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RESEARCH

Molecular Features Associated with Resilience to Ocean Acidification in the Northern Quahog, *Mercenaria mercenaria*

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Abstract

The increasing concentration of CO_2 in the atmosphere and resulting flux into the oceans will further exacerbate acidification already threatening coastal marine ecosystems. The subsequent alterations in carbonate chemistry can have deleterious impacts on many economically and ecologically important species including the northern quahog (*Mercenaria mercenaria*). The accelerated pace of these changes requires an understanding of how or if species and populations will be able to acclimate or adapt to such swift environmental alterations. Thus far, studies have primarily focused on the physiological effects of ocean acidification (OA) on *M. mercenaria*, including reductions in growth and survival. However, the molecular mechanisms of resilience to OA in this species remains unclear. Clam gametes were fertilized under normal pCO_2 and reared under acidified (pH ~ 7.5, pCO_2 ~ 1200 ppm) or control (pH ~ 7.9, pCO_2 ~ 600 ppm) conditions before sampled at 2 days (larvae), 32 days (postsets), 5 and 10 months (juveniles) and submitted to RNA and DNA sequencing to evaluate alterations in gene expression and genetic variations. Results showed significant shift in gene expression profiles among clams reared in acidified conditions as compared to their respective controls. At 10 months of exposure, significant shifts in allele frequency of single nucleotide polymorphisms (SNPs) were identified. Both approaches highlighted genes coding for proteins related to shell formation, bicarbonate transport, cytoskeleton, immunity/stress, and metabolism, illustrating the role these pathways play in resilience to OA.

Keywords RADSeq · RNASeq · Clam · Ocean acidification · SNPs

Introduction

The northern quahog (*Mercenaria mercenaria*) is an ecologically and economically important species within coastal and estuarine ecosystems along the Eastern coast of the United States. As benthic suspension feeders, clams filter algae and suspended particles from the water column which can improve water quality by increasing light penetration for seagrasses (Wall et al. 2008), prevent algal blooms (Cerrato et al. 2004), and contribute to nutrient cycling (Dame 2011). In addition to these beneficial services, *M. mercenaria* is also one of the most valuable aquaculture species for the east coast and supports both recreational and commercial fisheries. Total clam aquaculture in the US had an estimated value of $129 million in 2018 (NFMS 2019), and the fishery was valued at $52.8 million (NMFS 2019). Calcifying marine organisms, such as *M. mercenaria*, are particularly vulnerable to ocean acidification (OA). Understanding how clams will fare under predicted OA is important as this could have implications for local and national economies and estuarine ecology.

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Alterations in carbonate chemistry from elevated CO_2 in seawater will result in a reduction of carbonate (CO_3) and calcium carbonate saturation ($CaCO_3$), which will impact marine organisms that biomineralize $CaCO_3$ to form shells (Feely et al. 2004, 2009; Sabine et al. 2004). Changes to the external seawater chemistry can also affect the acid–base balance of bivalves (Michealidis et al. 2005; Fabry et al. 2008). Evidence suggests that bivalves can respond to OA via adaptive mechanisms including reallocating metabolic resources (Lanning et al. 2010; Thomsen and Melzner 2010; Xu et al. 2016; Gray et al. 2017); however, this can lead to trade-offs between competing physiological processes such as immunity (Burge et al. 2014; Sun et al. 2017; Schwaner et al. 2020).

Some species and populations of marine bivalves are already exposed to coastal waters with low pH and elevated pCO_2 (Wallace et al. 2014). Coastal acidification is a current problem in many estuaries because of excessive nutrient runoff that leads to algal blooms and subsequent microbial decay (Cai et al. 2011; Wallace et al. 2014; Baumann et al. 2015; Laurent et al. 2017). In natural populations of *M. mercenaria*, gonad maturation occurs when water temperatures begin to rise in the late spring and early summer and culminates in spawning when temperatures exceed 20 ℃ (Rice 1992). Spawning and larval development coincides with coastal waters reaching their extremes of acidification (Wallace et al. 2014; Baumann et al. 2015). Consequently, these animals are already challenged with high CO_2 and low pH during vulnerable stages of development, which will become more extreme with climate change.

Advances in omics have revealed many molecular responses to short term CO_2 stress that might enable organisms to tolerate variable pH (Timmins-Schiffman et al. 2014; Dineshram et al. 2015; Liu et al. 2016; Goncalves et al. 2017; Zhao et al. 2020). Studies of the oysters *Crassostrea gigas* (Timmins-Schiffman et al. 2014), *C. hongkongensis* (Dineshram et al. 2015), *Saccostrea glomerata* (Goncalves et al. 2017), and *Pinctada fucata* (Liu et al. 2016) revealed pathways underlying resilience to OA, including calcium homeostasis, acid base regulation, and metabolism. On the other hand, studies of the transcriptional response of bivalves to OA also reveal depressed pathways (Goncalves et al. 2017; Zhao et al. 2020). For example, the Korean mussel (*Mytilus coruscus*) downregulates gene expression levels of proteins involved in shell formation, resulting in weakened shells (Zhao et al. 2020).

While prior research has primarily focused on acclimation through transcriptomic analyses, studies are beginning to investigate the potential for adaptation to OA by looking at genetic variation and evidence of selection. Differences in the single nucleotide polymorphism (SNP) profiles of the mussel *M. galloprovincialis* between those reared in normal and acidified conditions suggest the potential for rapid adaptation to OA (Bitter et al. 2019). The authors identified hundreds of SNPs responding to each pH treatment and suggested that loci critical for increased fitness under OA are under selection in low pH environments (Bitter et al. 2019). Similarly, studies in the sea urchin *Strongylocentrotus purpuratus* (Pespeni et al. 2013a, b) reported significant allelic changes that suggested the potential for adaptation to OA. In contrast, there was little evidence to support adaptation potential in tropical sea urchin *Echinometra* sp. (Uthicke et al. 2019), highlighting variability between organisms and further demonstrating the need to expand these studies to encompass more species.

Although the physiological impacts of OA on *M. mercenaria* have been investigated (Talmage and Gobler 2009, 2011; Schwaner et al. 2020), the molecular mechanisms associated with resilience to OA in this species are relatively unknown. The objectives of this study were to determine if OA has a significant impact on the regulation of the transcriptome of *M. mercenaria* throughout various life history stages and to determine if OA-induced differential mortality allows the identification of SNPs associated with resilience. Here, potential for acclimation and adaptation to OA in *M. mercenaria* was explored. Impacts of acidification on gene expression and genetic variation throughout development in larvae and juveniles were investigated. Clams were continuously reared in control (pCO_2 of ~ 600 ppm, pH ~ 7.9) or acidified seawater (pCO_2 of ~ 1200 ppm, pH ~ 7.5) and samples were collected at 2 days (larvae), 32 days (postsets), 5 months (juveniles), and 10 months (juveniles) postfertilization for analysis of differentially expressed genes (DEGs). Clams that had survived 10 months of chronic OA exposure were deemed as "resilient" to OA, and samples were collected to determine differences in the frequency of SNPs between pCO_2 conditions. Furthermore, the function of differentially expressed genes or genes containing variants was investigated to identify molecular pathways associated with resilience to acidification.

Materials and Methods

Seawater Chemistry and Sample Collection

In order to increase genetic diversity of experimental animals, wild adult clams from four populations (20 clams from each of the following locations: Wellfleet, Massachusetts (41.9305° N, 70.0310° W); Northport, New York (40.9009° N, 73.3432° W); Hampton Bays, New York (40.8690° N, 72.5176° W); Riverhead, New York (40.9170° N, 72.6620° W) were maintained as broodstock and conditioned for spawning (Helm et al. 2004; Wallace et al. 2008; Schwaner et al. 2020). Females were moved into individual containers for egg collection, and after completion of spawning, eggs and sperm were mixed to ensure genetic heterogeneity. After allowing sufficient time for fertilization (1 h), around 30

million embryos were moved to aquaria with either ambient seawater (pCO_2 of ~ 600 ppm, pH ~ 7.9) or acidified seawater (pCO_2 of ~ 1200 ppm, pH ~ 7.5). Aquaria were continuously bubbled with 5% CO_2 mixed with air (via multi gas channel proportioners from Cole Parmer (Antylia Scientific; Vernon Hills, IL); Schwaner et al. 2020) or ambient air to maintain pCO_2 conditions. Seawater samples were collected and analyzed (Schwaner et al. 2020) using an EGM-4 Environmental Gas Analyzer ® (PP systems; Amesbury, MA) after acidification and separation of gas phases using a Liqui-cel® Membrane (3 M Membrana; Wuppertal, Germany). pCO_2, total alkalinity, carbonate concentration, and aragonite and calcite saturation were calculated using the R package *seacarb* (Gattuso et al. 2018), with known first and second dissociation constants of carbonic acid in seawater (Millero 2010). For quality assurance, certified reference materials were analyzed (provided by Andrew Dickson, Scripps Institution of Oceanography) with a 99.99% recovery rate (Tables S1 and S2). Larva rearing followed protocols outlined in previous publications (Helm et al. 2004; Wallace et al. 2008; Schwaner et al. 2020). After 2 days in either control or acidified conditions, about four million larvae from each condition (1 million/replicate) were sampled. Larvae were collected on a 50-micron sieve, rinsed to remove algae and detritus, checked for viability, and the resulting pellet was flash frozen and stored at -80 °C (sampling outlined in Fig. S1). Immediately after settling and metamorphosis (32 days post fertilization, hereby designated "postsets"), 10 juvenile clams (height 0.41 mm ± 0.1, length 0.44 mm ± 0.1) per replicate (40 per pCO_2 condition) were flash frozen and preserved at -80 °C. After settling, juvenile clams were transferred from a static system to a flow through system with water from Old Fort Pond in Southampton, NY (40°53′07.2"N 72°26′31.4"W). For the acidified condition, seawater flowed into an acidification chamber where 100% CO_2 was mixed with air via multi gas channel proportions and continuously bubbled to maintain a delta of 0.4 units between the two conditions (carbonate chemistry in Tables S3 and S4). After the seawater reached target values, it flowed into a header tank and the four corresponding replicate tanks using a "downweller" setting. Clams were held on 212 µm nylon mesh sieve and seawater flowed from the top to the bottom compartment of the tank (Schwaner et al. 2020). For the control condition, water flowed into an aerated head tank where it then continuously flowed into each of four replicate tanks. After five months of continuous exposure to either control or acidified conditions in the flow through system, a third sample was collected (10 juveniles/replicate, 40/pCO_2 condition; length 3.65 mm ± 1.21; height 3.34 ± 1.07). Then after 10 months, samples for both RNA sequencing (10 juveniles/replicate, 40/pCO_2 condition) and DNA sequencing (12 juveniles/replicate, 48/pCO_2 condition, 96 total) were collected (length 5.50 mm ± 1.39; height 4.86 mm ± 1.23). Before DNA extraction, juvenile clam tissues were removed from the shells and added to 100% molecular grade ethanol.

RNA and DNA Extraction

RNA was extracted from samples collected at 2 and 32 days, 5 and 10 months using NucleoSpin® RNA Plus Kit (#740,984.50; Macherey–Nagel; Düren, Germany) where DNA was removed with DNA-free™ Kit (#AM1906; Ambion, Inc.; Austin, TX), following manufacturer's protocols. For larval samples, RNA was extracted from a pellet of ~ 100,000 larvae, and for juveniles RNA was extracted from individual whole bodies. RNA quality and quantity were checked using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA). At 10 months, samples were also collected for DNA Sequencing. DNA was isolated from samples using standard phenol:chloroform:isoamyl extraction (Farhat et al. 2020; Boutet et al. 2022). DNA quality and quantity were checked using the Qubit dsDNA HS Assay Kit with assays read on a Qubit 2.0 fluorometer (Life Technologies/Agilent 2100; Waldbronn, Germany) and by agarose gel electrophoresis.

RNAseq Library Preparation, Sequencing, and Analysis

Extracted RNA was sent to Novogene Corporation (Sacramento, CA, USA) for sequencing. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (# E7760L; New England Biolabs; Ipswich, MA), following manufacturer's instructions. Libraries were sequenced on Illumina platform (Novaseq 2X150) and 150 paired-end length reads were then demultiplexed. The final reads received were inputted into the CLC workbench (version 11.0.1 (https://digitalinsights.qiagen.com), and trimmed based on quality scores (limit 0.05), ambiguous nucleotides (max 2 nucleotides per sequence), and adapters. Trimmed reads were mapped onto the *M. mercenaria* gene prediction (Farhat et al. 2022) using the "map reads to reference" function with default parameters. The next in-silico analyses were performed on the computing cluster SeaWulf at Stony Brook University. Reads were sorted, indexed, and the number of reads mapping to transcripts was quantified using Samtools version 1.9 (Li 2011). Counts were normalized and compared between conditions at various times of exposure (2 and 32 days, 5 and 10 months) using *DESeq2* (1.36.0) from Bioconductor (Love et al. 2014). The p-values were corrected for multiple testing using the Benjamini and Hochberg method. DEGs with a log fold change $>|2|$ and adjusted p-value < 0.05 were deemed significant. Principal component analysis (PCA) was performed using *plotPCA* function in *DE Seq2* of the vsd-transformed (variance

stabilizing transformation) counts, volcano maps were created to visualize fold change (Fig. S2), and cluster heat maps were generated for the top DEGs (61–100 depending on number of DEGs) (Fig. S3). We used the functional annotation of *M. mercenaria* proteins generated from the gene annotation (Farthat et al. 2022). A hypergeometric test was performed to test for statistical enrichment (FDR adjusted p value < 0.05) of GO terms using the *TopGO* package in R.

ddRAD Library Preparation, Sequencing, and Analysis

ddRAD libraries were prepared using a protocol with steps modified from Peterson et al. (2012). Briefly, 200 ng of each DNA sample was double digested with PstI high fidelity restriction endonuclease and MseI (New England Biolabs). After 10 h at 37 °C, the enzymes were inactivated by heating at 65 °C for 20 min. DNA samples were individually identified through the ligation of specific P1-n barcoded adapters at 16 °C for 6 h in a 30 µl reaction containing P1 adapter, T4 DNA ligase, 1 mM ATP, P2 adapter, and 1X CutSmart reaction buffer (New England Biolabs). Samples were then purified and size selected using Nucleomag NGS clean-up and size select kit (#744,970.50; Machery-Nagel) at 1:1 ratio of beads to sample and then resuspended in Tris buffer. Purified adapter-ligated fragments were then PCR-amplified with 12 uniquely indexed PCR-P2 primer sequences for multiplexing, allowing for post-sequencing demultiplexing. Gel electrophoresis was performed on PCR products and visualized using GelRed Stain and additional PCR was run if necessary. Each PCR product was then quantified using Qubit dsDNA HS assay kit, and then size selected with Pippin-Prep (1.5% agarose dye free gel cassette, Sage Science; Beverly, MA) under a setting with a range of 350–800 bp. Each fraction (350–800 bp, and 380–800 bp) was quantified with Qubit and quality was checked on a Bioanalyzer with high sensitivity DNA chip (Agilent technologies). To have better distribution, a larger range of sizes, and better representativity of each size fraction we added 50% of the 350–800 bp, and 50% of the 380–800 bp cut to the library for sequencing. 5% of PhiX control libraries were used to create a more diverse set of clusters as is recommended by Illumina for our sample type. Samples were sequenced using Illumina NovaSeq 6000 System with an S4 flow cell (Dr. Graham Wiley, Oklahoma Medical Research Foundation) on one lane.

The resulting paired end reads were demultiplexed by index by Novogene and then demultiplexed by barcode into individual samples using CLC Workbench. Reads were trimmed as described above and then mapped onto the *M. mercenaria* reference genome (Farhat et al. 2022) using CLC "map reads to reference" function with default parameters. This was followed by running the "Local Realignment" tool (selecting to realign unaligned ends with 3 multi passes using guiding variants). Bam files were exported from CLC to Stony Brook's high performance computing cluster, SeaWulf (Stony Brook University 2016) and variants were identified using Stacks v2 with the ref_map pipeline (Rochette et al. 2019). Data filtering for detection of high-quality SNPs included: minimum allele frequency 0.05 (–min-maf), a minimum percentage of samples per population (75%), a HWE p-value of < 0.05 (–hwe) (the probability that this variant deviates from HWE). After filtering, only SNPs that had significantly higher F_{ST} between the compared populations values were selected (corrected AMOVA $F_{ST} > 0.05$ and $p < 0.05$). The position of each variant in the genome (intergenic, untranslated region, coding sequence, or intron) and the type of consequence the variant has on the resulting protein (missense, synonymous, stop gained) were inferred as described by Farhat et al. (2020). To visualize population structure, we performed a principal components analysis to see variation between samples, using the *glPCA* function in R.

Results

We firstly tested the hypothesis that OA induces a significant regulation of the *M. mercenaria* transcriptome and identified genes that are differentially regulated in response to OA. At all life history stages investigated 2 day (larvae), 32 days (postsets), 5 months (juveniles), 10 months (juveniles) there were DEGs between clams in acidified (pH 7.5) and control conditions (pH 7.9). In addition, we explored the possibility that OA leads to selection that can be detected in the SNP profile of survivors and that certain genotypes are resilient to OA. Genetic difference between acidified and control clams at 10 months was overall low; however, there was significant enrichment of SNPs in clams surviving acidified conditions. The significant SNPs and DEGs were found in genes coding for proteins involved in biological processes known to be impacted by OA. These included genes related to shell formation, bicarbonate transport, cytoskeleton, immunity/stress, and metabolism, highlighting the role these pathways play in resilience to OA. The following sections provide thorough results from gene expression and SNP identification for clams under acidification stress.

Differentially Expressed Genes

Gene expression analysis revealed variations in the transcriptome of clams reared in acidified and control conditions in every life stage investigated. While there was evident variation from pCO_2, there were also differences between the developmental stages. The fewest DEGs were found in larvae that were exposed to acidified conditions for only 2 days and juveniles in acidified conditions for 10 months. T he

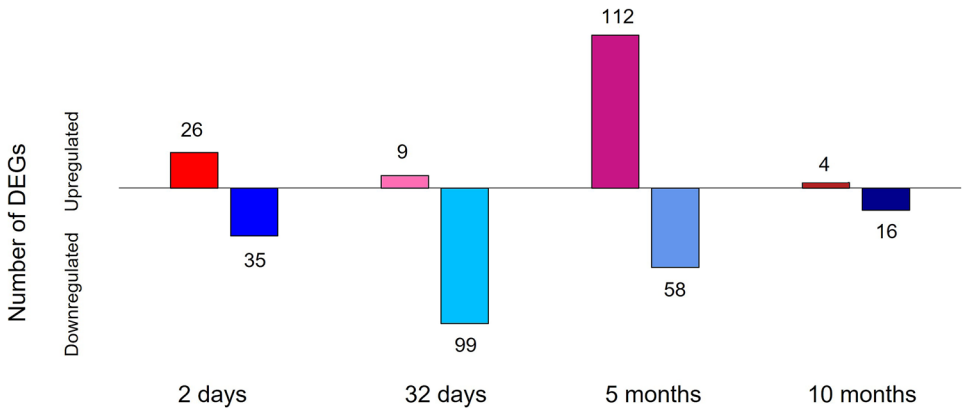


Fig. 1 Differentially expressed gene counts of *M. mercenaria* at 2, 32 days, 5 and 10 months of OA exposure. Number of genes upregulated under acidification stress (shades of red) and downregulated (shades of blue) at each sampling time point

greatest number of DEGs were detected in the 5 month old juvenile clams (Fig. 1). In the postset juveniles (32 days), 92% of their DEGs were downregulated in the acidified condition. Similarly, for 10 month old juveniles 80% of the DEGs were downregulated in the acidified condition.

PCA showed clear separation between pCO_2 treatments, with the 32 day old post set juveniles having the greatest separation between control and acidified clams (Fig. 2). PCA resulted in clustering of samples by age, showing distinct segregation between larval and juvenile samples and the most overlap between 5 and 10 months (Fig. 2).

Several genes upregulated under acidified conditions were similarly expressed between the different life stages (Fig. 3). In addition, many genes upregulated under acidified conditions coding for genes with the same predicted protein, were similarly expressed between life stages (Fig. 4). These genes and proteins were primarily involved with biomineralization and acid–base regulation.

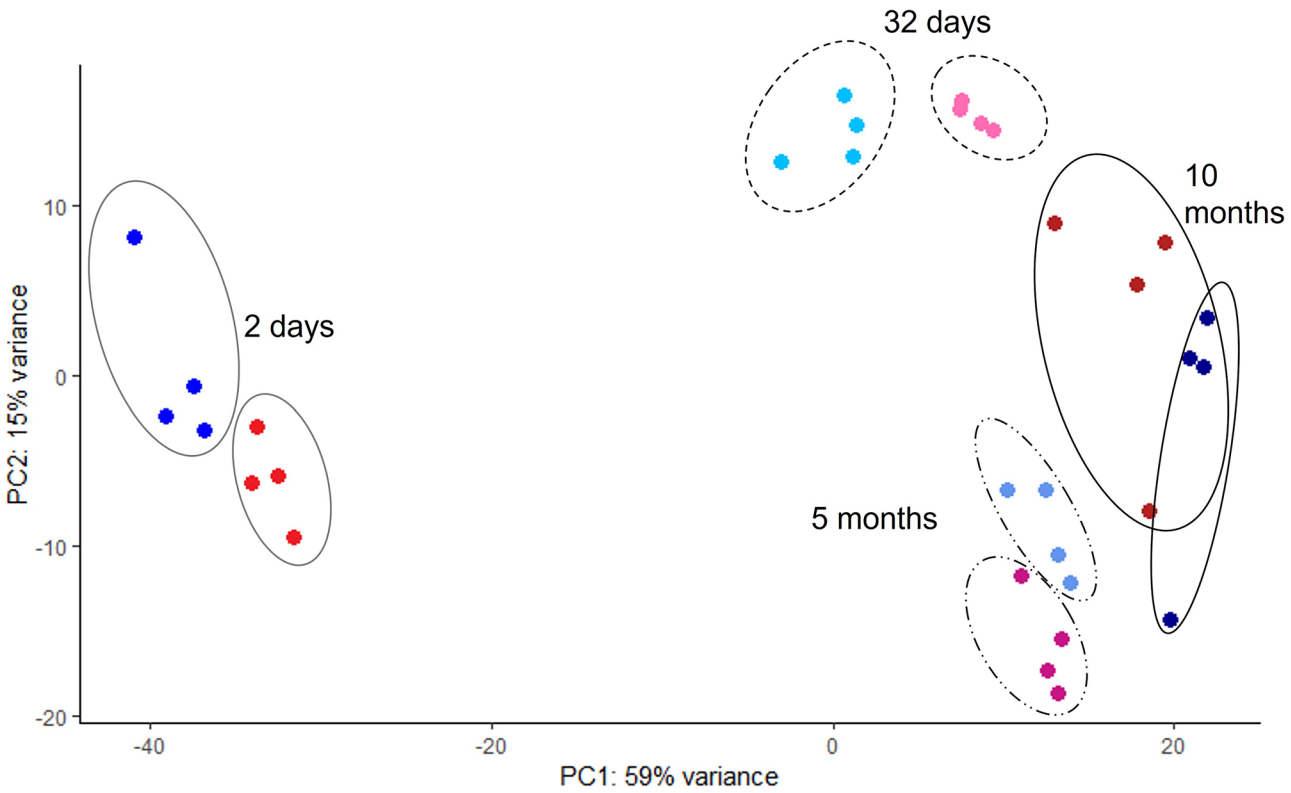


Fig. 2 Principal component analysis (PCA) of normalized gene expression data at 2 days, 32 days, 5 months, and 10 months between clams grown under different pCO_2 conditions (red acidified; blue control)

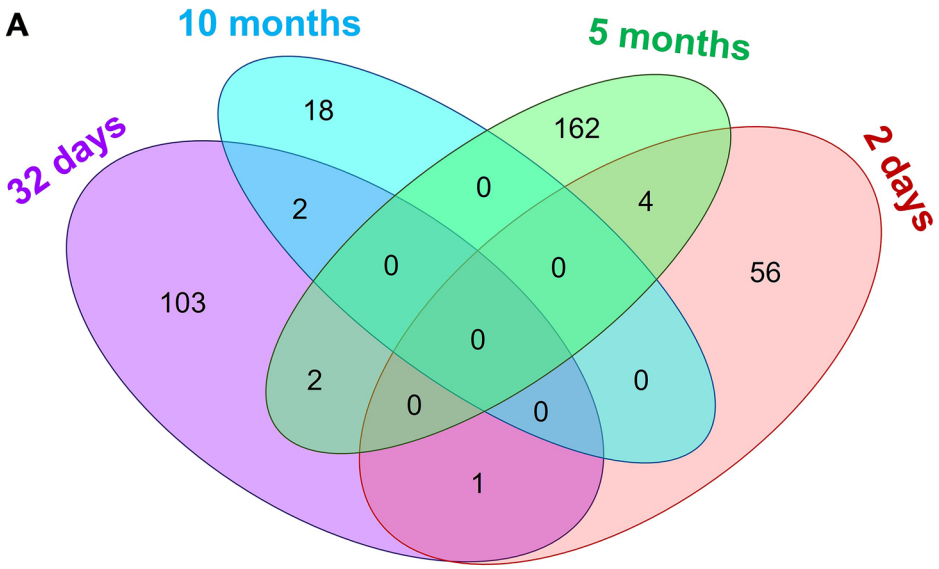


Fig. 3 **A** The Venn diagram presents the number of genes uniquely expressed within each group with the overlapping regions showing the number of genes that are expressed in 2 or more groups **B** DEGs in common/shared between comparisons, * is next to group with higher expression for the gene in acidified conditions. Life stages are 2d (2 days), 32d (32 days) 5 m (5 months) and 10 m (10 months)

B

Gene ID	Protein	Life stage
mRNA.chromosome_12.2645.1	Uncharacterized	2*, 5*
mRNA.chromosome_6.995.1	VWFC domain	2*, 5*
mRNA.chromosome_5.710.3	sterol regulatory element-binding transcription factor 1	2*,5*
mRNA.chromosome_12.282.1	Chitin-binding type-2 domain profile	2*, 5*
mRNA.contig_3016.1.1	Uncharacterized	2, 32
mRNA.chromosome_12.297.1	Collagen, type XII, alpha	32*, 10
mRNA.chromosome_1.1199.2	MFS transporter, OCT family, solute carrier family 22	32*, 10
mRNA.chromosome_12.2684.1	Histone H4	32, 5
mRNA.chromosome_12.2682.1	Histone H4	32, 5*

These included genes with Von Willebrand Factor (VWF) domains, chitin-binding domains, epidermal growth factor (EGF) domains, and solute carriers (Figs. 3 and 4).

Two Day Old Larvae

Genes coding for proteins related to the cytoskeleton, shell formation, protein binding, and bicarbonate transport were upregulated in two day old larvae in the acidified condition (Tables 1, 2 and S5). Moreover, chitin metabolic process was a significantly enriched GO term within the upregulated genes (Table S6). Transcription related genes (such as gene with Zinc finger CCHC-type profile and aryl hydrocarbon receptor nuclear translocator) were downregulated and transcription factor complex was the only significantly enriched GO term in downregulated genes.

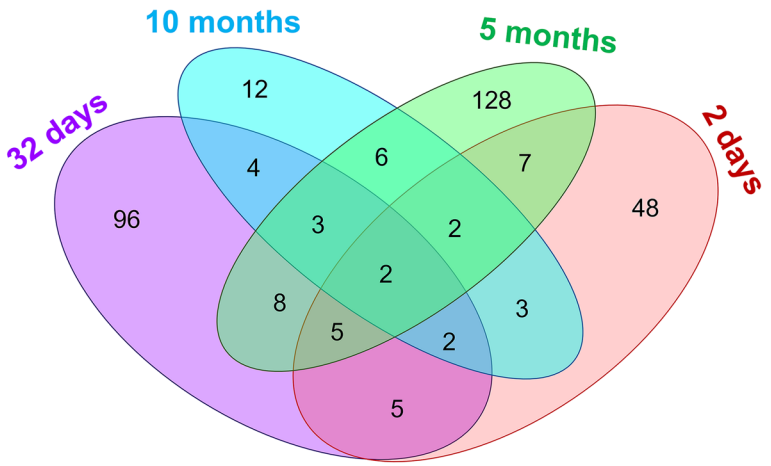
32 Day Old Juveniles

Postset juveniles had almost twice as many DEGs as larvae (Table 1), with the majority downregulated. Upregulated genes included those related to shell formation, bicarbonate transport, and immune/stress processes (Tables 3 and S5). Similar to 2 day old larvae, chitin metabolic process was also a significantly enriched GO term within upregulated genes (Table S7). Genes related to cytoskeleton and immune/stress processes were downregulated (Table 3).

Five Month Old Juveniles

After five months of exposure to OA, the juveniles had the greatest number of DEGs and the greatest percentage upregulated (Table 1). Upregulated genes included those related to cytoskeleton, shell formation, bicarbonate

A



B

Protein/Domain	Life stage
Solute carrier	2*, 32*, 5 (4-2up), 10
Chitin binding; Chitin synthase	2*, 32*, 5(3)*, 10
EGF	2*, 32*, 5(2)*
Zinc Finger CCHC	2 (4-1up), 5(2-1up),
VWF	2(2)*,32(2)*,5(5-3up)
Sterol regulatory binding element	2*,5*
GTP binding protein	2*,10*
Vitelline	32*, 5*,10
Collagen	32*,10
Cathepsin	32*,5(3)*
Histone h4	32(2),5(2-1up)
G protein	32,5*
Serine/threonine-protein	5*, 10*
TNF	5*, 10
Cytochrome p450	5*, 10

Fig. 4 **A** The Venn diagram presents the number of genes coding for unique proteins within each group with the overlapping regions showing the number of genes coding for similar proteins that are expressed in 2 or more groups **B** DEGs coding for proteins in common/shared between comparisons, * is next to group with higher expression for the gene in acidified conditions, the number of genes having the subject domain, including those upregulated under acidified conditions, is given between parentheses when more than one is found in that life stage

Table 1 Number of total DEGs between comparisons and percent of DEGs up or down regulated

	DEGs	Upregulated	Percent upregulated	Downregulated	Percent downregulated
2 day old larvae	61	26	42.6%	35	57.4%
32 day old post sets	108	9	83.3%	99	91.7%
5 month old juveniles	170	112	65.9%	58	34.1%
10 month old juveniles	20	4	20%	16	80%

Table 2 Representative significantly upregulated genes in acidified larvae. Genes presented here were selected based on log2foldchange > 2, adjusted p-value < 0.05, and relevance of function

ID	Predicted Protein	Log2FC	Padj	Functional Group
mRNA.chromosome_19.1142.1	Coadhesin	2.7	0.05	Cytoskeleton
mRNA.chromosome_17.1806.1	Putative GTP-binding protein6	2.2	0.02	Cytoskeleton
mRNA.chromosome_12.824.1	Fibronectin type-III domain profile	2.4	0.04	Shell formation
mRNA.chromosome_6.995.1	VWFC domain profile	2.4	0.00	Shell formation
mRNA.chromosome_12.1499.3	Aggrecan 1 protein	2.0	0.03	Shell formation
mRNA.chromosome_12.282.1	Chitin-binding type-2 domain profile	2.0	0.02	Shell formation
mRNA.chromosome_14.664.1	Calcium-binding EGF-like domain	2.0	0.03	Shell formation
mRNA.chromosome_3.211.2	VWFD domain profile	2.0	0.01	Shell formation
mRNA.chromosome_15.2198.2	F-box only protein 38 isoform X2	2.3	0.03	Protein binding
mRNA.chromosome_5.62.1	Translation initiation factor 5B	2.3	0.02	Protein binding
mRNA.chromosome_1.3867.1	RNA-binding protein 25	2.7	0.03	RNA binding
mRNA.chromosome_8.323.1	Zinc finger CCHC domain-containing protein 9	2.3	0.03	RNA binding
mRNA.chromosome_10.427.1	Solute carrier family 34	3.6	0.00	Bicarbonate transport

transport, and immunity (Tables 4 and S5). Transmembrane transporter activity and transporter activity were significantly enriched GO terms among upregulated genes (Table S8). Downregulated genes in juvenile clams included those related to cytoskeleton, ion transport, metabolism, and immune/stress processes (Table 4). GO terms of downregulated genes were enriched for metabolic processes (Table S8).

10 Month Old Juveniles

The number of DEGs of 10 month old juveniles was the fewest with the majority downregulated. Upregulated genes in 10 month old clams exposed to OA included those related to the cytoskeleton, DNA and nucleotide repair, and RNA binding. (Tables 5 and S5). The downregulated genes were related to the shell formation, immune/stress processes,

Table 3 Representative significantly upregulated genes in acidified postset juveniles. Genes presented here were selected based on log2foldchange > 2, adjusted p-value < 0.05, and relevance of function

ID	Predicted Protein	Log2FC	Padj	Functional Group
mRNA.chromosome_12.297.1	Collagen, type XII, alpha	6.6	0.01	Shell formation
mRNA.chromosome_14.667.1	EGF-like domain profile	6.0	0.02	Shell formation
mRNA.chromosome_15.1602.1	WAP-type 'four-disulfide core' domain profile	5.6	< 0.001	Shell formation
mRNA.chromosome_15.1544.1	Von Willebrand factor type A domain	5.7	0.01	Shell formation
mRNA.chromosome_7.1094.1	Chitin-binding protein	4.6	0.02	Shell formation
mRNA.chromosome_13.1301.1	Von Willebrand factor type C domain	2.0	< 0.001	Shell formation
mRNA.chromosome_1.1199.2	MFS transporter, OCT family, solute carrier family 22	3.0	0.02	Bicarbonate transport
mRNA.chromosome_15.882.1	Vitelline membrane outer layer protein 1	6.7	< 0.001	Immune
mRNA.chromosome_10.766.1	Heat shock protein 70 kDa protein 12A like	-4.8	< 0.001	Stress/immunity
mRNA.chromosome_9.410.1	Acidic leucine-rich nuclear phosphoprotein 32 family member A/C/D	-4.2	< 0.001	Stress/immunity
mRNA.chromosome_13.2893.1	Heat shock protein 70	-3.5	0.002	Stress/immunity
mRNA.chromosome_12.2682.1	Histone H4	-3.2	0.001	Stress/immunity
mRNA.chromosome_12.2684.1	Histone H4	-3.0	< 0.001	Stress/immunity
mRNA.chromosome_2883.2.1	Mucin 5B	-4.6	< 0.001	Cytoskeleton
mRNA.chromosome_5.2802.1	tRNA modification GTPase	-4.0	< 0.001	Cytoskeleton
mRNA.chromosome_19.81.1	Rem2- and rab-like small GTPase 1	-3.7	< 0.001	Cytoskeleton
mRNA.chromosome_3797.3.1	Rem2- and rab-like small GTPase 1	-3.1	< 0.001	Cytoskeleton
mRNA.chromosome_6.871.2	Tubulin polyglutamylase TTLL2	-2.7	< 0.001	Cytoskeleton

Table 4 Representative significantly upregulated genes in 5 month old juveniles. Genes presented here were selected based on log2fold-change > 2, adjusted p-value < 0.05, and relevance of function

ID	Predicted Protein	Log2FC	Padj	Functional Group
mRNA.chromosome_12.726.1	Actin beta/gamma 1	6.7	< 0.001	Cytoskeleton
mRNA.chromosome_1.2647.1	Dynein light chain	5.7	0.00	Cytoskeleton
mRNA.chromosome_5.1141.1	Myosin heavy chain	5.3	0.00	Cytoskeleton
mRNA.chromosome_3.1998.1	Actin beta/gamma 1	5.9	0.00	Cytoskeleton
mRNA.chromosome_2.2738.1	Actin beta/gamma 1	4.4	0.01	Cytoskeleton
mRNA.chromosome_13.1054.1	Tubulin monoglycylase TTLL3/8	3.1	0.01	Cytoskeleton
mRNA.chromosome_11.1042.1	Actin beta/gamma 1	2.5	0.01	Cytoskeleton
mRNA.chromosome_13.2773.1	Chitin-binding type-2 domain profile	6.9	< 0.001	Shell formation
mRNA.chromosome_4.1315.1	Chitin-binding type-2 domain profile	6.7	< 0.001	Shell formation
mRNA.chromosome_1.1197.1	EGF-like signature	6.4	0.00	Shell formation
mRNA.chromosome_6.995.1	VWFC domain profile	6.4	0.00	Shell formation
mRNA.chromosome_7.1323.1	EGF domain	6.1	0.00	Shell formation
mRNA.chromosome_12.282.1	Chitin-binding type-2 domain profile	6.0	0.00	Shell formation
mRNA.chromosome_3.2434.1	Von Willebrand factor type A domains	5.8	0.00	Shell formation
mRNA.chromosome_9.961.1	EF-hand calcium-binding domain profile	5.7	0.00	Shell formation
mRNA.chromosome_6.581.1	EF-hand calcium-binding domain	5.2	0.05	Shell formation
mRNA.chromosome_5.1518.1	Phosphorylase kinase alpha/beta subunit	3.6	0.01	Shell formation
mRNA.chromosome_6.997.1	Von Willebrand factor type A domains	3.3	0.01	Shell formation
mRNA.chromosome_13.1112.1	C-type lectin -type domain	3.0	0.01	Shell formation
mRNA.chromosome_1.284.1	Calmodulin	2.7	00.01	Shell formation
mRNA.chromosome_15.1517.1	Solute carrier family 16	6.1	0.00	Bicarbonate transport
mRNA.chromosome_7.136.1	Solute carrier family 16	6.3	< 0.001	Bicarbonate transport
mRNA.chromosome_9.1391.1	Vitelline membrane outer layer protein 1	6.7	< 0.001	Immune
mRNA.chromosome_13.1968.1	Galectin	6.3	0.00	Immune
mRNA.chromosome_4.788.1	Baculoviral IAP repeat-containing protein 2/3	6.3	0.00	Immune
mRNA.chromosome_11.833.1	Phage lysozyme 2	6.2	0.00	Immune
mRNA.chromosome_12.2682.1	Histone	5.7	0.00	Immune

metabolism, DNA binding, and bicarbonate transport (Table 5). GTP binding was an enriched GO term among upregulated genes (Table S9).

Genetic Variation

Mapped reads for each clam averaged 4,125,916 ± 973,563 with 91% mapping to reference genome for controls and 4,714,029 ± 1,026,685 with 95% mapping to the reference genome for acidified clams. Survivors of 10 months of exposure to acidified conditions were contrasted with clams maintained in control conditions. SNPs were identified in each group and filtered for significant variants (Table S10). The F_{ST} value between the control and acidified groups was 0.008 (Table S11) and Phi_{ST} was 0.003 (Table S12) and PCA showed little difference between pH treatments except in the lower two quartiles.. Summary genetic statistics of each group (acidified or control) based on the entire set of SNPs are summarized in Table S12.

The position of each SNP in the different regions of the genome (coding sequences, untranslated region, intron, intergenic region) was identified (Table S13). Variants in CDS regions represented 4.3% of the total variants, representing about twice as much as CDS coverage in the genome (Farhat et al. 2022). Predicted proteins of significant variants are listed in Table S10 and the consequences of the variants (missense, stop-gained, synonymous) detected in CDS are given in Table S14. We identified 1,668 variants in the CDS regions, including 398 missense, 718 stop-gained, and 552 synonymous variants (Table S14). There were three unique SNPs (2 missense and 1 synonymous) that were only detected in the acidified population and none were unique to control oysters.

SNPs in 233 genes were found with a higher frequency in clams surviving the acidified condition (Table S10). These genes were related to cytoskeleton, calcium binding, shell formation, growth/development, immune/stress, metabolism, signaling, protein synthesis, and bicarbonate transport (Tables 6, S10). Only three of these SNPs were

Table 5 Representative significantly downregulated genes in 10 month old juveniles. Genes presented here were selected based on log2fold-change > 2, adjusted p-value < 0.05, and relevance of function.

ID	Predicted Protein	Log2FC	padj	Functional Group
mRNA.chromosome_4.841.1	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A	8.28	0.05	DNA repair and recombination
mRNA.chromosome_7.1744.2	Cullin 4	8.02	0.01	Nucleotide excision repair
mRNA.contig_3511.13.1	Cleavage stimulation factor subunit 1-like	7.87	0.03	RNA binding
mRNA.chromosome_17.349.1	ADP-ribosylation factor 6-like	7.10	0.05	Cytoskeleton
mRNA.chromosome_11.487.1	Cytochrome P450, family 4, subfamily B, polypeptide 1	-7.24	0.02	Immune/stress
mRNA.chromosome_16.585.1	Vitelline membrane outer layer protein 1 homolog	-7.47	0.01	Immune/stress
mRNA.contig_3987.2.1	Plasminogen	-6.91	0.05	Immune/stress
mRNA.contig_3781.1.1	TNF(Tumour Necrosis Factor) family	-6.82	0.05	Immune/stress
mRNA.chromosome_15.1640.2	Insoluble matrix shell protein 5-like	-8.20	0.05	Shell formation
mRNA.chromosome_12.297.1	Collagen, type XII, alpha	-7.06	0.03	Shell formation
mRNA.chromosome_1.1199.2	Solute carrier family 22	-7.58	0.01	Bicarbonate transport
mRNA.chromosome_5.3241.1	Dopamine beta-monooxygenase; temptin-like isoform X1	-3.78	0.05	Metabolism
mRNA.chromosome_9.1656.1	Fibroblast growth factor receptor 1	-6.95	0.05	Growth factor
mRNA.chromosome_11.2178.1	Serrate RNA effector molecule homolog isoform X2	-7.74	0.01	DNA binding
mRNA.chromosome_1.412.1	Serrate RNA effector molecule homolog isoform X2	-7.64	0.01	DNA binding
mRNA.chromosome_2.93.1	Serine/arginine repetitive matrix protein 5-like isoform X2	-6.88	0.05	DNA binding

unique (absent in control clams). These included a missense SNP in triple functional domain protein, a missense SNP in relaxin family peptide receptor 1, and a synonymous SNP in an uncharacterized gene. Genes containing SNPs had predicted proteins that were the same as DEGs upregulated under acidification (Table 6). These included a SNP in the same exact gene "mRNA.chromosome_17.1526.1" which codes for adapter protein CIKS-like. Overrepresented GO terms of SNPs significantly enriched in acidified clams are listed in Table S15.

SNPs in 222 genes were found to be depleted in clams reared in acidified conditions (Table S10). None of the SNPs depleted in acidified clams were unique to the control population. In general, the SNPs were found in genes with the same broad functional groups as those enriched in acidified conditions (Table S10). GO terms enriched in depleted genes are listed in Table S15.

Discussion

Life History Stage and Length of pCO_2 Exposure

Larvae collected after 2 days of OA exposure had a surprisingly low number of DEGs when compared to the postset and juvenile clam samples. This was unexpected because the first 48 h of larval development is associated with increased vulnerability to OA (Waldbusser et al. 2013). A potential reason for the low number of DEGs might be because early larval development includes many short-lived ontogenetic stages, characterized by unique gene expression. For example, Lopez-Landavery et al. (2021) found differences in gene expression between control geoduck (*Panopea globosa*) larvae and acidified larva, but with differences between days. At two days, *M. mercenaria* should all be at the fully shelled D-stage; however, many studies have demonstrated delays in development under OA conditions (Waldbusser et al. 2015; Gray et al. 2017; De Wit et al. 2018). Because larval samples were pooled, there might have been intrinsic variations related to staggered developmental stages within each pCO_2 treatment that lowered the signal from pH treatments themselves. In fact, the surviving acidified larvae were actually larger than the controls five days post fertilization (Schwaner et al. 2020), suggesting that the smaller size cohort may not have survived. This cohort was sampled in the two day time point and could have contributed to variability within the pCO_2 treatment. Despite the lower-than-expected number of DEGs, this information is still useful in understanding the molecular mechanisms of resilience to OA in larval clams, particularly since molecular responses to OA can be species-specific. Upregulated DEGs included genes coding for proteins related to the cytoskeleton, shell formation, and bicarbonate, suggesting an upregulation of cellular growth and calcification to form the fully shelled D-stage larvae despite the alterations in carbonate chemistry of the external seawater.

The second sampling point immediately followed metamorphosis, one of the most dramatic physiological processes during bivalve development (Garcia-Esquivel et al. 2001; Miller et al. 2009). Metamorphosis is energetically

Table 6 SNPs in representative genes with higher frequencies in acidified clams. * indicates similar predicted protein as a previously identified DEG.

ID	Predicted Protein	SNP
mRNA.chromosome_14.727.1	VWFA domain profile*	Missense
mRNA.chromosome_1.3056.1	VWF type C domain*	Stop gained
mRNA.chromosome_19.1514.1; mRNA.chromosome_2.361.1	Calcium-binding EGF-like domain signature*	Missense; stop gained
mRNA.chromosome_10.1978.1; mRNA.chromosome_18.1515.1	Multiple EGF-like domain protein 11;6	Stop gained; Synonymous
mRNA.chromosome_1.1227.1	EGF-like domain profile*	Synonymous
mRNA.chromosome_13.2030.2	Cadherin EGF LAG seven-pass G-type receptor 1	Stop gained
mRNA.chromosome_15.2234.2	Laminin EGF domain	Missense
mRNA.chromosome_8.1485.1	Chitin synthase	Missense
mRNA.chromosome_18.845.1	Chitin-binding type-2 domain profile*	Stop gained
mRNA.chromosome_14.668.1	WAP-type (Whey Acidic Protein) 'four-disulfide core'*	Missense
mRNA.chromosome_2.2663.1; mRNA.chromosome_5.1350.1	Protocadherin Fat 1/2/3; protocadherin delta 1	Stop gained; synonymous
mRNA.chromosome_5.349.1	Receptor-type tyrosine-protein phosphatase beta	Missense
mRNA.chromosome_15.2303.1	Collagen alpha-6(VI) chain *	Synonymous
mRNA.chromosome_18.87.1	IQ calmodulin-binding motif	Stop gained
mRNA.chromosome_16.372.1	Vitamin D3 receptor-like isoform	Synonymous
mRNA.chromosome_2.611.1; mRNA.chromosome_7.210.1; mRNA.contig_4409.2.1	MFS transporter, LAT3 family, solute carrier family 43, member; solute carrier family 16 member 9; solute carrier family 12 member 8-like*	Stop gained
mRNA.chromosome_1.3787.1	GTP-binding protein 2-like*	Synonymous
mRNA.chromosome_4.1477.1	Baculoviral IAP repeat-containing protein 2/3*	Synonymous
mRNA.chromosome_10.55.1	Ankyrin	Synonymous
mRNA.chromosome_12.2299.1; mRNA.chromosome_12.2378.1; mRNA.chromosome_1.3528.1; mRNA.chromosome_5.2557.1	Tripartite motif-containing protein 71; 2/3;24; 2/3	Stop gained; stop gained; synonymous; missense
mRNA.chromosome_3.830.1; mRNA.chromosome_3.1067.1	Apolipoprotein B	Stop gained
mRNA.chromosome_1.1767.1	NF-kappa-B inhibitor alpha	Missense
mRNA.chromosome_18.733.1	Nuclear factor interleukin-3-regulated protein-like	Missense
mRNA.chromosome_7.466.1	Interleukin-1 receptor-associated kinase 4	Missense

demanding, resulting in clams with a reduced energy budget (Videla et al. 1998; Garcia-Esquivel et al. 2001). There was a general downregulation of gene expression in postsets from acidified conditions with over 90% of DEGs with significantly lower expression than controls. This was the greatest percentage of downregulated genes, and PCA revealed that the postsets had the greatest spatial separation from all other time points and between pCO_2 treatments. A general trend of downregulation of gene expression under OA conditions is not surprising. Sea urchin larvae (*Lytechinus picitus*) exposed to high pCO_2 had significantly different gene expression than sea urchins maintained in control conditions, with 80% of the genes with the largest changes in gene expression being downregulated. These genes were involved in energy supply and demand, which suggests metabolic depression (O'Donnell et al. 2010). Many studies have shown that it is energetically costly to develop under OA (Timmins-Schiffman et al. 2012; White et al. 2013), and differing allocation of energy reserves can result in delayed development and metamorphosis (Talmage and Gobler 2009; Timmins-Schiffman et al. 2012; Wessel et al. 2018). Impacts from OA incurred during the larval stage, such as depletion of energy reserves, can have carry over effects in later juvenile stages, possibly as a result of epigenetic modifications. In the Olympia oyster (*Ostrea lurida*) impacts from OA during the larval stage lasted 1.5 months after juveniles were transferred to a common environment (Hettinger et al. 2012). The larval clams from acidified conditions overexpressed many genes associated with biomineralization resulting in significantly larger larvae. However, this trend reversed at the juvenile stage, with significantly smaller postset clams from the acidified conditions (Schwaner et al. 2020). This might suggest depleted energy reserves at the larval stage which carried over into the juvenile stage and manifested in reduced growth.

Contrary to the general downregulation of DEGs at 32 days, the five month old juvenile clams had the greatest number and percentage of upregulated DEGs. This sampling point was right before winter, when food would be abundant and temperatures warm enough to increase metabolic activity. During winter, clams are less active, pump less, and deplete energy stores for metabolism. When water temperatures increased in the spring, food levels would still be low (there was no supplemental feeding on the flow through) and metabolic demand would be high, resulting in clams with reduced physiological condition (Zarnoch and Schreibam 2008). The 10 month old clams had 80% of their DEGs downregulated and had less clustering by pCO_2 treatment from PCA than samples from the other time points. The added stress of overwintering might be the cause for this decrease in DEGs and general trend of downregulation as they need to conserve more energy. In addition, studies have demonstrated a threshold for upregulating genes under stressful environmental conditions. For example, heat shock protein 70 was upregulated under OA in the starfish *Asterias rubens*; however, it was reduced after long term exposure (Henroth et al. 2011). This might suggest it is too costly to overexpress genes for extended periods and that the clams may have reached their threshold sometime between five- and 10 months. The 10 month old clams also downregulated genes related to shell formation, immunity, and bicarbonate transport, potentially indicating costs of chronic exposure to OA. Alternatively, by this point there might have been a selection of resilient genotypes rather than differential gene expression.

Genetic Differentiation

Previous research has demonstrated that OA significantly increases mortality of *M. mercenaria* larvae (Talmage and Gobler 2010, 2011) suggesting that there could be a selection of "OA-resilient" clams. Selection of OA-resilient bivalves has been found in other species (Thomsen et al. 2017; Bitter et al. 2019). Here, there were differences in allele frequencies between clams from control and acidified conditions; however, genetic differences between controls and survivors of OA were relatively low. There were also no unique SNPs in the control treatments and only three (2 missense and 1 synonymous) in the acidified condition. Visualization of SNP data showed clustering, but not by pCO_2. Four parent populations were spawned to produce the pooled larvae used here. A study of clam population structure along the east coast of North America showed genetically distinct populations between Massachusetts and Mid-Atlantic (New York) populations (Ropp 2020). Within each of the four groups, there appeared to be separation by pCO_2 treatment; however, the genetic structure from source population may have overshadowed the effect of pCO_2 treatment.

Two months after the last sampling point (1 year post fertilization), a reciprocal transplant was performed to test recovery potential and acclimation to OA on the remaining clams (Schwaner et al. 2020). Juvenile clams that had survived the acidified condition for one year appeared to have a broad pH tolerance and performed well under both acidified and control conditions, suggesting they possess a great deal of plasticity. While this could mean there was a selection of animals with this characteristic, it also demonstrates the importance of plasticity under variable pH conditions. The stressful pH conditions were within the range of pH values measured in the environment the parent clams were collected from (Fig. S5). While there were clear phenotypic differences (Schwaner et al. 2020) and a significant regulation of the transcriptome, perhaps a more extreme acidification regime would have induced a higher selective mortality as experimental clams used here appeared to be able to acclimate (i.e., reciprocal transplant) to the lower pH used.

Pathways

Overall trends were similar for all life history stages investigated and between RNA and DNA Sequencing. DEGs and SNPs were among genes related to biomineralization, acid–base regulation/ion transport, cytoskeleton, immunity/stress, and metabolism, highlighting the role these pathways play in resilience to OA.

Biomineralization

The impacts on biomineralization are probably the best understood consequences of OA. The upregulation of shell formation genes in this study might represent a mechanistic response to maintain calcification despite OA conditions. Arivalagen et al. (2017) described proteins with domains that are common to the shell matrix of four bivalve species, and they suggested these domains are evolutionary maintained as part of the "basic tool kit" for construction of the $CaCO_3$ molluscan exoskeleton. These domains included chitin binding-2 and Von Willebrand Factor-A (VWA) (Arivalagan et al. 2017). Chitin binding genes were upregulated under acidification in 2-day, 32 day, and 5 month old clams and a gene with chitin-binding type 2-domain contained a SNP with a higher frequency in acidified clams. Chitin plays a key role in development and functionality in larval bivalve shells (Weiss and Schonitzer 2006). Studies have demonstrated that OA can result in a loss of the quantity of chitin (Mustafa et al. 2015; Zhang et al. 2019), which might explain upregulation of chitin binding genes under OA (Zhao et al. 2020). Another domain of the biomineralization toolkit is VWA, and these domains were found in genes upregulated under OA stress in 2 day, 32 day, and 5 month old clams and a gene with Von Willebrand factor type C domain contained a SNP with higher frequency in acidified clams.

Arivalagan et al. (2017) identified additional proteins (beyond the basic toolkit) that are important for the nucleation and arrangement of $CaCO_3$ polymorphs, which is particularly interesting because OA causes the dissolution of $CaCO_3$ polymorphs. These included proteins with epidermal growth factor (EGF), whey acidic protein (WAP), and Fibronectin-3 (FN3) domains (Arivalagan et al. 2017). Genes with EGF domains were upregulated in 2 day, 32 day old, and 5 month old clams under OA stress. SNPs enriched in acidified clams were found in genes containing EGF domains as well. A gene with a WAP domain contained a missense SNP that was enriched in acidified clams and a gene with WAP domain was upregulated in 32 day old postsets. Lastly, c lam larvae upregulated a gene with a FN3 domain.

Aggrecan 1 was upregulated in larval clams raised under OA conditions, and this gene encodes for a calcification related protein similar to perlucin which forms $CaCO_3$ crystals (Foulon et al. 2019). Receptor-type tyrosine-protein phosphatase had a missense SNP enriched in acidified clams and tyrosine-protein phosphatase is involved in biomineralization (Shi et al. 2012), specifically the formation of the periostracum (Nagai et al. 2007; Zhang et al. 2012) which is a protective layer of shell that is sensitive to OA (Rodolfo-Metalpa et al. 2010; Gazeau et al. 2013; Ramajo et al. 2015). Collagen, type XII, alpha was upregulated in 32 day old postsets from acidified conditions and Collagen alpha-6(VI) chain contained a SNP enriched in acidified clams. Collagens are extracellular matrix proteins that are important for shell matrix formation (Agbaje et al. 2021). Calmodulin has been shown to be important for calcification in the pearl oyster (Yan et al. 2007) and was over expressed under OA in larval sea urchins (Evans et al. 2013) and larval oysters (Dineshram et al. 2015) to sustain calcification under OA. Calmodulin contained a SNP enriched in acidified clams and was upregulated under OA stress at 5 months. Lastly, Vitamin D3 contained a SNP enriched in acidified clams and Vitamin D3 is a stimulator of transepithelial calcium movement during shell mineralization (Lopes-Lima et al. 2012).

Acid–Base Regulation/Ion Transport

The supply of calcium and bicarbonate to the site of calcification via transepithelial transport is critical for calcification. Bivalves remove hydrogen ions and transport calcium and bicarbonate ions via membrane bound ion transport proteins (Ramesh et al. 2017; Sillanpaa et al. 2018). Intracellular pH is also regulated through similar mechanisms, making it hard to distinguish between the two processes. The chemistry at the site of calcification dictates the individual response to OA. For example, research has shown that adult bivalves can elevate pH of their calcifying fluids to sustain biomineralization in adverse conditions (Michaelidis et al. 2005). Solute carrier family is a major group of bicarbonate transporters essential to acid–base homeostasis and ion transport in bivalves (Romero et al. 2013). Solute carrier family 26 was identified as an ion transporter involved in providing calcium and bicarbonate for larval calcification (Ramesh et al. 2019). This study showed upregulation of solute carriers in 2 day, 32 day, and 5 month old clams and a solute carrier gene contained a SNP with a higher frequency in clams from low pH conditions. Solute carriers found in this study are part of the major facilitator superfamily (MFS). The MFS has been demonstrated to be involved in acid resistance and intracellular pH homeostasis of *Penicillium funiculosum* (Xu et al. 2014). Transmembrane transporter activity and transporter activity were enriched GO terms among upregulated DEGs. Upregulating these genes might be a compensatory mechanism to sustain calcification and maintain acid–base regulation despite exposure to low pH external seawater.

Cytoskeleton

Similarly, the upregulation of genes related to cytoskeleton could be to maintain cellular integrity and growth in unfavorable conditions. GTP binding protein 6 was upregulated at two days, GTP binding was overexpressed GO term in upregulated genes at 10 months, and GTP binding protein 2 contained a SNP with higher frequency in acidified clams. In a study of larval geoducks, the GO term GTP binding was overexpressed at low pH (Timmins-Schiffman et al. 2019), and c ytoskeletal genes were also overexpressed under OA in the blood clam (*Tegillarca granosa;* Su et al. 2018). Genes associated with cytoskeleton may also be important for immune function such as cytoskeleton mediated phagocytosis via the actin-myosin contractile system (Allen and Aderem, 1995). In fact, OA was found to decrease the abundance of cytoskeletons in hemocytes from blood clams, resulting in a weakened immune response (Su et al. 2018). Upregulation of cytoskeleton genes could represent a mechanism to increase the ability to produce cytoskeleton components under OA stress.

Immunity

Studies have demonstrated that OA can negatively impact bivalve immunity (Bibby et al. 2008; Cao et al. 2018; Su et al. 2018; Schwaner et al. 2020). Baculoviral IAP repeat-containing protein 2/3, an apoptosis gene, was upregulated under OA stress in 5 month old clams and contained a SNP with a higher frequency in acidified conditions. Ankyrin, which is important to hemocyte function (Wei et al. 2017), contained a SNP enriched in acidified clams. Tripartite motif-containing proteins are involved in pathogen recognition and host defense and are associated with hemocyte function of the scallop *Pecten maximus* (Pauletto et al. 2014). In

this study, tripartite motif-containing protein contained a SNP enriched in acidified clams. Similarly, Apolipoprotein B-like contained a SNP with a higher frequency in acidified clams. Apolipoprotein was found in the transcriptome of hemocytes from Manila clam *Ruditapes philippinarum* and is associated with inflammation (Moreira et al. 2012). Activation of this inflammation pathway is mediated by NF-kappa B, and there are several conserved NF-kappa-B sites in the apolipoprotein D promoter. NF- kappa-B actually contained a missense SNP in acidified clams as well. It was downregulated under OA in the blood clam, making them more vulnerable to viral infections as it is important in signaling pathway that induces innate antiviral responses (Liu et al. 2016). Interleukin-1 receptor-associated kinase 4, also associated with hemocytes (Mateo et al. 2012), contained a missense SNP enriched in acidified clams.

In contrast, there were many immune genes downregulated such as peroxidase, a gene with tumor necrosis factor domain, gene with Immunoglobulin domain, and big defensin. Subsets of the same clams used in this study were challenged with pathogenic *Vibrio spp*. and were found to have a marked reduction in immunity under OA stress (Schwaner et al. 2020). Heat shock protein 70kDA 12A-like was downregulated in 32 day old post set juveniles. While heat shock proteins have been upregulated under OA stress in corals (Moya et al. 2015) and bivalves (Goncalves et al. 2017), studies have also shown downregulation in urchins (Todgham and Hofmann 2009), oysters (Wang et al. 2016; Schwaner et al. 2020), and starfish (Henroth et al. 2011). This might be due to the fact that extended over-expression of heat shock proteins is not sustainable. Reduced expression of heat shock proteins could weaken an organism's ability to mitigate the impacts of OA stress at the cellular level.

Metabolism

Other downregulated genes which could demonstrate physiological costs of tolerance to OA were related to metabolism. In 5 and 10 month old juveniles, several metabolic genes displayed decreased expression in acidified conditions. Like with 32 day old clams, 10 month old clams had a general trend of downregulation, which might be due to metabolic depression. Metabolic depression as a mechanism to combat OA has been demonstrated in other marine invertebrate species, including blood clam *T. granosa* (Zhao et al. 2017), mussels *M. chilensis* (Navarro et al. 2013) and *M. gallo-provincialis (*Michaelidis et al. 2005). When exposed to acidification, the coral polyp (*Acropora millepora*; Moya et al. 2012), larval sea urchin (*Strongylocentrotus purpuratus*; O'Donnell et al. 2010), and larval oyster (*C. gigas;* Liu et al. 2020) downregulated genes involved in metabolism. Metabolic depression might be an energy saving strategy for surviving under OA conditions. However, this might have associated costs in addition to immune depression. The 10 month old clams downregulated solute carrier and insoluble shell matrix protein, which could come at the cost of shell formation.

Conclusions

This study underlines the resilience of *M. mercenaria* to OA and identifies the associated molecular features. As hypothesized, OA appears to significantly influence the transcriptomes of clams and there were DEGs between control and acidified clams at every life stage investigated. While the genetic differentiation between clams surviving 10 months of exposure to OA and control clams was lower than expected, there was convergence of evidence of DEGs and SNPs in similar genes and pathways. Specifically, shell formation, bicarbonate transport, and cytoskeleton were associated with resilience to OA, while reduction in immunity and metabolism might represent costs.

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Data Availability Statement The original contributions presented in the study are publicly available. Data can be found at: https://www.ncbi.nlm.nih.gov/sra under BioProject PRJNA887696 accession numbers SAMN31181489- SAMN31181584 (RADSeq) and BioProject PRJNA887747 accession numbers SAMN31183563- SAMN31183594 (RNASeq).

Declarations

Conflict of Interest The authors declare no conflict of interest.

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