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Transcriptomics, proteomics, and physiological assays reveal immunosuppression in the eastern oyster *Crassostrea virginica* exposed to acidification stress

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ABSTRACT

Ocean acidification (OA) is recognized as a major stressor for a broad range of marine organisms, particularly shell-building invertebrates. OA can cause alterations in various physiological processes such as growth and metabolism, although its effect on host-pathogen interactions remains largely unexplored. In this study, we used transcriptomics, proteomics, and physiological assays to evaluate changes in immunity of the eastern oyster *Crassostrea virginica* exposed to OA conditions (pH = 7.5 vs pH = 7.9) at various life stages. The susceptibility of oyster larvae to *Vibrio* infection increased significantly (131 % increase in mortality) under OA conditions, and was associated with significant changes in their transcriptomes. The significantly higher mortality of larvae exposed to pathogens and acidification stress could be the outcome of an increased metabolic demand to cope with acidification stress (as seen by upregulation of metabolic genes) at the cost of immune function (down-regulation of immune genes). While larvae were particularly vulnerable, juveniles appeared more robust to the stressors and there were no differences in mortality after pathogen (*Aliiroseovarius crassostrea* and *Vibrio* spp.) exposure. Proteomic investigations in adult oysters revealed that acidification stress resulted in a significant downregulation of mucosal immune proteins including those involved in pathogen recognition and microbe neutralization, suggesting weakened mucosal immunity. Hemocyte function in adults was also impaired by high pCO_2, with a marked reduction in phagocytosis (67 % decrease in phagocytosis) in OA conditions. Together, results suggest that OA impairs immune function in the eastern oyster making them more susceptible to pathogen-induced mortality outbreaks. Understanding the effect of multiple stressors such as OA and disease is important for accurate predictions of how oysters will respond to future climate regimes.

1. Introduction

Oysters are among the most ecologically and economically important species within coastal and estuarine ecosystems. As ecosystem engineers, oysters provide vital services including enhancing water quality, controlling nutrient cycling, coupling the benthic and pelagic zones, and providing critical habitat for a diversity of species [1]. The reefs built by the settlement of oysters can have profound impacts on local systems. However, oyster reefs are considered one of the most threatened marine habitats due in part to anthropogenic effects, overfishing, and disease [2], with habitat extent decreasing 64 % and oyster biomass decreasing by 88 % just within the 20th century [3]. The predicted effects of climate change may serve to worsen the loss of these organisms which could lead to reverberating economic impacts including investing billions of dollars into their recovery and restoration. In 2017, eastern oyster (*Crassostrea virginica*) landings in the US amounted to over $220 million [4]. A decline in the abundance of the eastern oyster could result in severe damage to local and national economies and severely alter ecosystem functioning.

Oysters typically spawn in the summer when water temperatures are warmest and pH is lowest meaning sensitive larvae are spawned when environmental conditions are least opportune. In situ pCO_2 measurements of a coastal salt marsh in Long Island, NY reached ~2200 μatm by mid-June and then up to ~4000 μatm by the end of July [5]. Larvae

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released at this time can face many challenges from the high pCO_2 levels. Saturation of aragonite and calcite, the biomineralized forms of $CaCO_3$, are lowered with increasing pCO_2. Oyster larvae biomineralize aragonite, the more soluble form of $CaCO_3$, making them particularly sensitive to CO_2 induced acidification (*i.e.*, ocean acidification, OA).

Eastern oyster larvae face additional stressors besides acidification, such as exposure to pathogenic bacteria. *Vibrio* species are a major concern and larval oysters are particularly susceptible to infection [6]. *Vibrio* spp. are ubiquitous and opportunistic pathogens of oysters, and are associated with devastating losses of aquaculture stocks [7]. It is important to understand the consequences of OA on susceptibility to *Vibrio* spp. and whether there is a synergistic effect of these stressors. Further underscoring the need to evaluate these co-occurring stressors is that *Vibrio* responds positively to low pH. For example, a series of laboratory studies demonstrated that *Vibrio* spp. were found at higher concentrations in raw seawater injected with CO_2 (pH = 7.5) and displayed better survivorship and growth when added to acidified seawater (pH = 7.6) compared to their respective controls (pH = 7.8 and 9) [8]. Previous work also reported higher levels of *Vibrio* in hemolymph from blood clams cultured in acidified seawater as compared to controls [9].

Juvenile oysters are more resistant to *Vibrio* infection than larvae; however, at this age they are plagued by *Aliiroseovarius crassostreae*, the causative agent of Roseovarius oyster disease (ROD; formerly Juvenile Oyster Disease). ROD is enzootic and occurs annually at grow-out sites in the Northeastern United States, with losses sometimes exceeding 90 % of production [10]. Oysters <25 mm in shell length are more susceptible to ROD than larger individuals [10], which might be because they do not have the required metabolic resources to successfully launch immune responses, including melanization and conchiolin deposition [11,12]. Surviving in acidified conditions could further deplete metabolic resources, increasing their susceptibility to ROD. Acidification is known to impact bivalve development, resulting in significantly smaller juveniles [8]. Therefore, OA might extend the amount of time oysters are vulnerable to ROD. ROD initiates infection and colonizes the inner shell surface and extrapallial space [11], which is the site of shell formation. The immune cells, hemocytes, present in the extrapallial fluid also function in biomineralization. There could be a trade-off between mounting an immune response and maintaining calcification under OA conditions.

Adult eastern oysters are also exposed to notorious infectious agents such as *Perkinsus marinus* and *Haplosporidium nelsoni*. The ability for a pathogen to establish infection in its host largely depends on the immunocompetency of the host which is partially controlled by environmental factors. In this context, studying adult shellfish under longer-term acidification stress is important to understanding potential physiological costs and trade-offs of acclimation to OA. Many OA studies, even on adult organisms, are short-term experiments and fail to look at long-term exposure to OA, ignoring the potential cost of chronic OA stress and potential trade-offs of surviving under OA conditions. As mentioned above, hemocytes have multiple functions and play central roles in immunity and biomineralization, and there might be OA-induced trade-offs. Furthermore, many proteins expressed in bivalves, particularly some calcium-dependent molecules, have dual functions in biomineralization and immunity. For example, previous studies in bivalves showed that complement 1q domain containing proteins (c1qDC) function as pattern recognition receptors and can initiate innate immune responses [13], while members of the c1qDC proteins are also involved in biomineralization and shell repair [14]. How bivalves will respond to impaired biomineralization from exposure to pCO_2 while still maintaining critical immune function remains uncertain. It might be that surviving in acidified seawater or responding to extreme fluctuations in the carbonate chemistry of seawater could be at the cost of immune functioning. Previous work showed that elevated levels of CO_2 in seawater impacted immune performance of the Pacific oyster *C. gigas* [15]. Similar studies demonstrated that the combined effects of *Vibrio* infection and acidification led to immunosuppression in *Mercenaria mercenaria* [8]. Most research on bivalve immunity focuses on cellular and humoral factors present in the hemolymph. While these are critical defenses, mucosal immunity is just as important, as it is one of the first lines of defense. For instance, mucosal secretions covering mollusk soft tissues create a physical barrier to microbes, contain numerous immune effectors, and can regulate pathogen growth and virulence [16,17].

This study investigated the cost of resilience to acidification in the eastern oyster throughout various life history stages, specifically focusing on immunity. Experiments were designed to determine whether susceptibility to *Vibrio* infection (for larvae) and ROD (for juveniles) was increased in oysters maintained in acidified seawater compared to those from ambient conditions. In addition to pathogen challenges, levels of naturally-occurring pathogens were determined in adult oysters after 10 months of exposure to pCO_2 conditions. Simultaneously samples were collected for transcriptomics and proteomics to better understand how acidification impacts immune processes at the molecular levels. Furthermore, circulatory hemocytes and mucus covering soft tissues were collected to assess both cellular and mucosal immunity for a holistic understanding of the impacts of OA on oyster immunity.

2. Materials and methods

2.1. Seawater carbonate chemistry manipulation

Because of logistical constraints (experiments performed in different field stations), seawater used in this work was collected from various locations surrounding Long Island, NY, including Great South Bay, Old Fort Pond, and Stony Brook Harbor (only one source used for each experiment). Prior to use, seawater was filtered up to 0.2 μm and sterilized by UV light. Two different systems were constructed to accommodate both static and flow through designs. For the larval cultures (static system) the high pH/low pCO_2 condition (ambient) was achieved by bubbling ambient air into 43-liter culture vessels to maintain a pH of 7.98 (pCO_2 of ~480 ppm). The seawater was not buffered, as to reflect ambient conditions in coastal estuarine environments. For the low pH/high pCO_2 condition (*i.e.*, acidified seawater), 5 % CO_2 was mixed with air using multi-channel gas proportioners (Cole Parmer® Flowmeter system, multitube frame, Antylia Scientific, Vernon Hills, IL) and bubbled into the seawater for a resulting pH of ~7.5 and pCO_2 1300 ppm (as described in Refs. [8,18]). Seawater was equilibrated to target pH/pCO_2 values 24 h prior to water changes to reduce fluctuations in pH.

Juvenile and adult oysters were held in a flow through system. For the acidified condition (~7.43 pH, 2220 ppm), water flowed into an acidification chamber where 100 % CO_2 was mixed with air and bubbled to maintain a delta of 0.5 units between the two pCO_2 conditions. The acidified treatment had a greater delta than larvae, as juveniles and adults are less vulnerable to OA. Water from the chamber flowed into a header tank and then into four replicate tanks corresponding to the pCO_2 condition using a "downweller" setting. For the ambient condition (~7.93 pH, 490 ppm), natural seawater flowed into an aerated header tank and then into each of the four replicate tanks corresponding to the pCO_2 condition (as described in Refs. [8,19]). The four replicate tanks per pCO_2 condition were designated as the experimental units ($n = 4$). pCO_2 condition was considered the fixed effect and the tank considered as the random effect, not a fixed factor, which was accounted for in analyses. The target low pH was selected based on end-of-the-century predictions and taking into consideration the current pH conditions in coastal waters of Long Island where this species inhabits. The pH was monitored daily using an Ohaus ST300 portable pH meter (precision of 0.01 pH). Samples for dissolved inorganic carbon (DIC) analysis were collected and read using a VINDTA 3D (Versatile Instrument for the Determination of Total inorganic carbon) delivery system coupled with a UIC Inc. (Joliet, IL, USA) coulometer (model CM5017O). Bicarbonate standards were used and for quality assurance certified reference

material was analyzed (provided by Andrew Dickson, Scripps Institution of Oceanography) with a 99.99 % recovery during every run. pCO_2, $\Omega_{aragonite}$, $\Omega_{calcite}$, DIC, CO_3, and alkalinity were calculated from pH, temperature, and salinity using the *seacarb* package [20] for R statistical software v3.6.1 with known first and second dissociation constants of carbonic acid in seawater [21]. Carbonate chemistry and seawater parameters are presented in Tables S1–S2.

2.2. Bivalve husbandry

Adult oyster broodstock were conditioned for eight weeks before planned spawning following recommendations of [22]. Oyster broodstock was acquired from aquaculture facilities in Shinnecock Bay, NY (for larval pathogen challenges) and Great South Bay, NY (for juvenile pathogen challenge). Oyster shells were scrubbed and placed in a flow through sea table held around 20 °C, the optimum temperature for their reproductive development [22]. In addition to natural algae in seawater, oysters were continuously drip-fed cultured algae: *Tetraselmis* spp., *Isochrysis galbana, Pavlova lutherii*, and *Chaetoceros muelleri*. To induce spawning, thermal stimuli was used and mature animals were placed in a spawning tank and exposed to cycles of hot water (25–28 °C) and ambient water, following thermal cycling recommendations of [22]. Individuals that released eggs were identified as female and separated from spawning males into a separate sea table for egg collection. The separation of spawning males and females ensures genetic heterogeneity as sperm from all males can be added at once to collected eggs for fertilization. After allowing sufficient time for fertilization (1 h), embryos were transferred to the control condition or the acidified condition described above, at a stocking density of 10 larvae/ml. The larvae culture tanks were partially submerged in a temperature-regulated sea table (set at 25 °C as this is the optimal growing temperature for *C. virginica* larvae as recommended by Ref. [22]) that serves to maintain constant temperature for all vessels. Larvae were fed *ad libitum* daily with fresh cultures of *Isochrysis* spp. for the first week and then a mixture of *Isochrysis* spp. and *Pavlova lutheri* until metamorphosis. Algae clearance was monitored daily, and feeding was adjusted to account for mortality and larval growth. Larvae received 100 % water changes every 24 h for the first two weeks and every 48-h thereafter, using 0.2 μm filtered seawater [22]. When oysters reached approximately 200 μm long, a PVC sieve was inserted into the large vessels with shell chips (*i.e.*, "cultch") layered on the sieve, so that oysters were able to settle out of the water column and attach to shell pieces. After settling and metamorphosis, juveniles were moved out of their initial culture tanks. Juvenile oysters were moved to the open flow through system described above ($n = 4$ replicates per treatment). Oysters received only the algae in the raw water without supplemental feeding.

Adult oysters (83 ± 8 mm length; 67± 9 mm width) were obtained from a commercial source (Frank M. Flower and Sons Inc., Oyster Bay, NY). Upon arrival, shells were washed and scrubbed to remove epibionts. A time zero sample of 21 oysters was collected. The remaining oysters were placed into each replicate ($n = 2$) of the flow through system and split between the two pCO_2 treatments (25 per bucket; 50 per treatment). Oysters remained in these conditions for 10 months.

2.3. Bacterial challenge in larvae

2.3.1. Vibrio challenge

Following the experimental procedures outlined in Ref. [8], three experiments were conducted to test the effects of acidification on oyster larvae susceptibility to *Vibrio* infection. In the first trial (Exp. 1), *Vibrio* challenge was performed in 16.8 mL 6-well microplates with no bubbling of air or CO_2. A series of four wells contained larvae (30 larvae/well in 15 mL 0.2 μm filtered seawater, ~20 °C) from each original larva culture vessel (2 treatments; 4 replicates treatment^{-1}), totaling 16 wells. Half the wells received a bacterial cocktail made of *Vibrio tubiashii, V. coralliilyticus, V. splendidus,* and *Listonella anguillarum* (~10^3 colony-forming units (CFUs) mL^{-1} per strain; a concentration slightly below the LD50 for *V. tubiashii* and *V. coralliilyticus* for bivalve larvae; [6]) while the other half received sterile seawater. pH in the acidified well plates reached control levels within 3 h, indicating that if there was increased mortality under acidification and pathogen stress it was a result of sustained immune suppression in bivalves cultured in acidified seawater as opposed to a direct effect of acidification during the challenge itself.

For the second experiment (Exp. 2), ~500 six-day old larvae were added to a series of 250-mL vessels ($n = 8$ treatment^{-1}) bubbled with 5 % CO_2 mixed with air (for the acidified treatment, pH = 7.5, pCO_2 ~1300 ppm, ~20 °C) or air only (for the ambient treatment, pH = 7.8, pCO_2 ~600 ppm, ~20 °C). Half of ambient and acidified vessels then received bacteria (same cocktail and concentrations as above).

For both experiments, the temperature was maintained at 20 °C and larvae were not fed throughout the duration of the challenge. Viability of experimental animals was assessed microscopically 24-h (Exp. 1 and 2) and 48-h (Exp. 2) post-exposure and oysters were considered dead if there was a lack of ciliary movement, swimming, or empty shell. Mortality data was compared using a two-way ANOVA with a post-hoc Tukey test.

A third trial (Exp. 3) was conducted following the design of Exp. 2 (250-mL vessels and bubbling, maintained at 20 °C) to evaluate the transcriptomic responses of oyster larvae to acidification and bacterial challenge. A greater number of larvae (~4000 larvae per replicate) was added so that there would be enough biological material to collect for RNA sequencing. The same bacterial cocktail at the same concentration was added. Pools of larvae were collected at 6 h and three days after exposure to pathogens from both control and acidified conditions ($n = 3$ replicates treatment^{-1}). Samples were flash-frozen and stored at −80 °C until RNA extraction and processing for RNA sequencing.

2.3.2. RNA extraction, sequencing, and analysis

RNA was extracted from larval pools using RNeasy Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Sequencing, read mapping, quantification of gene expression, and analyses followed are similar to those described in Ref. [18]. RNA quantity and quality were initially checked with a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware). Extracted RNA was sent for sequencing to Novogene Corporation (UC Davis, Sacramento, California). RNA degradation and contamination was monitored on 1 % agarose gels, purity was assessed using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA), and RNA integrity and quantity were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). For library preparation, 1 μg RNA per sample was used as input and sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) with cDNA fragments of 150–200 bp in length preferentially selected for. AMPure XP system (Beckman Coulter, Beverly, USA) was used to purify fragments. 3 μl of USER Enzyme (NEB, USA) was added to size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina, USA). Library construction was quantified using a Qubit 2.0 fluorometer (Life Technologies) and then diluted to 1 ng/μl before checking insert size on an Agilent 2100 and quantifying to greater accuracy to qPCR, before sequencing on an Illumina platform where 150 bp paired-end reads were generated. Reads were cleaned by removing reads containing adapters or reads of low quality. Reads were discarded if they had adapter contamination, contained more than 10 % of uncertain nucleotides (N>10 %), or had low quality nucleotides (base quality <20) that constitute more than 50 % of the read. Error Rate, Q20, Q30, and GC

content of the cleaned data were calculated. Cleaned reads that passed quality control were used in downstream analyses. HISAT2 [23] was used to map filtered sequenced reads to the reference genome (NCBI Accession GCA_002022765.4). HTSeq v0.6.1 [24] was used to analyze gene expression levels using the union mode. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) was calculated using the length of the gene and the number of reads mapping to that gene. Correlation between samples in the same treatment was calculated using Pearson correlation coefficient. Differential expression analysis was performed using DESeq2 from Bioconductor [25]. P-values were adjusted using the false discovery rate (FDR) and were only kept if $<$ 0.05. Cluster analysis of gene expression differences was performed using hierarchical clustering. Gene IDs were converted to gene names using NCBI and *C. virginica*-3.0 (GCF_002022765.2). The function of the gene was identified using UniProtKB. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by GOseq [26], in which gene length bias was corrected and GO terms with corrected *p*-value less than 0.05 were considered significantly enriched. KOBAS (KEGG Orthology-Based Annotation System) software was used to test the statistical enrichment of differentially expressed genes in KEGG pathways [27].

2.4. Bacterial challenge in juveniles

Juvenile oysters (~12 ± 4 mm L and 10± 3 mm W) were placed in sixteen 3 L aquaria (30 oysters per aquarium). Aquaria were submerged in a temperature regulated sea table to maintain a constant temperature of 26 °C (optimal temperature for *A. crassostreae* growth). Half of the aquaria (8) were equilibrated to low pH/high pCO_2 (pH = 7.55, pCO_2 ~1138 ppm) and half received ambient seawater (pH = 7.92, pCO_2 ~458 ppm). The oyster pathogen *A. crassostreae* was added (at ~10^6 CFU mL^{-1}) to half of the acidified aquaria and half of ambient replicates (n = 4 per treatment). Mortality was assessed daily, by looking for gaping. Partial water changes were conducted three times a week and after each water change a new inoculation of bacteria was added. Juveniles were fed algal paste (Shellfish Diet 1800, Reed Mariculture, Campbell, CA), and feces were pipetted out of the tank daily. After a month of the pathogen challenge, the previously described *Vibrio* cocktail (at ~10^6 CFU) was added to the tanks that previously received *A. crassostreae*. After a total of nine weeks of exposure, the experiment was terminated. Remaining samples were preserved to look for signs of ROD infection that may have occurred without associated mortality (*i.e.*, conchiolin deposition and cupped shells). Data was not normal so a Kruskal Wallis rank sum test was used to compare differences in mortality between treatments.

2.5. Effect of OA on adult oyster immunity

2.5.1. Hemocyte parameters

After 10 months of exposure to each pCO_2 treatment, oysters (16 per treatment) were notched and bled using a 3 ml syringe prefilled with chilled filtered artificial seawater (FASW, 27 PSU) so that raw hemolymph samples were diluted 1:3. All tubes, animals, and syringes remained on ice throughout the duration of processing. The diluted hemocytes in each tube were sampled to evaluate the number and type of hemocytes, as well as hemocyte viability and phagocytic activity. These assays were conducted using a BD FACSCalibur flow cytometer following the protocol outlined in Ref. [19]. Briefly, each sample of hemolymph was aliquoted into two subsamples. One aliquot (450 μl of diluted hemolymph) received propidium iodide (PI) at the final concentration of 20 μg/ml. Samples were incubated for 10 min in the dark at room temperature, and 10,000 cells were read on the flow cytometer to identify live and dead cells. PI is not able to enter live cells but will bind to DNA of dead cells which fluoresce in the orange channel (FL2). Subpopulations of hemocytes (agranulocytes and granulocytes) were separated based on forward (FSC) and side light scatter (SSC) patterns and the total number of cells was enumerated. The second aliquot served to evaluate hemocyte phagocytic activity where 2 μm yellow-green latex beads were added to 450 μl of diluted blood and incubated at room temperature for 1 h. Ten beads were added per hemocyte and 10,000 cells were analyzed. Hemocytes were considered phagocytic if associated with beads (FL1 signals). Nested ANOVAs were used to analyze differences of hemocyte counts, viability, and percent of phagocytic cells between pCO_2 treatments.

2.5.2. Oyster mucomics

The effect of OA on oyster mucosal immune factors was evaluated. After 10 months of exposure, three adult oysters from each replicate (6 per pCO_2 treatment) were collected from the flow through system. All oysters were cleaned of sediment and numbered. Oysters were carefully opened and organs were rinsed with FASW (28 PSU). Mucus from mantle, gills, and labial palps was collected as described by Ref. [28]. Briefly, mucus was collected using a sterile cotton swab (wrapped around forceps) by gently swabbing each organ. The cotton swab was immersed in 1 ml of ice cold FASW and 50 μl of protease inhibitor cocktail (SIGMA*FAST*™ Protease Inhibitor Tablets; prepared according to the manufacturer's recommendations for 1× solution; Sigma-Aldrich, Inc., St. Louis, MO, USA). Tubes containing cotton and associated mucus were placed on a rotating shaker at 4 °C for 1 h. Cotton was removed and the fluid was centrifuged (3000 g, 30 min, 4 °C) and then filtered through 0.8 μm and 0.22 μm syringe filters. The filtrate was stored at −80 °C until protein extraction for the proteomics analysis.

Pales Espinosa et al. (2016) reported that oyster mucus contained a major protein called dominin [29]. To improve the resolution of the proteomic analysis and enable detection of less abundant proteins, a pre-separation step on gel was performed to exclude dominin. Peptides were generated and analyzed by LC-MS/MS. Oyster mucus extracts were solubilized in 5 % SDS, 100 mM TEAB, 10 mM DTT at 55 °C for 30'. Reduced cysteines were alkylated with 20 mM iodoacetamide for 30' at room temperature in the dark, proteins acidified with phosphoric acid, proteins precipitated with 90 % methanol, 50 mM TEAB, and bound to S-Trap solid phase cartridges as described [30]. Protein precipitates were washed with 90 % methanol, 50 mM TEAB and digested with trypsin at 47 °C for 2 h and eluted with sequential 50 mM TEAB, 0.2 % formic acid and 50 % acetonitrile, 0.2 % formic acid elution steps by centrifugation at 4000×*g* for 1 min each. Peptides were analyzed by C18 reverse phase LC-MS/MS. HPLC C18 columns were prepared using a P-2000 CO2 laser puller (Sutter Instruments) and silica tubing (100 μm ID x 20 cm) and were self-packed with 3μ Reprosil resin. Peptides were separated by reverse-phase on an Eksigent 415 nanoHPLC with a flow rate of 300 nl/min, using a 10-min isocratic loading step 0.1 % formic acid/water), a gradient elution step with acetonitrile (ACN), 0.1 % formic acid (0.23 %/min) over 90 min, followed by 5 min 70 % ACN wash and 25 min re-equilibration steps. Electrospray ionization was achieved using spray voltage of ~2.4 kV. Information-dependent MS and MS-MS acquisitions were made using a 0.25 s survey scan (m/z 400–1600) followed typically by 20 consecutive second product ion scans of 0.1 s each (m/z 100–1400), using rolling collision energy. Parent ion with charge states of 2+, 3+ and 4+ were selected with a 15-s exclusion period. MS data was collected using Analyst (absciex.com). Fourfold MS and twelve fold MS/MS time binning was used to increase sensitivity. Data was collected throughout the HPLC cycle.

Protein abundance was established by protein database searching using ProteinPilot v5.01, followed by statistical analysis using JMP12. Two missed tryptic cleavages were allowed and posttranslational modifications considered included cysteine derivatization, STY phosphorylation, deamidation, carbamylation, oxidation. Database searches used the GCF_002022765.2_C_virginica-3.0_protein FASTA database. When multiple protein isoforms were identified, Protein Pilot allowed only peptides specific to each detected isoform to be used, which factored in ion counts for weighting in the protein ratio calculation. Parsimony of protein results was assured by rigorous protein inference with the

ProGroup algorithm.

To determine how the mucus proteome was influenced by different pCO_2 conditions, proteins were considered differentially expressed if either there was 1) log2foldchange $\geq |1|$ between pCO_2 treatments or 2) a p-value<0.10. Fold change could be calculated when proteins were detected in more than one oyster, but p-values were only generated when proteins were expressed by all oysters within a treatment group. Proteins in only one treatment were included by using both approaches.

2.5.3. Disease diagnostics

After bleeding (2.5.1), oysters were dissected and a portion of rectum and the mantle near the labial palps was placed in a tube containing Ray's fluid thioglycolate medium for *P. marinus* diagnostics. Fifty µl of lipid mixture, 10 µl of chloramphenicol (to prevent bacterial growth) and 50 µl nystatin (to prevent fungal growth) were added to each tube. The pieces of tissue were left to incubate for one week in the dark at room temperature. Subsequently, a cross section of soft tissue from each oyster (including digestive gland, gonad, mantle and gill) was placed into a cassette and fixed in formalin. Tissues were embedded in paraffin wax, sectioned (5 µm thick), mounted to slides, and stained with Hematoxylin and Eosin. Slides were read on a compound light microscope to assess disease status using standard histological techniques. After the week-long incubation, the rectum and mantle pieces were placed on a slide, the tissue was minced with a scalpel, and then stained with Lugol's iodine. *P. marinus* infection intensity was ranked based on the Mackin scale. Animals that died during the experiment were processed immediately. A time 0 sample of 21 oysters was processed before introduction to pCO_2 treatments in the same way. Data was not normal so a Kruskal-Wallis rank sum test was used to compare infection intensity of *P. marinus*. Number of oysters with and without signs of infection (from histology) between the pCO_2 treatments was compared using a G-test of independence, followed by post-hoc pairwise G-tests with adjusted p-value for multiple comparisons where needed.

3. Results

3.1. Larvae and juvenile survivorship

The hypothesis that exposure to OA conditions increases susceptibility to bacterial infection was tested. For larvae in well plates without continuous air or CO_2 bubbling (Exp. 1) bacteria and pCO_2 treatment had a significant effect on mortality ($p = 0.01$; Table S3), and the mortality in larvae exposed to bacteria in the acidified treatment (93.4 %) was greater than that measured in any other treatment (Fig. 1, Table S4). Differences between larvae in acidified treatment without bacteria (44.1 %), larvae in ambient seawater exposed to bacteria (40.5 %), and control larvae (no bacteria) in ambient seawater (33.8 %) were not significantly different from each other (Fig. 1, Table S4).

For the second experiment (Exp. 2), larvae in aquaria continuously bubbled with air or CO_2 were sampled at 24- and 48-h after addition of pathogens. Results mirrored those from Exp. 1. After 24 h, there was a significant effect of bacteria and pCO_2 treatment on larvae survivorship ($p < 0.001$; Fig. 1, Table S5). The overall greatest mortality was in the larvae in the acidified seawater that were challenged with bacteria (88.4 %). This was significantly higher (Table S6) than mortality in all other treatments, and was followed by mortality in acidified seawater

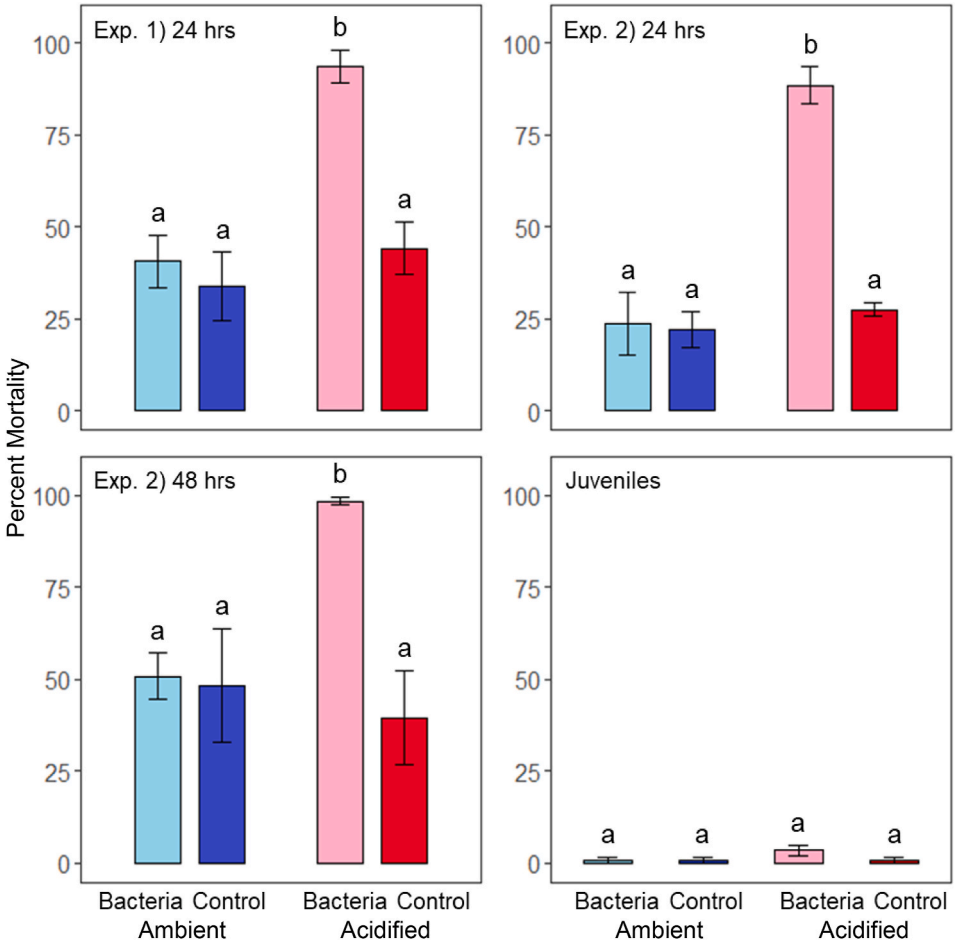

Fig. 1. Percent mortality of larvae (Exp. 1 and 2) and juveniles (mean ± standard error) from ambient (blue) and acidified (red) seawater with bacteria (lighter shades) and without bacteria (darker shades). Different letters denote significantly different mortality levels between treatments within each experiment.

without bacteria (27.4 %), ambient seawater with bacteria (23.6 %), and finally ambient without bacteria (22.1 %) (Fig. 1). After 48 h, trends were similar (Fig. 1, Table S7 and S8) with larvae in acidified seawater exposed to bacteria having the greatest mortality (98.3 %). This was followed by mortality of larvae in ambient seawater with (50.8 %) and without (48.2 %) bacteria, and larvae in acidified seawater without bacteria (39.3 %).

For juveniles, after 2 months of continuous exposure to ROD, and with the addition of the *Vibrio* cocktail, there were minimal mortalities overall and no significant differences were noted between treatments (Kruskal Wallis rank sum test, $p = 0.27$) (Fig. 1), even though mortality in juveniles exposed to OA and challenged with bacteria tended to be higher (3.33 %) as compared to that from the other treatments (0.8 %).

3.2. Transcriptomic responses in larvae

Transcriptomes of larvae exposed to OA and challenged with bacteria and their respective controls (acidified without bacteria, and ambient conditions with and without bacteria) were compared. Pearson's correlation coefficient comparisons of expression profiles demonstrated a moderate correlation ($R^2 > 0.42$) for samples derived from the same treatment (Fig. S1). Treatments clustered based on time (*i.e.*, 6 h of exposure and 3 days of exposure clustered together) (Fig. 2). Within the three days, samples clustered by pCO_2 treatment (*i.e.*, acidified clustered together and ambient clustered together) (Fig. 2).

The only comparisons with statistically significant differentially expressed genes (DEGs) were in larvae sampled three days after the initiation of the pathogen challenge. There were significant DEGs between larvae in the acidified seawater and exposed to bacteria compared to larvae in ambient seawater with bacteria. There were also significant DEGs between larvae with and without bacteria in the acidified condition (Table 1).

There were 578 upregulated (78 %) and 159 downregulated DEGs (22 %) in larvae challenged with *Vibrio* spp. under acidified conditions for three days as compared to challenged larvae maintained at ambient conditions (Tables 1–3; Table S9). However, there were no significantly enriched GO terms detected (Table S10), although an enriched KEGG pathway ("metabolic pathways") was found (Fig. 3, Table S11).

The effect of 3-day bacterial challenge on larvae maintained in acidified seawater yielded 1179 upregulated (78 %) and 328 downregulated (22 %) DEGs (Tables 1, 4 and 5; Table S12). Upregulated genes accounted for numerous physiological functions although there were no significantly enriched GO terms (Table S13) or KEGG pathways (Table S14).

3.3. Effect of OA on mucosal immune factors

After filtering, 408 proteins were considered differentially abundant, with only 47 proteins (12 %) upregulated and 361 proteins (88 %) downregulated in mucus from adult oysters maintained in acidified conditions (Table S15). These molecules included proteins from intracellular origin, such as actins, elongation factors, and ribosomal proteins, in agreement with a previous study reporting that such proteins may derive from hemocytes or epithelial cells found in mucosal secretions [29]. The under expressed proteins were grouped into major functional groups (Fig. 4; Table S15), and many were related to various aspects of innate immunity. These included proteins responsible for stimulating the immune response (macrophage migration inhibitory factor), pathogen recognition particles (fucolectin and galectin), proteins involved in host microbe interactions (such as adhesion proteins), and proteins involved in microbe neutralization via antimicrobial peptides (e.g., cystatin-B, metalloproteinase, alpha-aspartyl dipeptidase). Signaling is a crucial component of the host defense system in bivalves, and proteins involved in signaling pathways were also downregulated at OA conditions. These included kinases (galactokinase), G proteins, ras related protein rabs, and GTPAses. They also included components of

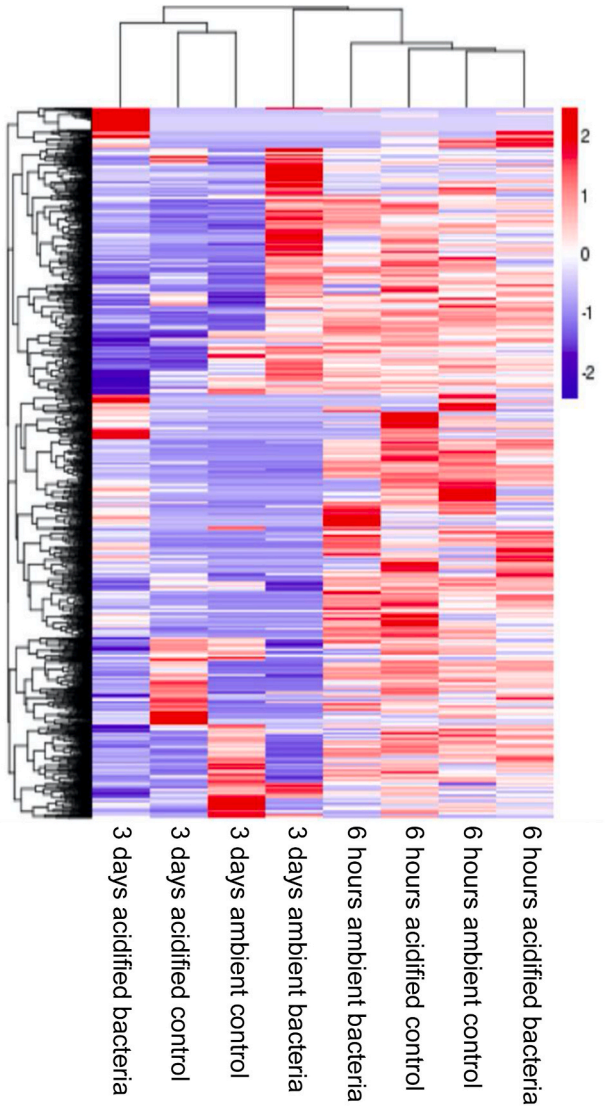

Fig. 2. Hierarchical cluster analysis of differentially expressed genes. Over-and under-expressed genes are represented in red and blue, respectively. Data are normalized by average expression in all samples ($n = 3$ replicates per treatment).

Table 1
Number of significant DEGs among larvae from the pathogen challenge experiment corresponding to each comparison. Direction (up- or downregulated) corresponds to 3 days acidified with bacteria.

Comparison	Upregulated DEGs	Downregulated DEGs
6 h ambient + bacteria vs 6 h acidified + bacteria	0	0
6 h ambient vs 6 h ambient + bacteria	0	0
6 h ambient vs 6 h acidified	0	0
6 h acidified vs 6 h acidified + bacteria	0	0
3 days acidified + bacteria vs 3 days ambient + bacteria	578	159
3 days ambient vs 3 days ambient + bacteria	0	0
3 days acidified vs 3 days ambient	0	0
3 days acidified + bacteria vs 3 days acidified	1179	328

Table 2
Seventeen characterized genes were among the top 25 (based on adjusted p-value) DEGs upregulated in larvae challenged with *Vibrio* spp. and maintained in acidified seawater for three days as compared to challenged larvae maintained in ambient seawater. See Supplementary Table S9 for the full list including those with unknown function.

Gene ID	Protein	Function
111099040	Lymphocyte cytosolic protein 2-like	Cytoskeleton
111099068	Protein unc-93 homolog A-like	Unknown
111101365	SH3 domain-binding protein 2-like	Unknown
111104148	polycomb protein EED-like	Histone methylation
111104314	Isobutyryl-CoA dehydrogenase, mitochondrial-like	Metabolism
111107765	Protein FAM107B-like	Unknown
111108057	Structural maintenance of chromosomes protein 1A-like	DNA repair
111108348	Pleckstrin-like	Cytoskeleton
111112705	Sodium/potassium/calcium exchanger 3-like	Calcium transport
111113160	Vacuolar protein sorting-associated protein 11 homolog	Transport
111114946	SH3 domain-binding protein 5-like	Signal transduction
111115947	Protein white-like	Transport
111118665	Phenylalanine–tRNA ligase alpha subunit-like	ATP binding
111119392	Ubiquitin carboxyl-terminal hydrolase 22-like	Histone ubiquitination
111120680	Dual specificity testis-specific protein kinase 2-like	Apoptotic processes
111121973	Forkhead box protein J1-B-like	Cilium assembly
111122329	Lipoxygenase homology domain-containing protein 1-like	Unknown

Table 3
Sixteen characterized genes were among the top 25 (based on adjusted *p*-value) DEGs downregulated in larvae challenged with *Vibrio* spp. and maintained in acidified seawater for three days as compared to challenged larvae maintained in ambient seawater. See Supplementary Table S9 for the full list including those with unknown function.

Gene ID	Protein	Function
111100266	V-type proton ATPase subunit E-like	Immunity
111100865	Myophilin-like	Actin crosslink formation
111101814	8-Oxo-dGDP phosphatase NUDT18-like	Hydrolase activity
111101848	Protein XRP2-like	Cell morphogenesis
111103230	Homeobox protein Hox-B3a-like	Transcription
111103987	Glutathione S-transferase P 1-like	Oxidation-reduction process
111104800	Perlucin-like	Biomineralization
111107502	Ctenidin-3-like	Immunity
111107754	cystatin-A5-like	Immunity
111108501	Kelch-like protein 15	Immunity
111108710	Prostaglandin reductase 1-like	Metabolism
111109163	Protein limb expression 1 homolog	Unknown
111109360	Ankyrin repeat, PH and SEC7 domain containing protein secG-like	Unknown
111113270	ADP-ribosylation factor-like protein 5B	GTP binding
111119681	Dedicator of cytokinesis protein 7-like	Signal transduction
111119746	Glycine dehydrogenase (decarboxylating), mitochondrial-like	Electron transfer activity

the ubiquitin pathway and cell surface receptors (14-3-3 proteins). Signaling cascades can lead to apoptotic processes, and several proteins involved in this function were downregulated (Rho proteins, cell death proteins). While not directly classified as immune proteins, other classes of proteins can protect against pathogen stress including nonspecific stress response proteins, and they were also found to be downregulated in oysters exposed to OA (heat shock proteins, calreticulin, peroxiredoxins, superoxide dismutase). Calcification related/calcium binding proteins were another group of proteins downregulated in mucus from oysters in the OA treatment. These included protocadherins, calmodulin, sarcoplasmic calcium binding proteins, and troponin T-like.

On the other hand, mucus from oysters maintained in acidified seawater did have some proteins upregulated compared to control oysters, however, this only accounted for 12 % of differentially expressed proteins. These included universal stress proteins, proteins involved in microbe neutralization, and proteins that function in adhesion. Complement c1q-like was upregulated in mucus from oysters in acidified conditions. Shell formation proteins Von Willebrand domain and transgelin-3 like were also upregulated under acidification.

3.4. Effect of OA on hemocyte activities

Phagocytic activity differed significantly between oysters maintained at different pCO_2 conditions. The percent of phagocytic hemocytes was three times lower ($p = 0.0176$; $n = 2$) in oysters from the acidified treatment (18.2 %) as compared to oysters derived from the ambient conditions (54.4 %; Fig. 5). In contrast, viability of hemocytes was similar in oysters from both treatments (87.5 and 86.1 % among acidified and control oysters, respectively; $p = 0.42$; Fig. 5).

While the overall hemocyte count was similar, the hemocyte composition (granulocyte and agranulocyte subpopulations) was markedly different between oysters from acidified and control conditions. Notably, there were significantly more agranulocytes (3.2×10^6 vs. 1.6×10^6; $p = 0.0015$) and less granulocytes (3.4×10^5 vs. 1.9×10^6; $p < 0.001$) in oysters from the acidified conditions.

3.5. Effect of OA on adult oyster health

At time 0 (before exposure to experimental treatments) a subset of oysters was processed for disease diagnostics. The average stage of *P. marinus* infection was <1 (0.62) (71 % prevalence). After maintaining these animals in the flow through system (water derived from Old Fort Pond, Southampton, NY) for 10 months, the level of infection increased significantly ($p < 0.05$), reaching 3.48 (3.63 weighted intensity, 93 % prevalence) and 2.74 (85 % prevalence) in the ambient and acidified treatment, respectively (Fig. 6). Difference between both treatments was, however, not statistically significant ($p = 0.19$).

Oysters collected at Time 0 displayed trematode infections reaching a prevalence of 33.33 % (Fig. 6; Fig. S2 represents a micrograph displaying trematode infection). Trematode prevalence decreased significantly after 10 months in the flow through system, reaching 10.7 % in oysters from the acidified treatment ($p = 0.05$) and 0 % in control oysters ($p = 0.001$). Differences in trematode prevalence between both pCO_2 treatments were also statistically significant ($p = 0.05$). No other abnormal conditions were noted.

4. Discussion

Many studies have explored the impacts of OA on bivalve growth and calcification [8,31], but far fewer have investigated the interaction between OA and pathogens, or if and how OA might lead to immunosuppression. It is well established that environmental factors can influence host ability to resist infections, and the pathogens themselves can be impacted by changes in environmental conditions [8]. This study exposed oysters to acidification and challenged them with *Vibrio* spp. (larvae) and *A. crassostreae* (juveniles) to determine if there was change in susceptibility to infection. Furthermore, transcriptomic and proteomic analyses were performed to determine molecular mechanisms underlying the observed responses to OA. Phagocytosis of hemocytes and differences in mucosal immune factors were also examined to have a more holistic view of differences in immunity between oysters under normal and acidified conditions. Overall, this study suggests impaired immune function of oysters caused by exposure to OA and increased risk of infection.

Findings presented here suggest that oyster larvae have compromised immunity under acidified conditions and are more vulnerable than juvenile stages. The greatest level of mortality was amongst larval

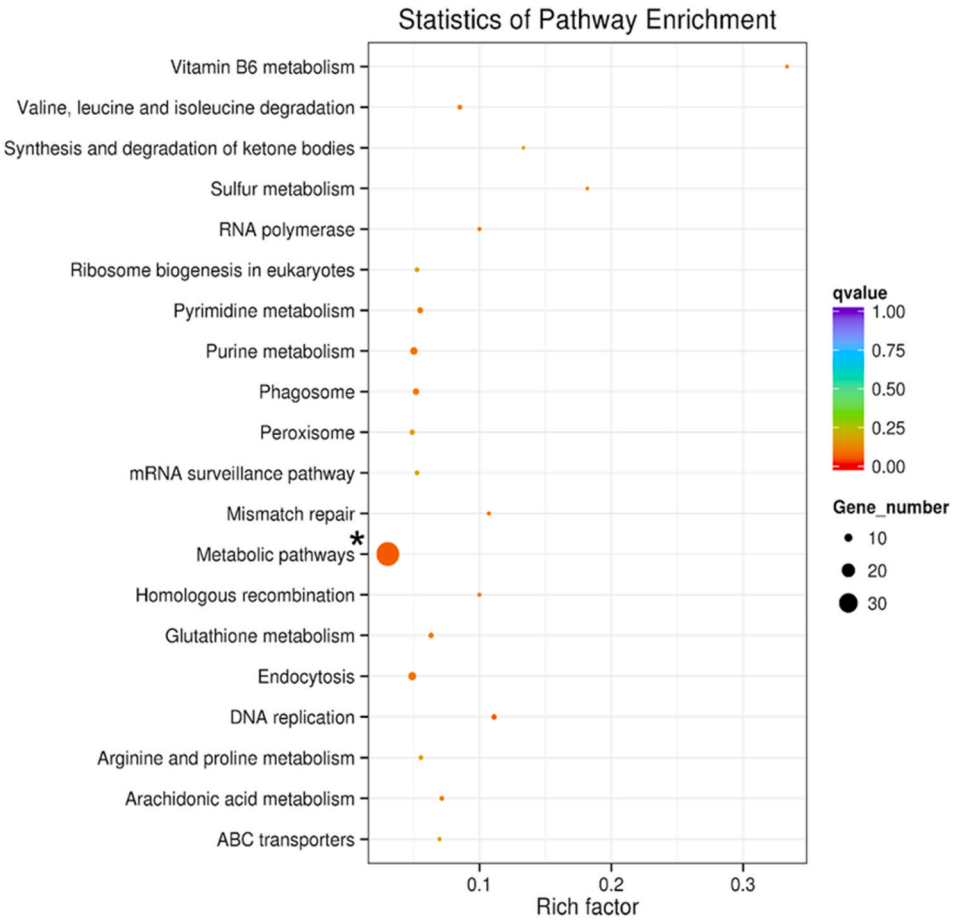

Fig. 3. KEGG pathways identified in larvae challenged with bacteria and maintained in acidified seawater for three days as compared to challenged larvae maintained in ambient seawater. Only one pathway ("metabolic pathways") was significantly overrepresented (*).

Table 4
Fifteen characterized genes were among the top 25 (based on adjusted *p*-value) DEGs upregulated in larvae maintained in acidified seawater and challenged with *Vibrio* spp. as compared to unchallenged controls maintained at the same pCO_2 conditions. See Supplementary Table S12 for the full list including those with unknown function.

Gene ID	Protein	Function
111119361	Isovaleryl-CoA dehydrogenase, mitochondrial-like	Metabolism
111099068	protein unc-93 homolog A-like	Unknown
111112705	Sodium/potassium/calcium exchanger 3-like	Calcium transport
111115947	Protein white-like	Transport
111120680	dual specificity testis-specific protein kinase 2-like	Apoptoic processes
111121538	Enoyl-CoA delta isomerase 1, mitochondrial-like	Metabolism
111122726	Heat shock 70 kDa protein 12A-like	Stress
111124391	Matrilin-2-like	Extracellular matrix
111125860	UPF0428 protein CXorf56 homolog	Unknown
111126198	Heat shock 70 kDa protein 12A-like	Stress
111127949	Probable serine/threonine-protein kinase DDB_G0278509	Signal transduction
111128414	Dentin sialophosphoprotein-like	Shell formation
111128550	UPF0696 protein C11orf68 homolog	Unknown
111128940	2-Oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase-like	Metabolism
111138213	Polyadenylate-binding protein 4-like	mRNA metabolism

oysters reared in acidified conditions and exposed to bacteria. Seawater in larval culture devices that did not have continuous CO_2 bubbling reached control water chemistry conditions relatively quickly. This design was employed to see if immune suppression was a direct effect of acidification during the pathogen challenge itself or if it was a consequence of sustained immune suppression in oysters cultured in acidified seawater. Larvae derived from the acidified treatment and exposed to *Vibrio* had the greatest mortality in both experimental designs. This trend was also seen in *M. mercenaria* larvae tested previously [8]; however, juvenile *M. mercenaria* remained susceptible to infection under acidified conditions while juvenile oysters appeared to be more robust to the effects of acidification. These findings agree with previous work demonstrating that larval bivalves are the most vulnerable life stage to OA [31], and underline the importance of host-pathogen interactions in the reduction of survivorship among bivalve larvae exposed to OA. It should be noted, however, that the exceptional resistance to ROD reported in juvenile oysters here may not be representative of how eastern oyster in general responds to infection by *A. crassostreae* under acidification stress. Indeed, ROD is endemic in New York and juveniles used in this study derive from an aquaculture stock that has a long history of exposure to the disease, which may translate into a high resistance to ROD therefore explaining the low mortality (<5 %) in oysters across all treatments. In addition, the juvenile oysters used in the ROD pathogen challenges were reared in acidified conditions since fertilization, and there could have been acidification-induced selective mortality during larval development, resulting in a cohort of survivor juveniles that are more resistant to the impacts of OA and/or, incidentally, ROD.

Results in this study for oyster larvae supported previous research demonstrating increased mortality in clam (*M. mercenaria*) larvae

Table 5
Twenty-one characterized genes were among the top 25 (based on adjusted *p*-value) DEGs downregulated in larvae maintained in acidified seawater and challenged with *Vibrio* spp. as compared to unchallenged controls maintained at the same pCO_2 conditions. See Supplementary Table S12 for the full list including those with unknown function.

Gene ID	Protein	Function
111119382	Nipped-B-like protein	Chromatid binding
111117721	Phosphatidylinositide phosphatase SAC1-like	Phosphatidylinositol biosynthetic process
111123828	Zinc finger protein 704-like	Transcription
111120164	Multidrug resistance-associated protein 1-like	Cellular response to oxidative stress
111107277	Cell wall protein RBR3-like	Cell wall organization/ remodeling
111109749	Transmembrane protein 45B-like	Transmembrane
111110102	Optineurin-like	Immunity
111128769	Serine protease inhibitor Cvsi-2-like	Immunity
111135171	CAP-Gly domain-containing linker protein 1-like	Cytoskeleton
111111886	cAMP-responsive element modulator-like	Immunity
111132909	Protein FAM45A-like	Unknown
111134083	Cyanophycinase-like	Cellular macromolecule metabolic process
111099573	26S proteasome non-ATPase regulatory subunit 2-like	Protein homeostasis
111123702	Tetratricopeptide repeat protein 16-like	Unknown
111109834	Caveolin-1-like	Calcium ion homeostasis
111133022	Eukaryotic translation initiation factor 4 gamma 1-like	Cell proliferation
111121711	UPF0505 protein C16orf62 homolog	Unknown
111112942	Polypeptide N-acetylgalactosaminyltransferase 1-like	O-Glycan processing
111123983	Rap guanine nucleotide exchange factor 1-like	Signal transduction
111129030	Uridine diphosphate glucose pyrophosphatase-like	Metabolic process
111100384	Transmembrane protein 164-like	Transmembrane protein

exposed to *Vibrio* spp. challenge under acidified conditions [8]. The current work further explored the mechanisms underlying increased susceptibility by evaluating the effect of OA on cellular and molecular immune factors. To do so, the combined impact of OA and pathogen challenge on larvae was evaluated at the molecular level to see if there were changes in pathways or genes critical to the innate immune response of bivalves. RNAseq results clearly demonstrated that the impact of pathogen challenge is greatly amplified under OA conditions as the greatest changes in the transcriptome were noted among larvae exposed to *Vibrio* spp. under acidification stress. Ultimately, this treatment induced the greatest mortality.

Larvae in the acidified treatment and exposed to bacteria displayed a significant upregulation in genes related to actin-cytoskeleton organization as compared to larvae exposed to bacteria under ambient conditions. The cytoskeleton of hemocytes is critical for phagocytosis, which is one of the most important defense mechanisms of innate immunity in bivalves. Formation of hemocytes have been documented as early as 17 h post fertilization in *C. gigas* larvae [32], and proper development of immune mechanisms is essential to combat pathogens. Su et al. (2018) demonstrated that genes activating the actin cytoskeleton of blood clam were significantly upregulated under OA conditions, even though cytoskeletal components and phagocytic activity of hemocytes were reduced [33]. The authors suggested that this might be a feedback mechanism to compensate for reduced cytoskeleton components from OA exposure [33]. In this study, there was reduced phagocytosis in adult oysters maintained in OA conditions. The significant upregulation of genes related to actin cytoskeleton might suggest that, like in the blood clam, acidification negatively impacts the cytoskeleton of oyster hemocytes and hinders phagocytosis.

After KEGG enrichment analysis, metabolism was the only significantly overrepresented pathway in larvae challenged with bacteria and maintained in acidified seawater as compared to challenged larvae held under normal conditions. Upregulation of metabolic pathways suggests higher energetic needs to tolerate acidification stress (acclimation). This was seen in *C. hongkongensis* oyster larvae that increased expression of proteins related to metabolic processes under high pCO_2 which enabled

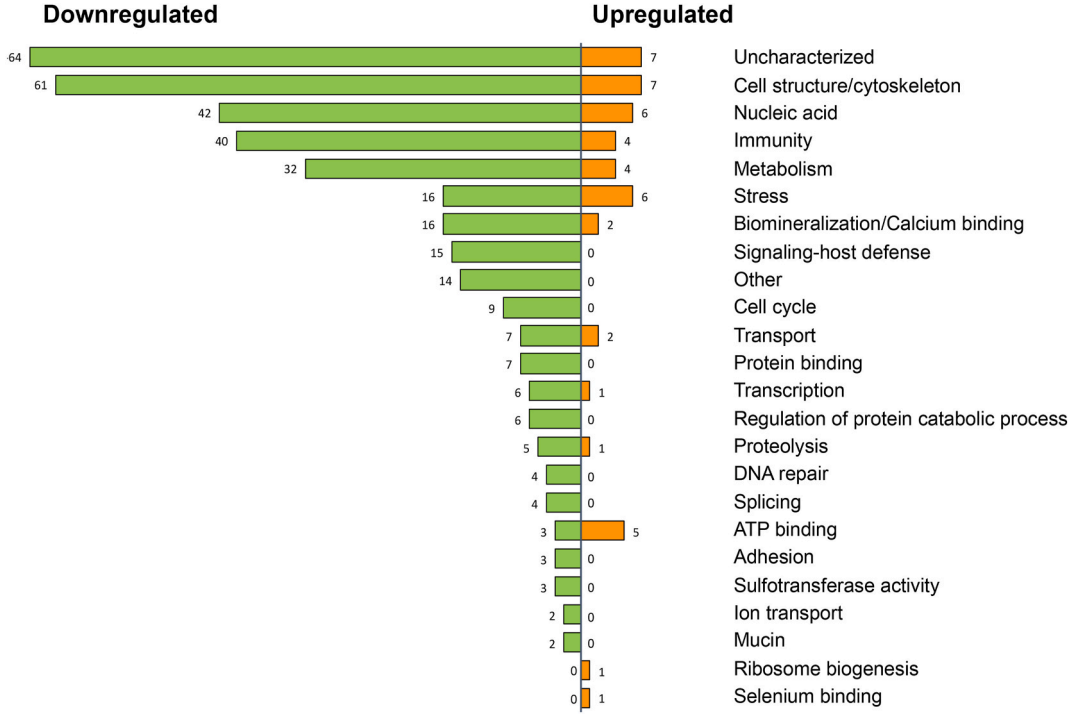

Fig. 4. Number of downregulated (left hand side) and upregulated (right hand side) mucosal proteins in oysters maintained under acidified conditions as compared to those held at ambient conditions.

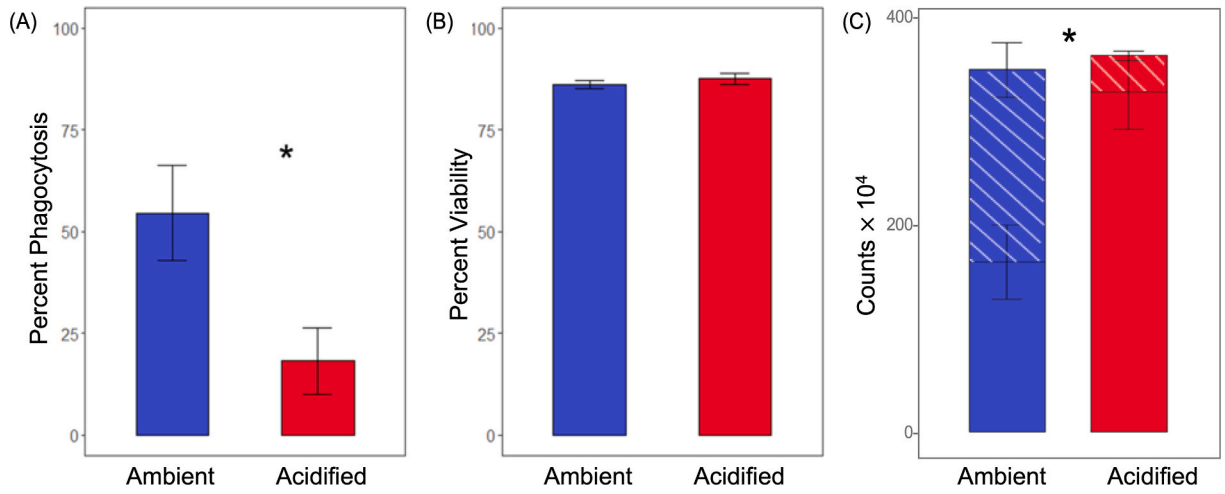

Fig. 5. (A) Phagocytic activity (B) viability and (C) counts of granulocytes (lined bars) and agranulocytes (plain bars) in oysters exposed to low and high pCO_2 conditions (mean ± standard error). * denotes significant difference between pCO_2 treatments; *t*-tests (nested for C), $n = 2$.

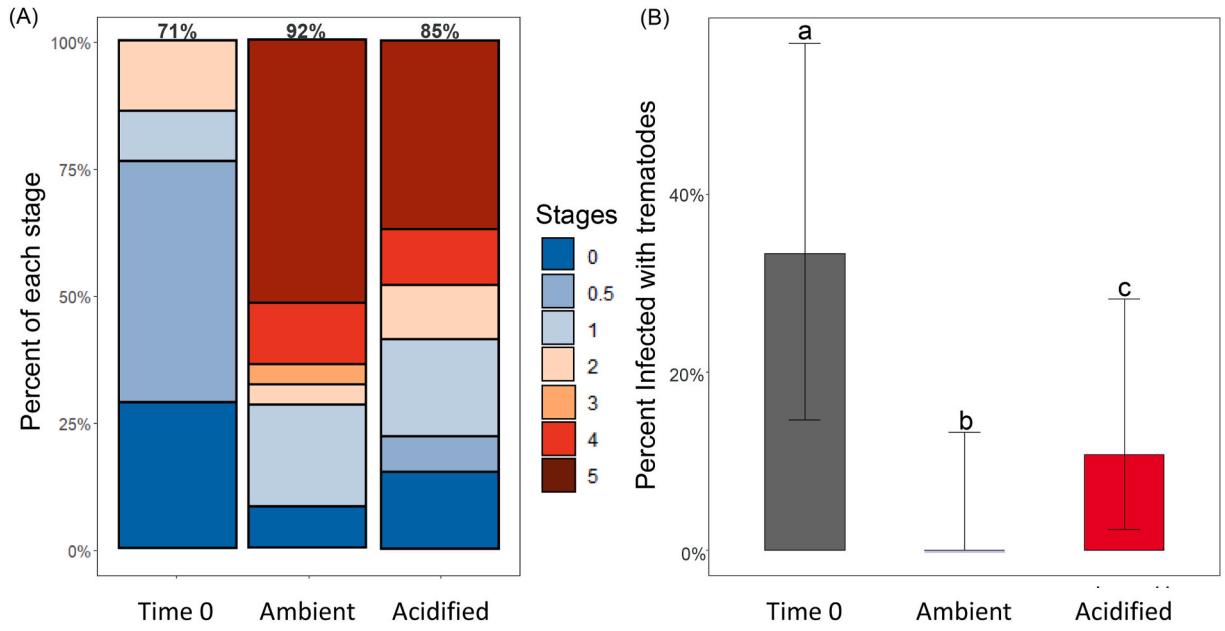

Fig. 6. A: *P. marinus* infection prevalence (% value shown above each bar) and intensity (stages) in oysters from the different pCO_2 treatments (no significant differences were detected between treatments). B. Prevalence of trematode infection in oysters from different pCO_2 treatments (error bars are 95 % confidence intervals). Different letters denote significant differences (G-test of independence with post-hoc pairwise tests, p = 0.05).

them to acclimate to the stressful environmental conditions [34]. Normally reallocating energy and upregulating metabolic pathways comes at the cost of competing physiological functions. For example, there was a significant upregulation of genes related to metabolism in sea urchin larvae under acidified conditions which corresponded to elevated metabolic rates [35]. While metabolism increased, growth rate decreased [35]. The authors suggested that the decrease in growth observed was because energy was reallocated to processes such as ion homeostasis that enabled urchins to survive under acidified conditions [35]. The upregulation of genes related to metabolic pathways reported here suggests increased stress under acidified conditions. The increased mortality when exposed to *Vibrio* might be from reduced immunity because of energy partitioned towards maintaining homeostasis rather than immune processes. During the pathogen challenge experiments, larvae were not fed, which could contribute to the limited energy available for immune response. Juvenile oysters were fed during pathogen exposure and had less mortality. Acclimation to acidified conditions is already energetically costly and mounting an immune response to resist *Vibrio* infection serves to further drain energy in challenged larvae. Energy must be allocated towards maintaining homeostasis and reacting towards the combined stress of OA and pathogen exposure. This is reflected in the gene expression data, with immune related genes downregulated under OA.

The immune genes cystatin-A, ctenidin-3-like, V-type proton ATPase subunit E-like, and kelch-like protein 15 were downregulated in larvae exposed to OA and bacteria compared to the larvae in the ambient pCO_2 bacteria challenge. A study of the mussel *Mytilus galloprovincialis* exposed to *Vibrio splendidus* identified immune mechanisms employed to respond to bacterial exposure [36]. Cystatin-A was one of the top 25 upregulated DEGs after bath exposure to *Vibrio* and was most likely associated with recognizing the bacterial infection and triggering immune mechanisms [36]. Ctenidin-3-like is rich in antimicrobial glycine and plays a role in defense against bacteria [37]. Kelch-like proteins have been documented to play a role in *C. virginica* response to the

pathogen *Perkinsus marinus* [38]. V-type proton ATPase subunit E-like is important for the phagosome signaling pathway and phagocytosis [39]. The downregulation of these genes could help to explain an inability to effectively resist *Vibrio* spp. infection compared to the oysters exposed to the same bacteria in ambient pCO_2.

In addition to weakened immune response contributing to higher mortality, the downregulation of a key ocean acidification resilience gene, perlucin, could make them more vulnerable. Perlucin is a key biomineralization gene and functions in the nucleation of $CaCO_3$ in bivalves [40]. Perlucin has been shown to be upregulated under acidification stress in eastern oyster [41]. In fact, silencing perlucin (via RNAi approaches) in *C. virginica* was shown to lead to less calcium carbonate formation and smaller deformed shells [41]. Therefore, downregulation of this gene could hamper oyster ability to mitigate negative impacts of OA, making them more vulnerable to additional stressors such as infectious diseases.

The only other comparison that yielded significant DEGs was between bacteria-challenged and -unchallenged larvae held under acidified conditions. The addition of bacteria had a significant impact on the transcriptome with 1179 genes upregulated in the "multistressor" condition. Many genes related to metabolism, immunity, biomineralization, cytoskeleton organization, and ion transport were differentially expressed indicating the dual stressors influence multiple physiological pathways. Within the acidified condition, the additional stress of pathogen exposure significantly impacted gene expression, and this treatment induced significantly greater mortality than OA alone (in previous pathogen challenge experiments). The upregulation of 1179 genes, if translated to protein synthesis, could require a significant amount of energy that may impose a burden on available resources, contributing to the death of these larvae. It should be noted that upregulated genes included multiple heat shock proteins, proteins related to the general stress response, and genes related to metabolism. This suggests high metabolic demand and stress which could have contributed to the observed mortality.

Of the downregulated genes, many were related to immunity, including serine protease inhibitor Cvsi-2-like, optineurin-like, and cAMP-responsive element. For example, serine protease inhibitor Cvsi-2-like was demonstrated to play a role in the immune response to *P. marinus* and bacterial exposure in the eastern oyster [12]. While information on optineurin-like is limited in bivalves, it is known to play a role in vertebrate inflammatory responses and immunity, and specifically functions in pathogen recognition and elimination through autophagy [42]. cAMP-responsive element is involved in immune recognition and induction of macrophages and dendritic cells in vertebrates [43]. Less information is known in invertebrates but recently it was found that cAMP response element binding protein mediates immune recognition in the silkworm *Antheraea pernyi* [44]. The downregulation of these genes in our study may imply impaired immune functioning.

While *Vibrio* might be more pathogenic under elevated pCO_2 exposure [8], the increased mortality reported here in *Vibrio*-challenged oysters from OA conditions still appears to be at least partially explained by immune suppression of the host. The hypothesis that oysters have reduced immunity when maintained in OA conditions is further supported by the reduction in hemocyte phagocytic activity in adult oysters maintained under OA conditions as well as the reduction in immune proteins of oyster mucus. Other studies have shown a reduction in phagocytosis as a result of elevated pCO_2 exposure in blood clam [33], but this is the first paper to incorporate mucosal immune factors to generate a more comprehensive evaluation of the effect of OA on bivalve immunity.

The role of mucosal immunity under OA conditions is important as previous research has shown that the concentration of marine heterotrophic bacteria increases in seawater with elevated pCO_2 [8]. Most research on the impacts of high pCO_2 on immunity have focused exclusively on cellular and humoral factors present in the hemolymph. The number of microbes reaching the circulatory system is much smaller than the fraction of microbes bathing mucosal tissues. Mucus is the first physical barrier to foreign cells and most relevant host-microbe interactions occur at mucosal interfaces [16,17]. A proteomic characterization of mucosal secretions of the eastern oyster found that mucus contained proteins related to immune recognition, immune activation, host-pathogen interactions, and proteins involved in repair of damaged molecules [29]. Energy allocated towards mucus production in mollusks is high and can exceed 15 % of energy gained from food [45]. If bivalves are limited by energy under acidification stress (as was suggested by DEG analysis), perhaps less energy is available for mucus protein production. However, this would create a mismatch since the growth of waterborne bacteria increases under acidified conditions. Results reported here show significant downregulation of mucosal proteins in oysters exposed to chronic acidification stress, including for proteins involved in various immune functions such as immune activation, immune recognition, signaling, defense against pathogens, microbe neutralization, and general stress response.

Signaling pathways control the defense system of bivalves, and members of this pathway were found at a lower abundance in oyster mucus from OA conditions. Signal transduction proteins such as kinases, phosphatases, G proteins, GTPAses, ras-related protein rabs, and 14-3-3 proteins were all downregulated in acidified conditions. Downregulation of these proteins suggests impairment of signal transduction pathways from exposure to high pCO_2, which could weaken the ability of oysters to mount an effective immune response.

Oyster exposure to acidified conditions also impacted the ubiquitin pathway. Previous studies have demonstrated that ubiquitin related proteins play a role in invertebrate immune response [46]. Components of the ubiquitin pathway such as elongin-B-like, E3 ubiquitin protein ligase TRIM 71, ubiquitin-conjugating enzyme, E3 ubiquitin-protein ligase mind bomb like, and ubiquitin carboxyl-terminal hydrolase 5-like were downregulated in mucus from acidified conditions.

An important protein in bivalve defense is cystatin. Cystatin is a protease inhibitor and the function of protease inhibitors in bivalve defense includes the inactivation of proteases produced by invading pathogens [47]. Cystatin-B-like was downregulated in mucus from oysters in acidified conditions, and cystatin-A was downregulated under acidification and pathogen stress in the oyster larvae (RNAseq results).

Proteins that serve to mitigate cellular stress, prevent protein degradation, or mediate correct folding of proteins are important parts of the general stress response in bivalves. These include heat shock proteins, alpha crystallin, and calreticulin which were all downregulated in mucus under acidified conditions. Previous research has shown that elevated pCO_2 elicits a stress response in bivalves. For example, *C. hongkongensis* had an increased abundance of stress tolerance proteins in response to high pCO_2 [34]. Cellular stress response protects bivalves from environmental stressors, and without this, they are more vulnerable to the harmful impacts of elevated pCO_2.

Antioxidant proteins including superoxide dismutase and peroxiredoxins were also downregulated in mucus from oyster maintained under OA. Past work has suggested that OA leads to an increase in production of reactive oxygen species (ROS) [15]. An overproduction of ROS can lead to apoptosis and disruption of phagocytosis in clam hemocytes [33]. Antioxidant molecules are produced to control ROS and maintain oxidative system balance, but an under expression of these proteins could impact the regulation of the ROS pathway and lead to reduction in phagocytosis. Allograft inflammatory factor-1-like was also downregulated in mucus from oysters maintained in high pCO_2 conditions. This protein functions in immune cell activation, inflammatory response, and can stimulate phagocytic ability of granulocytes. The reduction in phagocytosis under acidified conditions might be from the downregulation of the aforementioned proteins.

Similarly to findings of the proteomic analyses, the transcriptomics results revealed significant changes in the expression of immune regulated genes, highlighting the impact of OA on self-defense processes.

Future studies could benefit from incorporating transcriptomics and proteomics on the same animals, as multi-omics approaches can likely offer a more comprehensive understanding of the impacts of OA on immune regulation.

Mucosal tissues are irrigated by the circulatory system and hemocytes associated with mucosal secretions can phagocytize and transport materials across the epithelial barrier [17]. Hemocytes associated with mucus are thought to play sentinel roles as a first alert system and to secrete humoral factors into the mucus [16]. Other pockets of hemocytes, such as those populating the extrapallial fluid are known to play roles in biomineralization. While hemocytes in mucus are not known to be associated with shell formation processes, in this study, shell matrix proteins were identified in mucus (upregulated under acidified conditions). Pales Espinosa et al. (2016) found that the mucus covering the mantle of eastern oyster had proteins related to shell mineralization. It should be noted that hemocytes from oysters exposed to OA had lower phagocytic activity as compared to controls, which might be a tradeoff for increasing biomineralization processes under acidification stress [29]. This might explain the upregulation of shell matrix proteins and downregulation of immune proteins under acidified conditions. In fact, hemocyte associated lectin and macrophage mannose receptor, both of which being pattern recognition receptors that can enable non-self recognition and trigger phagocytosis, were downregulated in acidified conditions.

Granulocyte (the most phagocytic hemocyte sub-population in *C. virginica*) counts were significantly lower in hemolymph samples from oysters in acidified conditions, and the percent of hemocytes displaying phagocytic activity was also significantly lower. Liu et al. (2016) also demonstrated reduced phagocytosis in *T. granosa* under acidified conditions [48]. Phagocytosis is an effective innate immune response for defense against many pathogens, so lowered phagocytosis under acidified conditions would lead to greater susceptibility to infection. However, *P. marinus* is a protozoan parasite that is thought to use hemocytes to infiltrate the host and avoid elimination by oxidative killing as they are protected within the hemocyte [49]. Perhaps the observed reduction in phagocytosis lessened the spread of *P. marinus*, so the oyster's overall lower immunity did not contribute to greater infection. We tested the hypothesis that there would be increased *P. marinus* under OA conditions due to immunosuppression of the host, however, there were no significant differences between pCO_2 conditions. On average, oysters in the acidified conditions actually had lower *P. marinus* abundance than controls. No studies have investigated the impact of OA on the pathogen itself, but *P. marinus* is known to be affected by temperature and salinity of the seawater [50]. Oyster plasma bathes tissues where *P. marinus* can also proliferate extracellularly, and oyster pH of plasma can be low under elevated pCO_2. For example, the extracellular pH (pH_e) of hemolymph fell to 7.1 in the Pacific oyster *C. gigas* after one month of elevated pCO_2 exposure [51]. Assuming that pH_e in eastern oyster also decreases under elevated pCO_2 conditions, such environment might not be optimal for *P. marinus* proliferation. Future research on pathogen (e.g., *P. marinus*) specific response to OA would shed light on host-pathogen interactions under future climate regimes.

The only other evidence of infection found from histopathology was the presence of trematodes. Thirty-three percent of oysters collected at the beginning of the experiment were infected with trematodes, and prevalence decreased to 0 % after 10 months in oysters maintained at normal conditions while 11 % of oysters in acidified conditions were infected. Since there was no mortality throughout this experiment, the difference in prevalence between both treatments suggests a reduction in oyster ability to eliminate trematodes under OA conditions. Response to trematodes in the eastern oyster includes increased hemocyte concentration and infiltration (*i.e.*, inflammation; [52]). High pCO_2 might have suppressed the inflammatory response, favoring trematode persistence, which is supported by the observed alterations in hemocytes from oysters exposed to OA.

5. Conclusions

Together, the results presented here demonstrate that oysters, especially at larval stages, have an increased susceptibility to bacterial infection when exposed to acidified seawater. A possible explanation for the increased mortality of larvae maintained in acidified seawater and challenged with pathogenic bacteria could be the increased metabolic demand (inferred from upregulation of metabolic genes) at the cost of immune functions. Immune genes in larvae were downregulated, and interestingly, mucosal immune proteins and hemocyte phagocytic activity in adult oysters were also reduced under acidified conditions supporting our hypothesis that OA impairs immune functions. This study has begun to explore the underlying molecular mechanisms impacted by seawater acidification which may provide useful information for selective breeding programs to identify resilient stocks for restoration and aquaculture. Understanding the implications of OA on susceptibility to disease is important as it could have significant ecological and economic ramifications. It is likely that combined with acidification stress, these losses will be greater, leading to an uncertain future for aquaculture.

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CRediT authorship contribution statement

Caroline Schwaner: Formal analysis, Investigation, Methodology, Writing – original draft. **Michelle Barbosa:** Investigation, Methodology. **Emmanuelle Pales Espinosa:** Conceptualization, Funding acquisition, Writing – review & editing. **Bassem Allam:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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