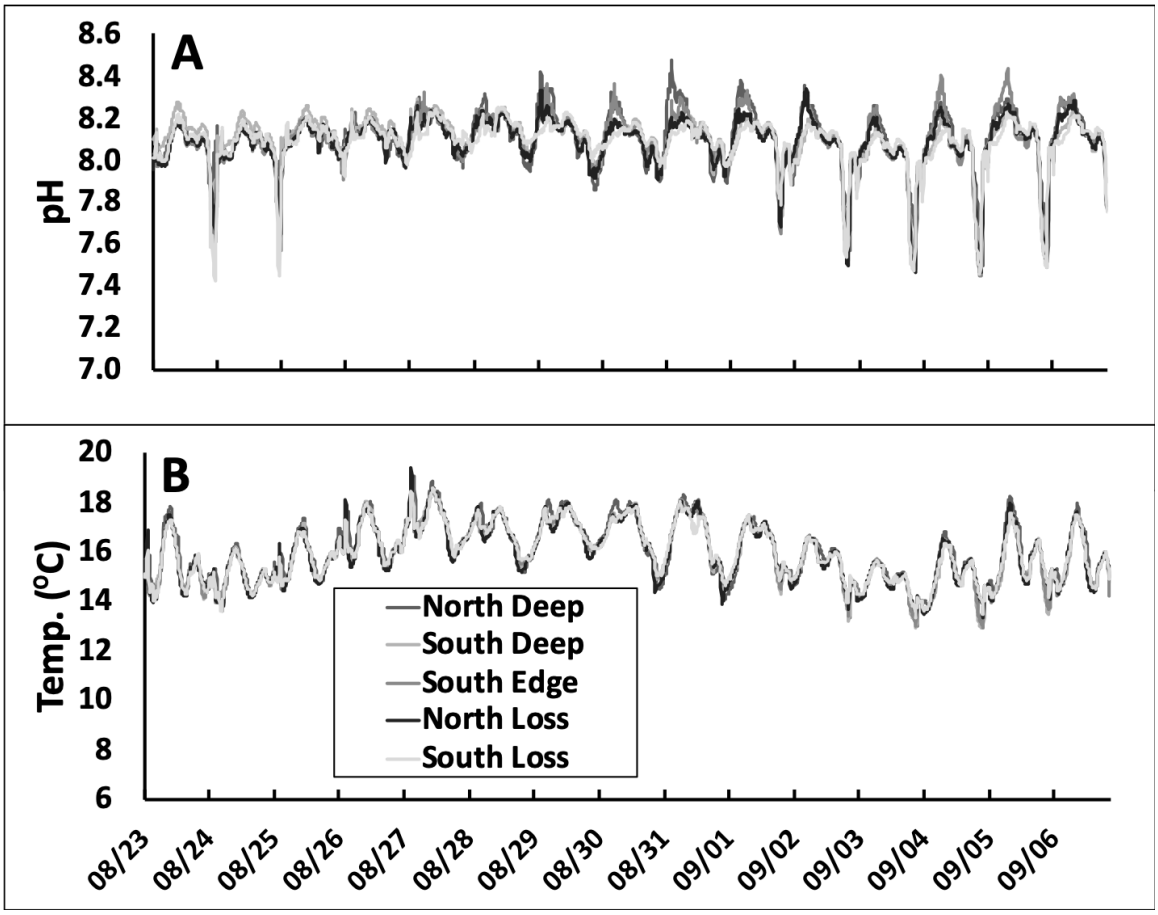

Figure 13. Field trial 2 timeseries data of (A) pH and (B) temperature at transect locations and positions within.

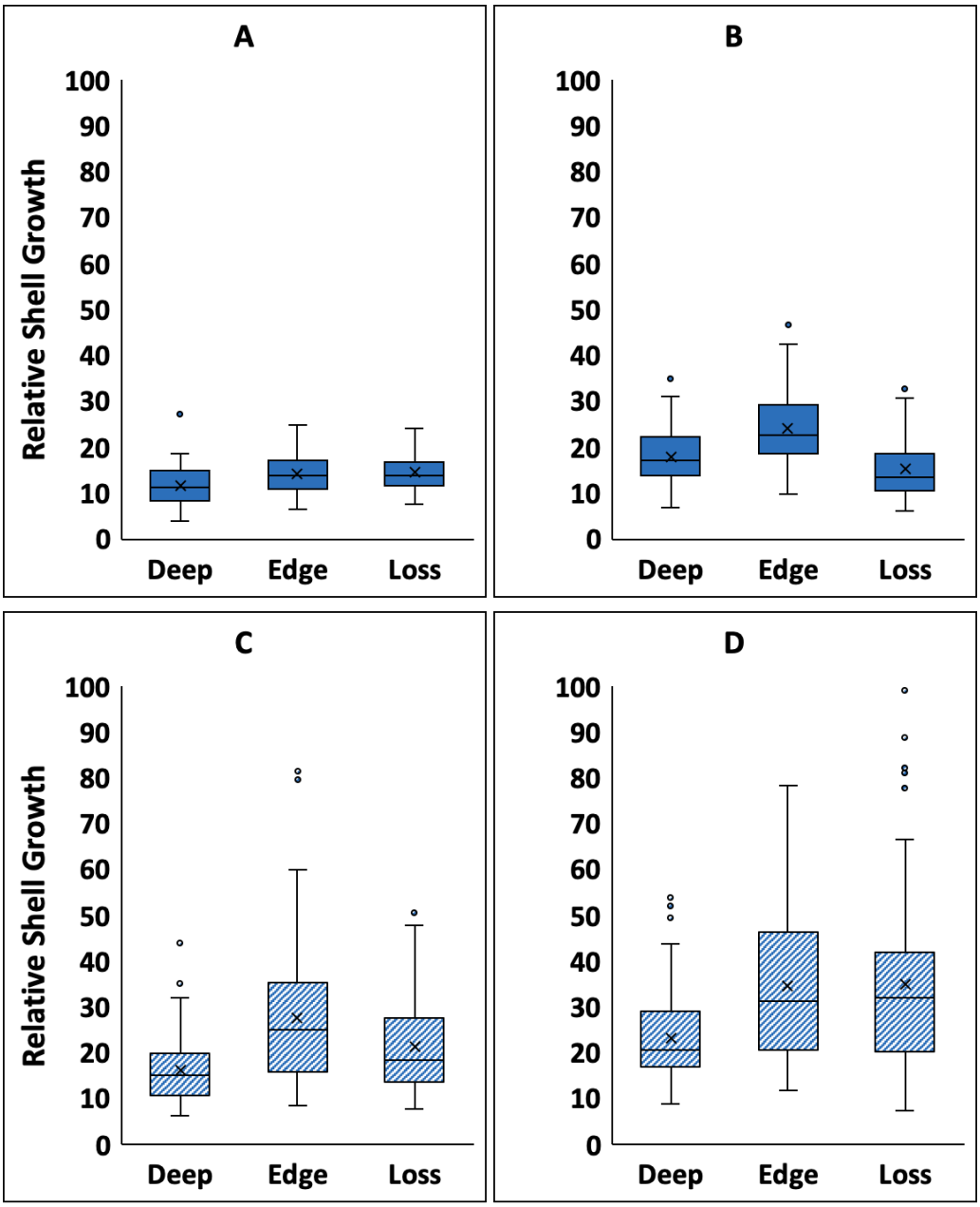

Figure 14. Box and whisker plots of relative shell growth by transect position, with panels representing trial and location;(A) for field trial 1 North and (B) for field trial 1 South; and (C) for field trial 2 North and (D) for field trial 2 South. Boxes span the interquartile range (IQR); horizontal lines within boxes represent medians, and means are represented with an 'X.' Whiskers extend to minimum and maximum values within 1.5 times the IQR of the box; points beyond the whiskers are outliers.

DISCUSSION

Intensifying OA means that it is crucial to investigate impacts on sensitive calcifying species, especially commercially important ones, as well as potential means of mitigating these impacts. My study aimed to provide important insight into how much eelgrass modifies carbonate chemistry and how much this may mitigate OA impacts on cultivated oysters. In my laboratory experiment I found that eelgrass increased seawater pH via net photosynthesis during the day, while net respiration at night decreased pH (though to a lesser extent), and that both effects grew stronger as the pH of incoming seawater decreased. However, there was no obvious effect of eelgrass on pH in the field, nor did eelgrass have a detectable effect on oyster growth in the laboratory or in the field. I will compare these outcomes with similar recent work and propose possible reasons for differences in findings including generally modest eelgrass effects. In the lab, oyster growth decreased with pH on both settlement substrates. Though oysters on shell flour grew fastest overall, they also saw greater and statistically significant decreases in growth in response to reduced pH. I will interpret these results, propose potential explanations, as well as further research needed to distinguish them. Finally, I provide some conclusions and practical implications of this research.

Methodological Issue: Differing Initial Sizes

The differing initial sizes of juvenile oysters in my laboratory experiment is a problematic source of experimental error and a confounding variable that significantly complicates interpretation of the results. The most parsimonious explanation for this is

likely accidental particle size sorting while dividing oysters into treatments. However, initial sizes based on calcein staining could not be independently corroborated because samples of oysters frozen at the beginning of this trial were lost due to a laboratory freezer failure. Such size sorting can result from granular convection (also known as the 'Brazil nut effect') in which smaller particles in a mixture tend to settle to the bottom leaving larger ones on the surface. To counteract this effect and ensure that initial oyster sizes are equal among treatments, juvenile oysters should be carefully resuspended, thoroughly mixed, and then divided into sub-samples using a plankton splitter or Stempel pipette.

While the use of relative shell growth as the metric of growth in my study corrects for different initial sizes to some degree, it cannot account for changes in juvenile oyster growth with size and development which are known to occur (Ernande et al. 2003). Thus, groups of oysters with different initial sizes should grow at different rates even in the absence of treatment. The difference in initial sizes is therefore a confounding variable that weakens the strength of inference that can be drawn about the effects treatments, but may also make detection of treatment effects more difficult. Future research should ensure homogenization during subsampling to avoid this problem.

Eelgrass Effects on pH and Oyster Growth

Lab experiment

In my laboratory experiment, I found that the effect of eelgrass on seawater pH (increased pH from net photosynthesis during the day and a smaller decrease pH at night

when there was net respiration) grew stronger as the pH of incoming seawater decreased (and the concentration of CO_2 increased). This is consistent with the hypothesis that seagrasses will benefit from the increasing carbonation of seawater (Beer and Rehnberg 1997; Zimmerman et al. 1997; Koch et al. 2013; Palacios and Zimmerman 2007; Hendriks et al. 2014). The range of eelgrass pH effects I observed is comparable to that seen in other studies (Koweek et al. 2018, Ricart et al. 2021b). My eelgrass aquaria density of ~190 shoots/m^2 lies intermediate to densities used by Ricart et al. (2021b) while the average seawater pH in their study (7.93) was closest to my High pH treatment (7.78). They saw pH increases of 0.04 and 0.06 units in their medium (115 shoots/m^2) and high (259 shoots/m^2) eelgrass density treatments. In the most comparable treatment in my study (High pH), I saw an average eelgrass-driven pH increase of only 0.01 units (Table 2); the eelgrass effect on pH seen in my study was only about 20% of what might be expected based on Ricart et al. (2021b). This difference cannot be explained by differences in residence time because both studies had similar time to complete water exchange: 153 min. in this study (110L aquaria with 12mL/s flow rate) versus 133 min. in Ricart et al. (2021b; 200L tanks with 25mL/s flow rate). Of the methodological differences between my study and that of Ricart et al. (2021b) including their use of outdoor tanks, raw flowing seawater, and rooting eelgrass in natural mud substratum the most plausible explanation for the difference in eelgrass effect is probably light intensity. While the artificial lighting in my indoor experiment provided an average daytime PAR of 124 $\mu E/m^2s$ at the surface, the outdoor tanks used by Ricart et al. (2021b) received natural sunlight and even the bottom of the tanks had PAR sufficient to support

maximum eelgrass photosynthesis (~200 μE/m^2s) in the bottom of the tanks; the PAR of sunlight on a clear day around noon in our region is around 2000 μE/m^2s. The comparatively low light level achieved by the artificial lighting (and consequently slower photosynthesis) in my experiment, likely explains the generally smaller effect of eelgrass on seawater pH and the lack of effect on oyster growth. Nitrate levels (~10 mg/L; G. Eberle, unpub. data) in the laboratory recirculating seawater system during my experiment were greater than the 20-30 μM (~1.2-1.9 mg/L) typical seen in Humboldt Bay (Swanson 2015) and the coastal waters of this region (Biller et al. 2013).

Even given the limited light availability in my study, the Low pH and especially the Extra Low pH treatment (with higher CO_2 levels) had stronger eelgrass effects and increased pH by 0.02 and 0.13 pH units, respectively (Table 2). Despite this, eelgrass did not drive a significant effect or even obvious trend in oyster growth in the laboratory. Though eelgrass in my Extra Low pH treatment increased pH more than Ricart et al. (2021b) saw at similar eelgrass densities, even with the increase in pH, the average was only 7.76 and so not conducive to shell-building. Generally faster eelgrass photosynthesis and larger increases in pH along with overall higher pH and carbonate saturation observed by Ricart et al. (2021b) were likely responsible for their finding of significant eelgrass effects on oyster growth, in contrast to the results of this study.

Another difference between the methods of Ricart et al. (2021b) and this study that may be relevant to oyster growth is the food provided; while they provided flowing natural raw seawater, I added microalgae concentrate because only recirculating filtered

seawater is available at the Cal Poly Humboldt Telonicher Marine Laboratory. Raw seawater is closer to natural conditions and may have contributed to faster oyster growth.

Field experiment

The lack of eelgrass effects in the field may be due in part to the small spatial extent of the study resulting in very similar conditions throughout the experimental area (pH, temperature, flow). The proximity of positions to one another (transects were only 50m long) may have resulted in all experiencing conditions too similar to drive any significant variation in oyster growth. A consequence of this small spatial scale is that tidal currents and mixing may have transported parcels of water across positions too quickly for significant differences in pH to arise among them. The effect of eelgrass on seawater pH and oyster growth was also likely reduced by the timing of the field study in late summer when eelgrass metabolic activity (both photosynthesis and respiration) is generally declining and the system is shifting to net respiration as eelgrass and other primary producers senesce and decompose (Abell et al. *in prep.*) due to falling light levels (shorter days, lower sun angle) and decreasing upwelling and nutrient levels. Stronger effects of eelgrass might be seen if this field experiment were repeated in the spring or early summer when upwelling is stronger and eelgrass productivity is generally higher.

Effects of pH and Substrate on Oyster Growth

Consistent with my hypothesis (that juvenile oyster growth would increase with increasing pH and carbonate saturation), there was a trend of declining oyster growth as

pH decreased, though this effect was only significant on the oysters settled on shell flour (Table 6). This pattern was expected because as pH decreases, so does the availability of carbonate required by calcifiers to build their shells, making growth slower and more energetically costly (Waldbusser et al. 2013, 2015). Another result that stands out is the contrasting pattern of oyster growth seen in flour versus chunk substrates: though oysters on shell flour grew fastest in the highest pH treatment, they also saw greater and statistically significant decreases in growth in response to low pH. Small-scale hydrodynamics including boundary layers may have played a role in both of these patterns.

Because boundary layer thickness varies as the square root of the characteristic dimension (e.g. length), the shell chunks settled with oysters (with a characteristic dimension of 20-30mm) should have a boundary layer that is 4-5 times as thick as that of oysters settled on shell flour (with a characteristic length of ~1mm) when subjected to similar, low flow levels (Schlichting 1979). Oysters are suspension feeders, and their growth has been found to vary directly with flow, which determines food availability (Lenihan et al. 1996). Thus, a thicker boundary layer with slower flows and reduced food availability was almost certainly a significant factor in the slower growth of oysters on shell chunks. Oysters on shell chunks may also experience greater competition for food (as well as space) among their closely spaced neighbors (Sebens et al. 2017).

However, one possible interpretation of my results is that the thicker boundary layer around the shell chunks may have provided some level of protection from low pH. Under low pH conditions, boundary layers may have elevated carbonate saturation that

protects calcifiers (Hurd et al. 2011, Cornwall et al. 2014; Hendriks et al. 2015) and their shells (Sulpis et al. 2022). Boundary layers with elevated pH have been found in coralline algae (Hurd et al. 2011, Cornwall et al. 2014), the application of shell hash to intertidal mudflats was found to mitigate OA in porewater in some situations (Doyle and Bendell 2022), and modeling suggests that aragonite (a more soluble form of calcium carbonate) may create a more saturated benthic boundary layer that protects calcite in seafloor sediments from dissolution (Sulpis et al. 2022). However, the hypothesis that shell chunks provide a protective boundary layer with enhanced carbonate saturation and may mitigate OA impacts remains a speculative possible explanation for my results, but suggests interesting avenues for further research. Such research could include explicit sampling and quantification of boundary layer chemistry around shell chunks, as well as measuring the growth of juvenile oysters in a range of pH treatments settled on chunks of calcium carbonate substrate with differing solubility (e.g. high-magnesium calcite, aragonite, low-magnesium calcite) as well as insoluble chunks of similar size (e.g. non-calcium stone) as a control.

Such further investigation is necessary to more conclusively test the hypothesis of a beneficial, carbonate-enriched boundary layer, because there are alternative explanations for the differing effect of pH on oyster growth on shell chunks versus shell flour. A thicker boundary layer that limits food availability appears to be the most likely explanation for the overall slower growth on shell chunks. However, the fact that oysters on shell chunks saw less reduction in growth as pH decreased has other potential explanations besides the protection of elevated carbonate saturation in the boundary

layer. Instead, pH effects on chunks could be small because food availability was too low to allow much increase in growth when pH was higher. Similarly, pH effects on chunks could be appear small because the pH effects on already food-limited oysters were so dire that their growth rate decreased only slightly toward some minimum value. However, I consider both of these alternative hypotheses relatively unlikely for the following reasons. Since the effects of OA and food on bivalve growth are largely energetic, within some range, the effects of low pH can be overcome with sufficient food (Waldbusser et al. 2013). Yet even on shell chunks, food was sufficient to allow oysters to increase their shell area by 7.6–11 times over the course of three weeks (Table 4, Figure 9). Lastly, rather than exhibiting reduced growth response toward some minimum value, juvenile oysters experiencing low pH and too little food would likely experience mass mortality (Barton et al. 2012, 2015, Waldbusser 2013). All this said, conclusively distinguishing among these hypotheses will require further research, such as the growth and pH experiment with oysters settled on chunks of substrate with different solubility suggested above.

Regardless, my results suggest that research conducted on shell chunk substrates or other calcium carbonate substrates that are large relative to the settled oysters may not accurately predict how industry oysters settled on shell flour will perform in low pH conditions. If additional studies provide more conclusive evidence that soluble carbonate substrates provide a high-carbonate boundary layer that protects oysters from OA, this could lead to the development of novel OA adaption and mitigation strategies for bivalve mariculture operations. One potential approach that might enhance boundary layer

carbonate saturation without greatly reducing flow or food availability would be to settle oysters on shell flour but mix in additional high-solubility shell flour particles. It might be possible to find an optimal quantity of such particles that would not greatly reduce flow or food delivery, but that, through close contact with growing oysters, would boost carbonate availability – particularly if the shell particles were composed of a form of calcium carbonate more soluble than the calcite of the juvenile oyster shells (e.g. aragonite or high-magnesium calcite).

This also has implications for the benefits of using oyster shell bags to create reefs for native bivalve restoration, shoreline protection, and sea level rise adaptation. There have already been some promising results from this kind of restoration effort in San Francisco Bay, Monterey Bay, San Diego Bay, Chesapeake Bay, and others (Waldbusser et al. 2011; Graham et al. 2016; Wasson et al. 2015, 2020). Oysters readily settle on oyster shell, a known settlement cue (e.g. Turner et al. 1994; Vasquez et al. 2013); my findings provide suggestive evidence that this shell may provide beneficial chemical modification. This is yet another area that could be informed by further research of the hypothesis that shell chunk dissolution in low pH creates a protective boundary layer with high carbonate saturation.

Conclusions

1) Eelgrass increased seawater pH via net photosynthesis during the day, while net respiration at night decreased pH (though to a lesser extent), and both effects grew stronger as the pH of incoming seawater decreased. As pH

declines with OA, and the effect of eelgrass on carbonate chemistry increases, eelgrass beds may become more important as refugia and their OA-mitigating ecosystem services may become more valuable. This is consistent with expectation that elevated CO_2 levels will allow faster eelgrass growth.

2) The increase in pH due to eelgrass is unlikely to completely counteract OA and may not always produce strong enough effects to enhance the growth of oysters and other calcifiers. Water flow and mixing including tidal currents may obscure eelgrass effects on pH, especially at relatively small spatial scales.
3) The growth of oysters and other calcifiers settled on large chunks of calcium carbonate substrate may be less sensitive to low pH due to the development of a boundary layer with enhancement carbonate saturation. Though consistent with the results of my laboratory experiment, the degree to boundary layer saturation was enhanced and their benefit to oysters remain hypothetical explanations – though they merit further investigation. If confirmed, this could suggest potential mariculture industry OA mitigation strategies, inform the use of shell reefs and hash for shoreline protection and restoration efforts for native bivalves and other calcifiers.
4) Oyster growth was slower on shell chunks than on flour across all pH treatments. This may be due to reduction in the availability of food due to the thicker boundary layer around the shell chunks, and potentially competition for space. Regardless of its true cause, this apparent tradeoff between space

and food competition with the likely enhanced carbonate saturation and reduced impact of low pH on growth, which reduces overall growth, may complicate efforts to reduce calcifier sensitivity to low pH through association with large chunks of calcium carbonate.

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