***Abstract—***

***Introduction—***

***Methods—***

**Study species—** The Eastern oyster, Crassostrea virginica, settle in dense aggregations forming subtidal coastal reefs along the eastern coast of North America (Gulf of St Lawrence to the Gulf of Mexico) (cite), along with the Chesapeake Bay and tributaries (as the only native oyster species) (cite). C. virginica prefer brackish or marine seawater in subtidal habitats, however, in warmer location they also form reefs intertidally (NOAA). Natural oyster reefs create important habitat for hundreds of species (cite), while commercial aquaculture growers favor C. virginica for X reasons (cite). We sourced oysters used in experiments from X brood location (settled X) that were grown to X size at a local shellfish farm (Hog Island Oyster Company, Tomales Bay, CA). Oysters were transported in chilled seawater from HIOC to the Bodega Marine Lab, where they were placed on flow-through seawater for 30 days and fed X, daily. Prior to arrival at BML, conditions in Tomales Bay were marine-dominated due to upwelling (cite), with many oysters still characterized as ‘dormant’ to professional farmers (personal comm.). We adhered oysters to X type plates using X marine silicone, which has negligible impact on mortality and growth rates (cite). We waited another 3 days following attachment to check for mortality and dislodgement.

**Seawater treatment conditions—** We exposed oysters to one of two salinity treatments and a unique [TA] in a pseudo-factorial design, where oysters (n = 49 per bin) were grown in tanks (ntotal = 12) of replicated treatments (n=2 per treatment). Within either ambient (S = 34) or low (S = 27) salinity conditions, oysters were exposed to one of three TA treatments. Oysters in ambient salinity treatments, were grown in elevated, X, or X TA conditions (X, X, X + SEs) whereas, oysters in low salinity treatments were exposed to one of three [TA]s (X, X, and X) likely to occur when seawater salinity is reduced by freshwater endmembers (High TA river [X], low TA river [X], hurricane/precipitation [X]) mixing with ambient seawater ([X] conditions).

**Experimental setup—** We quantified the relative influence of osmotic stress versus carbonate system stress on surface area shell growth in oysters by growing them in unique treatment conditions for 5 weeks and measuring shell and tissue growth responses in individuals. Exposure to treatment conditions was refreshed every three days (see X), at which time, measurements of seawater salinity, temperature, pH, and dissolved oxygen % saturation were measured with calibrated sensors (see X). Experiments occurred in a temperature-controlled room and largely in the dark, to prevent any influence of light changes to oyster gaping, thus feeding, behavior (cite). Oysters were exposed to light conditions similarly, once every three days when growth bin seawater was refreshed. We adhered individual oysters of similar size distributions (range: X – X, mean = X, SD = X) to individual plexiglass? plates (n = 49 per plate, n = X total) using marine epoxy (cite) and placed one plate in each growth bin, i.e., a unique seawater condition. Growth bin (13-L X brand) seawater was circulated continuously with aquarium pumps (Xgph) to ensure continuous access to food and oxygen. We fed oysters daily with X (concentration), following feeding guidelines from industry growers (personal comms.) to prevent food-limitations to growth.

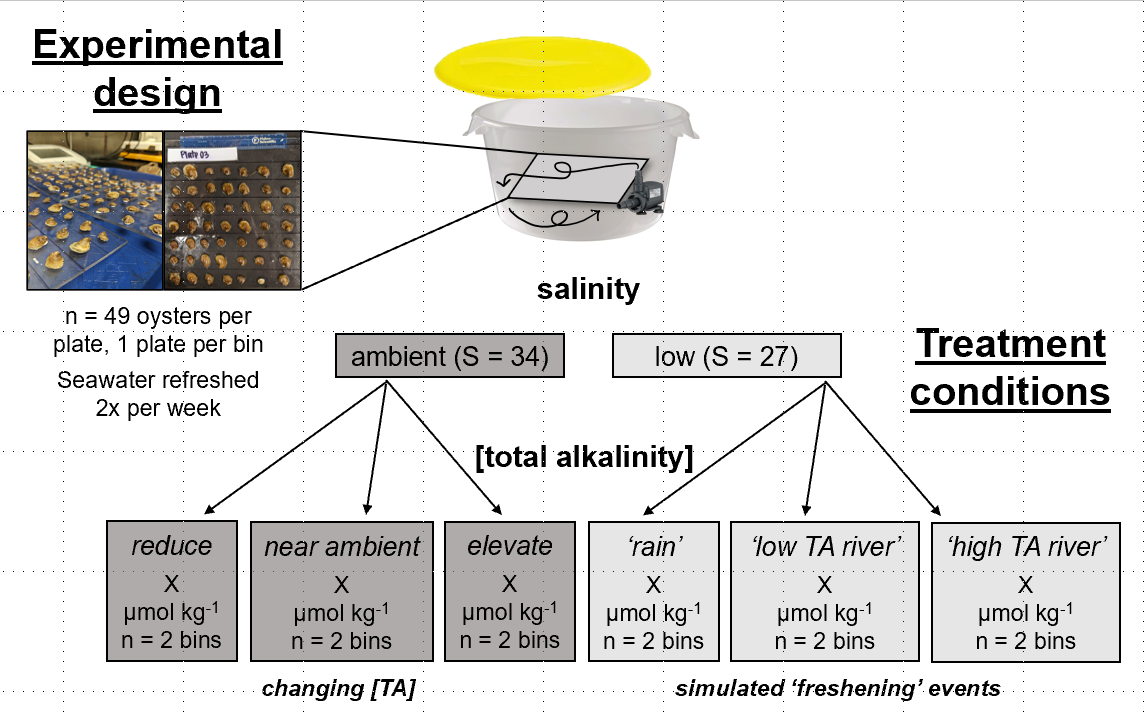


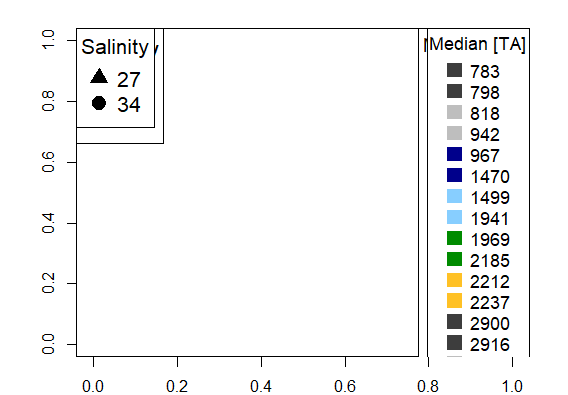
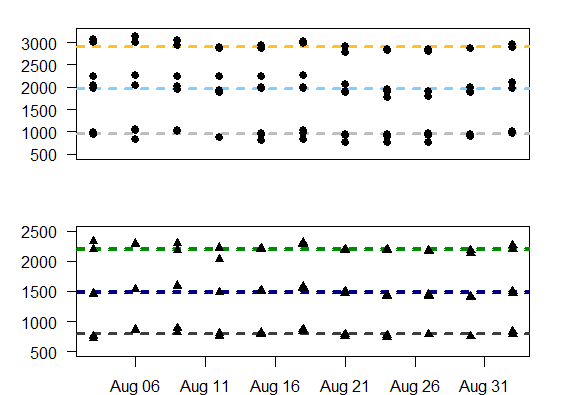
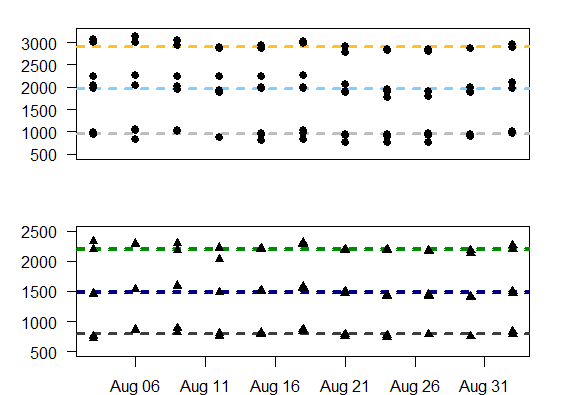
Figure X. Placeholder screenshot for optional schematic outlining experimental designs and showing photos of adhered oysters

**Estimating shell and tissue growth—** We measured shell growth as the surface area of the X side of the oyster, using Image J software to analyze photos of individuals. Photos of oysters were captured prior to the experiment (day 1), following 2 weeks (day 18) and after 5 weeks of exposure (day 36) using a XMP camera to capture top-view photos of each plate with a scale bar included for size. We used measurements of oyster shell growth (surface area mm2) to compute net growth rate and shorter, incremental growth rates between 0-18 dps (days post start), and 18-36 days. We sacrificed oysters after the experiment, and dried the shell separate from the tissue at 60°C for 48-hr. We measured dry tissue mass and dry shell mass on a microbalance (LOD = 0.0000mg) and used it to compute individual condition index (CI) values (Okumuş and Stirling 1998). We quantified individual condition index values as X, where CI values may indicate the relative availability for energetic effort (cite).

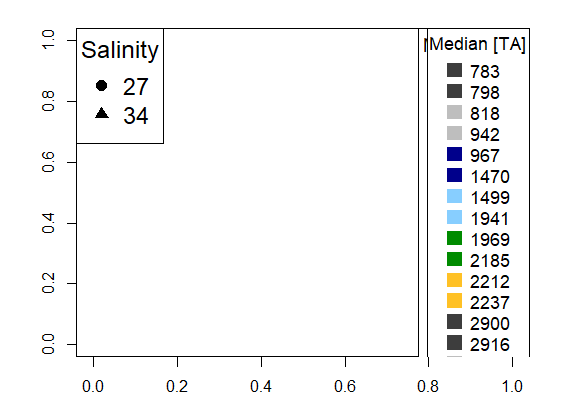
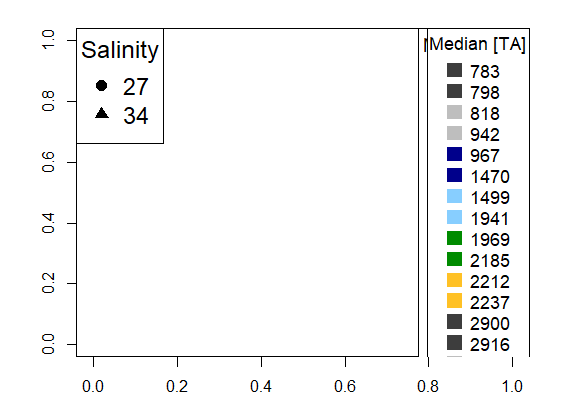
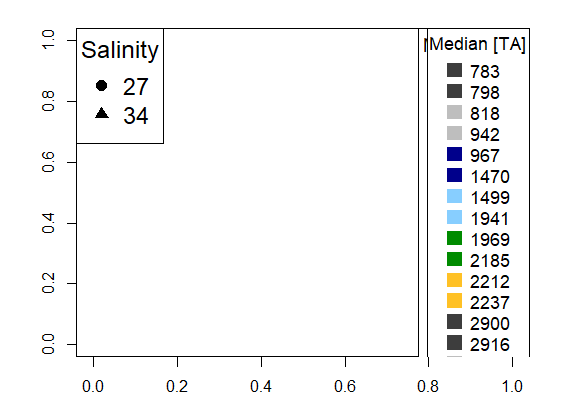
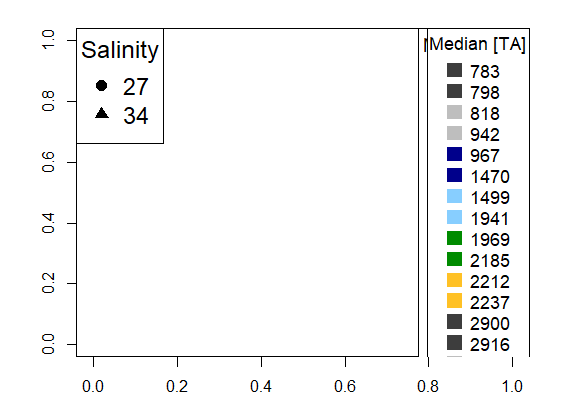
**Chemical manipulation of seawater—** We refreshed experimental conditions in the growth bins with chemically-adjusted seawater every three days for the duration of the experiment (n = 12 growth, n = 2 control bins). Prior to incubation adjustments, we reduced [TA] to undetectable amounts in filtered seawater (filter size) sumps (n = 4 sumps per water change date) by adding hydrochloric acid (HCl), to convert TA to CO2(gas), and then bubbling vigorously for 48-hrs, to off-gas CO2 and equilibrate seawater with atmospheric concentrations. In low salinity treatments, we diluted prepared seawater with deionized freshwater (milli-Q) to lower the salinity, and then adjusted the carbonate system with predetermined amounts of chemicals (NaHCO3 (sodium bicarbonate) + Na2CO3 (sodium carbonate) and HCl) to target specific [TA], while controlling seawater pH (Waldbusser et al. 2015, Ninokawa et al. In prep). Salinity minimally fluctuated over the duration of the experiment in both ambient and reduced treatments ( ) due to natural variations in lab flow through seawater. The reduced salinity treatments was roughly X% of the ambient treatment. In both salinity levels we were able to target three individual [TA]s, while keeping the refreshed pH above X, and average measured pH (between refreshed and day 3) above X in all treatments. Reported as the saturation state, treatments in low salinity, X, whereas treatments in ambient salinity X. Although temperature was largely controlled, temperatures were slightly cooler in refreshed seawater (X) than in day 3 seawater (X) conditions, however, these changes were observed in all of the growth bins. Oxygen concentrations remained high (mean = X SE) over the course of 3 days, ensuring oysters were free from stress that accrues in hypoxic conditions (cite).

**Characterizing experimental seawater conditions—** Prior to and immediately after refreshing individual bin seawater, we measured seawater temperature, salinity, and dissolved oxygen concentration with calibrated sensors (YSI blah blah blah) and pH spectrophotometrically and with the handheld multiparameter probe (YSI: pH blah blah). To report pH in total scale, we measured absorbance, calibrated daily with m-cresol dye standards (Easley and Byrne 2015). We measured pH absorbance in half of our sampling events at the same time as electrode charge measurements and applied the relationship to convert voltage measurements to pH. Concurrently, we collected and froze 250 mL of seawater for later total alkalinity concentration determination. We analyzed [TA] on a Metrohm 855 Titrosampler, correcting titration acid concentration daily using daily certified reference materials from the laboratory of Dr. Andrew Dickson (Scripps Institute of Oceanography). With two known parameters of the seawater carbonate system, in addition to salinity and temperature, we estimated the remaining carbonate system parameters (DIC, Omegacalcite, pCO2) using seacarb in RStudio with X coefficients (Table X, supplementary materials).

**Statistical analysis—**

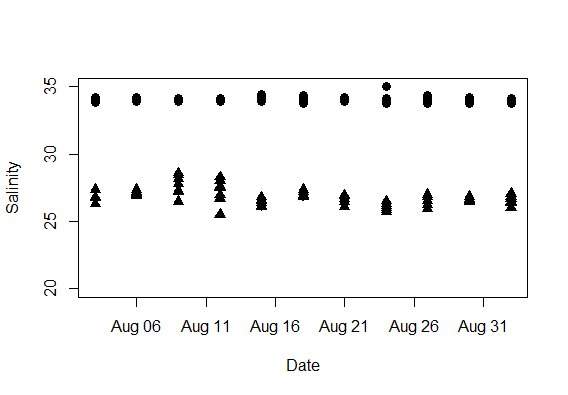
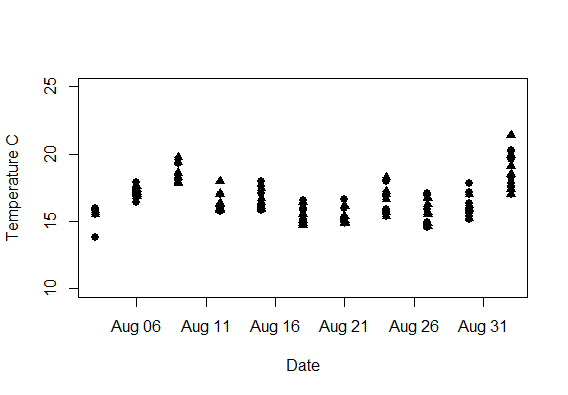


Total alkalinity (umol kg -1)



Temperature C

Salinity



Date (2022)

Figure X. Panels for total alkalinity conditions

Panels for T and S

***Results—***

**positive effect of [TA], no Influence of salinity, on net growth—** Our **net growth data appear to have a log relationship with [TA],** common in growth data (cite); in order to test with a linear model, we log transformed the response variable (net growth) before running the model. When including salinity, TA as fixed predictors alongside bin number as a random effect (grouping) we found that when comparing a wide range of [TA] and salinity, there is an effect of [TA] on net growth, but the effect of TA does not differ between high and low salinity exposure.

We can also see that over the duration of the experiment, there **is no significant influence of salinity** across the range of [TA]. No impact on CI. Net growth grate declined with [TA]

CI unimpacted by either factor.

**Effect of [TA] only observed in low salinity treatment ranges—** Net growth rates (unit defined) of juvenile *C. virginica* oysters were higher when seawater [TA] was elevated yet did not decline in severely low [TA] (Figure X, a vs. b). There was no difference in the growth rates of oysters grown in [TA] that are X% (X umol kg-1) of ambient seawater (2250 umol kg-1) and oysters exposed to [TA] that are X% (X umol kg-1) of ambient, indicating that oysters maintain similar, net growth in low [TA] with food available.

Now considering alkalinity change from a frequent source that also changes salinity (multiple stressor); for example, DI (hurricane/rainwater), diluted [TA], maintained [TA]; **to what extent does [TA] influence net growth during acclimation to low S conditions. Compare the highest growth ave with those from amb salinity?**

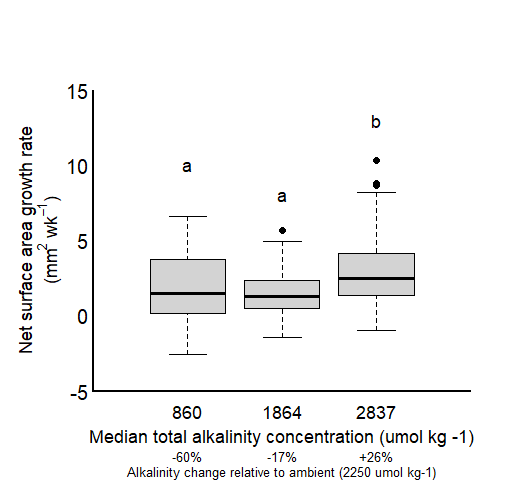
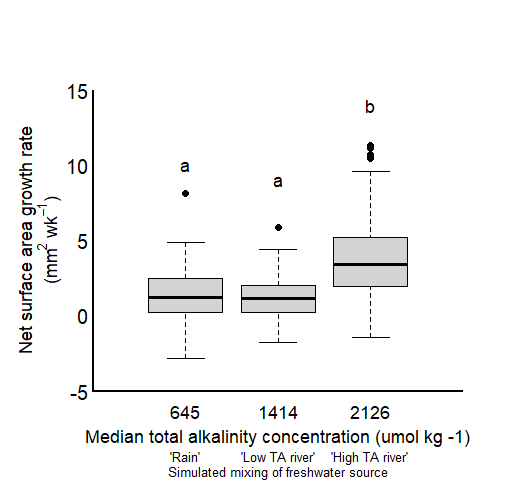


Figure X

**Incremental growth versus net growth rates—**

What does incremental growth say about how oysters are responding to environmental conditions?

Incremental growth declined in the second half of the experiment and did so regardless of treatment. The change in incremental growth was equal in all treatments. Therefore, the effect that we saw in our treatments was similar in both time intervals. Oysters, therefore responded to chemical conditions X, however, changed their growing patterns (intercept) for an unmeasured reason?

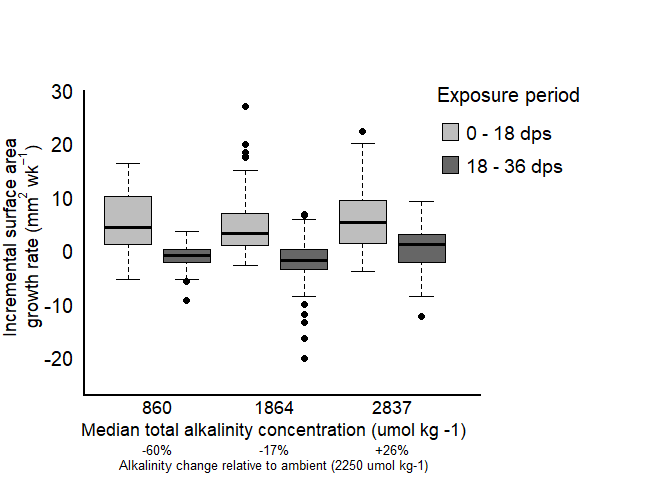
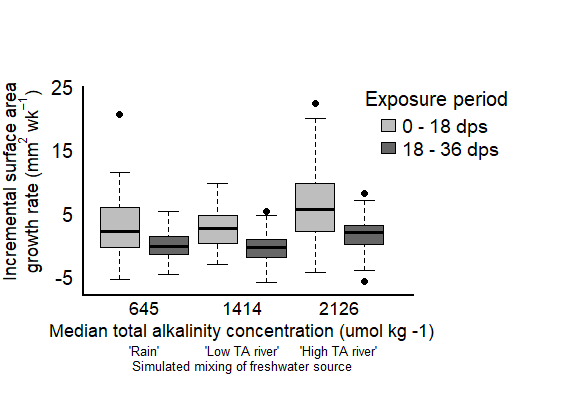


Figure X

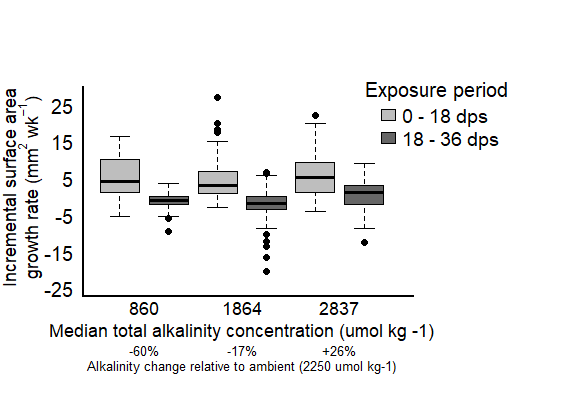
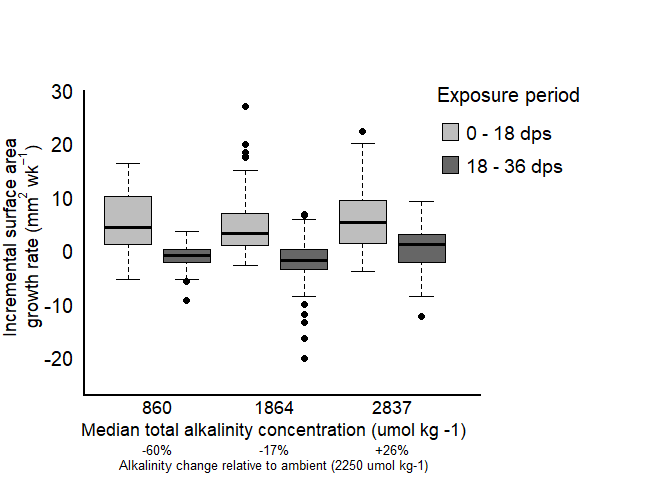


Figure X



***Discussion—***

Oysters repurpose periostracum material as ‘cement’ to adhere to firm bottom when settling naturally.-->changes in perio under OA or changed food/more maintenance costs.

coastal ‘living wall’ protection from sea level rise along X coast (cite). Shells have been posed as a potential mitigation tool for coastal acidification (cite) and are the preferred substrate for growers (cite), thus extending their importance after death.

Oyster growth was positively influence by [TA], in ambient and low salinity conditions

1. When changes in [TA] occurred in ambient S conditions X happened
   1. Which was largely driven by high incremental growth in the first two weeks, with incremental growth declining between week 2-5 in the ambient and high TA conditions…(Fig 5)
2. Oyster growth is maintained when alkalinity is severely reduced below ambient conditions (60% reduction) in ambient salinity(Fig 2)
   1. Which was largely driven by an increase in incremental growth **after** 2 weeks of exposure to low TA conditions (Fig 5)
3. We also saw X effect of changes to [TA] coinciding with abrupt acclimation to low S conditions.
   1. Oysters exposed to freshwater inputs that have elevated [TA], have higher growth than those exposed to rainfall/low TA rivers (Fig3)
      1. Which was largely driven by increased incremental growth in the first two weeks, with incremental growth declining between week 2-5…(Fig 5)
4. We do not observe an additional influence of salinity on top of the positive relationship with [TA], which may occur because oysters were fed often and not energetically limited.
   1. Often times we see a non-additive effect when stressors are combined. We would look at the first two week growth rate in the single vs multi stressor experiment to test this relationship. In fact, we see X in reduced [TA] with no change in S versus X when S was reduced with [TA].

Effect of TA in low salinity… how was this similar or different to mussel work?

Comparison:

Stevens and Gobler: CV

* low pH lowered growth rate
* low pH took away the negative effect of low DO when coupled (ie only see pH effect)
* *low DO coupled with warmer temp decreased survival*
* *higher temp led to lower tissue wt*
* *low pH, low DO and high temp led to lower tissue weight*
* **across high and low salinity data combined, we did not see an effect of pH on net shell growth in oysters (even though some treatments had med omega calcite values less than one). This suggests that they were well fed and robust. However, we do see a change in net growth when TA is elevated in contribution to TA. In our case, low S interacting with TA produced a higher influence than either change alone.**

Parter: Saccostrea glomerata

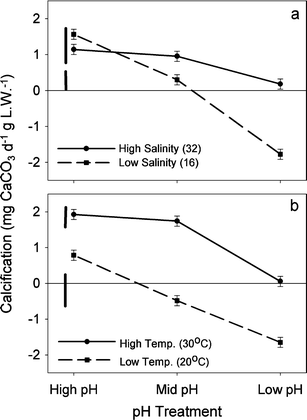
* high co2 acclimation led to significant metabolic depression
* acute exposure to elevated temperature and reduced S (and low TA) with the higher CO2 treatment led to extracellular acidosis

Dickinson 2012: CV

* A graph of different types of dry mass

  Description automatically generated with medium confidenceexposure to elevated pCO2 and or low salinity (and low TA) led to significant increase in mortality, reduction of tissue energy stores and negative soft tissue growth
* responses to high CO2 and or low salinity (and low TA) indicate
* oysters respond to these stressors by becoming energy deficient, or, juvenile oysters maintain their cellular energy status at the expense of energy stores
* Under the conditions of our experiment, low salinity (and low TA) is a greater single stressor than high PCO2, whereas the combination of these two factors produces greater changes in the physiology and shell properties of these mollusks than each of the factors alone
* **We did not see an influence of Ta on mortality. Low salinity and low TA (di or low TA river) trended to have reduced survival, but it was not significant at the salinities we tested (was not the aim of our study). We saw no impact of S or TA on energy stores, tissue growth, even though others have seen impacts of pco2 on these components. We did not see oysters becoming energy deficient (maintained similar gut tissue, higher than controls), but we did see activity decline. They found that their low S (and low TA) treatment was stronger than any of the high pCO2 treatments. When coupled with high pco2, low S (and low TA) had a greater impact on physio and shell properties than each factor alone. (impetus for researching?)**

Waldbusser 2011: CV

* comparing the effects of salinity (and TA) along pH treatments and temp treatments.
* High pH high the highest calcification (low S slightly higher and amb S), this also occurred at the highest TA
* The effect of high pH at high S is less than when coupled with decreasing S (and TA)
* *High Temp elevated calcification regardless of pH, and there was an independent decline in calcification from pH (similar to sal curve)*.
* The effect of pH at high S was minimal (black solid line)
* The effect of pH between S (and low TA) treatments were strong (dashed line)
* **We saw highest growth rates in the low salinity coupled with high alkalinity treatment, suggesting that oysters were able to optimize on their preferred condition quickly (effect was apparent by two weeks), with elevated TA becoming more important following the two week period, marginally.**
* **They didn’t see a strong effect of pH at ambient salinity; we didn’t see a strong effect of TA (and subsequently omega) at ambient salinity. The effect of diluting TA with freshwater (rain/hurricane) will also decrease the pH, and they showed it decreased calcification. We see that there is elevated growth when TA is elevated in low S, suggesting that the effect of low pH may not be as strong.**

Dickinson 2013: Hard shell clams

* Low S (and low TA) had profound effects on survival, energy metabolism and biomineralization of hard-shell clams
* Low S (and low TA) modulated clam response to high CO2
* Negative effects of low salinity (and low TA) were mostly due to the strongly elevated basal energy demand, indicating energy deficiency, that led to reduced growth, elevated mortality and impaired shell maintenance (evidenced by the extensive damage to the periostracum).
* Moderate hypercapnia (similar to 800 mu atm P-CO2) increased shell and tissue growth and reduced mortality high salinity exposures
* these effects were abolished under the low salinity (and low TA) conditions or at high P-CO2 (similar to 1500 mu atm)
* **similar: we saw trends of decreased survival in low S and low TA conditions only (but not profound effects like them). Difference: We did not see effects of low S and low TA on energy metabolism (ie gut wt). New material: we did see an elevated shell growth response to low S conditions coupled with high TA (they didn’t test). If low S doesn’t necessarily mean low TA, then estuaries may not experience the harmful consequences of high CO2. Moderate elevations in CO2 raised shell and tissue growth rates (maybe some sort of physio priming), like we see in low S and high TA?**

Gazeau 2013: Review

* in Benaish 2010 shell area was not impacted by high CO2 but shell mass lowered. This suggests that shells were thinner under high co2 conditions. As a note, their treatments spanned below omega calcite threshold of 1.
* (B) They suggest that shell area or length may not be sufficiently accurate as indicators of the effects of OA without measuring mass as well.
* **Our shell area was also not impacted by varying TA (and omega), which correlate with CO2. We did NOT see an effect of low TA on shell mass (ie shells were not thinner). Both our treatments and their span a range of omega values above and below 1. Suggest that just area or length may not be sufficient without understanding of mass.**

Hollarsmith 2019): Ostrea lurida and Crassostrea gigas

* the influence of carbonate system parameters, temperature, salinity, dissolved oxygen (DO) gradients is contingent upon the location in the estuary as well as seasonal timing (Hollarsmith 2019).
* During upwelling events (dry season), temperature, carbonate chemistry, and DO had the greatest impact on oyster performance. (Hollarsmith 2019)
* During runoff events (wet season), gradients in salinity, nutrient concentrations, and total alkalinity driven by river discharge were comparatively more important. (Hollarsmith 2019)
* the spatial importance *of carbonate chemistry and temperature* are seasonally variable and are two of several other factors that determine oyster performance. (Hollarsmith 2019)
* **Context for performance patterns overall: Performance was highest in upwelling season and declines following. We were between upwelling and river events and so there is some sort of transition in physio that is occurring such that, salinity, nutrients and TA are becoming more important. Future work should consider interval effects at different seasons, or consider outplants like these for CV in TX. Maybe because we were in a transition point, we saw depleted growth rates with time (coming down from upwelling?)**