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Shell hash promotes growth in Pacific littleneck clams (*Leukoma staminea*) by altering pore water chemistry

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Abstract. Bivalves that build calcium carbonate skeletons are at particular risk from ocean acidification, and mitigation strategies will be needed to keep coastal populations healthy. It can be energetically costly for organisms like clams and mussels to build their shells under low pH conditions, and acidification can lead to shell dissolution. Adding crushed shells (shell hash) to beach sediments, a practice used by some Indigenous communities and aquaculturists, may mitigate the negative effects of ocean acidification by altering the chemistry of the pore fluids they live in. We tested the hypothesis that mixing shell hash into the sediment improves the growth and physiology of infaunal Pacific littleneck clams (*Leukoma staminea*). Juvenile clams (pre-sexual maturity) were raised for 90 days under four conditions: control seawater with sediment, acidified seawater with sediment, control seawater with sediment plus shell hash, and acidified seawater with sediment plus shell hash. Pore water and overlying seawater were sampled three times a week for pH, alkalinity, salinity, temperature, and dissolved oxygen. Clam shell weight, soft tissue weight, and new shell growth were measured, and mantle tissue RNA was collected for gene sequencing after three months. Our results demonstrate that the addition of shell hash increased the pH of porewater relative to the control, and animals exposed to acidified water plus shell hash grew larger than animals exposed to acidified water alone. Gene expression profiling suggests that animals in acidified seawater with shell hash were largely indistinguishable from animals in non-acidified water. Our experimental results suggest that adding shell hash to sediments alters the chemistry of pore fluids, thus buffering against acidic conditions that can negatively affect the growth of economically and culturally important shellfish like littleneck clams.

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1 Introduction

Anthropogenic carbon dioxide (CO_2) inputs are disrupting the natural acid buffering feedbacks of the Earth system— leading to a ~30% increase in ocean acidity globally since pre-industrial times (e.g., Zeebe and Wolf-Gladrow, 2001; Caldeira & Wickett, 2003,; Sabine et al. 2004; Zachos et al., 2008; Doney et al., 2009; Hönisch et al., 2012). As the ocean absorbs excess anthropogenic CO_2, it also shifts carbonate system equilibria towards lower concentrations of carbonate ions, an important building block for calcium carbonate shells/skeletons (Caldeira and Wickett, 2003; Kleypas et al. 1999; Orr et al., 2005). Ocean acidification therefore makes it energetically costly for calcifying marine invertebrates, including animals like corals and shellfish, to build their shells and skeletons, and acidic conditions can dissolve these structures (e.g., Kleypas et al. 1999; Green et al., 2004, 2009; Orr et al., 2005; Waldbusser et al., 2015; Bednaršek, 2019). Given the threats ocean acidification poses to global ecosystems, mitigation strategies are of growing interest to scientists, managers, and the public (e.g., Curtin et al., 2022; Mackenzie et al., 2022; Macreadie et al., 2019; Renforth & Henderson, 2017; Saderne et al., 2019).

Despite the concern, the impact of ocean acidification on particular species can be hard to predict, particularly on infauna—animals that live within the sediment and are not directly exposed to overlying waters (Ries et al., 2009; Andersson and Mackenzie, 2011; Widdicombe et al., 2011; Dodd et al., 2021; Gold & Vermeij, 2023). This is in part because of the complexity of carbonate chemistry in pore water (i.e. water within the sediment grains), which is clearly related to the overlying seawater chemistry but is generally lower in pH (Widdicombe et al., 2011). Processes that impact pore water pH include microbially-mediated redox reactions, abiotic mineral precipitation/dissolution, and mixing of sediments by macrofauna (Burdige et al., 2008; Dashfield et al., 2008; Kindeberg et al., 2020; Widdicombe et al., 2011). On the one hand, these naturally low pH environments may suggest infaunal bivalves are well-adapted to deal with ocean acidification (Gold and Vermeij 2023). Alternatively, if shellfish fail to cope with the rapid pace of anthropogenic climate change, coastal ecosystems, commercial aquaculture, and communities dependent on shellfish for food security will suffer (e.g., Doney et al., 2020).

Attempts have been made to mitigate the effects of ocean acidification on bivalves. In the early 2000s, Pacific Northwest commercial oyster hatcheries experienced widespread collapse associated with coastal acidification (Feely et al., 2012; Barton et al., 2015). In response, hatchery, government and academic scientists began to explore methods to buffer seawater used in shellfish culture. For example, this included adding soda ash (Na_2CO_3) to seawater in larval rearing tanks (Barton et al., 2015; Clements & Chopin, 2017; C. L. Mackenzie et al., 2022). In the northeastern United States, adding pulverized shell hash to mudflats was shown to increase the pH of sediment pore waters, and increase settlement of the clam *Mya arenaria* (Green et al., 2009, 2013). Similarly, Ericson and Ragg (2021), demonstrated that rearing tanks enriched with mussel (*Perna canaliculus*) shell hash enhanced *P. canaliculus* larval shell development under high pCO_2 conditions. Despite these promising studies, others have found little to no effect of shell hash on sediment chemistry or infauna. It therefore remains unclear how/if chemical buffering of acidification will improve growth and survival under future ocean acidification scenarios, and may depend on

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what species is of interest, their habitat preferences, and regional oceanography (Greiner et al., 2018; Beal et al., 2020; Doyle & Bendell, 2022). Controlled experimental studies of the impacts of shell hash and acidification on growth, physiology, and survival of infaunal clams of the same age and genetic stock are needed to help disentangle these variables.

There is also significant evidence from traditional ecological knowledge that shell hash could be beneficial to bivalve health. Adding shell material back into intertidal sediments and/or creating spaces where shell hash naturally accumulates is one component of complex Indigenous mariculture systems (Lepofsky et al., 2015; Tadlock, 2019; Toniello et al., 2019), particularly along the Northwest coast of North America. Indigenous "clam gardens" promote healthy coastal ecosystems and provide food security (Deur et al., 2015; Groesbeck et al., 2014; Jackley et al., 2016; Lepofsky et al., 2015; Toniello et al., 2019). Although exact management techniques vary by location, clam gardening often involves the purposeful addition of pulverized shell material, or "shell hash," into beach sediments (Deur et al., 2015; Greiner et al., 2018; Groesbeck et al., 2014; Salter 2018). The existing published research on clam gardens demonstrates that they host larger populations of clams and greater intertidal biodiversity (Groesbeck et al. 2014; Toniello et al., 2019; Cox et al., 2019; Jackley et al., 2016).

For this study, we examine the impacts of acidification and shell hash addition on infaunal Pacific littleneck clam (*Leukoma staminea*) calcification and genetics. All individuals were collected from the Bodega Bay region in Northern California. *L. staminea* is an excellent focal species, as it is geographically widespread, relatively abundant, and ecologically and culturally significant (Fraser and Smith, 1928; Lepofsky et al., 2015; Schneider et al., 2018). It is a marine venerid bivalve that burrows 5-15 cm beneath the sediment surface along nearly the entire coast of western North America, ranging from Baja California, Mexico to Alaska (Fraser & Smith, 1928). Found in wide-ranging habitats, including exposed rocky beaches and low-energy mudflats, *L. staminea* have been managed and harvested, along with other clam species, by Indigenous peoples across the northeastern Pacific coast for millennia (Lepofsky et al., 2015; Schneider et al., 2018; Toniello et al., 2019) and remain commonly collected and eaten by humans today. It is also an important food source within intertidal food webs, and is preyed upon by drilling gastropods, sea stars, crabs, birds, and sea otters (Feder et al., 1979; Hiebert, 2015). Like many bivalves, *L. staminea* is a suspension feeder, metabolizing ambient water that they take in with their siphons, while living in an infaunal environment where pore fluids affect their development once shell formation commences (Fraser and Smith, 1928). *L. staminea* grows a shell composed of the calcium carbonate polymorph aragonite, and contains light and dark banding patterns in the outer shell layers (Fraser & Smith, 1928; Takesue and van Geen, 2004; Kempf et al., 2023). The shells' banding patterns correspond with geochemical and microstructural changes in the shell, and significant size variability can exist within populations of the same age (Kempf et al., 2023). Recent research has documented *L. staminea* population decline in some parts of the British Columbia coast, perhaps due to competition with invasive species (Bendell, 2014). Additional research suggests that some *L. staminea* populations have declined at least partly in response to oceanographic changes in salinity and temperature (Barber et al., 2019), but information regarding the specific impacts of acidification on *L. staminea*, particularly

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in juveniles, is lacking in the current literature. We hypothesized that shell hash would increase the pH, total alkalinity and saturation state with respect to aragonite (Ω_{Ar}) of pore fluids, resulting in enhanced calcification and a loss of pH-stress genetic signatures in juvenile *L. staminea* exposed to experimental acidification.

2 Materials and Methods

2.1 Broodstock collection and husbandry

Juvenile clams used in this study were sampled from a single genetic cohort from a controlled spawn in the lab, with parent individuals collected live from Bodega Harbor in Bodega Bay, CA (Kempf et al., 2023). Bodega Bay is located approximately 60 miles north of San Francisco on the coast of California, and sits within the California Current System (CCS) where seasonal upwelling creates large natural fluctuations in pH (Hickey & Banas, 2003; Feely et al., 2008; Checkley & Barth, 2009; Davis et al., 2018; Largier, 2020).

Adult broodstock *L. staminea* collected from Bodega Harbor during the winter of 2021 (n = 22) were brought to the UC Davis Bodega Marine Laboratory (BML) in Bodega Bay, CA for conditioning. Seawater is supplied to the laboratory via intake lines approximately 200 ft offshore. Intake elevation is approximately six feet below mean low tide, and water is sand-filtered to ~30 um before being gravity-fed to the facilities. Broodstock were placed in an open plastic basket (10” x 6”) and set within a water table filled with circulating seawater. During the 6-week conditioning period clams were fed twice a day with a dense mixture of *Nannochloropsis oculata* and *Isochrysis spp.* algae. These species were chosen by BML aquaculturists as their preferred diet for raising juvenile bivalves. During the first week, seawater temperature gradually increased ~1°C/day from 11°C to 17°C to condition the clams for spawning. The system was maintained with a steady influx of filtered seawater, and waste was siphoned from the holding containers daily. To check if clams were gravid, two animals were chosen, and the visceral mass was gently scraped with a scalpel. The gonadal fluid from these clams was pipetted onto a microscope slide and mixed with a small amount of seawater and checked for the presence of eggs or sperm.

Once gravid, all conditioned clams (n=20) were rinsed with ~20°C seawater and placed in clean baskets inside a 10-gallon container. The seawater was then slowly heated to ~24°C using a heated stirring rod. After reaching this temperature, 30 L of dense *N. oculata* was added, and clams fed for one hour while adjusting to room temperature (~18 °C). Two additional feedings of algae heated to 30°C followed at one-hour intervals. Each time the clams fed for a total of 2 hours in darkness. As spawning had still not occurred, one clam was opened, sperm was confirmed, and gonadal fluid was pipetted into the setup to induce spawning. After two more hours at ~24 °C, clams were fed a final time with 10 L of *N. oculata* and *Isochrysis*, then left overnight as the system gradually cooled to room temperature (~20 °C). By morning, the remaining 19 clams had spawned.

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Planktonic larvae were removed from the spawning container the following morning with a siphon, and transported into 400 L tanks in the Bodega Marine Laboratory Shellfish Hatchery. Once larvae began to settle at the bottom of the tank (three weeks post-fertilization), they were transferred onto a 250 micron settling screen and placed in a shallow water table supplied with filtered seawater in the same hatchery room (18-20 °C). Lights were kept on during the day and turned off at night, and salinity was maintained between 33-36 ppt and checked approximately every 3 days. The entire cohort was fed twice weekly with a 30 L mixture of *N. oculata* and *Isochrysis* algae. All individuals were kept in identical laboratory conditions during the juvenile rearing period (200 days). At 200 days old, juveniles were transported to the experimental conditions outlined below.

2.2 Experimental design and treatments

Juvenile clams were raised in four experimental conditions for 90 days where the pH of the overlying fluids was maintained in the following ways: (1) control filtered seawater (pH= ~7.8), (2) experimentally acidified seawater (pH= ~7.4), (3) control seawater with shell hash (pH= ~7.8), and (4) acidified seawater with shell hash (pH= ~7.4) (**Fig. 1**). Treatments were run in duplicate, totalling eight ~22.7 L food-grade plastic buckets. Buckets were fed with filtered seawater held in header tanks kept at the ambient temperature of the wet lab space (~15°C), in which pH conditions were controlled using Pinpoint® pH controllers and regulators and a cylinder of compressed air with 1% CO2 (Airgas) (**Fig. 1**). Probes were briefly removed every Monday and Friday in order to recalibrate using Pinpoint® recalibration fluids (pH = 4.0 and 10.0, NIST scale). Probe measurements were not used to calculate carbonate chemistry parameters, but to maintain approximate target pH conditions for the experiments. Each header tank was supplied with air via an airstone.

The buckets were filled with filtered seawater for three days prior to the start of the experiment. Buckets were then filled with 5 L of unconsolidated medium-grained quartz sand (AquaQuartz; porosity of 38.8%) prior to adding seawater. This silica-based sand was chosen because it has comparable grain size to Bodega Bay sediment, and to minimize confounding variables that could significantly influence carbonate chemistry of the pore fluids via geochemical reactions (e.g., precipitation and dissolution) and/or microbial processes. Disarticulated clam shells were gathered in Bodega Bay, CA, bleached in 6 vol% NaOCl for 30 min to minimize the effects of microorganisms, rinsed thoroughly with fresh water, and air dried for 48 hours. Shells were then crushed using a mortar and pestle, and shell hash pieces were size-graded to 0.25-0.5 cm. All treatments with shell hash contained quartz sand with 10 wt% pulverized shells of *L. staminea* and *Saxidomus nuttalli*, species native to Bodega Bay. The addition of *S. nuttalli* shells, rather than just *L. staminea*, were used to ensure enough aragonitic shell material was available for the experimental setup. Juvenile clams (n=30 per bucket) were placed in each bucket at the beginning of the experiment. Eight liters of cultured *N. oculata* was added every Monday and Friday to the header tanks to feed the animals. Seawater was replaced in the header tanks approximately every three weeks (days 21, 42, 63, and 80). At the end of 90 days, four individuals were sampled from each bucket for genetic analyses, and the remaining living individuals were collected for growth analyses.

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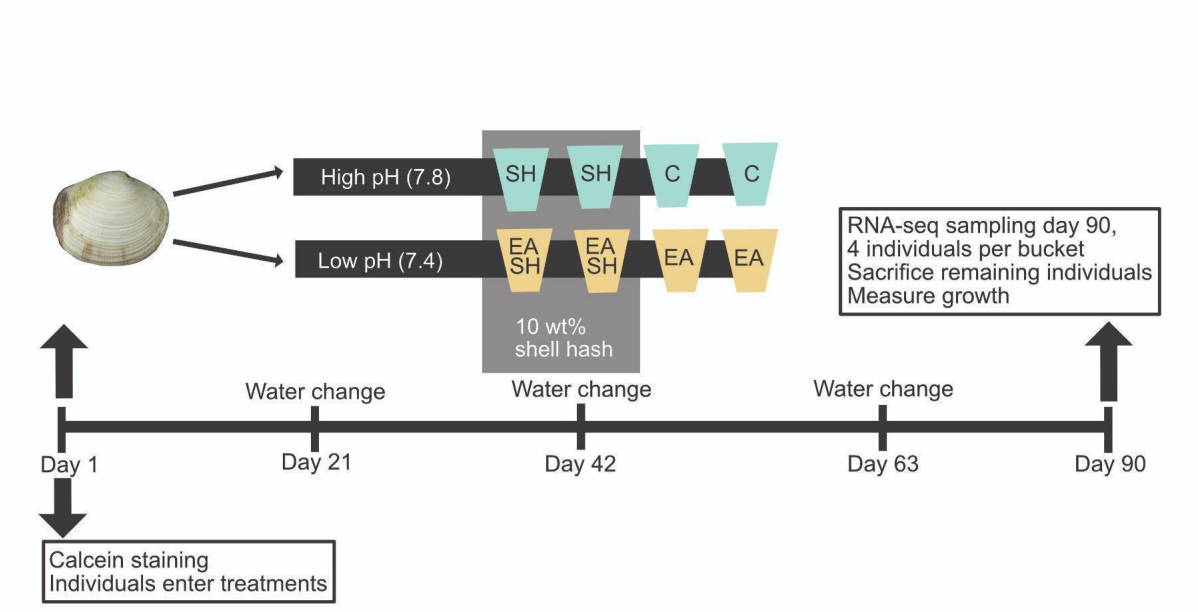

Figure 1. Design of 90-day experiment. SH = shell hash, C = control, EASH = experimental acidification with shell hash, EA = experimental acidification.

2.3 Calcein staining and fluorescence

All individuals were fluorescently stained with calcein immediately before the experiment to mark the animals' shell length at the start of the experiment. Following the protocols of Moran and Marko (2005), calcein powder and DI water were combined to make a concentrated stock solution of 6.25g/L calcein, and the pH of the stock was adjusted to ~7.5 using sodium bicarbonate to increase the solubility of the calcein. The stock was added to 1L of filtered seawater (final concentration of 100 mg/L) (Moran & Marko, 2005). After spending 24 hours in this solution, clams were transported to the experimental buckets, and the 90-day experiment began.

2.4 Seawater chemistry

Throughout the duration of the 90-day experiment, seawater in the buckets was sampled for salinity, temperature, pH, and dissolved oxygen every Monday, Wednesday, and Friday using a Pinpoint® pH probe, a Pinpoint® dissolved oxygen probe, a thermometer, and a VeeGee refractometer for salinity. Pore water in each bucket was sampled for carbonate chemistry once per week. Pore water samples were taken using pore water wells (MHE products) from 8 cm depth in the substrate. Pore water

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was sampled less frequently than overlying fluids to allow the pore fluids more time to interact with shell hash before removing them for sampling.

Water samples for total pH and total alkalinity were collected for pore water and overlying water in 125 mL glass bottles with a positive meniscus once per week during the mornings (between 7:00 and 10:00 am) to characterize water chemistry. All pH samples were poisoned with mercuric chloride to stop microbial respiration and stored at 4°C or analysed immediately. pH samples were run in duplicate on an Ocean Optics Jaz Spectrophotometer EL200 (SD +/- 0.003) using m-cresol purple dye (Dickson et al., 2007). A calibration regression was produced for m-cresol and calibrated against Tris for a <0.1 pH offset. At the same time, total alkalinity (TA) samples were collected in 125 mL Nalgene with a small amount of headspace, frozen at -20°C, and later analysed by Gran titration using an automatic titrator (809 Titrando, Metrohm) in triplicate. Drift due to changes in temperature was measured with drift catchers every twelve samples. Outliers were removed, and measurements were corrected against Dickson certified reference material (Batch #s 189 and 197). The average precision of the certified reference materials was +/- 3.11 µmol/kg. For runs 4, 5, 6, and 8, where certified reference materials were not analyzed due to instrument malfunctions, samples were secondarily calibrated to known drift catcher values, for which the average precision was +/- 9.2 µmol/kg. Alkalinity data from one run (run 9) was discarded due to poor accuracy and precision. Uncertainty of these measurements was included in our statistical analyses. Salinity, pH and alkalinity, and temperature were used to calculate aragonite saturation state (Ω_{Ar}) using the "seacarb" package in R (vers. 4.1.3). Constants for K1 and K2 according to Leuker (2000) and total boron according to Lee et al. (2010) were used.

2.5 RNA extraction and quality control protocol

Total RNA was extracted from clam mantle tissue on day 90 of the experiment. Four biological replicates (i.e., four individuals) were chosen from each of the eight buckets at day 90 (n = 32). Total RNA was extracted from the mantle tissue using a TRIzol protocol. Approximately 50-100 mg of mantle tissue was removed from the mantle edge of each clam (i.e., the ridge of tissue near the ventral margin of the shell) using a surgical scalpel equipped with a new blade for each animal. Extractions were performed at ambient room temperature (~19°C) in a fume hood to avoid contamination. 1 mL of TRIzol was added to each tissue sample within five minutes of collection, and the sample was homogenized using an electric pestle. Next, 200 µl chloroform was added and the samples were centrifuged for 15 minutes as 12,000 × g and 4°C to separate the molecular phases. The top aqueous layer was transferred to a new sample tube, and an equal volume of isopropyl alcohol and 1 µl GlycoBlue (ThermoFisher cat #AM9516) was added. After centrifuging the samples again for 10 minutes at 12,000 × g and 4°C, the resulting RNA precipitate was washed with 75% ethanol and air dried before being dissolved in RNase free water.

Concentration and quality of the RNA extractions were first evaluated using a NanoDrop spectrophotometer and QuBit fluorometer. Once assessed, samples were sent to the UC Davis DNA Technologies and Expression Analysis Core Laboratory,

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where the raw RNA was then run on an Agilent BioAnalyzer 2100. The highest quality samples (i.e., those with BioAnalyzer RNA integrity numbers > 7) were used for cDNA library prep and sequenced using Illumina High Throughput Sequencing (n=24 total, three per bucket). Samples were run on a NovaSeq 6000 S4 flow cell to produce ~25 million 150 base pair paired-end reads per sample for all 24 individuals. The quality of raw sequencing data was evaluated using fastQC to identify whether adapter sequences were present (FastQC, 2015). Because adapters were present, we then used Trimmomatic to trim the first 15 base pairs from each read to remove adapters (Bolger et al., 2014). These reads are available through the National Center for Biotechnology Information (NCBI), accession PRJNA1011264.

2.6 Mantle transcriptome assembly

As there is no published reference genome for *L. staminea*, we assembled de novo (i.e., reference-free) transcriptomes with the raw Illumina reads in Trinity (Grabherr et al., 2011). The amount of data produced made it computationally infeasible to create a reference transcriptome from all data simultaneously. We therefore created two transcriptomes, each using half of the data, and combined the two datasets using Corset (Davidson & Oshlack, 2014). We mapped the reads back to the transcriptome using Salmon (Patro et al., 2015), with ~75-82% of reads mapping per dataset.

The transcriptome was then functionally annotated using Trinotate (Bryant et al., 2017), a comprehensive annotation suite designed for de novo transcriptomes, including a sequence similarity search (Uniprot/SwissProt), protein domain identification (HMMER and PFAM), and eggNog mapper (Cantalapiedra et al., 2021). We also used the Blast2Go program (OmicsBox 3.1.9) to get an additional set of annotation predictions for our differentially expressed transcripts by comparing our sequences against the non-redundant protein sequences database on NCBI.

2.7 Differential gene expression analysis

To determine which genes were differentially expressed across replicates and treatments, we used the R package edgeR (Robinson et al., 2010) which is part of the Trinity pipeline. Pairwise comparisons of gene expression were performed for all genes in all samples, and correlation heatmaps were produced to compare overall gene expression profile similarity across biological replicates. Genes were considered "differentially expressed" if there was a logarithm of fold change (logFC) $> |2|$ (i.e., a four-fold change in gene expression between experimental conditions) and a false-discovery rate adjusted p-value $<$ 0.001. Differentially expressed genes were also annotated using the program Blast2Go (OmicsBox 3.1.9), which searches the National Center for Biotechnology Information (NCBI) protein database using a translated nucleotide sequence, and identified the top 100 protein hits for each of our differentially expressed transcripts.

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2.8 Shell morphology and growth

On day 90, all surviving juvenile clams (n=137) were collected. Soft tissues were removed, immediately frozen at -20 °C and later freeze-dried. Shells were gently cleaned with fresh water and air-dried. We measured the following traits of clam shells: (1) total axis of maximum growth (AMG), (2) new growth along the AMG from the calcein mark, (3) shell dry weight and (4) freeze-dried soft tissue weight (**Fig. 2**). Furthermore, in order to account for differences in shell sizes and growth rates, we calculated the percentage of AMG represented by new growth during the experiment as (5) % new growth = [(new growth (mm) / total axis maximum growth (mm))*100]. Shell weight and soft tissue dry weight were then measured using a Mettler Toledo ME104E scale (0.0001 g). Shell length and AMG were measured using digital calipers (0.001 mm). New growth along the AMG was measured in Fiji (formerly ImageJ) using images taken on a Nikon AZ100 fluorescent microscope (Schindelin et al., 2012) (Supplemental Figure 1).

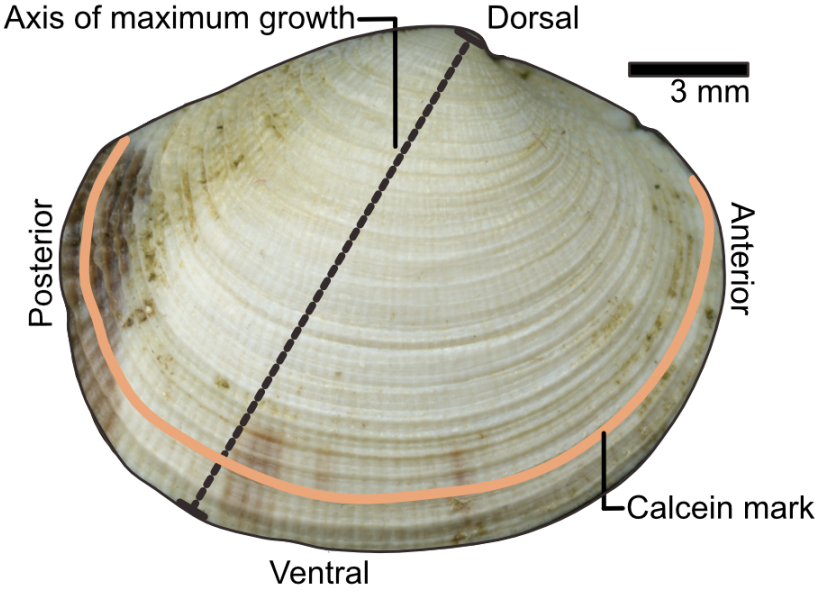

Figure 2. **Diagram illustrating the measurements taken on *Leukoma staminea* shells**. Image taken on the right valve of a juvenile clam used in this study.

2.9 Statistical analyses

Two-way analysis of variance (ANOVA) tests were performed to evaluate the statistical relationships between shell growth, carbonate chemistry, and treatment types. Here, two-way ANOVAs tested whether the overlying water pH and/or shell hash affected the pore water pH, pore water alkalinity, and pore water Ω_{Ar}. For morphological characteristics, we tested whether overlying water pH (independent variable 1) and shell hash (independent variable 2) affected the percent of new growth and

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shell weight (dependent variables). When data were not normally distributed, we transformed them using a log function. For all comparisons, we first ran three ANOVAs: (1) an additive two-way ANOVA (without any interaction or blocking variable), (2) a two-way ANOVA with interaction (sediment type and pH level) but with no blocking variable (bucket ID), and (3) a two-way ANOVA with interaction (sediment type and pH level) and a blocking variable (bucket ID). We then calculated Akaike information criterion (AIC) to determine which ANOVA model best fit the data and reported F-statistics and p-values from that model. As two-way ANOVAs only describe which parameters (i.e., sediment type and overlying pH) are significant, post-hoc Tukey Honestly-Significant-Different (HSD) tests were conducted to test for pairwise differences between groups (C, SH, EA, EASH). Lastly, to investigate bucket effects on growth, a linear mixed effects model was applied using restricted maximum likelihood in the lme4 package in R. The model predicted the percent new growth with treatment as a fixed-effect parameter and bucket ID as a nested random-effect within treatment. The call for this model in lme4 was: percent_new_growth ~ treatment + (1 | bucket.id/treatment). All statistical tests were performed in R (version 3.5.3).

3 Results

3.1 Terminology

For readability, we will use the following terms for our experimental conditions: “C” = control (seawater pH ~7.8, no shell hash in the sediment); “SH” = shell hash (seawater pH ~7.8; shell hash added); “EA” = experimental acidification (seawater pH ~7.4, no shell hash in the sediment); “EASH” = experimental acidification plus shell hash (seawater pH ~7.4, no shell hash in the sediment).

3.2 Seawater chemistry

Salinity, temperature, and dissolved oxygen remained relatively constant over the course of the 90-day experiment (**Table 1**; **SI Table 1**). All pH values of the overlying water demonstrate the experimental design was successful in maintaining the desired values around 7.4 and 7.8. The pH of the pore water was generally stable through the course of the experiment (**Figure 3**) although there was a notable drop in pore water pH in the control condition over time. pH values did not vary significantly among technical replicates (T-test: $p > 0.05$), and we therefore pooled replicate data for calculating means and standard deviations (**SI Table 1**).

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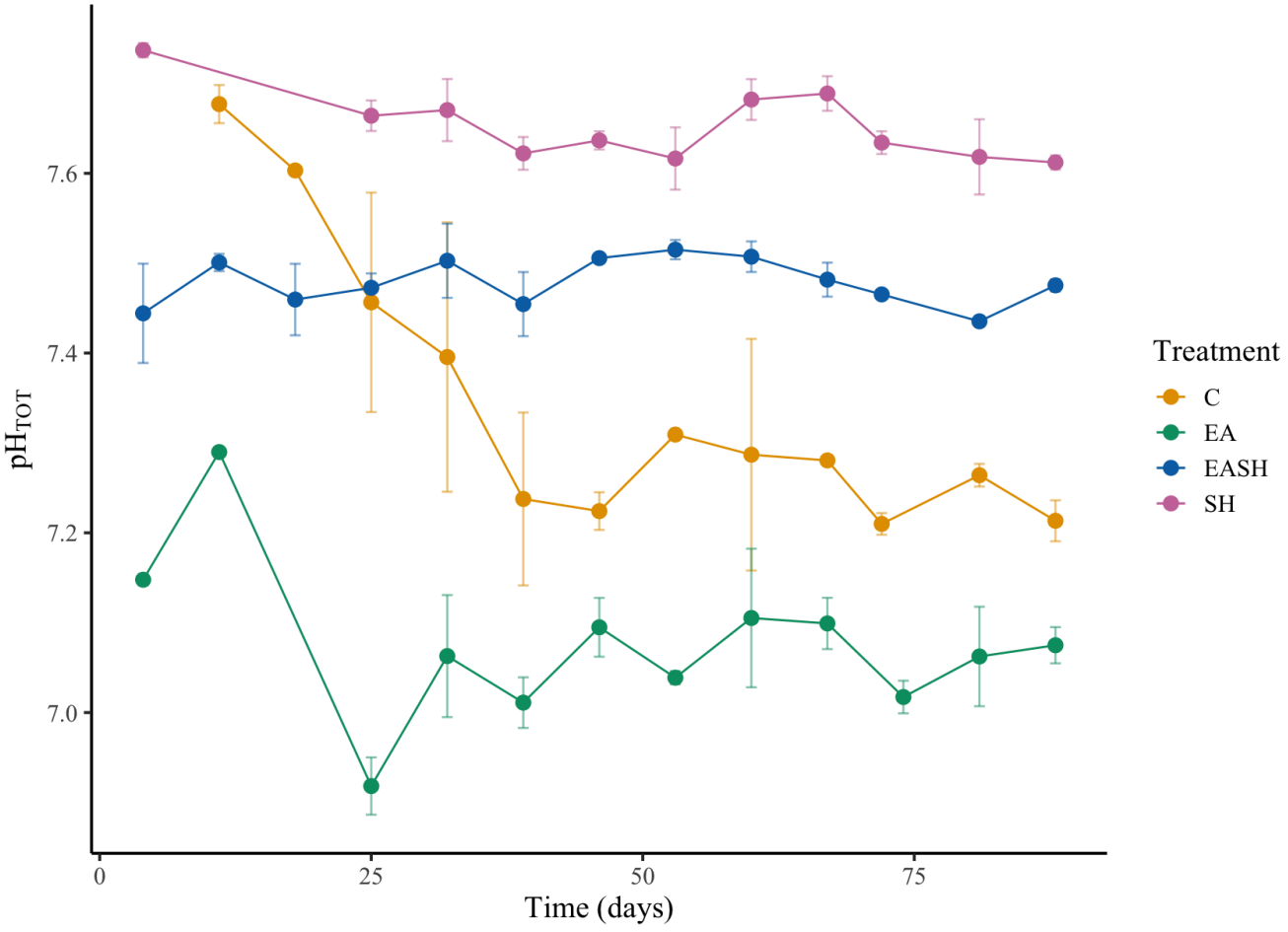

Figure 3. Porewater pH_{TOT} over time (days). Points on the plot show the mean values, and error bars denote one standard deviation. A set of samples from the beginning of the experiment are missing due to sampling error.

Table 1. Means and standard deviations for salinity, temperature, dissolved oxygen, and pH measured in the header tanks (acidified and control) over the course of the 90-day experiment.

Header tank	Salinity (ppt)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH (in situ)
Acidified	34.6 +/- 0.500	15.7 +/- 0.490	8.22 +/- 0.569	7.80 +/- 0.039
Control	34.6 +/- 0.510	15.7 +/- 0.490	8.25 +/- 0.471	7.31 +/- 0.028

The different treatments show markedly different seawater chemistry. Box plots of the data are provided in **Fig. 4** and are consistent with expectations. In the control, pore water pH and Ω_{Ar} values are markedly lower than the overlying seawater. Adding shell hash raises these values in the pore water, while experimentally acidifying the overlying water lowers these

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values in the pore waters. Adding shell hash to experimentally acidified treatments increases pH and Ω_{Ar} values in the pore water, with Ω_{Ar} values comparable to what is seen in the non-acidified treatment. Using two-way ANOVA tests, we confirmed that the sediment type (shell hash vs. no shell hash) and overlying pH (acidified or non-acidified) had a significant effect on pore water alkalinity, pH and Ω_{Ar} (**Table 2**) and Tukey post-hoc tests for all carbonate chemistry parameters revealed significant pairwise differences between many treatments based on presence/absence of shell hash and overlying pH (**Table 3**). In summary, pore water pH decreased when the overlying seawater had a low pH, but that was countered by the addition of shell hash to the sediment.

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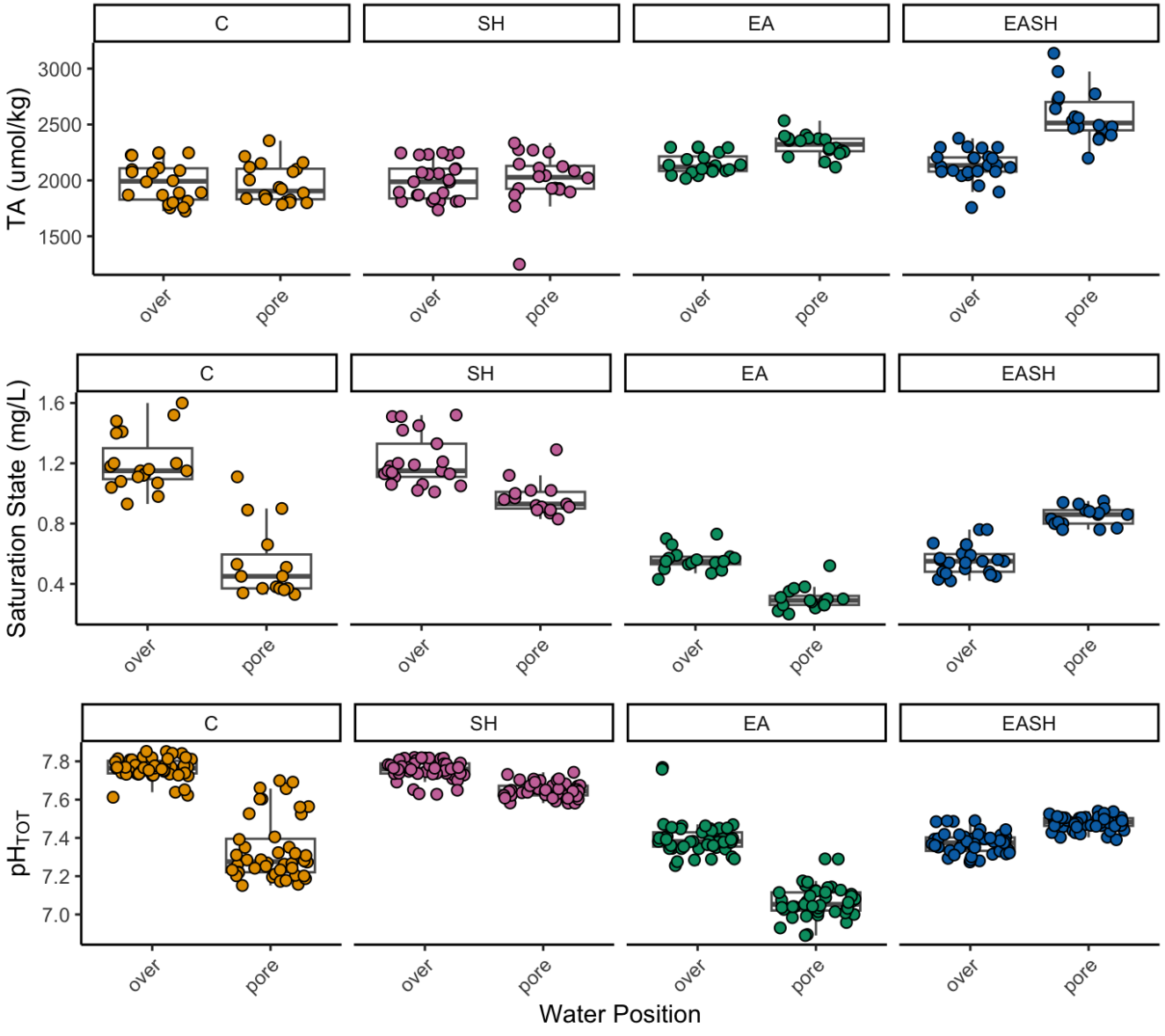

Figure 4 Box plots depicting total alkalinity (TA), aragonite saturation state, and pH_{TOT} for all treatments. Results are separated between pore water and overlying water measurements. C = control; SH = shell hash; EA = experimental acidification; EASH = experimental acidification + shell hash.

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	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Alkalinity					
sed_type	1	0.0473	0.0473	5.050	0.0281
over_pH	1	0.7360	0.7360	78.625	9.66e-13
sed_type:over_pH	1	0.0462	0.0462	4.931	0.0299
Residuals	64	0.5991	0.0094		
pH					
sed_type	1	0.07884	0.07884	196.999	< 2e-16
over_pH	1	0.02975	0.02975	74.339	3.83e-15
sed_type:over_pH	1	0.00172	0.00172	4.298	0.0396
Residuals	175	0.07004	0.0004		
Saturation State (ΩAr)					
sed_type	1	8.465	8.465	112.44	2.78e-14
over_pH	1	1.751	1.751	23.25	1.42e-05
sed_type:over_pH	1	0.707	0.707	9.39	0.00354
Residuals	49	3.689	0.075		

Table 2. ANOVA tests for water alkalinity, pH and Ω_{Ar}. Interaction models are reported here; see the associated code for results from all ANOVA model tests. Key: "sed_type" = sediment type (shell hash vs. no shell hash); "over_pH" = overlying seawater pH (normal vs. experimentally acidified seawater).

Tukey HSD Results for Pore Water Chemistry ($p < 0.001$)		
pH	**Alkalinity**	**Saturation State**
SH:C EA:C EASH:C EA:SH EASH:SH SH:EA	SH:C EA:C EASH:C EA:SH EASH:SH EASH:EA	SH:C EA:C EASH:C EA:SH EASH:EA

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Table 3. Tukey post-hoc tests for all pore water carbonate chemistry parameters analyzed with two-way ANOVA. Significant pairwise differences between treatments based on presence/absence of shell hash (sand vs. sand with shell hash) and overlying pH (low vs. high) are shown for pH, alkalinity and aragonite saturation state.

3.3 Shell morphology and growth

At the end of 90 days, 137 clams survived. Calcein stains were reliably produced in most individuals, with only a small subset lacking distinct lines (n=7). Therefore, a total of 130 clams were measured and compared statistically at the end of the experiment. Average percent new growth varied markedly across treatments (**Table 4**), with those in shell hash (SH) treatment growing the most (9.38 +/- 7.24 % new growth) and those in the experimental acidification (EA) treatment growing the least (5.65 +/- 3.50 % new growth). Two-way ANOVAs revealed that differences in average percent new growth were statistically significant due to overlying pH [$F(1)=5.82$, $p = 0.0173$], but not presence/absence of shell hash [$F(1)=3.01$, $p = 0.085$], and the interaction between these metrics was not statistically significant ($p = 0.066$). However, Tukey HSD post-hoc tests for percent new growth suggest the EA treatment had significantly lower growth when compared to the other treatments. Two-way ANOVAs and Tukey HSDs did not identify statistically significant differences in shell weight or tissue weight across treatments with overlying pH or sediment type ($p > 0.05$). Bucket effects did not affect growth metrics, with only 0.154% variance explained by bucket ID (SI Table 2). Our results suggest that, despite high variation in growth across treatments, animals experiencing experimental acidification and shell hash had a higher rate of shell growth than those experiencing experimental acidification only, and that these animals were statistically indistinguishable from the control.

	Shell length (mm)	Total AMG (mm)	Growth on AMG (mm)	% New growth	Shell weight (g)	Tissue dry weight (mg)
C	12.4 +/- 4.21	11.5 +/- 3.76	1.05 +/- 0.815	8.30 +/- 4.24	0.604 +/- 0.485	47.5 +/- 39.7
SH	13.2 +/- 4.33	11.1 +/- 3.45	1.13 +/- 0.963	9.38 +/- 7.24	0.611 +/- 0.538	49.7 +/- 44.3
EA	12.8 +/- 3.53	10.7 +/- 2.91	0.670 +/- 0.510	5.65 +/- 3.50	0.468 +/- 0.374	35.7 +/- 27.9
EASH	12.8 +/- 3.97	11.0 +/- 3.15	0.873 +/- .500	7.46 +/- 3.35	0.486 +/- 0.414	37.5 +/- 31.5

Table 4. Morphological metrics measured on day 90. Averages and standard deviations are shown. AMG = axis of maximum growth, C = normal pH conditions, SH= normal conditions with shell hash, EA = experimental acidification, EASH = experimental acidification + shell hash.

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3.4 Gene Expression Analysis

Our transcriptome of *L. staminea* mantle tissue included 638,598 transcript models, which clustered into 530,958 gene models. This number is likely far higher than the actual number of genes present, but this is not unusual for a *de novo* transcriptome assembly (Haas et al., 2013; Raghavan et al., 2022). 284 genes were found to be differentially expressed, based on a cutoff of a p-value < 0.001 and a > 4-fold change in expression between two or more conditions. Notably, the experimentally acidified (EA) treatment looked markedly different from all other treatments. The principal components plot (**Fig. 5A**) and sample correlation matrix (**Fig. 5B**) demonstrate that the replicates from the EA treatment cluster together, while the other conditions are intermixed. These plots are consistent with the numbers of differentially expressed genes recovered; excluding the EA condition, no more than 11 of the 284 genes were differentially expressed in any pairwise comparisons. This suggests that the addition of shell hash to experimentally acidified water resulted in a gene expression profile largely indistinguishable from animals raised in non-acidified conditions, with or without shell hash.

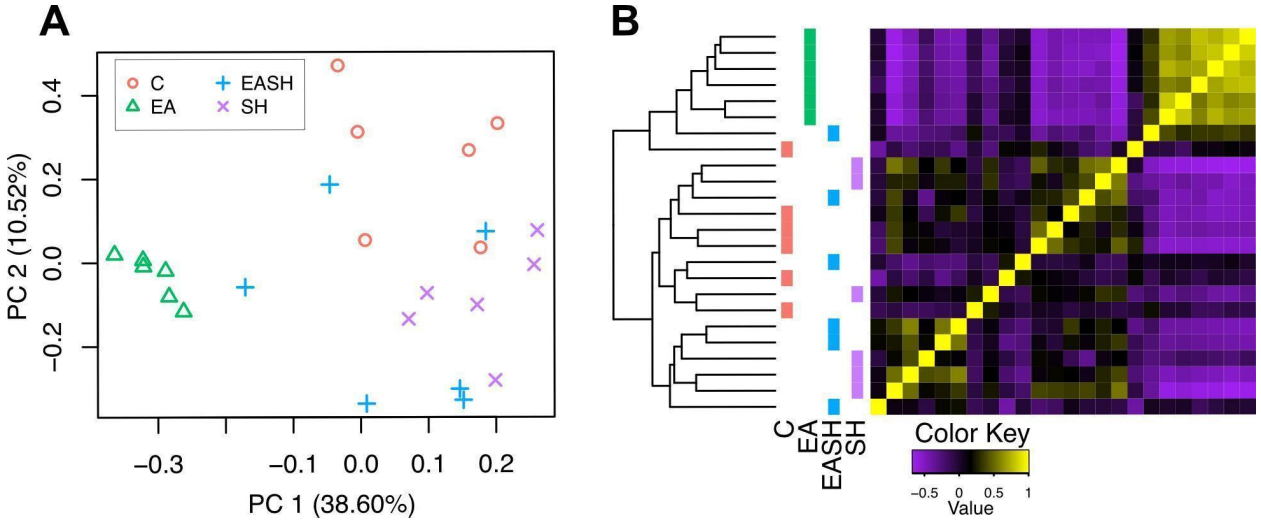

Figure 5. Overview of differential gene expression analysis. (A) PCA plot demonstrating similarity among the replicates, based on the count of differentially expressed genes. (B) A correlation matrix of the same data, with the count data log transformed and centered around the mean. Note how, in both analyses, the EA replicates cluster together, while samples from all other conditions are intermixed.

While the broad pattern of gene expression supports our hypothesis that adding shell hash would result in a loss of pH-stress genetic signatures, we were only able to annotate a small subsample of the genes, which limited our ability to understand the range of biological processes being impacted in our experiment. The vast majority of our 284 differentially expressed genes failed to receive an annotation in the Trinotate pipeline; a second approach using BLAST2GO did only marginally better (see **SI Table 3** for details). Attempts to identify enriched biological pathways based on gene annotations failed to recover any

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statistically significant results. We therefore performed several additional analyses to look for possible genes that play a role in response to ocean acidification.

Firstly, we tested if any differentially expressed genes were shared between two or more treatments, looking for genes generally involved in tolerance to acidified seawater. Twelve genes were differentially expressed in two or more pairwise comparisons. Of these, 10 genes were found in multiple pairwise comparisons against the "EA" condition, suggesting these genes play a general role in acidification tolerance. Unfortunately, only one of these genes was annotated in our pipeline, showing ~79% similarity to "DNA repair protein complementing XPA cells homolog" of the Manila clam *Ruditapes philippinarum*. In mice, the XPA protein plays a critical role in nucleotide excision repair in response to ultraviolet and chemical damage (de Vries & van Steeg, 1996; Pan & Lee, 2009); it is unclear whether the *L. staminea* gene plays a similar role, but we note that four additional differentially expressed genes were similarly annotated as XPA-like proteins, and the role of XPA proteins in *L. staminea* in response to ocean acidification is worth exploring further.

We also tested how many of our differentially expressed genes could be identified in other bivalves, with the thought that these genes, though potentially unannotated, could reveal important clade-specific regulators of acidification tolerance. We downloaded a reference proteome for every bivalve genus in the UniProt database (https://www.uniprot.org/), as well as a selection of non-bivalve animal outgroups. We then used BLASTx to compare our differentially expressed *L. staminea* transcripts against these proteomes, using an e-value cutoff of 10e-10. The results of this analysis are shown in **Fig. 6** (and detailed in SI Table 4). We tested 299 transcripts predicted from the 284 gene models (Trinity attempts to predict isoforms, so there are often more transcripts than gene models), yet only 49 matched to a sequence in one or more proteomes. There is some phylogenetic signal in the data, with the highest number of matches coming from *Dreissena polymorpha*–which, like *L. staminea*, is part of the taxonomic superorder Imparidentia–and bivalves generally showed a larger number of matches than other animals (Figure 6A). Fourteen transcripts were found across all datasets. Most of these appear to be general cell maintenance genes, but one notable exception is a perlucin-like protein, which is known to regulate calcium carbonate precipitation in some molluscs and is necessary for proper shell growth in oysters raised in acidified seawater (Schwaner et al., 2023). We also identified a putative monocarboxylate transporter, which has been recovered in other experimental acidification studies on bivalves, and is hypothesized to transport ions to the site of biomineralization (Li et al., 2022; Sleight et al., 2020). Another two transcripts from *L. staminea* matched sequences present in all analyzed bivalve proteomes. Both had annotations, including a putative NADPH oxidase 5-like gene, which has also been identified in Atlantic surf clams (*Spisula solidissima*) exposed to acute heat stress (Acquafredda et al., 2024). A final nine sequences were uniquely shared between *D. polymorpha* and *L. staminea*; most of these were unannotated and we found no evidence that the few genes with annotations had been identified in previous bivalve RNA-Seq studies. These results do not support the hypothesis that our dataset is

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enriched in bivalve-specific genes, but this exercise did reveal some important genes thought to regulate environmental tolerance and/or biomineralization in other species.

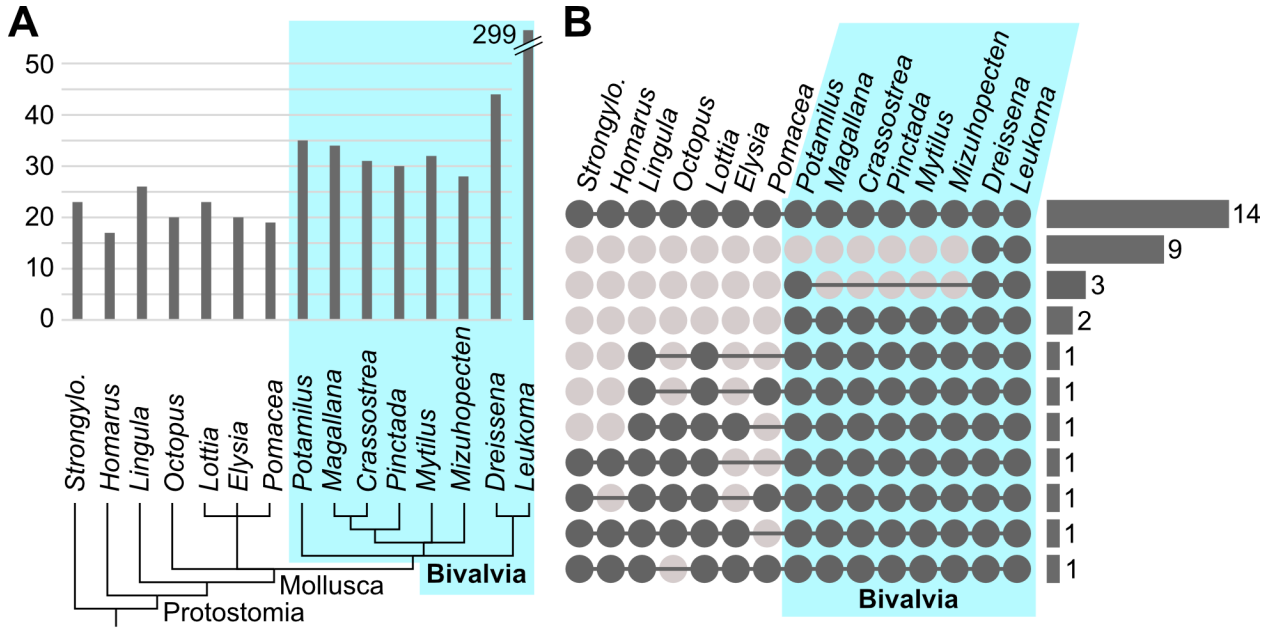

Figure 6. Summary of sequence similarity between the differentially expressed genes in this study and proteomes from other species. (A) A plot showing the number of matching sequences between *L.staminea* and other genera, with a phylogenetic tree at the bottom showing evolutionary relationships. (B) An UpSet plot visualizing the shared number of transcripts found in *L.staminea* and other organisms. All other combinations resulted in one or fewer shared genes. In both images, the bivalves are highlighted in blue; "Strongylo." = *Strongylocentrotus*.

4 Discussion

Our results suggest that shell hash can lessen the effects of acidification by buffering the chemistry of pore fluids, thus providing a potential acidification mitigation strategy for infaunal organisms like clams (Curtin et al., 2022; Doyle & Bendell, 2022; Ericson & Ragg, 2021; Green et al., 2013; Salter, 2018). Gene expression profiling of clam mantle tissue suggested that shell hash was beneficial for the clams, as the "acidification" signal present in animals grown under low-pH conditions disappeared when shell hash was added. Further, despite high variability in growth metrics across individuals—which complicated our statistical analyses—our results suggest the addition of shell hash to experimentally acidified water resulted in greater *L. staminea* shell growth, to the point that it was statistically indistinguishable from the control population. In the future, additional studies using animals of the same size—alongside age and genetic cohort—may help disentangle individual variability from the effects of acidification on shell growth.

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Additional research should investigate shell hash buffering in natural settings. Existing studies on the role of shell hash in moderating pore water chemistry have had variable results, and local environmental conditions appear critically important (Beal et al., 2020; Curtin et al., 2022; Doyle & Bendell, 2022; Green et al., 2009, 2013; Greiner et al., 2018). Still, our research provides clear support for the hypothesis that shell hash can buffer pore water chemistry, and mitigates a natural tendency for pore water pH to decrease over time (Figure 3). Therefore, this study warrants continued research investigating scalability, efficacy, and safety of using shell hash to buffer acidification. Further research is also needed to assess the ability of shell hash to buffer acidic conditions at the water-sediment interface where larvae first settle and clams' siphons extend (Green et al., 2009; Waldbusser et al., 2015). This research is needed for many infaunal species, as compounding environmental stressors could yield conditions that are too corrosive for larval settlement and development (Doyle & Bendell, 2022; Green et al., 2009; Waldbusser et al., 2015).

5 Conclusions

In conclusion, this experimental study suggests that shell hash buffers acidic conditions in pore waters and benefits clam growth. Implementation of this technique in coastal settings, however, would best be considered on a site-by-site basis, and include partnerships with Indigenous communities and other experts with an understanding of local practices. The controlled conditions of the laboratory ignore variables that will likely dictate the success of field projects, such as the chemical and microbial complexity of natural sediments and tidal influences. Finally, our findings further point towards the need for research and stewardship explored in collaboration with Indigenous communities who continue to visit, gather from, and care for intertidal spaces. This study, like all research, is conducted on Indigenous land, and "place always matters" (Justice 2016, 21). As documented elsewhere on the Pacific Coast (Groesbeck et al., 2014; Lepofsky et al., 2015), tending coastal Californian clam beds is grounded in Indigenous systems of knowledge. We draw from current calls to braid Indigenous science with western science, and other "boundary spanning" approaches, that support the goals and interests of Indigenous communities, not science alone (Whyte et al. 2016; Atalay, 2020; Hatch et al., 2023; Kimmerer & Artelle, 2024 White et al., 2024; Wickham et al., 2022). In all, this experiment provides significant evidence of shell hash buffering capacity, and provides promising results to prompt future collaborative research in coastal settings on clam bed tending in the face of continued climate change.

Data Availability

The genetic data produced in this study is accessible on NCBI (Accession: PRJNA1011264). The code used to analyze the data is provided on GitHub at https://github.com/DavidGoldLab/2025_Leukoma_RNA-Seq (Zenodo DOI 10.5281/zenodo.19488331). Shell images are available on Harvard Dataverse at https://doi.org/10.7910/DVN/DL8BY0.

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Author contributions

HLK: conceptualization, investigation, model experiments, funding acquisition, formal analysis, visualization, and writing (original draft preparation). AJG: methodology and writing (review and editing). TMH: methodology and writing (review and editing). TDS: supervision, conceptualization, and writing (review and editing). SJC: project administration, supervision, conceptualization, and writing (review and editing). DAG: project administration, supervision, conceptualization, formal analysis, funding acquisition and writing (original draft preparation).

Competing interests

The contact author has declared that none of the authors has any competing interests.

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7 Supplemental Figure Legends

Supplemental Figure 1. Example fluorescent image of a calcein-stained shell. Area of the shell within the blue lines indicates shell growth during the duration of the experiment. Red line indicates the length of new growth. All shell images are available on Harvard Dataverse at https://doi.org/10.7910/DVN/DL8BY0.

Supplemental Figure 2. pH measurements from Pinpoint® probes in the overlying water plotted over time for all buckets. Dotted lines show average for acidified and non-acidified treatments.

8 Supplemental Table Legends

Supplemental Table 1. Mean values and standard deviations for pH and aragonite saturation state (**Ω**) of overlying water and pore waters in the bucket treatments. Sample results here were taken once weekly for spectrophotometric pH analysis and alkalinity titrations. OA = acidification, OASH = acidification + shell hash, C = control, and SH= control pH with shell hash.

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Supplemental Table 2. Summary of linear mixed effects model fit to percent new shell for clams at the end of the 90-day experimental period. Fixed effects (i.e., treatment) have constant, systematic influence on the dependent variable (here, percent new shell). Random effects (i.e., bucket replicate ID) account for random variability not directly related to independent variables.

Supplemental Table 3. Annotation of differentially expressed genes in the L. staminea transcriptome. Full data can be found on GitHub, file "DE_Genes_Summary.xlsx".

Supplemental Table 4. Results of BLASTp analyses comparing the differentially expressed L. staminea protein models with proteomes from select animals. Full results can be found on GitHub in folder "7_DE_Genes".

9 Supplemental Data

Supplemental Sheet 1. Complete list of cleaned total pH data.

Supplemental Sheet 2. Complete list of cleaned total alkalinity data.

Supplemental Sheet 3. Complete calculated carbonate system data using the R package 'seacarb'.

Supplemental Sheet 4. Complete list of pH measurements using Pinpoint® probes.

Supplemental Sheet 5. Complete list of salinity measurements using VeeGee refractometer.

Supplemental Sheet 6. Complete list of dissolved oxygen measurements using Pinpoint® probes.

Supplemental Sheet 7. Complete list of temperature measurements using a digital thermometer.

Supplemental Sheet 8. Table with raw clam growth data measured day 90 of the experiment.

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