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Experimental acidification increases susceptibility of *Mercenaria mercenaria* to infection by *Vibrio* species

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ABSTRACT

Ocean acidification alters seawater carbonate chemistry, which can have detrimental impacts for calcifying organisms such as bivalves. This study investigated the physiological cost of resilience to acidification in *Mercenaria mercenaria*, with a focus on overall immune performance following exposure to *Vibrio* spp. Larval and juvenile clams reared in seawater with high pCO_2 (~1200 ppm) displayed an enhanced susceptibility to bacterial pathogens. Higher susceptibility to infection in clams grown under acidified conditions was derived from a lower immunity to infection more so than an increase in growth of bacteria under high pCO_2. A reciprocal transplant of juvenile clams demonstrated the highest mortality amongst animals transplanted from low pCO_2/high pH to high pCO_2/low pH conditions and then exposed to bacterial pathogens. Collectively, these results suggest that increased pCO_2 will result in immunocompromised larvae and juveniles, which could have complex and pernicious effects on hard clam populations.

1. Introduction

Based on current trajectories, elevated pCO_2, reduced pH, and lower calcium carbonate ($CaCO_3$) saturation are all expected to occur in our oceans by the end of the century (Caldeira and Wickett, 2003; Haugan and Drange, 1996; IPCC et al., 2014; Orr et al., 2005). Disruption of oceanic carbonate chemistry processes is known as ocean acidification (OA). OA has been studied extensively over the past decade. Studies have increasingly identified the high susceptibility of economically and ecologically important calcifying estuarine species such as marine bivalves to acidified environments (Fabry et al., 2008; Gazeau et al., 2013; Kroeker et al., 2013; Lemasson et al., 2017). For instance, increasing pCO_2 in seawater was shown to affect several physiological processes such as acid-base regulation (Fabry et al., 2008; Michaelidis et al., 2005), growth (Frieder et al., 2017; Milano et al., 2016; Omoregie et al., 2019; Parker et al., 2011), development (Dupont et al., 2008; Waldbusser et al., 2015, 2016; Wessel et al., 2018), survival (Dupont et al., 2008; Huo et al., 2019; Talmage and Gobler, 2009), and alter energetic demands of bivalves (Gray et al., 2017; Lanning et al., 2010; Thomsen and Melzner, 2010; Xu et al., 2016).

Although there is a general trend of adverse impacts of OA on bivalves, responses are species-, population-, and often process-specific. For example, some bivalve species exhibited lowered growth and development under acidification conditions (Berge et al., 2006; Miller et al., 2009; Ringwood and Keppler, 2002; Talmage and Gobler, 2009; Timmins-Schiffman et al., 2013) while other species grew faster and developed better (Guo et al., 2016; Miller et al., 2009) or, alternatively, did not show any discernible impact (Range et al., 2011). Responses can also differ across life stages within an organism (Talmage and Gobler, 2010). Acidification's effects also varied by population, with certain aquaculture lines of bivalves performing better under OA stress than others (Goncalves et al., 2017; Parker et al., 2011; Stapp et al., 2018). Other studies also highlighted local adaptation in wild organisms (Thomsen et al., 2017).

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The variability between and within species can arise from the fact that many of these organisms are found in highly variable coastal environments with a high degree of environmental heterogeneity (Bernatchez et al., 2019; Boch et al., 2018; Boyd et al., 2016; Kapsenberg and Cyronak, 2018). pH and pCO_2 are much more variable in coastal estuaries that experience acidification from anthropogenic sources in addition to atmospheric CO_2 (Baumann et al., 2015; Cai et al., 2011; Laurent et al., 2017; Wallace et al., 2014). Estuarine areas can be impacted by excessive nutrient loading and enhanced algal productivity and subsequent microbial decay that further increases the concentration of pCO_2 in seawater. This is often distinguished as coastal acidification (Cai et al., 2011; Wallace et al., 2014). In some cases, hyper-eutrophic estuaries are already reaching acidic levels (pH < 7.0) during late-summer months (Wallace et al., 2014), with daily pH amplitudes in some estuaries averaging 0.22 to 1.0 (Baumann et al., 2015). Future acidification scenarios will most likely push environments from a state of net $CaCO_3$ precipitation to net dissolution through a reduction in seawater pH and aragonite saturation state (Ω_a) (Cyronak et al., 2018). Predicted OA in conjunction with natural biogeochemical processes and anthropogenic disturbances can dramatically influence the carbonate chemistry of seawater in estuarine environments and inflate the scale of natural fluctuations. This could lead to greater variability in pH, pCO_2, and Ω_a. Natural fluctuations means periods of stress and release from stress, but this could actually indicate higher metabolic costs of repeated recovery (Saba et al., 2019). There is a debate in the current literature if marine organisms will respond to reduction in average pH, occurrences of extreme pH, changes in the scale of variability, or a combination of all these factors (Clark and Gobler, 2016; Mangan et al., 2017; Saba et al., 2019; Vargas et al., 2017).

The variability seen in responses to acidification might reveal the role of local adaptation or phenotypic plasticity (Vargas et al., 2017). This might be indicative of selection over the years for populations living in a mosaic of carbonate chemistry or could be a plastic response. Populations are able to react to environmental change within their lifetimes through plasticity such as changes in phenotypes that do not depend on changes in their genotype. Organisms have the ability to alter gene expression and to produce different phenotypes in response to changes in the environment. It has been suggested that marine organisms can differentially regulate gene expression as a compensatory response to acidification (Hüning et al., 2013; De Wit et al., 2018). But, this is not a panacea, as plasticity is often associated with fitness costs (Thor and Dupont, 2015; Ertl et al., 2016; Fox et al., 2019), and there is still a physiological tipping point or threshold of tolerance (Dorey et al., 2013). While there might be upregulation of genes associated with resilience to environmental stressors, there are almost always genes that are downregulated indicating depressed pathways (David et al., 2005; Ertl et al., 2016). So while organisms may seem tolerant or resilient to OA, this might come with a fitness cost or a physiological trade-off between specific processes such as immunity.

Tolerance and resistance to disease requires specific allocation of resources and are energetically costly. Surviving in low pH or extremely variable pH might come at the cost of immune functioning (Rauw, 2012). Environmental stressors can promote disease emergence through increasing host susceptibility, introducing novel pathogens, or enhancing virulence of ubiquitous pathogens (Burge et al., 2014; Engering et al., 2013). Host immunity is the first line of defense, and can be strongly influenced by environmental factors (Gajbhiye and Khandeparker, 2017; Hooper et al., 2014; Mydlarz et al., 2006). The degree to which a host resists infection can determine if a pathogen or altered environment will lead to disease outbreak (Harvell et al., 1999). Climate-mediated physiological stresses can decrease a host's resistance to disease and increase their susceptibility to opportunistic infection (Harvell et al., 1999). Furthermore, an often overlooked aspect is the pathogen response to environmental changes. Asplund et al. (2013) demonstrated that *Vibrio tubiashii* may actually have a positive response to acidification, highlighting the importance of understanding host-pathogen interactions in changing environments. In this framework, the ability to understand how multiple stressors (e.g. pathogens and acidification) affect bivalves is important for the management of bivalve stocks under future environmental conditions.

This study was designed to assess the physiological cost of resilience to acidification in the hard clam (*M. mercenaria,* also known as northern quahog). This species is economically important to the eastern coast of the United States and is the most important marine fishery in the state of New York (NMFS, 2017). We examined growth and susceptibility to bacterial infection of larval and juvenile clams reared in seawater with altered pCO_2. We specifically focused on changes in immune performances, measured as alterations of resistance of larvae and juveniles towards opportunistic *Vibrio* spp., among clams exposed to acidified seawater. By examining different life history stages, we were able to document differences in exposure time to acidification as well as potential carry-over effects. To determine if increased susceptibility to infection was direct or indirect, we examined the response of the *Vibrio* species used in the bacterial challenge to different pH treatments and their native presence in the treatment tanks prior to bacterial challenges. Furthermore, we determined if reduced immune function induced by exposure to acidification was permanent or if clams can rebound and restore immunity after transfer to low pCO_2/high pH conditions (indicating a plastic response and/or a selection event) and if early survival in OA conditions was related to success at later stages (through either acclimation or carry over effects). The observed results are discussed in light of the current knowledge of physiological trade-offs in bivalves and potential for acclimation and adaptation to future climate conditions.

2. Materials and methods

2.1. Bivalve husbandry and water chemistry regulation

To increase genetic diversity of the offspring, wild adult clams from four populations (about 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. Data on New York locations demonstrates large fluctuations in pH with NY clam populations regularly exposed to pH minima of ~7.5 in the summer months (Wallace et al., 2014). Collected clams were conditioned for spawning according to Wallace et al. (2008) and Helm et al. (2004). Briefly, adult clams were taken from the field, their shells scrubbed to remove fouling organisms and sediment, and they were placed in a flow through sea table held around 20 °C, the optimum temperature for *M. mercenaria* reproductive development (Helm et al., 2004). In addition to natural algae in seawater, clams were continuously drip-fed through a reservoir of cultured algae: *Tetraselmis* spp, *Isochrysis galbana, Pavlova lutherii*, and *Chaetoceros muelleri*. Clams were conditioned for 8 weeks before planned spawning (Helm et al., 2004), and the stage of gametogenesis was assessed by sacrificing an adult and checking gonad condition under a microscope. Mature clams were placed in a spawning tank, fitted with a standpipe and both cold and warm water hoses. Cycles of hot water (25–28 °C) and normal water were used as thermal stimuli following thermal cycling recommendations (Helm et al., 2004). Additional stimuli were added in the form of frozen sperm from a previous spawn. Seven females (2 Hampton Bays, NY; 4 Northport, NY; 1 Riverhead, NY) and twelve males (3 Hampton Bays, NY; 3 Northport, NY; 2 Riverhead, NY; 4 Wellfleet, MA) were observed releasing gametes. Individuals that released eggs were identified as female, and separated from spawning males into a separate seatable for holding and egg collection. The separation of spawning males and females ensured genetic heterogeneity as sperm from all males could be added to collected eggs for fertilization. After allowing sufficient time for fertilization (1 h), embryos were transferred to experimental tanks equilibrated with CO_2 to attain the following pCO_2 targets: low pCO_2/high pH (pCO_2 of ~600 ppm, pH~7.9) and high

pCO_2/low pH (pCO_2 of ~1300 ppm, pH~7.5) (Tables S4–S7) (Talmage and Gobler, 2009, 2011). The high pCO_2 treatment was selected based on the predictions made by the IPCC for the end of the century (IPCC et al., 2014), taking into consideration the seasonal and diel variability in pCO_2, pH, and Ω_a in *M. mercenaria* local environments (Baumann et al., 2015; Wallace et al., 2014). Larvae cultures were maintained in 43-L vessels held at target pCO_2 with four replicates per treatment. The target pCO_2 was adjusted by continuously bubbling ambient air for the low pCO_2/high pH condition (the water was not buffered as to reflect ambient conditions) or, for the high pCO_2/low pH condition, 5% CO_2 was mixed with air using multi-channel gas proportioners (Cole Palmer) and bubbled directly into the vessels. The seawater was monitored by a Durafet III pH probe (Honeywell, Morristown, New Jersey, USA). The larvae culture vessels were partially submerged in a temperature-regulated sea table (set at 25 °C as this is the optimal growing temperature for *M. mercenaria* larvae as recommended by Wallace et al., 2008; Helm et al., 2004) that served 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. Clearance of algae was monitored daily, and feeding was adjusted to account for mortality as well as larval growth. Larvae received 100% water changes every 24 h for the first two weeks and 48-h thereafter, using 1 μm filtered seawater (Helm et al., 2004). Viability was monitored during water changes. Temperature and pH were recorded daily. Dissolved Inorganic Carbon (DIC) samples were assessed using an EGM-4 Environmental Gas Analyzer ® (PP systems) after acidification and separation of gas phases using a Liqui-cel ® Membrane (Membrana), prior to the introduction of the larvae and throughout the experiment. Total alkalinity, aragonite and calcite saturation, carbonate concentration, and pCO_2 was determined in R using the software package *seacarb* (https://cran.r-project.org/web/packages/seacarb/index.html), with known first and second dissociation constants of carbonic acid in seawater (Millero, 2010). For quality assurance, before and after analyses of DIC samples certified reference material was analyzed (provided by Andrew Dickson, Scripps Institution of Oceanography) with a 99.99% recovery.

Juvenile clams (2 months old, length 0.74 mm ± 0.2, height 0.7 mm ± 0.2) were moved to an open flow through system with water sourced from Old Fort Pond in Southampton, NY (40.8853° N 72.4419° W). In this case, clams received only the algae in the raw water without supplemental feeding from laboratory cultures. For the high pCO_2/low pH treatment, water from Old Fort Pond flowed into an acidification chamber where 100% CO_2 was mixed with air as described above and bubbled to maintain a delta of 0.4 units between the two treatments. Water from the chamber then continuously flowed into four replicate vessels corresponding to the high pCO_2/low pH treatment using a "downweller" setting where clams were held on a sieve (212 μm nylon mesh) that allowed the equilibrated seawater to flow from the top to the bottom compartment of the vessels. For the low pCO_2/high pH treatment, water from Old Fort Pond flowed into an aerated head tank where it then continuously flowed into each of four replicate vessels corresponding to the low pCO_2/high pH treatment. For our aforementioned flow through system, we designated the 4 vessels per pCO_2 treatment to be our experimental units (n = 4). pCO_2 treatment was the fixed effect and the vessel was the random effect. According to Cornwall and Hurd (2016), tank identity should be a random factor, not a fixed factor, which is reflected in the analyses. Sieves holding the juveniles were regularly cleaned (approximately 3 times per week). Larvae and juveniles were sampled at various time points (24 h, 48 h, 5 days, 1 month, 2 months, and one-year post-fertilization) to assess growth or for use in the various bacterial pathogen challenge experiments.

2.2. Growth measurements

Larvae and juveniles were preserved in a solution made with 1 mL 10% buffered formalin and 10 mL seawater and stored at −20 °C until image acquisition. Briefly, larvae and juvenile bivalves were photographed under an inverted and a dissection microscope, respectively, before digital images were processed using the program ImageJ (Version 1.44, NIH). For larvae, length measurements were obtained from 100 larvae per replicate and are expressed in micrometers. For juvenile clams, height (distance between the tip of the umbo to the ventral edge of the shell) and length (anterior to the posterior edge of the shell) measurements were obtained from 100 clams per replicate and are expressed in millimeters. Growth data was analyzed for significant differences between pCO_2 treatments as described below.

2.3. Bacterial pathogen challenge

2.3.1. Larvae

Two experiments were designed to test the effects of acidification on clam larvae susceptibility to bacterial infection. In the first experiment (Exp. 1), bacterial challenge was performed in 16.8 mL 6-well microplates with no bubbling (Fig. S1). A series of four wells contained larvae (n = 30; two-day old larvae in 15 mL filtered seawater) from each original larvae culture vessel (2 treatments; 4 replicates treatment^{-1}), yielding a total of 16 wells. Half the wells received a bacterial cocktail made of *Vibrio tubiashii, V. coralliilyticus, V. splendidus,* and *Listonella anguillarum* (yielding ~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; Richards et al., 2015) initially grown on marine agar and suspended in sterile seawater while the other half received sterile seawater. A control plate containing only control (pCO_2 ~600 ppm or pH = 7.8) and acidified seawater (pCO_2 ~1300 ppm, pH = 7.5) was prepared to follow changes in pH over time. For the second experiment, (Exp. 2) (Fig. S2) ~500 six-day old larvae in 150 mL of filtered (0.2 μm) seawater were added to a series of 250-mL vessels (n = 8 treatment^{-1}) bubbled with 5% CO_2 mixed with air (for the high pCO_2/low pH treatment, pCO_2 ~1300 ppm, pH = 7.5) or air only (for the low pCO_2/high pH treatment, pCO_2 ~600 ppm or pH = 7.8). Half of low pCO_2/high pH and high pCO_2/low pH vessels then received bacteria (same cocktail and concentrations as above). In this setting, larvae derived from each original larvae culture vessel were represented by two vessels: one with bacteria suspended in seawater and one with seawater, yielding a total of four replicates per treatment. 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 post-exposure. Clams were considered dead if there was a lack of ciliary movement, swimming, or empty shells.

2.3.2. Juveniles

As was done for larvae, experiments were conducted with and without continuous CO_2 bubbling on ~ five week old clams using higher bacterial concentrations (~10^4 CFU mL^{-1} per strain). Briefly, 16, 250-mL experimental flasks containing 450 juvenile clams (slightly above recommended culture density; Helm et al., 2004) each received either bacterial cocktails or seawater (controls), yielding four replicates/pH condition (low pCO_2/high pH pCO_2 ~600 ppm, pH = 7.85; high pCO_2/low pH pCO_2 ~1400 ppm, pH = 7.52). Mortality was assessed microscopically at multiple time points for the experiment with bubbling (Fig. S3) and at 48-h post-exposure for the experiment without bubbling (Fig. S4).

2.3.3. Reciprocal transplant experiment

This experiment was designed to evaluate the ability of clams grown under acidified conditions to recover their immune performances and resist bacterial infections after transplant to low pCO_2/high pH conditions as well as to assess the effect of exposure to acidification on immune performances in juvenile clams. After 1 year of continuous exposure to high pCO_2/low pH (pCO_2 ~1166 ppm, pH = 7.57) or low pCO_2/high pH (pCO_2 ~576 ppm, pH = 7.9) conditions, 100 clams (~10

mm in length) were taken from each replicate culture vessel, transferred to a small mesh bag (mesh size = 800 μm) held inside a Pyrex Petri dish and moved to the opposed condition (e.g. high pCO_2/low pH moved to low pCO_2/high pH, and vice versa). Another subset (also 100 clams) was placed in mesh bags and returned to the original vessel (to control for the effect of placing clams in mesh bags). Therefore, treatments for this transplant experiment included: clams initially grown under low pCO_2/high pH remaining in low pCO_2/high pH, clams from high pCO_2/low pH transplanted into low pCO_2/high pH, clams from high pCO_2/low pH remaining in high pCO_2/low pH, and clams from low pCO_2/high pH transplanted into seawater with high pCO_2/low pH. This transplant was for a total of six weeks before clams were divided into two groups with one group (30 clams/replicate; 4 replicates) challenged with bacteria as described above (~10^6 CFU mL^{-1} for each strain). In this experiment, 5% CO_2 mixed with air was bubbled to maintain the transplant pH conditions throughout the 5-day bacterial challenge: high pCO_2/low pH (pCO_2 ~1166 ppm, pH = 7.57) or low pCO_2/high pH (pCO_2 ~576 ppm, pH = 7.9).

2.4. Effect of acidification on bacterial abundance

This experiment evaluated bacterial concentrations in high pCO_2/low pH and low pCO_2/high pH seawater and clams. Samples of water and one-year old clams were collected separately from flow-through vessels. Water samples (1 mL) were obtained after thoroughly homogenizing the water in each vessel. Ten juvenile clams (~10 mm) from each treatment were sampled randomly. Seawater was serially-diluted in filtered artificial seawater (FASW) and plated on thiosulfate citrate bile salts sucrose media (TCBS: a selective media for *Vibrio* spp.) and Marine Agar (non-selective media). Clam tissues were homogenized in FASW (0.1 g of tissue into 500 μL of water) and 100 μL was plated on the TCBS and Marine Agar media. Plates were incubated at room temperature for 3 days and CFUs were enumerated on both culture media and compared for significant differences between treatments in seawater and clam tissue.

2.5. Effect of acidification on Vibrio spp. growth

Based on preliminary findings of increased susceptibility of clams to *Vibrio* spp. infection under acidified conditions, we also evaluated the effect of acidification on *Vibrio* spp. survivorship and growth in seawater. Experimental flasks ($n = 4$) contained 150 mL of filtered (0.2 μm) natural seawater continuously bubbled with either air or 5% CO_2 mixed with air: low pCO_2/high pH (pCO_2 ~487 ppm, pH = 7.95), or high pCO_2/low pH (pCO_2 ~1145 ppm pH = 7.59). The same bacterial cocktail (~10^6 CFU mL^{-1} per strain) used in the juvenile pathogen challenge experiments was added to each flask and seawater samples were collected after adding bacteria, at 6 h, 24 h, and 120 h (sampling times were based on clam mortality data). Seawater was serially-diluted in FASW and 100 μL was plated on TCBS. Plates were incubated at room temperature for 3 days and colonies were counted. The number of CFUs was compared for significant differences between treatments.

2.6. Statistical analysis

All statistical analyses were conducted using R version 3.3.2. Assumptions of a normal distribution and homoscedasticity were confirmed using Shapiro-Wilk and Bartlett's tests, respectively. Length of larval clams from high pCO_2/low pH and low pCO_2/high pH treatments was compared by using a Student t-test, after taking averages of clam size from each replicate. A Bonferroni correction was applied by dividing the alpha by 3 (0.05/3 = 0.0166). Post-set and juvenile clam height and length at one month and two months old were analyzed with a nested ANOVA, with pCO_2 treatment as a fixed effect and replicate (vessel) included as a random effect. The ratio of dead clams to live clams from the larval and juvenile pathogen challenges was analyzed using a two-way ANOVA (the two factors were pCO_2 treatment and bacteria). Post-hoc comparisons were performed and p-values were adjusted accordingly for multiple comparisons using Tukey Kramer post hoc test. Number of CFUs from clam tissue and seawater from each pCO_2 treatment were compared using a nested ANOVA after log transformation of the bacteria counts. Differences in *Vibrio* spp. growth between each pCO_2 treatment were compared using a Student's t-test, after CFU data was log transformed and an average was found from each replicate. The final seawater carbonate chemistry effect on clam mortality in the reciprocal transplant experiment was analyzed using a G-test of Independence and post hoc pairwise testing. Results were deemed significant at $\alpha \leq 0.05$.

3. Results

3.1. Growth

Size of larvae was not significantly different one ($n = 4$; p = 0.79; Student's t-test) or two ($n = 4$; p = 0.25; Student's t-test) days post-fertilization between pCO_2 treatments. At five days, larval clams reared in high pCO_2/low pH pCO_2 were significantly larger than larvae reared in low pCO_2/high pH pCO_2 ($n = 4$; $p = 0.012$; Student's t-test; Fig. 1).

At one month, height and length were significantly greater in clams grown in low pCO_2/high pH conditions as compared to those in clams from the high pCO_2/low pH condition (nested ANOVA $p = 0.037$ and 0.003, respectively; Fig. 2 and Table S1). The same trends were also noted at two months, with both height and length remaining significantly greater in clams reared in low pCO_2/high pH conditions as compared to clams maintained in high pCO_2/low pH ($p < 0.001$).

3.1.1. Susceptibility to infection by bacteria

3.1.1.1. Larval clams. For both experiments, significantly ($p < 0.05$; two-way ANOVA, Table S2) higher mortality of larval clams was observed following bacterial challenge under high pCO_2/low pH. In the well plate experiment (no continuous CO_2 bubbling), pH in wells taken with initially acidified seawater increased rapidly in the first hour and leveled out until reaching control levels within 3 h. Larval mortality was significantly greater (80 ± 5% after 24 h; $p = 0.036$, two-way ANOVA, $n = 4$; Fig. 3 Exp. 1) among clams challenged with both acidification and *Vibrio* spp. relative to clams from the low pCO_2/high pH treatment and

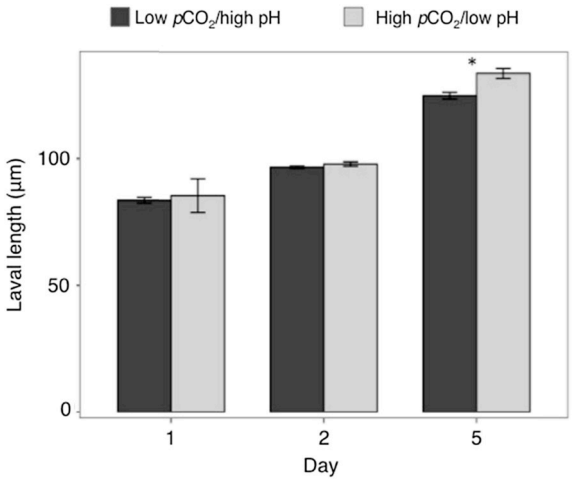

Fig. 1. Larvae reared in seawater with high pCO_2/low pH were significantly larger than those in low pCO_2/high pH five days post-fertilization ($n = 4$ replicates, with 100 individual larvae measured per replicate, error bars denote ± standard error of the mean, * denotes significant differences within a single time point, Student's t-test, $p = 0.012$.

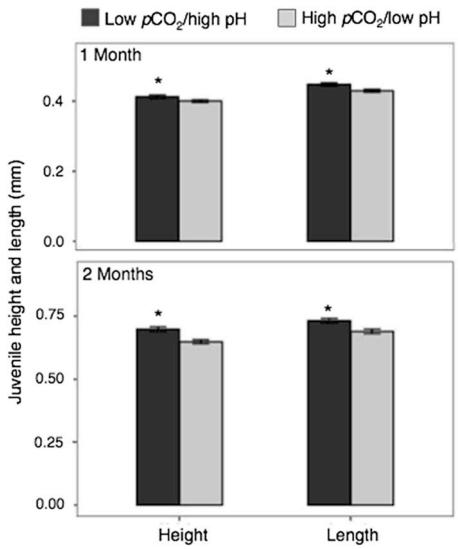

Fig. 2. Height and length of juvenile clams at one- and two-months post fertilization in varying pCO_2 treatments ($n = 4$ replicates with a minimum of 100 juvenile clams measured per replicate, error bars denote ± standard error of the mean, * denotes significant differences; $p < 0.05$; nested ANOVA).

challenged with bacteria (47 ± 3%). Larval mortality was low in clams from both the low pCO_2/high pH and high pCO_2/low pH treatments without bacteria (20 ± 7 and 25 ± 4%, respectively; $p > 0.05$, two-way ANOVA, $n = 4$). Similar results were obtained in the experiment with CO_2 bubbling, with larvae reared in high pCO_2/low pH pCO_2 and exposed to bacteria having greater mortality (82 ± 1%; $p = 0.014$, two-way ANOVA, $n = 4$; Fig. 3 Exp. 2) than in any other treatment (6 ± 3% among high pCO_2/low pH clams without bacteria to 72 ± 4% among low pCO_2/high pH clams with bacteria). In fact, results obtained with CO_2 bubbling in 6-day old larvae perfectly mirror the trends observed in the well plate experiment (2-day old larvae, no CO_2 bubbling) with highest mortality amongst larvae in high pCO_2/low pH pCO_2 seawater with bacteria, second highest mortality in larvae from low pCO_2/high pH seawater with bacteria, and no significant ($p > 0.05$; two-way ANOVA) differences in mortality for treatments without bacteria.

3.1.1.2. Juvenile clams. For the experiment with bubbling using 38-day old juvenile clams, there were no significant differences (all $p > 0.05$, two-way ANOVA, $n = 4$, Fig. 3 Exp. 3) in mortality at any time point (4 h, 24 h, 48 h, 96 h) between any treatments and overall mortality was low (9 ± 2% to 14 ± 1% after 96 h). For the experiment without bubbling using 33-day old clams, juveniles grown in acidified conditions and exposed to bacteria experienced significantly higher mortality (79 ± 5%; $p = 0.03$, two-way ANOVA, $n = 4$; Fig. 3 Exp. 4, Table S2) as compared to all other treatments 48 h post-exposure. Clams from low pCO_2/high pH and exposed to bacteria experienced the second highest level of mortality (56 ± 3%), with low levels of mortality amongst the controls (23 ± 3% and 30 ± 1% in low pCO_2/high pH and high pCO_2/low pH, respectively; $p > 0.05$, two-way ANOVA, $n = 4$).

3.2. Bacterial abundance

Both *Vibrio* spp. and total heterotrophic bacterial counts were significantly ($p < 0.001$, nested ANOVA, Table S3) greater in tissues of clams reared at high pCO_2/low pH (1.57×10^3 CFU g^{-1} of wet tissue and 5.32×10^4 g^{-1} of wet tissue, respectively) as compared to clams reared under low pCO_2/high pH conditions (5.95×10^2 g^{-1} of wet tissue in clam and 1.04×10^4 g^{-1} of wet tissue in clam, respectively; $p < 0.001$, nested ANOVA; Fig. 4a and b). These findings were also mirrored in bacterial counts in seawater, with significantly higher counts of *Vibrio* spp. and total heterotrophic bacteria in high pCO_2/low pH seawater (8.56×10^2 CFU mL^{-1} and 1.21×10^3 CFU mL^{-1}, respectively) as compared to seawater from the low pCO_2/high pH treatment (33 and 3.07×10^2 CFU mL^{-1}, respectively; $p < 0.001$, nested ANOVA; Fig. 4c and d).

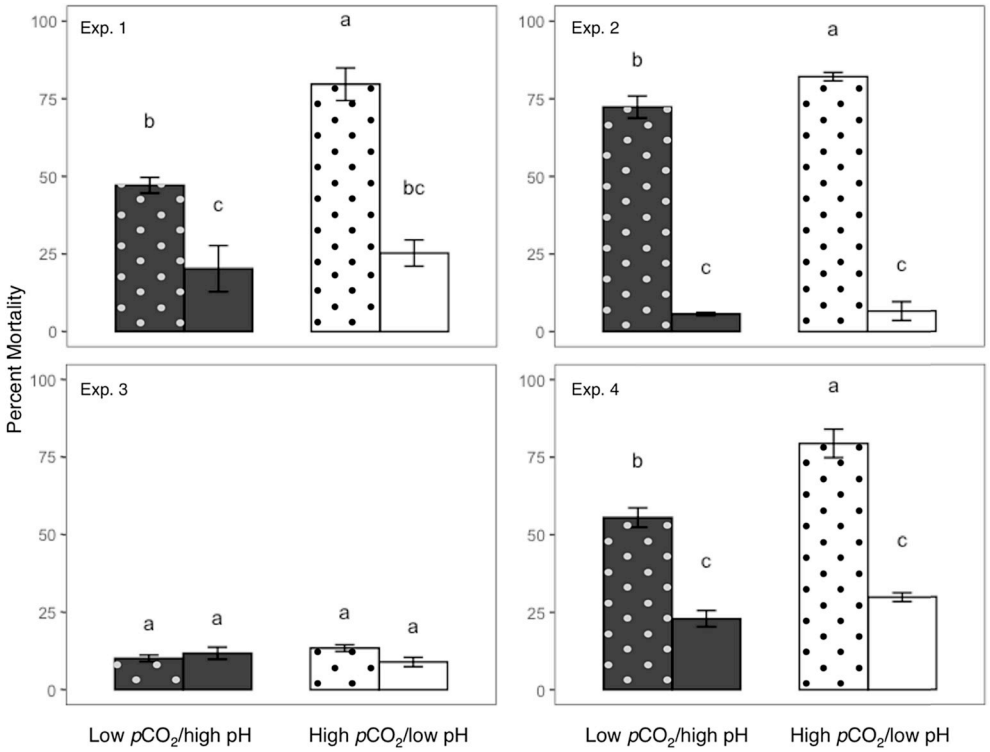

Fig. 3. Mortality of control (undotted) and bacteria-challenged (dotted) larval and juvenile clams (mean ± standard error of the mean, $n = 4$ replicates). Different letters denote significantly different mortality levels between treatments within each experiment (two-way ANOVA, Tukey Kramer post hoc test, $p < 0.05$). Experiment 1: 24 h post challenge, 2-day old larvae, no CO_2 bubbling. Experiment 2: 24 h post challenge, 6-day old larvae exposed to bacteria with bubbling. Experiment 3: 96 h post challenge, 38 day old juvenile clams with bubbling. Experiment 4: 48 h post challenge, 33-day old juvenile clams without bubbling.

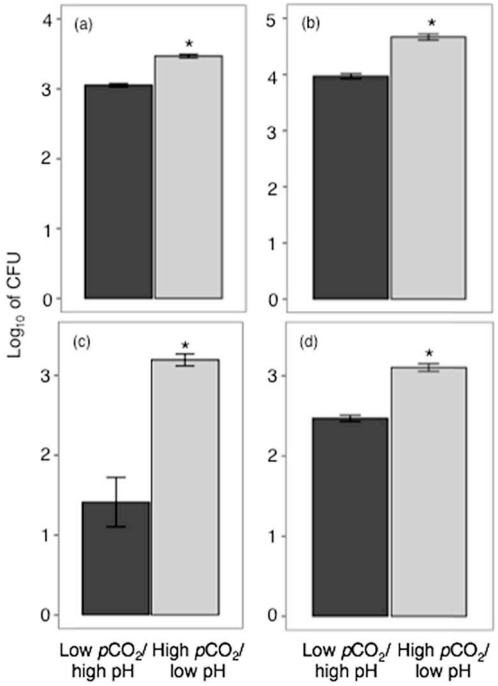

Fig. 4. Abundance of *Vibrio* species (a and c) and total heterotrophic bacteria (b and d) in clam tissues (a and b, Colony forming units (CFU) per gram of wet weight) and seawater (c and d, CFU per mL) from the high pCO_2/low pH and low pCO_2/high pH treatments (mean ± standard error of the mean), * denote significance, $p < 0.001$ nested ANOVA.

3.3. *Vibrio spp. growth*

Vibrio spp. cocktails were added to seawater with either high pCO_2/low pH or low pCO_2/high pH and growth was followed. No significant differences in growth at 6 h (CFU ranged from 1.09×10^7 to 8.03×10^7; Fig. 5) and 24 h (from 1.05×10^6 to 1.34×10^7 CFU; Fig. 5) were observed. After 120 h, the number of CFUs was significantly greater in the seawater from the high pCO_2/low pH treatment as compared to the low pCO_2/high pH treatment (7.45×10^8 as compared to 2.8×10^8, $n = 4$; $p < 0.001$, Student's t-test; Fig. 5).

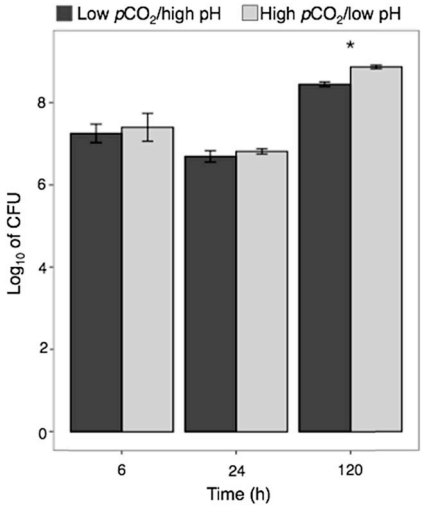

Fig. 5. *Vibrio* species growth (mean counts ± standard error of the mean, mL^{-1}) in high pCO_2/low pH and low pCO_2/high pH,* denotes significance; $p < 0.001$; Student's t-test, $n = 4$.

3.4. *Reciprocal transplant with pathogen challenge*

Final seawater carbonate chemistry did have a significant effect on mortality with and without bacterial exposure ($G = 26.094$, $p < 0.001$, G-test of Independence; Fig. 6). Clams transferred from seawater with low pCO_2/high pH into seawater with high pCO_2/low pH and exposed to *Vibrio* spp. had the greatest mortality overall (46%) and was significantly greater than clams transferred from high pCO_2/low pH to low pCO_2/high pH (31%) and clams that stayed in high pCO_2/low pH and were exposed to bacteria (24%). Clams that remained in the low pCO_2/high pH seawater and exposed to bacteria had the second highest mortality (38%) and had significantly higher mortality than those that stayed in high pCO_2/low pH and were exposed to bacteria. When comparing with and without bacteria, only clams that were transplanted had significantly greater mortality than their controls. For example, clams from low pCO_2/high pH transferred to high pCO_2/low pH with bacteria had 46% mortality compared to those without bacteria which only had 21% mortality, and clams transplanted from high pCO_2/low pH conditions into low pCO_2/high pH with bacteria had 31% mortality compared to those without bacteria which had 16% mortality.

4. Discussion

Previous studies have demonstrated the sensitivity of calcifying marine organisms, such as bivalves, to OA (Dupont et al., 2008; Fabry et al., 2008; Lanning et al., 2010; Parker et al., 2011; Talmage and Gobler, 2009; Thomsen and Melzner, 2010; Waldbusser et al., 2015). While many prior studies have investigated the impacts of OA on marine life, few have begun to examine the impacts of multiple stressors (Dupont and Pörtner, 2013) and often only examined acute exposure without evaluating the effect of long-term exposure to varying pCO_2 or potential carry-over effects throughout life stages. Our study provides a novel, long-term view of the effects of OA on marine bivalves reared from embryos to juveniles in acidified conditions. Under OA stress, larval- and juvenile-stages of hard clams face several potentially detrimental physiological stresses: growth responses demonstrate the differential effects of acidification on development at various life stages and, the most concerning result from our study, markedly increased susceptibility to bacterial infections in both larvae and juveniles exposed to OA.

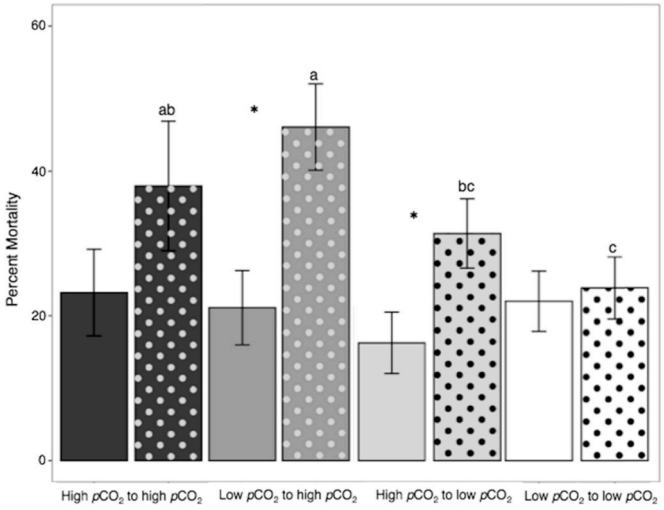

Fig. 6. Mortality of clams from the transplant experiment after exposure to *Vibrio* spp. (dotted) and control (undotted) ($p < 0.001$; G-test of Independence; $n = 4$, with 30 juvenile clams per replicate, mean ± standard error of the mean). Letters represent significant differences between final carbonate chemistry after transplant, * represents significant differences between controls and pathogen challenges.

Our findings demonstrate clam larvae experiencing chronic high pCO_2/low pH were significantly larger than those grown under low pCO_2/high pH conditions after five days of exposure, but the trends were inverted after one and two months. Many studies indicate that the growth of bivalves may be affected by OA, although trends appear to be species-specific. Some bivalve species exhibit lowered growth and development under OA, as is the case for *M. edulis* (Berge et al., 2006; Ventura et al., 2016), *M. mercenaria* (Ringwood and Keppler, 2002; Talmage and Gobler, 2009), *C. virginica* (Miller et al., 2009), *C. gigas* (Timmins-Schiffman et al., 2013) and *A. irradians* (Talmage and Gobler, 2009). In contrast, *Paphia undulata* grew faster and developed better under acidified conditions (Guo et al., 2016), *C. ariakensis* grew larger (Miller et al., 2009), and there was no difference in net calcification, size or weight in *Ruditapes decussatus* (Range et al., 2011). Furthermore, previous investigations in bivalves showed that the variability in response to OA is not only species-specific, but also population-specific (Parker et al., 2011). Changes in growth under OA conditions shown here may be the result of a genuine change in the response of different ontogenic stages of clams, with larvae growing faster and juveniles slower under OA. Additionally, whereas larvae were fed *ad libitum*, juveniles were maintained on a flow through system where algal biomass fluctuated seasonally and may have been limiting at times. Food availability has been demonstrated to be just as important as acidification stress for individual fitness; food limiting conditions in combination with acidification stress reduces growth rates of calcifying species (Pansch et al., 2014) whereas under abundant food supply survival and calcification remains high despite high pCO_2 (Thomsen et al., 2013). Fluctuations in food supply experienced by juvenile clams here may have contributed to the differences in growth observed between age classes if juveniles were food limited. An alternative scenario could potentially imply an initial selection where the larger larvae survived OA exposure increasing the overall average size. Furthermore, the clams used in this study were derived from various genetic backgrounds (Wellfleet, MA; Northport, NY; Hampton Bays, NY; Riverhead, NY), and we cannot rule out that genetic selection could have occurred over time in response to our extended OA exposure, therefore confounding our ability to determine whether the contrasted response to OA between clam larvae and later-life stages derives purely from food availability, age, concurrent genetic selection, or a combination of factors.

It is well established that small changes in abiotic conditions can gradually affect the fitness of an individual and compromise host defenses against infection (Studer et al., 2012). Our observations suggest that both larval- and juvenile-stages are increasingly vulnerable to pathogen infection in acidified conditions as their immune functions may be compromised. Similar trends in mortality data of larvae were observed in both experiments (with or without continuous CO_2 bubbling), with the greatest level of mortality amongst clams reared in high pCO_2/low pH and exposed to bacteria. These findings indicate that mortality under combined acidification and pathogen stress is a result of a sustained (at least for 24 h) immune suppression in bivalves cultured at low pH and not due to a direct effect of low pH during the bacterial challenge itself since pH in the well plate quickly (within 3 h) reached control levels. Similarly, juvenile clams reared in high pCO_2/low pH and exposed to bacteria (without continuous CO_2 bubbling) had significantly greater mortality. This data offers novel integrative information on the effects of acidification on bivalve immunity. While previous studies have indicated a reduction in some immune parameters under acidification, the work on bivalves was rather narrow in scope and provided information on a very limited number of immune parameters, such as the function of hemocytes (Sun et al., 2017). Bibby et al. (2008) demonstrated that an increase in pCO_2 suppresses phagocytosis by hemocytes in *M. edulis*. Liu et al. (2016) demonstrated a reduction in total hemocyte count, phagocytosis frequency, and red granulocytes in *Tegillarca granosa* under elevated pCO_2. However, other investigations found that pCO_2 did not have an effect on *C. gigas* phagocytosis after 28 days of exposure to OA (Wang et al., 2016). Furthermore, another study showed that phagocytosis increased in *M. edulis* under OA stress (Mackenzie et al., 2014), opposing Bibby et al. (2008) findings in the same species. Understanding the effect of OA on hemocyte function is important; however, using hemocyte parameters alone as a measure of immune competency fails to account for the fact that changes in hemocyte activities (e.g. hemocyte counts, phagocytosis, reactive-oxygen production, etc.) are often a general stress response (Allam and Raftos, 2015). Further, most prior studies focused on adult organisms which are less vulnerable to both OA and disease. Ellis et al. (2015) demonstrated an initial suppression of anti-bacterial activity in cell-free hemolymph derived from *M. edulis* at an extreme low pH of 6.5 and higher mortality at pH 6.5, however, the study examined only the adult life stage of the mussel. Even studies that showed alterations in immune activities did not test overall immune performances via exposure to infectious agents. In this framework, our study provides a more integrative understanding of the effects of OA on overall immune performances by examining the effects of low pH on clam susceptibility to pathogens. As a note, the similarity in trends between experiments with and without bubbling could, in addition to overall immunosuppression due to acidification stress, be attributed instead to an acute stress response. As described, pH equilibrated between treatments within approximately 3 h in the experiments without bubbling and this rapid change in pH may have elicited an acute stress response from the larvae and juveniles thus potentially obscuring results with regard to the true effect of pCO_2 condition on immune function. It should also be noted, however, that we did not feed our organisms while they were undergoing the pathogen challenge, and that might have implications for energy availability. OA and pathogen stress can have synergistic or additive effects. In other words, OA may cause an increase in energy demands and infection typically increases metabolic demands to allow the host to mount immune responses, thus both would serve to drain host energy reserves possibly leading to a weakened host. Under OA conditions, many physiological processes of bivalves such as acid-base balance, metabolism, and calcification are often impacted (Beniash et al., 2010; Michaelidis et al., 2005; Miller et al., 2009; Zhao et al., 2017). Energy must be allocated between the competing physiological processes under the combined challenge of OA and pathogen exposure. The energetic demands to maintain physiological homeostasis, metabolism, or calcification might be a key limiting factor and might divert energy away from processes such as immunity. Cao et al. (2018) demonstrated that despite depression of aerobic metabolism under OA, *C. gigas* uses energy modulation methods to compensate for pathogenic challenge, however in the long term the process would be unsustainable and eventually energy reserves would be depleted. In addition, a recent study by Frieder et al. (2017) demonstrated that regardless of the saturation state of aragonite the energy for initial larval shell formation was similar in *C. gigas*, suggesting that resilience to ocean acidification might be more convoluted and a better understanding of genotype-environment interactions is needed.

To better understand whether higher mortality rates in challenged clams were the result of better fitness of the bacteria, lower resistance of the host, or both, we investigated if OA impacts the growth of *Vibrio* spp. Native *Vibrio* spp. were more abundant in both the seawater and clam tissue from high pCO_2/low pH seawater in our open flow through system, which could be a result of enhanced growth in high pCO_2/low pH environments. Labare et al. (2010) demonstrated that a *Vibrio* spp. showed changes in morphology and inhibition of growth at a rather extreme pH of 5.2, but then recovered after 6 h of exposure, indicating a high tolerance to acute changes in pH. We also examined growth of *Vibrio* spp. over a period of five days (the duration of the longest pathogen challenge experiment). Bacterial growth was not significantly different between treatments until day five when it was significantly higher among OA conditions. However, the larval pathogen challenge experiments showed significant differences in mortality within 24 h, a period of time during which no significant effect of acidification was noted on *Vibrio* spp. growth. Altogether, these results support that the

main driver to higher susceptibility of clam larvae to *Vibrio* spp. infections under acidification stress is a decrease in host immunity.

After a year of exposure to high pCO_2/low pH or low pCO_2/high pH, we performed a six-week reciprocal transplant in which we transferred a subset of clams to a different pCO_2 treatment to evaluate if clams can restore immunity after transfer to low pCO_2/high pH conditions and if exposure at an earlier life stage could lead to acclimation or if the one year old clam survivors were more resilient to OA. The greatest mortality was in clams transferred from seawater with low pCO_2/high pH to seawater with high pCO_2/low pH and then exposed to *Vibrio* spp. In this case, the six-week exposure to pCO_2 had a greater effect on immune functioning in clams from low pCO_2/high pH conditions than life-long exposure to high pCO_2/low pH conditions. Clams from high pCO_2/low pH conditions that were transplanted to low pCO_2/high pH conditions did not have significantly less mortality than clams that were maintained in high pCO_2/low pH seawater. When comparing mortality between controls and pathogen challenges, only the transplanted clams had significantly greater mortality when exposed to bacteria. Both groups of clams that were maintained in their initial treatments had similar survival when exposed to bacteria. The low mortality in clams from high pCO_2/low pH conditions transplanted to both high pCO_2/low pH and low pCO_2/high pH conditions may suggest the clams that were selected for and initially survived the stressful low pH as larvae may have a broader tolerance range for pH than clams from low pCO_2/high pH conditions. Thus, clams from high pCO_2/low pH performed well under both pH regimes whereas clams from low pCO_2/high pH, which may have a narrower pH-tolerance, were more susceptible to high pCO_2/low pH. In this case, stressful conditions may not have selected for animals that performed better under only low pH but instead selected for more pH-tolerant animals overall. An alternative hypothesis is that the clams that stayed in high pCO_2/low pH conditions (and had low mortality) acclimated to adverse conditions, a finding consistent with prior longer term grow outs of *M. mercenaria* (Gobler and Talmage, 2013).

Of important note in experimental acidification work is the notion of local adaptation. To date, many studies set high pCO_2 conditions at levels that species or populations used within the study are already naturally experiencing (Vargas et al., 2017). As suggested by others (Griffiths et al., 2019; Vargas et al., 2017), local adaptation may explain some of the differences observed here with *Mercenaria mercenaria* in comparison to other bivalve species. Thomsen et al. (2017) demonstrated populations of *Mytilus edulis* obtained from regions experiencing naturally high CO_2 exhibited higher fitness under elevated pCO_2 in the laboratory. Here *M. mercenaria* were obtained from several populations with the aim of mixing populations upon spawning to eliminate any adaptive advantage. Some broodstock were obtained from sites already experiencing occasional pH minima reaching 7.6 which is nearly equivalent to our experimental high pCO_2/low pH. This may be impacting the results of this study, however, in the natural environment exposure of clams to such low pH levels is acute; this study exposed larvae and juveniles to high pCO_2/low pH over the long term and thus demonstrates a response to chronic acidification stress as one would expect under future ocean acidification conditions.

Many studies have begun to show that the environment during reproductive conditioning of an organism can influence offspring fitness (Hettinger et al., 2013; Munday, 2014; Parker et al., 2011, 2015). There are different methods through which parents can confer resilience to offspring, such as maternal effects due to nutritional provisioning (Mousseau and Fox, 1998), epigenetic controls on gene expression (Putnam et al., 2016), and selection through successive generations of particular traits (Sunday et al., 2014). Immediate selection for OA tolerant organisms does not necessarily translate into increased fitness (Thomsen et al., 2017). In *S. glomerata* larvae from adults conditioned in low pH that were exposed to multiple stressors (low pH, high pCO_2/low pH temperature, reduced salinity, and reduced food) only had positive carry over effects when the sole stressor was pCO_2 as compared to larvae from adults conditioned in low pCO_2/high pH. The larvae from parents exposed to low pH had reduced survival when challenged with multiple stressors, suggesting that exposure to low pH during reproductive conditioning is maladaptive when larvae experience multiple stressors (Parker et al., 2017). Future coastal marine ecosystems are expected to have multiple co-occurring stressors (OA, temperature, lower pH, harmful algae blooms, and lower dissolved oxygen) which could have additive, synergistic, or antagonistic impacts (Boyd, 2011; Gobler et al., 2014; Talmage and Gobler, 2011; Griffith and Gobler, 2019). Results presented here suggest that future bivalve populations experiencing acidification will be more susceptible to disease, and looking at the response to bacterial pathogens in combination with other co-occurring stressors is a crucial next step. In addition, further investigating the impact of OA on marine pathogens, such as how their survival or infectivity may be impacted, will provide a more comprehensive view of disease dynamics in our future ocean.

New environmental stressors have the potential to stimulate disease emergence, and understanding both the host's immune response and the response of known pathogens is vital to understanding how OA can impact vulnerable shellfish species. *Vibrio* spp. are important opportunistic pathogens that are ubiquitous and can cause disease outbreaks in a wide range of shellfish species, particularly in larval and juvenile stages grown in hatcheries (Dubert et al., 2017). We have demonstrated an increased susceptibility to infection by *Vibrio* spp. as well as increased growth of *Vibrio* spp. in clams under OA conditions. This may have implications for the health of future shellfish populations, their restoration, and aquaculture operations. Locales already exhibiting seasonal coastal acidification and concurrent hypoxia, including natural habitats of the hard clam, may be more vulnerable to accelerating acidification (Sunda and Cai, 2012) and climate change. As OA continues to intensify, both larval and juvenile clams will be at risk to increased bacterial infections. It is likely that prior estimates of declines in clam population due to acidification that have not considered their enhanced susceptibility to disease (e.g. Ekstrom et al., 2015) have likely underestimated the true losses for this fishery. At the same time, our results showing selection for clams that are more tolerant to acidification hold promise for the development of clam strains better suited to survive in our future oceans. Research is ongoing to identify genetic features associated with resilience to OA, with the aim of facilitating selection of resilient stocks.

Credit Author Statement

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2019.104872.

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