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Probing the role of carbonic anhydrase in shell repair mechanisms in the eastern oyster *Crassostrea virginica* under experimental acidification stress

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ABSTRACT

The reduction in pH from atmospheric inputs of CO_2 (ocean acidification, OA) threatens marine calcifiers, including the eastern oyster (*Crassostrea virginica*), that precipitate biogenic $CaCO_3$ for shell formation. Recent investigations have demonstrated that alterations in gene expression enable bivalves to respond to episodic low pH. Evidence generated from several studies highlighted the importance of upregulating genes related to biomineralization, ion transport, and acid-base balance such as carbonic anhydrase (CA) genes. Two experiments were designed to evaluate the effect of acidification on calcification processes and to probe the specific role of CA in oyster resilience to low pH. First, adult oysters were exposed to eight months of chronic acidification stress (pH ~7.3, pCO_2 ~3300 ppm) or control conditions (pH ~7.9, pCO_2 ~500 ppm) before shells were artificially damaged and shell repair monitored. Results showed a dramatic decrease in shell regeneration after chronic high pCO_2 exposure (only 30% of oysters regrew any shell) suggesting that mechanisms that promote calcification under high pCO_2 conditions may not be sustainable for extended periods of time. To further explore these mechanisms, a second experiment was designed by focusing on the role of CA in mitigating acidification stress. Here, adult oysters received an injection of acetazolamide in dimethyl sulfoxide (DMSO) to inhibit CA or DMSO (control) before rearing in control (pH ~8.1, pCO_2 ~340 ppm) or acidified (pH ~7.3, pCO_2 ~3300 ppm) conditions. After three weeks, oyster shells were damaged and shell repair monitored. Oysters incubated at low pH seawater with CA inhibition had the least amount of shell regeneration at the end of 21-day regrowth period. Interestingly, oysters were able to increase intracellular pH (pH_i) of hemocytes under low pH conditions; however, this ability was significantly diminished with CA inhibition. Results highlight the role of CA in maintaining calcification under low pH conditions by establishing an intracellular environment favorable to calcium carbonate precipitation.

1. Introduction

The eastern oyster *Crassostrea virginica* is an ecologically and economically important species, which provides many beneficial ecosystem services including water filtration, habitat formation, shoreline stabilization, and supports fisheries and aquaculture (reviewed in Beck et al., 2011). It is estimated that there has been an 85% loss of oyster reefs globally (Beck et al., 2011). While many conservation measures and restoration efforts of oyster reefs are in effect, these are undermined by the reverberating impacts of climate change, including ocean acidification (OA). As atmospheric carbon dioxide (CO_2) levels have risen since the Industrial Revolution, so has the flux of CO_2 into the ocean. This has resulted in alterations of the carbonate chemistry of seawater, including a marked reduction in the availability of carbonate ions (CO_3) and decreased saturation states of calcium carbonate ($CaCO_3$) minerals. The estuarine habitats of the eastern oyster experience additional coastal acidification from sources beyond atmospheric inputs including freshwater run-off and nutrient discharge (Wallace et al., 2014; Baumann et al., 2015). Acidification can negatively impact organisms that precipitate biogenic $CaCO_3$ to form their exoskeletons, including marine bivalves. Many studies have demonstrated the vulnerability of marine shell building organisms to changes in carbonate chemistry of seawater (Fitzer et al., 2016; Liu et al., 2016; Zhao et al., 2017; Su et al., 2018; Schwaner et al., 2020).

However, there is much variability in responses to OA, and calcifying organisms have shown negative, positive, or neutral impacts of acidification on calcification and growth (Duarte et al., 2015; Frieder et al., 2017; Kurihara et al., 2018; Cameron et al., 2019). Some organisms

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might be better suited to responding to OA because they are found in habitats that experience natural conditions of low pH or variable carbonate chemistry. The eastern oyster is found in estuarine environments that already experience coastal acidification (Wallace et al., 2014; Baumann et al., 2015). Measurements of pCO_2 in a tidal salt marsh on Long Island, NY fluctuated from ~330 ppm and ~1200 ppm (early May) to ~4000 ppm (end of July) (Baumann et al., 2015). In the extreme case, diel changes in pH exceeded one unit of magnitude ($\Delta pH \geq 1$) between June and September (Baumann et al., 2015). Animals already exposed to these conditions could be locally adapted and have mechanisms to mitigate the impacts of low pH. For example, the soft-shell clam (*Mya arenaria*) can maintain pH homeostasis despite low pH environments through active removal of protons in calcifying fluid to promote shell formation (Zhao et al., 2018). In a common garden experiment of mussels (*Mytilus edulis*) from two different populations, it was demonstrated that naturally and locally diverging carbonate chemistry parameters affected the response of the populations to elevated pCO_2. Populations derived from a high CO_2 environment showed higher fitness under increased pCO_2, suggesting acclimation or adaptation to local environmental conditions (Thomsen et al., 2017). Another example is a study by Dang et al. (2021) in a similar estuarine oyster species, *C. hongkongensis*, where oysters with a history of larval OA exposure displayed rapid local adaptative responses (via epigenetic modification) to cope with acidification stress.

Several studies have shown differential gene expression as a response to altered carbonate chemistry and have begun to shed light on the molecular mechanisms of resilience to acidified environments (Evans et al., 2013; Goncalves et al., 2017; Strader et al., 2020; Wright-LaGreca et al., 2022; Barbosa et al., 2022; Schwaner et al., 2022a, 2022b; Schwaner et al., 2023). Genes that appeared to be associated with resilience included carbonic anhydrase (CA) genes, which belong to the metallo-enzyme superfamily and catalyze the reversible reaction of carbonate hydration: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. CAs regulate intracellular pH in mantle epithelia cells of Pacific oyster (Ramesh et al., 2020), function in acid base regulation and ion transport in the crab *Callinectes Sapidus* (Henry and Cameron, 1983), and genes with CA domains are considered part of the basic toolkit for bivalve biomineralization (Arivalagan et al., 2017). The role of CA in the biomineralization process is thought to be highly conserved in metazoans (Le Roy et al., 2014). CAs are found in the shell nacre matrix (Marie et al., 2010), biominerals (Borelli et al., 2003), the mantle (Freeman and Wilbur, 1948; Henry and Saintsing, 1983), and the calcifying fluid (Schwaner et al., 2022a) of bivalves. CA can serve to maintain internal fluid pH homeostasis through ion transport, helping to promote calcification under low pH conditions. During periods of low pH/elevated pCO_2, when CO_2 enters the bivalve, it reacts with internal body fluids and buffers in equilibrium to form $HCO3^-$ and H^+ ions, increasing the internal concentration of CO_2, HCO_3^- and H^+, decreasing pH (Parker et al., 2013).

While organisms with environmental histories of fluctuations in pCO_2 might have mechanisms to combat low pH, such as upregulating genes like CA, there is still a need to understand how future OA conditions of more extreme pH and chronic exposures to low pH might alter their ability to respond. Variability in responses to OA might be artifacts of experimental design, including varying lengths of exposure to pCO_2. While studies have identified mechanisms to combat changes in carbonate chemistry of seawater (Zhao et al., 2018; Wright-LaGreca et al., 2022), it is still unclear if these are sustainable under long term exposures or have associated fitness costs.

The purpose of this study was to (1) assess the effect of chronic exposure to high pCO_2 on shell repair in adult oysters and (2) evaluate the role of CA in oyster response to low pH. We hypothesized that chronic exposure to high pCO_2 seawater will markedly reduce oyster ability to build new shells. We further hypothesized that CA promotes biomineralization under environments not favorable for mineral precipitation by regulating intracellular pH, whereas CA inhibition suppresses oyster ability to respond to low pH. Results are discussed with the aim of enhancing our understanding of mechanisms that enable eastern oyster resilience to OA.

2. Material and methods

2.1. Experiment 1: Effect of chronic exposure to high pCO_2 on shell repair

Twenty adult oysters (86 ± 10 mm height; 71 ± 10 mm width) were acquired from a commercial source (Frank M. Flower and Sons Inc., Oyster Bay, NY; −40.8760286°N, 73.5288112°W). Oysters were cleaned and acclimated for one week at ambient conditions (27 PSU, 23 °C, pH = 7.94, fed fresh algae cultures of *Tetraselmis spp, Isochrysis galbana, Pavlova lutherii*, and *Chaetoceros muelleri*. The oysters were then separated between four containers with two replicates per treatment (5 oysters per replicate): 1) elevated pCO_2 (pH ~7.3 and pCO_2 ~ 3300) or 2) ambient pCO_2 (pH ~ 7.94 and pCO_2 ~ 500) (Table S1). Experimental conditions were maintained using a flow through system with water sourced from Old Fort Pond in Southampton, NY (40.8852681°N, 72.4442576°W). For the elevated pCO_2 treatment, natural seawater flowed into an acidification chamber where 100% CO_2 was mixed with air (using multi gas channel proportioners from Cole Parmer, Schwaner et al., 2020, 2022a, 2022b) and bubbled into the seawater. Seawater from the acidification chamber flowed into a header tank and then continuously into the replicate containers corresponding to the elevated pCO_2 treatment. For the ambient pCO_2 treatment, natural seawater flowed into an aerated header tank and then flowed into each corresponding container. The experimental pH was selected taking into consideration local carbonate chemistry fluctuations (Baumann et al., 2015) as well as predictions made by the IPCC for the end of the century (IPCC, 2014). While held in the flow through system, oysters only received algae in the raw seawater without supplemental feeding. After eight months of chronic exposure to ambient or elevated pCO_2, oysters were selected for shell regrowth experiments. Oysters (5 per replicate, 10 per pCO_2 treatment) were sanded to create a flat surface and small plastic identification tags were adhered using superglue for monitoring of individual oysters (Fig. 1A). Shells were then notched using a Dremel drill with a circular burr of 2.5 cm in diameter without damaging the soft body (Fig. 1A), as described in Mount et al. (2004). Oysters were photographed immediately after the notch was made for a Time 0 photo and returned to their respective container. At 2, 4, 7, 11, and 21 days after notching, oysters were photographed and shell regrowth (repair) was monitored by assigning a stage based on the visual degree of regeneration at the notch site. This was done following the shell regrowth staging scheme defined by Sillanpää et al. (2016) and described in Table 1.

2.2. Experiment 2: Carbonic anhydrase inhibition

2.2.1. Animals and experimental treatments

Seventy-two adult oysters (89 ± 8 mm height; 71 ± 7 mm width) were obtained from the same commercial source (Frank M. Flower and Sons Inc.). Oysters were scrubbed to remove epibionts and sediment and acclimated for one week at ambient conditions (33 PSU, 14 °C, pH = 8.09, fed fresh algae cultures of *Tetraselmis spp, Isochrysis galbana, Pavlova lutherii*, and *Chaetoceros muelleri*. After acclimation, oysters were tagged with unique identification numbers as described above (Fig. 1A). At this point, oysters were divided (36 oysters in each group) and transferred to one of two pCO_2 treatments maintained in the flow through system described previously (Section 2.1): (1) ambient (pH ~ 8.1 and pCO_2 ~ 340 ppm) and (2) elevated (pH ~ 7.3 and pCO_2 ~ 3200 ppm) (Fig. 2; Table S2).

After three weeks of exposure to pCO_2 treatments, each oyster was notched twice to monitor shell regrowth and to create an access point to the adductor muscle for the injection of the CA inhibitor (or DMSO for controls) and the collection of hemolymph (Fig. 1B). Oysters were photographed immediately after the notches were made for a Time

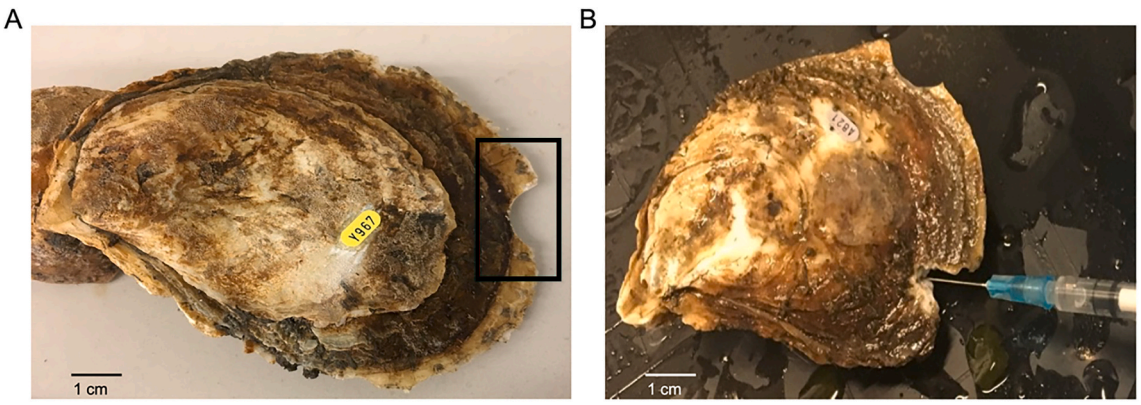

Fig. 1. (A) Plastic identification tags were adhered to oyster shells to enable monitoring of the individual oyster before shells were damaged with a rotary tool (red rectangle) to assess shell regrowth. (B) The oyster was notched again to access adductor muscle to enable injection of the CA inhibitor and withdrawal of hemolymph.

Table 1
Shell stages of regrowth from Sillanpää et al. (2016).

Stage	Description
I	No visible shell repair
II	Organic matter formation
III	Tanning of organic layer and increase in surface area of organic layer
IV	$CaCO_3$ deposition visible or overall change in color and white deposition

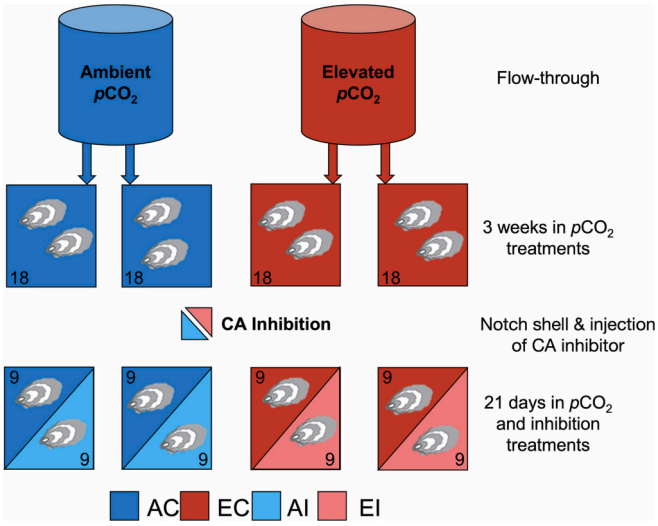

Fig. 2. Schematic representation of the experimental design of Experiment 2. Numbers refer to the quantity of individual oysters in each treatment. The first letter A (ambient) or E (elevated) refers to pCO_2 treatment and the second letter C (control) or I (inhibition of CA) refers to oyster injections.

0 assessment. Half of the oysters from each replicate (nine individuals) received an injection into the adductor muscle of 100 µl CA inhibitor acetazolamide (Sigma-Aldrich) at 10 mM (dissolved in dimethyl sulfoxide (DMSO), a dose that is 10-fold lower than the lowest dose used by Wang et al. (2017) but that was able to induce a response in *C. virginica* during our preliminary trials. The remaining oysters were injected with 100 µl of DMSO (control). This created the following treatments: ambient pCO_2 control (AC); ambient pCO_2 + CA inhibition (AI); elevated pCO_2 control (EC); elevated pCO_2 + CA inhibition (EI). Injections were repeated on day 3, 7, 10, 15 and 18. At each of these days as well as day 21 (end of the experiment), oysters were photographed, and shell regrowth stage was determined as described above (Section 2.1).

2.2.2. Evaluation of hemocyte parameters

After 21 days, the 72 oysters were bled through the same notch that served for injection using a 3 ml syringe prefilled with chilled filtered artificial seawater (FASW, 27 PSU) so that raw hemolymph samples were diluted 1:3. All tubes, animals, and syringes were kept on ice. The diluted hemocytes were then aliquoted to assess viability, intracellular pH (pH_i), phagocytic activity, and calcium concentration. These assays were conducted using flow cytometry (BD FACSCalibur) following protocols outlined in Schwaner et al. (2022a). Agranulocytes and granulocytes were separated based on light forward (FSC) and side (SSC) scatter parameters and treated separately for analyses (Schwaner et al., 2022a). Briefly, the diluted hemolymph was aliquoted into 450 µl sub samples. To assess hemocyte viability, propidium iodide (PI; ThermoFisher) was added at the final concentration of 20 µg/ml and incubated in the dark at room temperature before reading 10,000 cells on the flow cytometer. PI binds to DNA of dead cells and fluoresces in the orange channel (FL2). The second aliquot was to measure relative pH_i and the diluted hemolymph was immediately transferred to a sealed 0.5 ml microcentrifuge tube to minimize gas exchange and then centrifuged (800 *g* 4 °C for 10 min). The supernatant was removed and the pellet was resuspended in FASW and 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF AM; Sigma) at a final concentration of 1 µM and incubated at room temperature in the dark for 10 min, before reading on the flow cytometer (the probe fluoresces in the green channel (FL1)). To measure relative calcium content in hemocytes, the dye Fluo-3 (ThermoFisher) was added to the third aliquot at a final concentration of 5 µM, incubated in the dark for 20 min at room temperature, and read on the flow cytometer (FL1). The last aliquot was used to evaluate phagocytic activity and 2-µm yellow-green beads (Sigma) were added (1:10 hemocyte: bead ratio), incubated for one hour, and read on the flow cytometer. Hemocytes containing beads (FL1 signals) were considered phagocytic.

2.2.3. Calcium content and carbonic anhydrase activity in mantle tissue

Following bleeding, oysters were shucked, and two 50 mg pieces of the mantle tissue were collected. Calcium content in the first piece of mantle tissue was quantified using Calcium Colorimetric Assay Kit (ElabScience), following manufacturer's instructions. CA activity of a second piece of mantle tissue was measured using the Carbonic Anhydrase Activity Assay Kit-Colorimetric (Biovision) following manufacturer's recommendations. Protein concentration was quantified using Pierce BCA protein assay reagent kit, to standardize CA assay.

2.2.4. Disease diagnostics

A third piece of mantle tissue (collected near the labial palps) and a portion of the rectum were added to a tube of Ray's fluid Thioglycollate Medium for *Perkinsus marinus* diagnostics (Ray, 1966). Samples were

incubated in the dark at room temperature. After the week-long incubation, tissues were placed on a slide, minced, and then stained with Lugol's iodine. Slides were read on a compound light microscope to measure the *P. marinus* infection intensity which was ranked based on the Mackin scale (Mackin, 1962). In addition, a cross section of soft tissue from each oyster (including digestive gland, gonad, mantle and gill) was placed into a histological cassette and fixed in formalin, embedded in paraffin wax, sectioned (5 μm thick), mounted to slides, and stained with hematoxylin and eosin. Slides were subsequently read on a compound light microscope to assess disease status using standard histological techniques (Mackin, 1951; Ford and Haskin, 1982; Aguirre-Macedo et al., 2007; Sühnel et al., 2016).

2.3. Statistical analyses

All statistical analyses were performed in R version 3.6.3. Assumptions of normal distribution and homoscedasticity were tested using Shapiro-Wilk and Bartlett's tests, respectively. The ordinal categorical data from Experiment 1 (stage of shell regrowth) was compared between the two pCO_2 conditions over the different days (fixed factors), with random effects (oyster and tank), using the ordinal package (Christensen, 2022) for a mixed effect ordinal logistic regression model. *P. marinus* staging from Experiment 2 was analyzed similarly. For significant interactions between factors, Tukey post hoc analyses were performed (emmeans package; Lenth et al., 2017). For shell regrowth data from Experiment 2, a mixed effect ordinal logistic regression model was also used, but CA inhibition was added as an additional fixed factor. Linear mixed effect models were used to analyze the remaining data sets from Experiment 2. The hierarchical structure was taken into account, which involved nested observations with tanks. The lme function in nlme package was used (Pinheiro et al., 2023) to fit these models, specifying tank as a random effect and CO_2 condition and CA inhibition as fixed effects. Post hoc analyses were conducted as described above. Results were deemed significant at $\alpha \leq 0.1$.

3. Results

3.1. Experiment 1: Assessing shell repair after eight months in pCO_2 conditions

Shell regeneration differed significantly between oysters maintained in ambient and elevated pCO_2 conditions at all days (2, 4, 7, 11, and 21 post notching) regrowth was assessed (Fig. 3; $p < 0.001$; Table S3–4). At the end of the assessment period (21 days), 100% of oysters cultivated at ambient pCO_2 had at least partial shell regrowth, whereas only 30% of oysters from elevated pCO_2 conditions demonstrated any noticeable shell regrowth. Eighty percent of the oysters from the ambient pCO_2 treatment regrew shell to the final stage, whereas oyster shell regrowth in the elevated pCO_2 condition was characterized as the lowest stage of shell regeneration (i.e., Stage I: No visible shell repair).

3.2. Experiment 2: Carbonic anhydrase inhibition

3.2.1. Shell regeneration

Results from the model analysis (Table S5) revealed that day of sampling ($p < 0.0001$), pCO_2 condition ($p = 0.07$), and inhibition ($p < 0.01$) all had an impact on shell regeneration. Further examination, averaged over the days (Table S6), indicated significant differences in shell regeneration when contrasting the interaction of pCO_2 levels and inhibition treatment. There were significant differences between the following pairs: AC (ambient pCO_2, no inhibition) and EC (elevated pCO_2 no inhibition; $p = 0.02$); AC and EI (elevated pCO_2, CA inhibition; $p = 0.01$); AI (ambient pCO_2, CA inhibition) and EI ($p = 0.02$) (Fig. 4). At the end of the experiment, oysters in the AC condition had the highest percentage (41%) reach the final stage of shell regrowth, in contrast to AI (22%), EC (17%), and EI (6%). While CA inhibition within elevated pCO_2 treatment did not lead to a statistically significant reduction in shell regrowth, there was still a noticeable difference in their ability to repair shells. Specifically, 72% of oysters in the EI group had no shell repair compared to only 44% in the EC group (Fig. 4). In AI and AC groups, only 22% and 24%, respectively, failed to regrow their shells (Fig. 4).

3.2.2. Hemocyte parameters

Granulocytes collected from oysters maintained in the EC condition

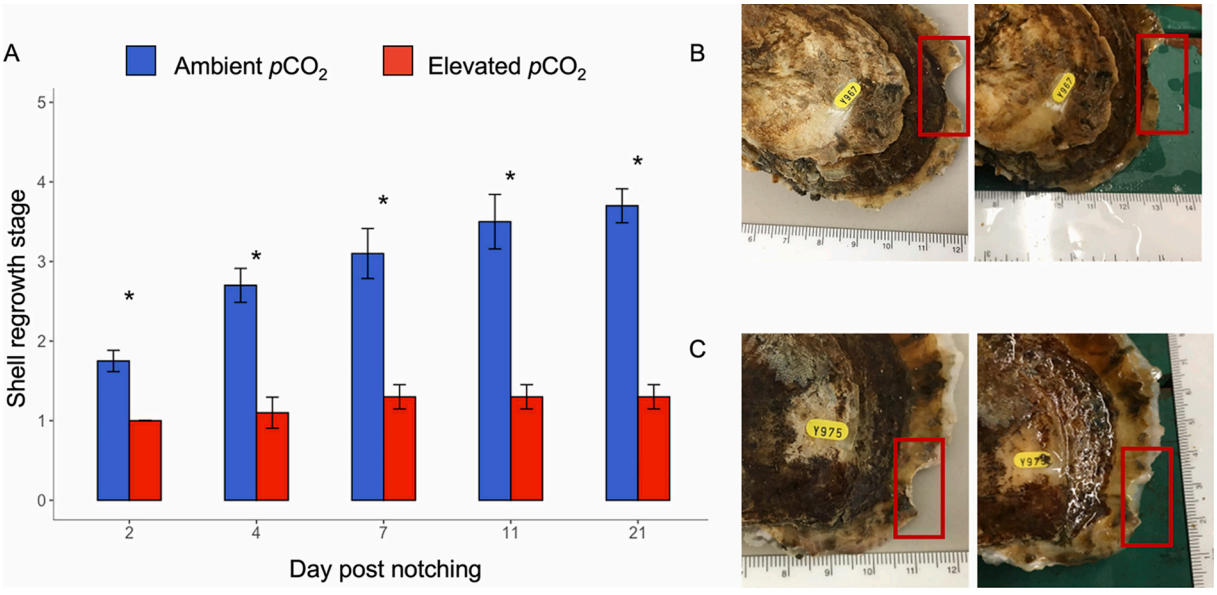

Fig. 3. (A) Shell regrowth (mean ± standard error) in oysters exposed to acidified or control conditions. The asterisks denote significant differences between treatments ($p < 0.01$). (B) Example of stage 1 (oyster from elevated pCO_2) and (C) stage 4 (oyster from ambient pCO_2) shell repair (for each oyster, left and right photographs represent pictures taken at Day 0 and Day 21, respectively).

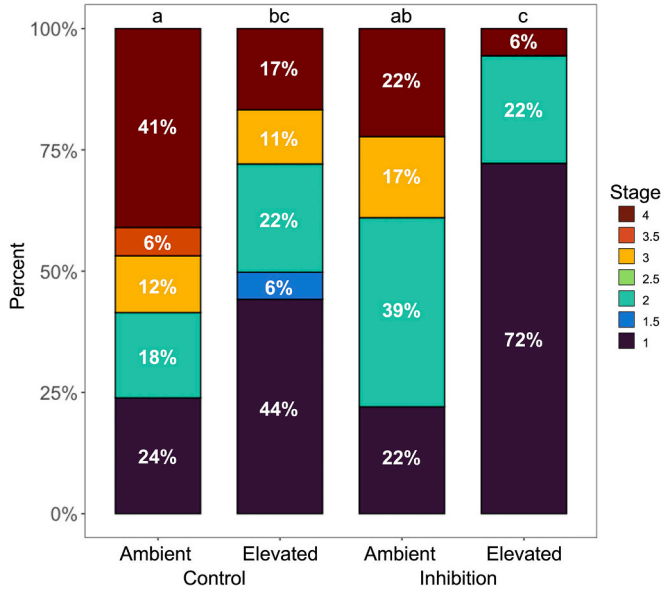

Fig. 4. Percentage of oysters at each stage of shell regrowth (1–4) 21 days post-notching, under different CO_2 conditions (Ambient, Elevated) and with or without CA inhibition (Inhibition, Control). Different letters above the bars indicate significant differences after pairwise comparisons based on post-hoc tests. Bars sharing the same letter are not significantly different from each other, while bars with different letters are significantly different.

had a significantly higher pH_i than granulocytes of oysters from the other treatments (Fig. 5A; $p < 0.001$; Table S7), demonstrating that in response to elevated pCO_2 the pH_i of granulocytes is increased. With CA inhibition, the pH_i of granulocytes was similar to the pH_i of granulocytes from oysters in the ambient pCO_2 conditions (AI, AC). There were no significant differences in the pH_i between any of the agranulocyte samples (Table S8).

The oyster treatments influenced the calcium concentration of the granulocytes (Fig. 5B; $p = 0.1$; Table S9–10). Calcium concentration in granulocytes from oysters maintained in EI was significantly higher than that measured in granulocytes from oysters in AC and AI treatments, however, differences were not significant with levels measured in oysters from EC which displayed intermediate concentrations. Trends were similar for calcium concentration in agranulocytes. For instance, agranulocytes from oysters in the EI treatment had the highest calcium concentration, but it was not significantly different from the other treatments (S9; S11). Oysters in the EC condition maintained intracellular calcium concentration, but with CA inhibition calcium concentration varied from ambient treatments.

There were no discernable differences in the phagocytic activity of granulocytes or agranulocytes collected from oysters in the different treatments (Fig. 5C; Table S12–13) and neither pCO_2 nor CA inhibition did appear to impact phagocytosis. Similarly, regardless of treatment, there were no significant differences observed in the viability of granulocytes or agranulocytes (Fig. 5D; Table S14).

3.2.3. *Calcium content and carbonic anhydrase activity in mantle tissue*

Model output revealed that CO_2 and CA inhibition had a significant interaction effect ($p = 0.03$; Table S15) on calcium concentration in mantle tissue. Calcium concentration was highest in mantle tissue extracted from oysters in the EI treatment, although, it was only significantly higher than that measured in oysters from the EC treatment (Fig. 6A; $p = 0.1$; Table S16). The model also showed that CO_2 treatment (at $p = 0.1$) and CA inhibition ($p = 0.03$) impacted CA activity in mantle tissue (Table S17). Supporting our assumption, CA activity measured in mantle tissue was significantly higher in oysters from the elevated pCO_2

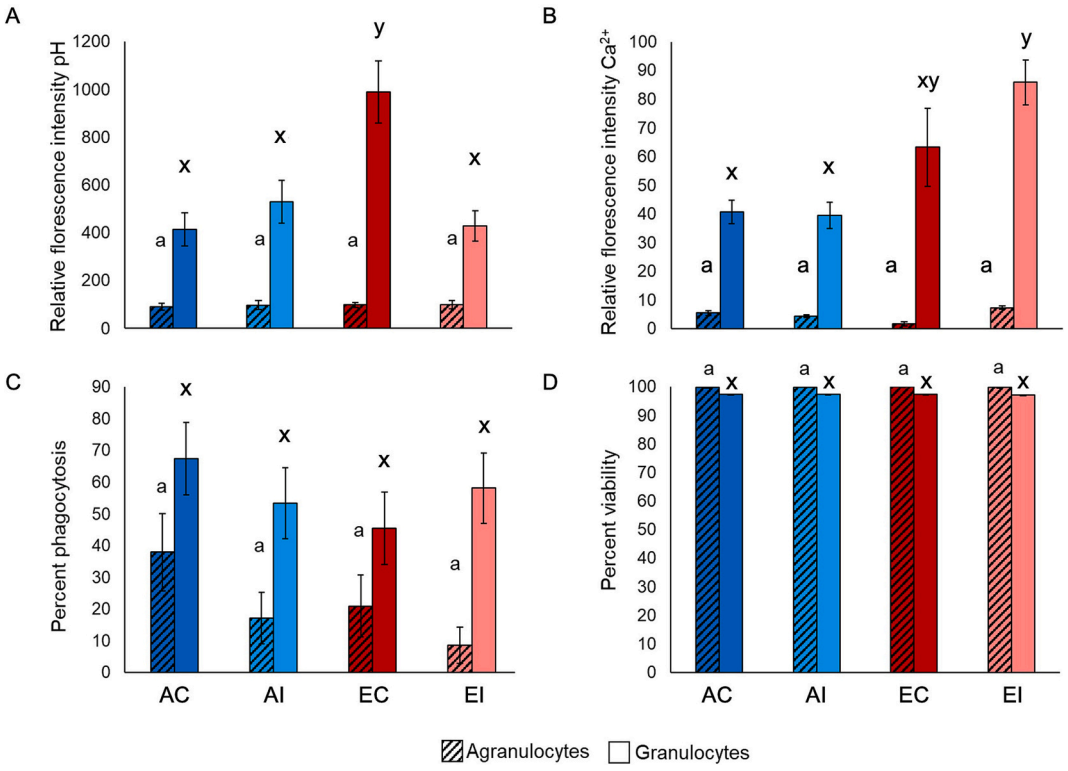

Fig. 5. Relative fluorescence intensity of (A) BCECF-AM (indicator of pH_i) and (B) Fluo-3 (indicator of Ca^{2+}), phagocytic activity (C), and viability (D) for agranulocytes (lined) and granulocytes of oysters from different treatments (AC- ambient pCO_2 control; AI- Ambient pCO_2 CA inhibition; EC- elevated pCO_2 control; EI- elevated pCO_2 CA inhibition). Mean ± standard error. For each parameter, letters (a and b for agranulocytes, and x and y for granulocytes) denote significant differences between treatments.

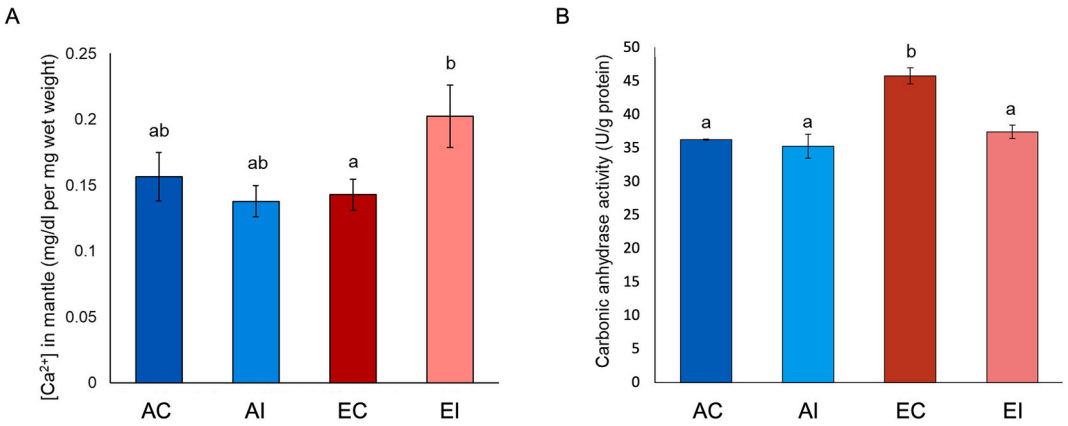

Fig. 6. Calcium content (A) and carbonic anhydrase activity (B) in mantle tissues. Mean ± standard error. For each parameter, different letters denote significant differences between treatments ($p < 0.1$). AC- ambient pCO_2 control; AI- Ambient pCO_2 CA inhibition; EC- elevated pCO_2 control; EI- elevated pCO_2 CA inhibition.

conditions compared to oysters in ambient pCO_2 (Fig. 6B; $p = 0.1$; S18). CA activity in mantle tissue was not fully suppressed with the chemical inhibitor; however, it was significantly reduced between EC and EI oyster mantle tissue (Fig. 6B; $p = 0.09$).

3.2.4. Disease diagnostics

Oysters were submitted to *P. marinus* and histopathology diagnostics. There were no significant differences between treatments (Table S19) for *P. marinus* intensity or prevalence. Overall, there was very little evidence of *P. marinus* with 72% of oysters having no detectable *P. marinus* cells. In the acidified CA inhibition (EI) treatment, 17% of oysters contained ciliates in their tissues, and these were not found in any other treatment (Fig. S1). There were no other signs of parasitic infections in histology samples.

4. Discussion

The eastern oyster is found in coastal estuarine environments with episodic low pH, reaching levels known to be deleterious to bivalve health in summer and fall. Thus, oysters have a capacity to tolerate environmental conditions of transient elevated pCO_2. To assess the impact of chronic acidification stress on oyster biomineralization processes, we evaluated oyster ability to repair shell damage after eight months exposure to elevated pCO_2 conditions. In addition, to better understand the regulatory mechanisms that could enable calcification under elevated pCO_2, we evaluated the role of CA in responding to short term acidification stress (1 month of exposure). Results underline a central role of CA in creating an intracellular environment that enables calcification under unfavorable seawater conditions, while a chronic exposure to high pCO_2 markedly altered oyster ability to perform shell repair.

4.1. Increased carbonic anhydrase activity mitigates impacts of low pH

Some mechanisms to mitigate the impacts of low environmental pH have been previously identified in various marine organisms. For instance, research on *C. hongkongensis* demonstrated the overexpression of genes related to Ca^{2+} binding and signaling to sustain biomineralization in low pH conditions (Chandra Rajan et al., 2021). In the pearl oyster, *Pinctada fucata*, transcriptomic analysis revealed compensation for low pH via transmembrane movement of H^+ and HCO^{3-}, although this process was hampered during extreme acidification (Li et al., 2016). Ramesh et al. (2017) found that *M. edulis* larvae were able to elevate pH and carbonate beneath their forming shell, which in turn led to a substantial increase in calcium carbonate saturation state. Coral exposure to low pH induces an upregulation of genes coding for proteins involved in calcium and carbonate transport, conversion of CO_2 into HCO_3^-, and organic matrix to promote biomineralization (Vidal-Dupiol et al., 2013). Furthermore, investigations in the hard clam *Mercenaria mercenaria* demonstrated an upregulation of genes related to biomineralization, calcium binding, and ion transport, including CA (Schwaner et al., 2022a, 2022b). CA was upregulated in the extrapallial fluid (EPF; site of shell formation) in clams maintained in high pCO_2 conditions, and this upregulation was associated with an increase in the pH both in hemocytes (pH_i) and in the fluid itself (extracellular pH or pH_e) as compared to those in clams under ambient pCO_2 conditions or to the surrounding seawater. In addition, weighted gene co expression network analysis of the transcriptomes of clam hemocytes suggested CA was involved in maintaining pH_i (Schwaner et al., 2022b). These studies have begun to reveal the role of upregulating processes related to biomineralization, acid-base regulation, and ion transport to combat low pH conditions.

Of particular interest is the upregulation of CA due to its biological importance in biomineralization and acid base regulation. CAs are catalysts of the carbonate hydration reaction and play crucial roles in acid-base regulation, a pathway associated with resilience to elevated pCO_2 (Moulin et al., 2014; Wang et al., 2016; Wang et al., 2017; Schwaner et al., 2022a, 2022b). *P. fucata* significantly upregulated CA genes after 60-days in a high CO_2 treatment (Li et al., 2016), and *C. gigas* had increased CA activity under OA as well (Wang et al., 2017). Other studies reported reduced CA activity under elevated pCO_2 conditions, including decreased CA activity in mussels, but this was measured after six months of exposure to high pCO_2 and shell regrowth was compromised (Fitzer et al., 2014). A decrease in CA activity in response to elevated pCO_2 was observed in the marine polychaetae *Sabella spallanzanii*, but this was because of physiological trade-offs related to increasing metabolism, and the authors suggested the decrease in CA is not sustainable long-term as it makes the worms more vulnerable to low pH (Turner et al., 2015).

Here, we utilized a commercially available CA inhibitor to compare responses to elevated pCO_2 between oysters with and without CA inhibition. We firstly confirmed that there was increased CA activity in mantle tissue from oysters in elevated pCO_2 compared to ambient pCO_2. Because of the role of CA in biomineralization, we evaluated if CA inhibition would impair oyster ability to regenerate shell in the low pH condition. Oysters can repair shells after incurring damage by deposition of new materials to maintain shell quality and thus protection against predators, pathogens, or mechanical damage (Taylor, 2016). This process is extremely important to the fitness of oysters and many studies have demonstrated that biomineralization will be affected by pH levels predicted for the end of the century (Meng et al., 2018). Oysters incubated at high pCO_2 with CA inhibition had the least amount of shell regeneration. Oyster shell regrowth in the elevated pCO_2 condition was not significantly different from shell regrowth in the ambient pCO_2 conditions. In contrast, significantly less shell regrowth was noted in

elevated pCO_2 with CA inhibition as compared to elevated pCO_2 alone supporting that CA is contributing to resilience.

There are multiple CAs in mantle tissue of bivalves (Cardosa et al., 2019; Malachowicz and Wenne, 2019), and an increase in general CA activity was noted in mantle from oysters in elevated pCO_2 as compared to ambient pCO_2. When acetazolamide was used to inhibit CA there was only a significant decrease in CA activity within the acidified treatment. This could be from differences in constitutive and inducible CAs, meaning the CAs upregulated (and inhibited) under elevated pCO_2 conditions may have remained constant in control conditions. Acetazolamide efficiency in inhibiting CA is different across different CAs (Hongo et al., 2013; Perfetto et al., 2017; Cardosa et al., 2019), and the inhibited CAs appear to belong to the same group upregulated in acidified conditions.

Previous studies have shown that elevated pCO_2 can have adverse impacts on hemocytes, which are important cells for biomineralization and immune processes in bivalves. Hemocytes play roles in biomineralization including transporting calcium intracellularly to the site of shell mineralization, acid-base regulation, and they contain many critical shell formation proteins (Mount et al., 2004; Schwaner et al., 2022a). Exposure to elevated pCO_2 conditions in the blood clam led to a reduction in the total number and phagocytic activity of hemocytes (Liu et al., 2016; Su et al., 2018). Li et al. (2015) suggested a potential impact of elevated pCO_2 and warming on hemocyte mediated biomineralization in *P. fucata,* specifically reporting altered hemolymph pH and outflow of calcium from hemocytes. Alterations in intracellular pH as a result of acidification can directly alter cellular metabolism of an organism, and if not compensated for, can impact an organism's ability to meet its energy needs for essential cellular functions (Pörtner et al., 1999).

Here, we investigated the impact of elevated pCO_2 and CA inhibition on hemocyte parameters relevant to both biomineralization and immunity. Hemocyte populations were treated separately because of prior knowledge on functional differences between the different subpopulations (Lau et al., 2017; Schwaner et al., 2022a). Granulocytes from oysters in the EC treatment had significantly higher pH_i compared to all other treatments, including those from oysters in the EI conditions. The increase of pH_i in hemocytes from oysters exposed to elevated pCO_2 suggests that oysters compensated for the acidosis caused by high pCO_2. When CA was inhibited, however, pH_i remained at control levels suggesting that CA is at least in part responsible for this response. Prior investigations have shown that bivalves exposed to elevated pCO_2 conditions compensated by active transport of acid-base relevant ions across cell membranes (Parker et al., 2013). Elevated CO_2 promotes acid base regulators such as CA to transport acid-base relevant ions such as H^+ and HCO_3^- across cell membranes which would lead to an increase of intracellular pH or accumulation of HCO_3^- (Wang et al., 2017). Contrarily, Zhao et al. (2020) demonstrated that the pH of hemolymph of Korean mussels was reduced by elevated pCO_2, and Thomsen et al. (2013) found a linear relationship between hemolymph pH and seawater pH in blue mussels. An organism's ability to modulate pH and keep internal pH elevated compared to seawater might determine tolerance to elevated pCO_2. There were no differences in the pH_i of agranulocytes from oysters in any treatment, but these hemocytes do not appear to be heavily involved in biomineralization (Mount et al., 2004) so sustaining pH optimal for biomineralization might not be as important as in the case of granulocytes. Similarly, granulocytes but not agranulocytes collected from *M. mercenaria* from elevated pCO_2 had higher pH_i than hemocytes from clams in ambient pCO_2 (Schwaner et al., 2022b).

Calcium content was higher in granulocytes of oysters in elevated pCO_2 compared to oysters in ambient conditions; however, this was exacerbated by CA inhibition. Calcium concentration in granulocytes from oysters in EI, but not EC, was significantly higher than calcium concentration of hemocytes from oysters in both ambient treatments. Similar trends were observed in mantle tissue, with highest calcium content in mantle tissue from oysters in the EI treatment. Dissolution of $CaCO_3$ shell from OA conditions will release Ca^{2+} leading to an increase in Ca^{2+} concentrations in the hemolymph (Michaelidis et al., 2005; Zhao et al., 2017), which could explain why calcium was higher in hemocytes from low pH conditions. The inhibition of CA might have caused the greatest dissolution and therefore higher calcium release from shell dissolution. An alternative scenario might be the inability of hemocyte and mantle cells to precipitate and release intracellular Ca^{2+} into shell $CaCO_3$ under low external pH conditions, leading to accumulation of Ca^{2+} in cells.

While the focus of this research was primarily on biomineralization, biomineralization and immunity in bivalves are intertwined (Schwaner et al., 2022a) so it was also important to investigate basic immune parameters. Hemocytes are the backbone of bivalve immunity and calcium concentration is important to immune signaling (Wang et al., 2020). There were no differences in viability or phagocytic activity of hemocytes between treatments. This is unexpected as many studies have indicated a weakened immune response as result of exposure to elevated pCO_2 (Bibby et al., 2008; Hernroth et al., 2011; Li et al., 2015; Liu et al., 2016; Su et al., 2018; Schwaner et al., 2020). Specifically, Liu et al. (2016) demonstrated a reduction in hemocyte phagocytic activity under high pCO_2 and downregulation of hemocyte immune genes. Su et al. (2018) also demonstrated changes to hemocytes from high pCO_2 such as decreased cytoskeleton components, reduced concentration of lysozyme activity, and increased production of nitric oxide which has negative implications for immune processes. There were also no differences in prevalence or intensity of *P. marinus* (however overall prevalence was very low across all treatments) and there were minimal histological differences. One month of exposure to elevated pCO_2 and CA inhibition did not appear to impact immune processes in adult oysters.

4.2. Chronic exposure to elevated pCO_2 impairs oyster ability to regenerate shell

In the chronic exposure trial, oysters were maintained in ambient or elevated pCO_2 conditions for eight months before shell repair abilities were assessed. The environmental history of the oysters used in this study includes natural periods of low pH and extreme acidification (Baumann et al., 2015), so acute exposure reflects what is naturally occurring. Research suggests that bivalves, especially those found in environments with variable pH, have mechanisms conferring some degree of acclimation to future carbonate chemistry conditions. During exposure to high pCO_2, genes related to stress, such as heat shock proteins, may be upregulated (Clark et al., 2013; Timmins-Schiffman et al., 2014; Moya et al., 2015); however, this might not be sustainable long-term. A study conducted by Hernroth et al. (2011) found that heat shock proteins were over expressed after one week of low pH exposure in the star fish *Asterias rubens*, but not after chronic exposure to low pH. Certain mechanisms might be too costly to maintain for long periods. The brittlestar *Amphiura filiformis* was able to increase calcification under low pH; however, this came at the substantial cost of muscle wastage and was not a sustainable solution (Wood et al., 2008). Acidified conditions can heighten the energy demands of acid-base and ion homeostasis, and prolonged exposure to elevated pCO_2 could result in physiological trade-offs due to energy limitations.

Oysters held at high pCO_2 conditions for one month had similar shell regrowth to those in ambient conditions (when CA was not inhibited), suggesting mechanisms to sustain biomineralization. However, eight months of exposure elicited a different result. Shell regrowth between oysters held in ambient and elevated pCO_2 conditions varied dramatically with only 30% of oysters in elevated pCO_2 displaying any shell regrowth compared to 100% in ambient conditions. Furthermore, the 30% of oysters that regrew shell in elevated pCO_2 conditions only had initial organic matter formation, whereas 80% of oysters in ambient conditions reached the final stage of shell growth. Findings suggest that chronic exposure to elevated pCO_2 hampers the ability of oysters to repair damaged shells and implies compromised calcification under

future low pH environments. Continuous exposure to low pH conditions and resulting shell dissolution may outpace the ability to repair damage. Low pH conditions can lead to deterioration of the periostracum or protective shell layers, making the shell more vulnerable to low pH (Peck et al., 2016). The oysters used in both trials were from the same aquaculture stock, so differences in shell regrowth were more likely due to time of exposure and not environmental history. This might potentially indicate that it is too costly to chronically maintain mechanisms (perhaps upregulation of CA) to sustain biomineralization in environments not favorable for mineral precipitation. It would have been interesting to investigate CA activity between oysters from different pCO_2 conditions in the second study, to see if there was a down-regulation of CA genes after long-term exposure.

There is great variation in species responses to shell damage and repair under low pH. Here, we saw a marked reduction in shell regeneration; however, Cross et al. (2016) found that the ability to repair shells was not affected by low pH conditions in the lampshell brachiopod *Calloria inconspicua*. A difference in experimental design in Cross et al. (2016) and our experiment is the length of exposure and pH level. Their design was a total of 12 weeks, and the experimental condition was a pH of 7.62. Coleman et al. (2014) studied two species of gastropods and found that the zebra top snail *Austrocochlea porcata* exhibited a reduction in shell regrowth under low pH while the common warrener *Subninella undulata* displayed no differences in shell regrowth, underscoring species specific responses. While there might not be obvious differences in shell regeneration, the repaired shell could still be compromised. For example, in *M. edulis,* shell regrowth under low pH was at the expense of structural integrity due to the disorientation of calcite crystals (Fitzer et al., 2016).

5. Conclusions

This study supports the role of CA in mitigating the negative impacts of acidification stress in the eastern oyster, particularly in maintaining intracellular hemocyte pH despite decreases in external seawater pH. This might help to promote biomineralization in conditions that do not favor mineral precipitation. The ability of an organism to regulate pH_i might be indicative of its tolerance to acidification and help to explain species-specific differences that are abundant in the literature. Animals from habitats with fluctuations in carbonate chemistry appear to have mechanisms to mitigate the impacts of elevated pCO_2, but there still might be a threshold or cost to chronic exposure to elevated pCO_2. The ability to regenerate damaged shells after eight months of high pCO_2 exposure was significantly reduced, indicating compromised calcification under future OA regimes. If acclimation to pH via processes such as gene regulation are not sustainable or represent only short-term solutions, then future studies should focus on identification of genotypes that might be better suited to low pH environments.

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Authors contribution

CS, MB, BA and EPE conceived the study. CS, BA, EPE secured the funding. CS and MB performed the experiments. CS analyzed the data and drafted the manuscript. All authors reviewed and approved the final manuscript.

CRediT authorship contribution statement

Caroline Schwaner: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Michelle Barbosa:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Emmanuelle Pales Espinosa:** Conceptualization, Funding acquisition, Writing – review & editing. **Bassem Allam:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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