Salinity stress cross-tolerance of *Ostrea lurida* after exposure to acidified ocean conditions in a simulated freshet discharge

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Abstract

Ocean acidification is widely regarded as a serious danger to marine organisms, especially calcifying invertebrates, but how this might interact with other abiotic stressors in realistic environmental events is less well modeled. Low salinity events in Washington state caused by snowmelt may increase in severity due to climate change, adding another stressor onto the backdrop of an already acidifying ocean. To test the effect of these asynchronous multi-stressors, we acclimated Olympia oysters *Ostrea lurida* to a gradient of pH conditions, after which we restored the pH and exposed them to low salinity, simulating a river freshet discharge. Our data indicate that shell thickness and respiration rates decrease with pH, regardless of salinity shock conditions. Calcification rates decreased with pH for oysters exposed only to ambient salinity, while calcification rates trended towards an increase in oysters exposed to the salinity shock. These findings could indicate a possible cross-tolerance in *O. lurida*, where exposure to increasingly acidic conditions could prepare them for short-term hyposaline shock.

Introduction

Increasing amounts of anthropogenic stressors are greatly impacting our ocean’s ecosystems. As the consequences of global warming start to take effect - such as increased temperature, low pH, high sea levels, and changes in precipitation- the organisms in our ocean are becoming exposed to increasingly taxing conditions (IPCC 2022). However, it is uncommon that organisms experience these stressors in isolation. Especially now in our changing world, stressors tend to act in tandem with each other, often inducing different effects on the organisms than if the stressor were isolated. Further, these effects also rarely occur simultaneously, instead usually being dictated by asynchronous natural events. For example, in San Francisco Bay,
temperatures during the winter and spring are expected to rise, consequently leading to earlier
snowmelt and increasing the frequency of low salinity events in the short term (Cloern et. al 2011). These low salinity events therefore will occur in areas that have already been exposed to
heat stress. It is incredibly important to understand how these interacting factors play a role in
the health of our ecosystems, yet most experiments studying multiple stressors tend to assume
that they happen synchronously. This does not provide applicable results, as it fails to consider
that an organism’s response to prior stressors may affect how it responds to subsequent stressors
(Agrawal and Jurgens 2023).

An organism’s response to multiple stressors can vary greatly depending on the time scale at which these stressors presented themselves in the environment (Gunderson et. al 2016). Effects can either be additive, synergistic, or antagonistic. Additive effects occur when the effect of multiple stressors is the sum of the individual effects. Synergistic effects of multiple stressors have a multiplicative impact, while antagonistic effects mitigate each other (Todgham and Stillman 2013). Synergistic effects most often arise when multiple stressors occur simultaneously or with very little time in between them. On the contrary, stressors that occur asynchronously over a long period of time tend to show additive effects, while asynchronous stressors that occur over a short period of time mostly show antagonistic effects (Gunderson et. al 2016). Antagonistic effects tend to arise when the time between the two stressors was long enough for the organism to repair the damage, yet short enough that the response to the previous one is still in place, better preparing it for subsequent stressors (Gunderson et. al 2016). This phenomenon is known as cross-tolerance, which has an incredibly important role in the interactions between biotic and abiotic factors (Todgham and Stillman 2013).
As global warming drives stressors past intensity thresholds, the concept of cross-tolerance is becoming increasingly relevant. The frequency of high stress events is drastically increasing, and with it, multi-stressors interactions are becoming more common (Perkin-Kirkpatrick and Lewis 2020, Deshpande et. al 2021). Ocean acidification is the backdrop for many of these interactions. As the ocean increases in carbon dioxide uptake, calcium carbonate concentration in the water declines. Organisms that use calcium carbonate to build their shells and skeletons such as molluscs or corals are particularly affected by the lack of building material (Fabry et. al 2008). Still reeling from the low pH, the introduction of new stressors on top of that could induce many synergistic, additive, or antagonistic effects (Gobler and Baumann 2016).

While ocean acidification is a well-documented inhibitor to many calcifying organisms, more multifaceted climate change events are less understood (Medeiros and Souza 2023). As anthropogenic carbon emissions and associated warming continue to increase, oscillation events driving atmospheric variability are expected to have increased severity in warmer months, in turn altering precipitation and nearshore hydrology (Hao et al. 2017). Climate indices explain up to 58% of hydrological variations, and with decreasing expected annual runoff, spikes in atmospheric temperature may create more severe snowmelt events and cause short-term hyposaline conditions in nearshore ecosystems (Rasouli et al 2020). While organisms are contending with other factors such as high pCO₂, a short-term event of relatively low salinity could prove synergistically stressful- yet such changes in salinity caused by altered freshwater inputs are expected in changing ocean conditions (Korhonen et al. 2013). While exposure to a single factor can increase tolerance or even exhibit cross-tolerance in a population, simultaneous exposures to stressors have been shown to significantly reduce bivalve growth rates and increase mortality (Gobler et al 2014).
*Ostrea lurida* is a severely threatened species in the Pacific Northwest, with a 40% reduction in historic range and most populations being over 90% lost or functionally extinct (Gillespie 2009, Beck et al. 2011). While they tend to live in estuaries and sheltered waters, they are more far more tolerant of full-strength seawater compared to brackish or freshwater, with up to 100% mortality when exposed to 5ppt salinity water for 2-3 weeks (Cook et al 2000, Pritchard et al. 2015). Despite commercial overexploitation, *O. lurida* are still important for local tribal subsistence and harvesting (Baker 1995, Groth and Rumrill 2009). They also form reef habitats that act as larval nurseries and promote biodiversity, while also mitigating shoreline erosion (Baker 1995, Lenihan et al. 2001).

*O. lurida* are known to show stunted growth in acidified conditions, but how they react to salinity stress post-acidification is still unknown (Storie 2016). The objective of this study is to observe the impacts of salinity shock events on *Ostrea lurida* calcification and condition in acidified environments as would be observed during a river discharge in the Salish Sea. We hypothesized that oysters in more acidic conditions will exhibit cross-tolerance towards acute salinity stress due to improved calcification pathways. Further, their net calcification rates will remain relatively unchanged before and after salinity shock, while oysters in ambient pH will have greater dissolution rates. While oysters may compensate for higher dissolution rates with greater calcification rates, it may come at the expense of gonad production. Therefore, condition index in these oysters - which is the visceral gut weight divided by the total weight of the oyster - may see more change than oysters in less acidic conditions. Likewise, we hypothesized that respiration would decrease with both acidic conditions and lowered salinity, due to stress inhibiting oysters’ gaping responses.
Methods

Our study was conducted from May 18th to May 25th, 2023, at the University of Washington Friday Harbor Labs. In order to determine oyster response to low salinity after exposure to acidic conditions, we established a gradient of pH treatments which would each experience a short term hyposaline shock event. We used linear models of calcification and respiration rates, condition index and shell thickness to compare oyster responses to salinity shock across the pH gradient. Prior to treatment administration, oysters were collected from Westcott Bay, San Juan Island on February 6th.

Seawater Conditions

Oysters (n=60) were divided into 6 flow-through water baths which each held 2 tubs of oysters (n=5) and allowed to acclimate to lab conditions for 1 week. Temperature was maintained at 15°C throughout the duration of the study. After the 1-week acclimation period, each bath was brought to a different pH (7.1, 7.25, 7.4, 7.55, 7.7, 7.95). The seawater pH was controlled with Honeywell controllers that monitored pH with Honeywell Durafet III’s calibrated on the total scale with simultaneous measurements of spectrophotometric pH. The controllers added compressed CO₂ via solenoids to maintain the target pH. Following three months in the pH treatments, oysters were returned to ambient pH (pH = 8.0) for 12 hours as pH in the region tends to increase during freshet discharge. Then, one tub in each bath was maintained at ambient salinity conditions (29.4 ppt) while the other was exposed to a low salinity shock event (22ppt) that lasted 30 hours.
Chemical Analysis

Oyster incubations were performed after the 3-month acclimation period to assess pre-shock condition, and again after the 30-hour salinity trial. Oysters were placed in sealed incubation vessels for 1.5-2 hours in a specialized water bath outfitted with integrated stir plates designed to keep the oysters at 15°C. Each oyster in a tub was incubated individually. A stir bar prevented the formation of boundary layers within the jars during the incubations, and oysters were suspended above the bar with rigid mesh. Control incubations (1 per tub) lacking an oyster were measured alongside each group of oysters. Oysters were kept in the dark during the incubations to improve their gaping behavioral response and ensure calcification and excretion.

Salinity was measured prior to the incubations with a handheld Hach HQ Series pH probe. Initial pH, temperature, and dissolved oxygen of the incubation water were measured with a PyroScience Firesting PRO probe kit. One sample was collected for all initial incubation data in a tub. Measurements for all incubation vessels were performed after incubations. Initial and ending ammonia samples were also collected to be analyzed in a Spectronic Genesys8 spectrophotometer. Additionally, starting and ending alkalinity was measured with a Mettler DL15 Titrator.

Respiration rates during the incubations were calculated by subtracting post incubation dissolved oxygen content from initial vessel dissolved oxygen and normalizing this change to the duration of the incubation and each individual’s biomass. Calcification rates were calculated as the change in alkalinity in the vessel water mass before and after the incubation and were then normalized to the duration and each individual’s biomass. After accounting for the effects of ammonia excretion during the incubation as described by Gazeau et al. (2015), these alkalinity measurements were used to create a model for calcification rate.
Biometrics

The buoyant weight of each oyster was calculated by submerging a hanging wire basket attached to a scale in 1000 mL of saltwater. A small glass weight of known density was used to calculate seawater density during the measurements. During this time, the length, width, and height of each oyster was also measured and used to scale oyster biomass.

Dissections were performed immediately after the second salinity shock trial in order to obtain condition index as a metric used to quantify individual oyster health. Oysters were shucked and the gonads and visceral guts were separated into weigh boats of known weight. Guts and shells were then placed into an oven for 12 hours so that dry weight could be measured for condition index, which was calculated by dividing the dry visceral gut weight by the dry weight of both the gut and the shell. Shell thickness was also measured at the margin of both the top and bottom shell halves using an iGaging double pointed micrometer.

To test the effect of pH and salinity exposure on calcification and respiration rates and on condition index and shell thickness, linear regression models were created in RStudio v 4.3.0. Dissolved oxygen concentrations data were processed using the packages marelac and seacarb.
Results

Physiological Processes

Oysters displayed a near significant change in condition index in response to the pH of the coolers (p = 0.0875), displaying a slight negative relationship between pH and condition index. However, there was no significant relationship between the condition index and the salinity of the treatments (Figure 1a). Shell margin thickness significantly decreased with pH (p < 0.001). Salinity also had a significant effect on thickness (p = 0.049), with the shell margin of oysters in low salinity water (22 ppt) being thicker on average than that of the oysters in ambient salinity (29.4 ppt) (Figure 1b).

Figure 1: Linear model comparing (a) condition index and pH (b) shell thickness and pH. Red data points and trendline indicate low salinity treatments and green data points and trendline indicate ambient salinity treatments.
**Figure 2:** Linear models comparing (a) calcification rate and biomass with a line delineating net calcification versus dissolution at y=0. (b) Respiration rate and biomass, and (c) calcification rate and respiration rate, with a line delineating net calcification versus dissolution at y=0. Red points and trendlines indicate low salinity exposed oysters and green represents ambient salinities.

**Figure 3:** Linear models comparing pH treatments to (a) respiration rate and (b) calcification rate, with a line delineating net calcification versus dissolution at y=0. Red points and trendlines indicate low salinity exposed oysters and green represents ambient salinities. Each set of oysters at a given pH treatment is represented by a mean value with standard error bars.

**Biochemical Processes**

Oysters showed a significant positive correlation between mass specific calcification rates and biomass, regardless of salinity treatment (Figure 2a, p=0.031). The oysters also showed negative correlations between mass specific respiration and biomass (Figure 2b, p=<0.001) and between calcification and respiration (Figure 2c, p=0.006). Respiration was also found to decrease with pH, regardless of salinity (Figure 3a, p=0.038). Calcification rates were found to be near significance, trending towards increasing with pH in ambient salinity conditions and...
decreasing with pH in low salinity conditions (p=0.054). As seen in Figure 3b, the effect of salinity on calcification rate was also near significance (p=0.052). Further, the interactive effect between pH and salinity was found to be near significance (p=0.052).

**Discussion**

**Physiological Processes**

According to the data, *Ostrea lurida* appears to be highly resistant to changes in pH and salinity, being able to maintain a mostly stable condition index throughout a high range of conditions. This does not necessarily support the cross-tolerance we hypothesized, as the oysters in low pH conditions seemed unaffected even before the salinity trials. This could also suggest that 30 hours in low salinity was not enough time to affect the condition index of the oysters. However, it is enough time to affect the shell thickness. The results for pH follow the typical trend of decreasing shell thickness by dissolution with decreasing pH, but the salinity results show an unexpected pattern; oysters in low salinity treatments show near significant rates of dissolution, yet high thickness at the same time. One possible explanation for this could be that with higher dissolution, the margins of the shell begin to erode. As a consequence, the area that was measured as the margin was actually less recent growth that the oysters had been building on for a long time.

**Biochemical Processes**

Our results indicate that mass specific calcification and respiration rates are inversely correlated, changing differentially with overall oyster biomass (Figure 2). In both salinity conditions, net calcification generally fell below 0, indicating overall shell dissolution during the
experiment. Any changes in this dissolution rate could be attributed to mitigation by an individual calcifying or by abiotic factors. Calcification was found to decrease with respiration, indicating that the efficiency of calcification pathways may increase with size, or that there are other factors that may govern both rates such as external morphology. While the energetic products of respiration are what stimulate the organic calcification process, our data show that these rates are not positively correlated (Nelson and Altieri 2019). By having a lesser surface area to volume ratio, a large oyster would be dissolving at a slower relative rate than a small oyster, and while it may respire at a lower rate, its perceived output of net calcification may be greater. Further study would be required to distinguish an abiotic dissolution signal from individual calcification efficiency.

A decreased demand per unit biomass of oxygen as well as their relatively higher net calcification means larger oysters may be favored in changing environments. Small oysters, including juveniles and especially larvae, may be especially at risk. In one 2019 study, oyster larvae were shown to have reduced growth rates (Venkataraman et al. 2019). Male gametes *O. luridia* have also been shown to develop less after high pCO$_2$ exposure, possibly indicating synergistic stress on oyster recruitment rates (Spencer et al 2019).

Neither respiration nor calcification as a function of biomass were directly impaired by salinity, supporting our null hypothesis (Figure 2). When only approaching this single variable, its non-significance can likely be attributed to the species’ ability to tolerate a range of pHs and brief exposure to lower salinities (Peter-Contesse and Peabody 2005). When viewed in the context of more acidic conditions, however, the effects of salinity may be relevant to oyster calcification. In both treatments, respiration decreased with pH (Figure 3a). This likely is due to
the greater overall stress and a reduced gaping behavioral response, which supports our hypothesis.

The insignificance of the salinity treatment on the results indicates that a freshet discharge has no impact on total oyster energy consumption. The use of this energy may differ between treatments, however. The near-significant p values indicate that with increased acidity and hyposaline exposure, calcification rates may be a function of these combined abiotic factors (Figure 3b). The oysters in ambient salinity conditions follow the expected trend of decreased net calcification with decreased pH. However, the oysters exposed to low salinity show the opposite trend, which could be due to a possible cross tolerance. When exposed to acidic conditions for a prolonged period, the oysters at lower pH may have developed more robust calcification pathways, and even when exposed to the different stressor of salinity, they are more able to sustain shell construction.

Notably, the salinity stress coincided with a pH change to ambient conditions. In this way, one stressor was removed and replaced with another, possibly allowing these more pH stressed oysters to rebalance their energetic budget and allocate more to energy towards calcification. Oysters kept at higher pHs which were then exposed to salinity stress may have been less developed, and thus their dissolution increased relative to more and to ambient-salinity oysters at the same pH. With this, we fail to reject our null hypothesis that oysters in more acidic conditions will not exhibit cross-tolerance towards acute salinity stress, but the near significance indicate the possibility of such a signal if exposed to greater stress.

Ecological Implications
Our results demonstrate that low salinity events may not prove entirely detrimental to the health of *Ostrea lurida*. Our experiment was designed to mimic a low salinity event that occurred in the Salish Sea during the duration of our experiment, meaning that our results directly reflected the response of the oysters in the wild. This is promising, as little significant change was observed over the 30 hour period, and the mean duration of low salinity events in the ocean is typically 2 days (Poppeschi et. al 2021). This is important from a conservation standpoint. As the severity of hyposaline events may increase in the future, it is crucial that *O. lurida* is able to withstand the stress from both the low salinity and pH, and our results seem to show that they are mostly unaffected by current conditions.

While oysters exposed to more acidic conditions may be better equipped to respond to hyposaline stress events, these results are less conclusive than the impact of size on calcification success. Especially given their teetering population statuses, competition with larger oysters could prove dangerous. Unlike our hypothesis stated, the stress caused by short term freshwater influxes is negligible by most metrics used in this study. Freshet discharges may come with other risks, however; a 2010 study on mussels suggests that areas prone to freshwater influxes, such as estuaries, are at greater risk of contaminant bioaccumulation due to the increase in water solubility of pollutants (Hamer et al 2010).

Further investigation of freshet discharge on oysters is required, but other metrics such as pollution may be of interest. By the 2040s, Spring snow water runoff is projected to decrease by approximately 38–46% in Washington State (Elsner et al. 2010). This could drastically alter the concentration of terrestrial elements in the nearshore environment or could possibly result in less frequent but more severe freshet events, which may carry agricultural pollutants (Danilov-Danilyan et al. 2020). Ultimately, *O. lurida* proved capable of withstanding the asynchronous
low pH acclimation and subsequent salinity shock event and was not stressed to the point cross-
tolerance became recognizable. This reflects their ability to endure *in situ* freshet events even in
future acidified conditions, but other dangers of pollution and recruitment failure still demand
careful monitoring of their populations in a changing ocean.
References


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