Microbiome response differs among selected lines of Sydney rock oysters to ocean warming and acidification

Elliot Scanes[1†,2*], Laura M. Parker[3], Justin R. Seymour[1], Nahshon Siboni[1], William L. King[1,4], K. Mathias Wegner[5], Michael C. Dove[6], Wayne A. O'Connor[6], Pauline M. Ross[2]

[1] Climate Change Cluster, University of Technology Sydney, Ultimo, New South Wales, 2007, Australia

[2] The University of Sydney, School of Life and Environmental Sciences, Camperdown, New South Wales, 2006, Australia

[3] The University of New South Wales, School of Biological, Earth and Environmental Sciences, Kensington, New South Wales, 2052, Australia

[4] Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, PA, 16802, U.S.A.

[5] Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Coastal Ecology, Wadden Sea Station Sylt, List, 25992, Germany

[6] New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, New South Wales, 2316, Australia

*Corresponding author

†Current address

Corresponding author address: Vicki Sara Building, University of Technology Sydney, Ultimo, New South Wales, 2007, Australia. Elliot.scanes@uts.edu.au

ABSTRACT

Oyster microbiomes are integral to healthy function and can be altered by climate change conditions. Genetic variation among oysters is known to influence the response of oysters to climate change and may ameliorate any adverse effects on oyster microbiome, however, this remains unstudied. Nine full-sibling selected breeding lines of the Sydney rock oyster (*Saccostrea glomerata*) were exposed to predicted warming (ambient = 24°C, elevated = 28 °C) and ocean acidification (ambient pCO_2= 400, elevated pCO_2 =1000 µatm) for four weeks. The haemolymph bacterial microbiome was characterised using 16S rRNA (V3-V4) gene sequencing and varied among oyster lines in the control (ambient pCO_2, 24

°C) treatment. Microbiomes were also altered by climate change dependent on oyster lines. Bacterial α-diversity increased in response to elevated pCO_2 in two selected lines, while bacterial β-diversity was significantly altered by combinations of elevated pCO_2 and temperature in four selected lines. Climate change treatments caused shifts in the abundance of multiple Amplicon Sequence Variants (ASVs) driving change in the microbiome of some selected lines. We show that oyster genetic background may influence the Sydney rock oyster haemolymph microbiome under climate change and that future assisted evolution breeding programs to enhance resilience should consider the oyster microbiome.

INTRODUCTION

Microbiomes of marine organisms perform a range of beneficial services for their host (McFall-Ngai et al. 2013; Person-Leary et al. 2020; de Vries et al. 2020). For example, microbiomes provide corals with energy and nutrients (Yellowlees et al. 2008), enable fish to alter behaviour (Soares et al. 2019), facilitate bio-luminesce in squid (Apprill 2017) and allow molluscs to resist infection (King et al. 2019a). Microbiomes also allow organisms to exist in hostile environments – for instance, bivalve molluscs living around hydrothermal vents rely on chemosynthetic bacteria to survive (Ponnudurai et al., 2017). Despite their potential importance, little is known about how climate change may influence the microbiome of marine species.

Climate change is increasing the frequency and severity of marine heatwaves, increasing average sea-surface temperatures, and reducing the pH and availability of carbonate ions in oceans and estuaries (Rhein et al. 2013; Scanes et al. 2020a). Over the coming decades, these changing ocean conditions will threaten the survival and success of marine organisms, especially calcifying species such as molluscs that produce shells and skeletons made from calcium carbonate (Hoegh-Guldberg et al. 2018). It has been shown that for some calcifying organisms, climate change will decrease calcification, reproductive rate, skew sex ratios, alter adult physiology, slow larval development, and increase larval mortality (Pörtner 2008; Ross et al., 2011; Parker et al. 2013; Gattuso et al. 2015; Thomsen et al. 2015; Parker et al. 2018).

Adaptation to changing ocean conditions, via the selection of more tolerant genotypes has been identified as a potential source of resilience for some marine species (Parker et al. 2011; 2012; Wright et al. 2014; Foo et al. 2016). For example, in the sea urchin *Centrostephanus rodgersii,* the tolerance of early embryos to ocean warming and acidification was found to differ among genotypes, with some genotypes, showing no effect of warming and only moderate effects of ocean acidification (Foo et al. 2016). In the Sydney rock oyster, *Saccostrea glomerata*, populations that were selectively bred for

enhanced growth/disease resistance, grew 65% faster and were better able to maintain their extracellular pH under ocean acidification compared to the wild population (Parker et al. 2011; 2012). This effect was also apparent among oyster breeding lines that were full-sibling families. Breeding lines of the oyster *S. glomerata* were found to experience up to 50 % mortality when exposed to an atmospheric heatwave of 50 °C in the laboratory, while there was less than 20 % mortality experienced by other lines (Scanes et al. 2020b). The mechanisms underlying the increased resilience of some genotypes to ocean warming and acidification have been linked to greater maternal provisioning (Foo et al. 2012), changes in the acquisition, production and allocation of energy and changes in energy metabolism (Melzner et al. 2009; Parker et al. 2012; 2015; Thomsen et al. 2015), as well as alterations in genes involved in biomineralization, ion regulation and membrane regulation (Pespini et al. 2013).

Resilience to climate change may also be facilitated by microbes and the microbiome. Climate change is likely to alter the microbiome of marine organisms (Scanes et al. 2021), but the level of this effect is potentially dependant on the host's genotype, with this variation potentially driving responses to disease (Wegener et al., 2013; Lokmer and Wegner 2015; Wendling et al. 2017; Qiu et al. 2019, King et al., 2019b; Nguyen et al. 2020) or environmental stress (Apprill 2017; Glasl et al. 2019; Green et al., 2019). Heritable genetic variation influencing the composition of microbiomes can therefore be an integral part of an organism's response to stress. For example, studies on coral have shown that microbiomes differ among coral genotypes, with heat resilient corals possessing a unique microbiome (van Oppen et al. 2018; Glasl et al. 2019; Osman et al. 2020). Microbiomes can also aid in organisms coping with adverse environmental conditions such as toxic pollutants (Apprill 2017). The interdependence of genotype-specific microbiomes and an organism's response to climate change can influence the resilience of marine organisms and play a decisive role in their survival.

To understand the influence of oyster genetic variation in the Sydney rock oyster (*S. glomerata*) microbiome's response to climate change, we characterised the oyster haemolymph microbiome in nine *S. glomerata* selected breeding lineages following exposure to ocean warming and acidification. Our rationale for studying the impact of climate change on the host microbiome in oysters is two-fold. Firstly, oysters have proven to be excellent models for studying both the role of microbiome (Lokmer et al. 2015; 2016a; Wendling et al. 2017; King et al. 2019a) and climate change resilience (Parker et al. 2012; 2015; Wright et al. 2014; Scanes et al. 2020b). Secondly, oysters are also ubiquitous on shorelines around the globe, including eastern Australia (Scanes et al, 2016), where they provide immense ecological services through building three-dimensional reef structures that shape hydrodynamics and

wave attenuation, as well as providing habitat (Coen et al. 2007). In addition, oysters filter the water column and cycle nutrients (Grabowski and Peterson 2007). Oyster aquaculture also provides income and sustenance for communities across the globe, with over 5.5 million tonnes of live oysters with a value of USD 12 billion produced each year (FAO 2018). In eastern Australia the Sydney rock oyster (*S. glomerata*) comprises over 90 % of total aquaculture production value worth USD $35 million annually (NSW DPI 2020) to the state of New South Wales (NSW). The economic cost of climate change on global aquaculture has been estimated to be USD100 billion (Narita et al. 2012). Understanding the mechanisms underlying resilience to climate change, is essential if we are to future-proof these ecologically, economically and culturally important organisms.

MATERIALS AND METHODS

Study organisms

As part of a wider oyster breeding program run by the NSW Department of Primary Industries, genetic lineages of *S. glomerata* have been selectively bred for three-generations at the Port Stephens Fisheries Institute (PSFI; Dove et al. 2020). The purpose of creating selectively bred lines was to build resilience to disease and fast growth to improve aquaculture profitability, however, previous research has shown that different oyster selected lines exhibit dissimilar responses to the effects of climate change (Parker et al. 2011; 2012; 2015; Scanes et al. 2020b). Seventy two oysters were obtained from each of nine distinct selected lines of *S. glomerata.* Each selected line used in this study was created at the PSFI in November 2017 using the culture methods of Dove et al. (2019). Each selected line is created by the pair mating of a male and female oyster from selected lines with known pedigree and traits. When juvenile oysters reached a shell length of 1-2 mm they were transferred to purpose built bags (SEAPA Co. Edwardstown South Australia, 600 x 250 x100 mm) and cultured on intertidal leases in Cromarty Bay, Port Stephens (152° 4'0.69"E, 32°43'19.69"S) where they remained for approximately two years until the beginning of the experiment. The nine selected lines from the breeding program were designated: A, B, C, D, E, F, G, H and I, for this study.

Oyster husbandry, acclimation and experimental exposure

All seawater used in acclimation and experimental exposure was collected from Little Beach, Port Stephens (152°9'30.00"E, 32°42'43.03"S), filtered through canister filters to a nominal 5 µm, and stored onsite in 38,000 L polyethylene tanks as a stock of filtered seawater (FSW).

Approximately 72 individual *S. glomerata,* from each of the nine selected lines (A-I) were collected from intertidal leases in Cromarty Bay, Port Stephens (152° 4'0.69"E, 32°43'19.69"S) in September 2019 for experiments, meaning all oysters were 22 months old when experiments began. Once collected, oysters were transported to the laboratory at PSFI and gently cleaned of any fouling organisms before being placed into a single 1500 L fibreglass tank containing aerated FSW. Despite being the same age, the dry tissue weight of oysters differed among selected lines. Mean (±SE; g) dry weight of selected lines was; A = 0.7 ± 0.06; B= 0.77 ± 0.05; C = 0.65 ± 0.03; D = 0.58 ± 0.02; E = 0.64 ± 0.03; F = 0.68 ± 0.04; G = 0.7 ± 0.04; H = 0.59 ± 0.03; I = 0.49 ± 0.02 g.

Oysters were maintained in a single 1500 L fibreglass tank for two weeks to acclimate to laboratory conditions at 24 °C and ambient pCO_2 (400 µatm). During all acclimation and experimental exposure, tanks containing oysters received a full water change every second day. This involved removing all oysters from the tank, gently rinsing them with freshwater to remove solid waste and un-eaten food, placing them into a new tank of FSW pre-equilibrated to their pCO_2 and temperature treatment. During all acclimation and experimental exposure, oysters were fed live algae cultured on site comprising of 25 % *Chaetoceros muelleri*, 25 % *Diacronema lutheri*, and 50 % *Tisochrysis lutea* at a rate of 1 x 10^9 cells/oyster/day.

Following acclimation, oysters from each selected line were divided among twelve 750 L polyethylene tanks filled with 400 L FSW at a density of 54 oysters per tank, with each selected line represented by six replicate individuals. Experimental treatments are described in detail in Table 1. Treatments consisted of orthogonal combinations of two pCO_2 concentrations (ambient [400 µatm]; elevated [1000 µatm]) and two temperature treatments (24 and 28 °C). Each combination was replicated across three tanks (Table 1). Treatments were selected to represent temperatures and pCO_2 concentrations predicted for 2080-2100 by the IPCC (Collins et al. 2013) and reflect measured changes in estuary temperatures reported from south eastern Australia (Scanes et al. 2020a). Oysters remained in experimental treatments for four weeks. Oysters were checked daily for mortality. However, no dead oysters were found in any tanks during the four-week exposure period.

The two pCO_2 levels used in this study (400 µatm, 1000 µatm) were based on the multi-model average projection by the IPCC for 2100 (Collins et al. 2013). These pCO_2 levels corresponded to a mean ambient pH_{NBS} of (8.18± 0.01) and at elevated CO_2 levels a mean pH_{NBS} of (7.84 ± 0.01). The elevated CO_2 level was maintained using a pH negative feedback system (Aqua Medic, Aqacenta Pty Ltd, Kingsgrove, NSW, Australia; accuracy ± 0.01 pH units). To determine the pH level corresponding to pCO_2 levels, total

alkalinity (TA) was quantified at each water change using triplicate Gran-titration (Gran 1952), and a CO_2 system calculation program (CO2 SYS; Lewis et al. 1998), using the dissociation constants of (Mehrbach et al. 1973), and the pH level corresponding with the desired pCO_2 level was calculated. Seawater variables including; pH_{NBS}, total alkalinity, and salinity were measured each water change (Table 1), the desired pH values corresponding with pCO_2 levels were then recalculated accordingly. Food grade CO_2 (BOC Australia) was bubbled directly into independent tanks via a CO_2 reactor to ensure proper mixing and reduce pH. A pH probe connected to a controlling computer was placed within each tank. Each tank set to elevated pCO_2 was controlled by its own independent pH controlling system. The pH values of each tank were monitored daily and checked daily against another calibrated pH probe (NBS buffers, WTW 3400i).

Once oysters were transferred to experimental tanks, the pCO_2 level and temperature were steadily increased in elevated exposure tanks over one week until the experimental treatment level was reached. Oysters were then exposed to their respective treatments for a further four weeks. This length of time has previously been used to elicit a stable response in *S. glomerata* physiology to warming and acidification (Scanes et al., 2017).

Sampling procedure

Following exposure to experimental conditions for four weeks, haemolymph was taken from three replicate oysters from each oyster selected line, from each tank for microbial analysis. This amounted to nine individuals from each selected line, in each treatment. To take haemolymph samples, each oyster was opened using an autoclave sterilised shucking knife, ensuring that the pericardial cavity was not ruptured. Excess fluid was tipped off the tissue surface and 200 – 300 µL of haemolymph was extracted from the pericardial cavity using a new sterile 1 mL needled syringe (Terumo Co.). Samples from three oysters were transferred to three new pre-labelled DNA/RNA free 1 mL tubes (Eppendorf Co.) and immediately frozen at -80 °C where they were stored until DNA extraction.

DNA extraction and sequencing

Bacteria have been identified as the most important component of the microbiome found in oyster haemolymph (Dupont et al., 2020). To characterise the bacterial microbiome of *S. glomerata* haemolymph, 16S rRNA gene sequencing was used. DNA was extracted from 324 oyster haemolymph samples (9 selected lines × 4 treatments × 3 replicate tanks × 3 replicate oysters per tank) using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Australia, Chadstone, VIC), according to the manufacturer's

instructions. Twelve samples did not contain enough haemolymph for successful DNA extraction, however, all selected lines in all treatments were still represented. The bacterial microbiome of the oyster haemolymph was characterised with 16S rRNA gene sequencing, using the 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) primer pair (Herlemann et al. 2011) targeting the V3-V4 variable regions of the 16S rRNA gene with the following cycling conditions: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplicons were sequenced on the Illumina Miseq platform (2x300bp) following the manufacturer's guidelines at the Ramaciotti Centre for Genomics, University of New South Wales. Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA663356.

Sequence analysis

Raw demultiplexed data was processed using the Quantitative Insights into Microbial Ecology (QIIME 2 version 2019.1.0) pipeline. Briefly, paired-end sequences were imported (qiime tools import), trimmed and denoised using DADA2 (version 2019.1.0), which also removes chimeras (Callahan et al. 2016). Sequences were identified at the single nucleotide threshold (Amplicon Sequence Variants; ASV) and taxonomy was assigned using the classify-sklearn QIIME 2 feature classifier against the Silva v138 database (Quast et al. 2012). Sequences were further cleaned by removing ASVs with reads below 0.005 % ASVs of the summed relative abundance across all 324 samples. Sequences identified as chloroplasts or mitochondria were also removed. Cleaned data were then rarefied at 6,500 counts per sample. Data from QIIME 2 was then exported to R v.4.0.1 (R Core team) for analysis and statistical testing using the packages, "Phyloseq" (McMurdie and Holmes 2013), "DESeq2" (Anders and Huber 2010) and "Vegan" (Dixon, 2003).

Data analysis

To establish a baseline and compare bacterial microbiomes among oyster selected lines at control conditions, a subset of data was created for only those samples taken from oysters in the ambient pCO_2, 24 °C treatment. Samples from this treatment contained 4086 unique ASVs. α -diversity metrics of the ambient pCO_2, 24 °C treatment subset including Simpson's index of diversity and Pielou's evenness were calculated and found not to be normally distributed as determined by Shapiro-Wilk normality tests. α-diversity indices were then analysed among selected lines using the Kruskal- Wallis test. For significant differences detected among selected lines, Kruskal-Wallis multiple comparison tests with Benjamini-

Hochberg adjusted p-values were done to determine the source of variation. To analyse β-diversity, Jaccard and Unifrac distances were created from the ambient pCO_2, 24 °C treatment subset and analysed using a single factor PERMANOVA with "Selected line" (A-I) as the fixed and orthogonal factor.

α -diversity metrics of the entire data set (all treatments) including Simpson's index of diversity and Pielou's evenness, were calculated from rarefied sequence data using the "Phyloseq" package in R software. α -diversity metrics were also calculated from non-rarefied data however the results were not different to the rarefied data and therefore are not presented. Indices were then analysed using a 4-way linear mixed model (LMM) with "Temperature" (24 or 28 °C), "CO_2" (ambient or elevated), and "Selected line" (A-I) as fixed and orthogonal factors, and "Tank" (n=3) as a random factor. ANOVAs (Type II Wald chi-square tests) were performed on models to determine difference among factors using the "lme4" package (Bates et al. 2014), and pairwise comparisons among levels of significant factors or interactions were made using estimated marginal means with Tukey's methods in with the "emmeans" package. Shapiro's test was used to confirm a normal distribution and Pearson residual plots were used to check model error.

Prior to beta diversity analysis, we removed low abundance ASVs to reduce the dataset complexity (<1 % total prevalence [Lokmer et al. 2016a; Callahan et al. 2016]), resulting in the removal of 1143 ASVs to give 4837 ASVs. Unifrac and Jaccard distance matrices were then created from the entire dataset and analysed using a three factor PERMANOVA using the Adonis method with "Temperature" (24 or 28 °C), "CO_2" (ambient or elevated) and "Selected line" (A-I) as fixed and orthogonal factors. These distance matrices were chosen because they are sensitive to low abundance taxa which have been previously shown to drive differences in the *S. glomerata* microbiome (Scanes et al., 2021).

To determine which selected lines were significantly affected by CO_2 or Temperature treatments, two factor PERMANOVA using the Adonis methods were run for each selected line on Unifrac and Jaccard distances with "Temperature" (24 or 28 °C) and "CO_2" (ambient or elevated) as fixed and orthogonal factors. This analysis was repeated for each selected line of oysters (Table S2). To determine significant differences in the abundance of ASVs between levels of CO_2 and Temperature treatments, the "DESeq2" package in R v4.0.1 was used. DESeq2 uses univariate Generalised Linear Models with a negative binomial distribution and a Benjamini-Hochberg adjusted P value to compare abundances of ASVs among treatments (Anders and Huber, 2010). The abundances of ASVs between levels of CO_2 and

temperature treatments were compared for each oyster selected line. ASVs identified by DESeq2 as significant were searched on the NCBI BLAST database.

To explore significant PERMANOVA results, Principal Coordinate Analyses (PCoA) and Canonical Analysis of Principal Coordinates (CAP, using Selected line, CO_2 or Temperature as constraining variables) plots were created for distance matrices to allow for comparisons of constrained and unconstrained ordination (Anderson and Willis 2003). Non-significant PERMANOVA factors were pooled in ordinations to allow for comparison of levels within significant factors. ANOVA was used to assess the significance of CAP constraints. Plots were created using the "Phyloseq" and "Vegan" packages (McMurdie and Holmes 2013). Homogeneity of multivariate dispersions were tested using "betadisper" function in the "Vegan" package for all PERMANOVA analyses. Where factors with non-homogenous dispersions were identified, they have been treated with necessary caution (Anderson 2001).

To determine the core bacterial microbiome, ASVs were identified that occurred in > 90% of samples from a given grouping, these were calculated using the "AmpVis2" (Albertsen et al. 2015) package in R v4.0.1 software.

To explore correlations among bacterial abundances, selected lines and experimental treatments, microbial network analyses were used and figures were created with the "NetCoMi" package (Peschel et al., 2021). Bacterial associations were calculated among selected lines that were "not-affected" (selected lines A, B, C, F, H) and were significantly "affected" (D, E, G, I) by treatments of either elevated pCO_2 or temperature. The SPRING (Yoon et al., 2019) method was used as the association method, correlations were transformed to dissimilarities via the "signed" distance metric and the corresponding (non-negative) similarities are used as edge (connecting line) weights. Node size indicated relative abundance of that taxa, and eigenvector centrality was used for defining hubs (nodes with a centrality value above the empirical 90% quantile). Data were filtered to only include the 85 most abundant taxa to simplify correlation figures.

RESULTS

Bacterial microbiome of different *S. glomerata* selected lines

The microbiome differed among selected oyster lines in the control (ambient pCO_2, 24 °C) treatment (Figure 1). The Simpson's Index of selected line G was significantly lower than that of selected lines A, E and F (Chi^2 = 18.49, P = 0.017) and there were significant differences in the bacterial community β –

diversity among selected lines (PERMANOVA; Jaccard- $F_{8,72}$ =1.1, P=0.003; Unifrac- $F_{8,72}$ =1.24, P=0.006; Figure 2). The core microbes present in control oysters mostly belonged to the bacterial families *Arcobacteraceae* and *Marinifiliacae*, with five ASVs from *Arcobacteraceae* present in the core microbiota of selected oyster line H (Figure 3).

Bacterial microbiome of *S. glomerata* selected lines exposed to elevated pCO_2 and warming

A total of 5980 bacterial Amplicon Sequencing Variants (ASVs) were identified across the entire dataset of 312 samples. Across all selected lines, the bacterial microbiome of *S. glomerata* haemolymph was predominantly composed of the classes Bacteroidia, Campylobacteria, Gammaproteobacteria and Spirochaetia. The most abundant ASVs in oyster haemolymph were assigned to the genera *Marinifilum*, *Halarcobacter* and *Neptuniibacter* which comprised 75% of the total bacterial microbiome (Figure 1, Figure S2). Four core ASVs were identified, cumulatively comprising 12.6% relative abundance and all members of the *Marinifilaceae.*

Overall within and between oyster diversity of hemolymph bacterial microbiomes differed among selected lines when exposed to elevated pCO_2 and temperature (Chi^2 = 16.43, P = 0.037; Table S1). Analyses of both α and β- diversity metrics revealed that elevated pCO_2 and temperature affected the bacterial microbiome of *S. glomerata* differently according to oyster selected line. The bacterial α-diversity of two selected lines, D and G, was significantly greater at elevated compared to ambient pCO_2 (LMM, CO_2 x Selected line; Simpson's Index; Figure 4). Pairwise tests also indicated the bacterial α-diversity of selected lines B and C was significantly different to those of D, H and I, but not A, E, F and G at elevated pCO_2 (Figure 4). There were no significant effects of treatments on Pielou's Index of evenness.

The β-diversity of haemolymph bacterial microbiomes were also altered by both elevated pCO_2 and temperature dependent on oyster selected lines. PERMANOVA analysis performed on both Unifrac and Jaccard distances also indicated significant Selected line × CO_2 and Selected line × Temperature interactions (Table 2).

There were significant effects of both CO_2 and temperature (Figure 5) on the bacterial microbiome of selected lines D (PERMANOVA; CO_2, $F_{1,32}$ =0.034, P=0.02; Temperature, $F_{1,31}$ =0.031, P=0.023) and E (CO_2, $F_{1,32}$ =1.7, P=0.029; Temperature, $F_{1,31}$ =1.8, P=0.014). In addition, selected line G was significantly affected by CO_2 ($F_{1,32}$ =0.3, P=0.04) and selected line I, by Temperature ($F_{1,32}$ =1.4, P=0.05). There were no significant Temperature × CO_2 interactions in PERMANOVA run for any selected lines.

The effects of CO_2 and temperature on selected line D were due to decreases in abundance of nine ASVs, all belonging to the genus *Malaciobacter*. Significant effects (DESeq2 GLM Padj <0.01) of CO_2 and temperature on selected lines E and G were driven by increases and decreases, respectively, in ASVs belonging to the family *Spirochaetaceae* (Table 3). Significant effects of temperature on selected line I were driven by decreases in two ASVs, one belonging to the genus *Mycoplasma* and the other to the genus *Marinifilum.* PCoA plots showed few clear patterns with each selected line displaying overlapping polygons representing treatments (Supplementary Figure S1). Canonical Analysis of Principal Coordinates (CAP) showed that CO_2 and temperature treatments affected each selected line in a unique way (Figure 5). Of the selected lines identified to be significantly affected by experimental treatments, there was clear separation of the treatment groups (Figure 5). CAP constraints were found to be significant (ANOVA P <0.05). Results from the NCBI BLAST database search of significant ASVs as identified by DESeq2 can be found in Table 4.

Experimental treatments caused changes in the core haemolymph microbiome of oysters (Figure 3). Six *Arcobacteraceae* ASVs were present in the core of oysters in the control treatment (Ambient CO_2; 24 °C), five ASVs present in oyster selected line H and one present in oyster selected line D. However, the presence of these ASVs was reduced to two in oyster selected line D in the Ambient pCO_2; 28 °C treatment and were entirely lost from the core of oysters in both elevated pCO_2 treatments. In the elevated pCO_2; 28 °C treatment there were increases in core microbes from the family *Marinifilaceae* for oyster selected lines E, F, G, H, I. Elevated pCO_2 increased the number of core members of the family *Nitrincolaceae*, with this ASV present in 7 selected lines in the elevated pCO_2; 24 °C treatment.

Network analysis showed different bacterial associations among the selected lines that were and were not-affected by elevated pCO_2. The two networks shared similar properties, there were five node hubs identified in both oyster selected line groups, two of which were shared between the family groups; *Aurantivirga_8e5d* and *Anaerovoracaceae(f)_735d*. There were also similar numbers of clusters in the not-affected and affected lines (11 and 10 respectively). The largest differences in closeness centrality between the selected line groups belonged to the taxa *Anaerovoracaceae(O)_aef4*, *Arcobacteraceae(f)_5970*, *Halarcobacter_faf8* (also from the family *Arcobacteraceae*), *Spirochaetaceae_84ds* and *Spirochaetaceae_ca08* . There were similar overall properties but differing importance of ASVs as nodes and hubs between the two selected line groups as indicated by network analysis (Figure S3).

DISCUSSION

Relationships between microbiomes and the genotypes of their host has so-far been unstudied in response to the ocean warming and acidification caused by climate change. Our study has shown that compositional shifts in the hemolymph bacterial microbiome of oysters was dependent on the oyster selected line, temperature, and ocean acidification. Elevated pCO_2 caused the strongest compositional shifts in microbiomes of some selected lines with negligible changes in others. The selected line dependent nature of microbiome shifts observed in this study may begin to help identify selected lines in which resilient traits are connected to microbiome composition. This may open potential links to the pattern of selected line dependent physiological responses seen in previous studies of climate change and selected lines of *S. glomerata* (Parker et al. 2011; 2012; 2015; Scanes et al. 2020b). The wellbeing of marine organisms is interconnected with their microbiome (McFall-Ngai et al. 2013; Person-Leary et al. 2020; de Vries et al. 2020).

Effects of climate change on oyster bacterial microbiomes across selected lines

This study supports other studies that have found that the oyster microbiome is influenced by host genotypes in addition to the environment (Lokmer et al. 2016a; King et al. 2019b; Nguyen et al. 2020; Dupont et al., 2020). We found that in the control treatment, the bacterial microbiome varied according to oyster selected line, a result that has also been found in the Pacific oyster*, Crassostrea gigas* (Lokmer et al. 2016a; King et al., 2019b) and *S. glomerata* (Nguyen et al., 2020), however none of these previous studies investigated the microbiome in a laboratory environment. Recent research on *C. gigas* suggests that the environment is a stronger determinant of bacterial microbiome rather than host genotype (Dupont et al., 2020). In our study, oysters were all reared in the laboratory as larvae, then reared in adjacent cages at the same site for 20 months before being transferred to the laboratory for this study. By keeping oysters together their entire life, we have strong evidence that differences in the microbiome can be driven by genetic variation among oysters (i.e. selected lines).

Marine organisms have been found to differ in their resilience to climate change dependent on genotype in urchins (Foo et al. 2016), oysters (Parker et al. 2010; Wright et al. 2014; Scanes et al. 2020b), mussels (Thomsen et al. 2017) and seagrass (Reusch et al. 2005). Our study has now shown that the genotype- dependent effects of climate change extend to the microbiome. Recent advances have begun to unravel the reasons for greater resilience of some populations or selected lines of oysters to climate change. Genotypes of oysters that can maintain their haemolymph pH (pH_e) often have greater metabolic rates and more energy efficient filtration rates (Parker et al., 2015; Stapp et al. 2018) which may enable resilience when exposed to climate change.

The findings from this study show that climate change-driven shifts in haemolymph bacterial microbiome of oysters is governed in part by the host genotype. Genotypic differences in physiological traits of oysters have been previously identified and suggested to contribute to the resilience of some oyster selected lines (Parker et al. 2015, 2017; Stapp et al. 2018). Such physiological traits may also be present in the selected lines which experienced small perturbations in microbiomes when exposed to elevated pCO_2 or temperature. Our experiment was not equipped to determine whether a microbiome resilient to warming and acidification improves resilience in oysters, or merely a side effect of an oyster with resilient physiology. Further experiments would be needed to determine this.

The microbiome of oyster selected lines in this study responded differently to that of wild oysters examined in a recent study (Scanes et al., 2021). Scanes et al., (2021) showed that the microbiome of *S. glomerata* was significantly altered by both elevated pCO_2 and temperature at levels comparable to this study. The microbiome of only two of nine selected lines in this study experienced similar effects as the wild cohort of *S. glomerata*. Many of the same taxa were present in the haemolymph microbiome of wild oysters and the selected lines from this study including *Marinifilaceae*, *Arcobateraceae* and *Spirochaetaceae* (Scanes et al., 2021). Direct comparisons between the microbiome of wild oysters and those of the selected lines used here must be treated with caution because of the differing life histories of wild oysters compared to hatchery reared selected lines and the likelihood that a cohort of wild-caught oysters would contain multiple genotypes from an unknown number of parental combinations (In et al., 2016).

This study identified elevated pCO_2 to be a stronger driver of shifts in microbiomes compared to elevated temperature. Elevated pCO_2 increased the α- diversity of bacterial microbiome associated with oysters from selected lines D and G, while some selected lines experienced significant decreases in select ASVs. Reduced diversity of microbiomes is generally considered to be indicative of a shift towards an "unhealthy" state in oysters (Green and Barnes 2010; Lokmer and Wegner 2015; de Lorgeril et al. 2019). There were shifts in the relative abundance of taxa dependant on genotypes, for example, elevated pCO_2 and temperature reduced the presence of the bacterial family *Arcobacteracea*, especially from oyster selected line D and the core of oyster selected line H. *Arcobacteraceae* were also suggested by network analysis as differing between the affected and not-affected selected lines. *Arcobacteracea* are identified as opportunistic pathogens and have been found in associated with disease causing bacteria such as *Vibrio* (Lokmer and Wegner 2015; de Lorgeril et al. 2019). Several members of the *Arcobacteracea* have previously been found associated with oysters (Delgado 2020) and are

386 microaerophilic (Vandamme et al. 1991), so it follows that changes in the relative abundance of
387 *Arcobacteracea* may be due to oysters increasing or decreasing their respiration and metabolic rates,
388 which are responses often reported in response to elevated pCO_2 (Lannig et al. 2010; Parker et al. 2012;
389 Scanes et al. 2017) and the direction of change (increase or decrease) can vary among genotypes (Parker
390 et al. 2012; Wright et al. 2014). Overall, *Arcobacteracea* comprised a large proportion of the bacterial
391 microbiome of *S. glomerata* haemolymph which is consistent with previous work suggesting the
392 *Arcobacteracea* are haemolymph-specific symbionts (Lokmer and Wegner 2015).

393 Previous studies have shown that temperature can influence the microbiome in other oyster species
394 such as *C. gigas* (Lokmer and Wegner 2015). However, the microbiome of wild *S. glomerata* was shown
395 to be more strongly influenced by elevated pCO_2 than temperature (Scanes et al., 2021). The strong
396 effects caused by exposure to elevated pCO_2 may be attributable to the internal physiological changes
397 elicited by elevated pCO_2 which are often greater than those caused by elevated temperature at the
398 levels used in this study (Parker et al. 2018). The warming and acidification scenarios used in this study
399 are not unusual for Australian estuaries (Scanes et al. 2020a) indicating that oysters may already be
400 experiencing such alterations to their microbiome in their natural habitat or on oyster farms.

401 There is evidence that the hemolymph microbiome of Pacific oysters, *C. gigas,* can vary according to
402 oyster genotype, however these inter-genotype differences can disappear with changing environmental
403 conditions (Wegener et al., 2013; Lokmer and Wegner 2015). Furthermore, environmental disturbance
404 can remove rare taxa from the microbiome (Lokmer and Wegner 2015). We found changes in the core
405 microbiome of all selected lines, with the strongest changes in the overall haemolymph microbiome
406 experienced by oyster selected lines D, E and G. Each selected line experienced changes in different
407 taxa; DESeq analysis showed that selected line D experienced decreases in the genus *Malaciobacter*, and
408 selected line E had increases in the ASV *Spirochaetaceae_cae5* at both elevated temperature and pCO_2.
409 Conversely, oyster selected line G experienced decreases in *Spirochaetaceae_cae5* when exposed to
410 elevated pCO_2 and temperature. BLAST searches of the NCBI database revealed that at least three ASVs
411 found to be significantly altered by our treatments have been previously identified in oysters.
412 *Spirochaetaceae_cae5* was identified in the tissues of *C. gigas* from Tasmania (Australia) in an
413 experiment that tested the effects of warming on dead oyster tissue (Fernandez-Piquer et al., 2012).
414 Two *Spirochaete* ASVs were identified by network analysis as different between the groups of not-
415 affected and affected selected lines. The prevalence of *Spirochaetes* in our study and that of others (e.g.
416 Fernandez-Piquer et al., 2012) suggests that *Spirochaetes* may be a common feature of the oyster

microbiome that respond to environmental change. Two ASVs identified as *Mycoplasma*; *Mycoplasma_e553* and *Mycoplasma_d15b* matched ASVs in the BLAST search from previous studies by Fernandez-Piquer et al., (2012) and Green and Barnes (2010). Green and Barnes (2010) found that *Mycoplasma sp.* were absent in *S. glomerata* collected from the Pimpama River, Queensland (Australia) that were infected with the protozoan *Marteilia sydneyi. M. sydneyi* is responsible for QX disease in *S. glomerata*, a disease that causes widespread oyster death and significant economic damage during outbreaks. We found *Mycoplasma_e553* to decrease in abundance at elevated pCO_2 indicating that this ASV may be more abundant in healthy oysters. The functional role of these bacterial taxa remains largely unknown and further testing would be required to discern whether changes in their abundance would have downstream consequences for oyster health and survival particularly in the context of climate change.

Conclusions

This study has shown that both elevated pCO_2 and temperature can cause significant shifts in microbiome composition. With four of the nine selected lines showing microbiome shifts, the microbiome's response to climate change is clearly dependent on the genetic background of the respective oyster selected lines. Such shifts have previously been associated with an immune compromised state (de Lorgeril et al. 2019), which may allow opportunistic bacteria to invade and result in death. Microbiome stability across environments might therefore correlate with climate change resilience, and preliminary findings suggest those selected lines with stable microbiomes may also be physiologically resilient to climate change (Parker et al., Unpublished data). Further research on the causal direction between physiology and microbiome composition will be needed to explore this connection.

Oysters contribute invaluable ecological services such as providing habitat for fish and invertebrates while filtering the water column (Grabowski et al. 2012). Oyster aquaculture also provides income and sustenance for communities across the globe, with over 5.5 million tonnes of live oysters with a value of USD $12 billion produced each year, much of which is produced by sustenance farmers in developing nations (FAO 2018). Producing invertebrates sustainably will be vital to meeting the ever-growing global demand for food. We therefore, must identify, understand and build resilience in oysters to maintain their economic, social and cultural value in the face of climate change. The response of oyster microbiomes is dependent on oyster genotypes which may hold the key to enhancing the production and survival of oysters in the face of intensifying climate change by assisted evolution and selective

breeding. Such approaches need to consider the microbiome if we are to future-proof marine organisms and habitats to climate change.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

FIGURE LEGENDS

Figure 1. Mean relative abundances of *S. glomerata* haemolymph microbial taxa at the Order level for each oyster selected lines A-I as well as temperature (24 or 28 °C) and pCO_2 (ambient or elevated) treatments at the end of four weeks experimental exposure. Each column represents a mean relative abundance for that selected line and treatment combination.

Figure 2. Ordination plots of microbial microbiome in *S. glomerata* haemolymph using Constrained Analysis of Principal Coordinates (CAP) on Jaccard (Panel A) and Unifrac (Panel B) distances of samples for each oyster selected line (selected lines A-I) in the control (ambient pCO_2; 24 °C) treatment. Polygons encircle oyster selected lines, colours and line type also identify oyster selected lines.

Figure 3. Presence/absence map of core microbial members that were present in >90% of samples from an oyster selected line (A-I) in temperature (24 or 28 °C) and pCO_2 (Ambient or elevated) treatments. Each row represents a unique ASV, and ASV rows are grouped into taxonomic families. Only ASVs that were in the core of the control (ambient pCO_2; 24 °C) treatment are included to simplify this figure.

Figure 4. Boxplot (n=54) in the style of Tukey of Simpson's Index calculated from *S. glomerata* haemolymph bacterial microbiome at ambient (white) or elevated (grey) pCO_2 treatments and each oyster selected line (selected lines A-I). The upper and lower "hinges" correspond to the first and third quartiles (the 25th and 75th percentiles) while whiskers extend from the hinge to the highest and lowest value that is within 1.5 × IQR of the hinge, data beyond the end of the whiskers are outliers and plotted as points. Asterisks below red-outlined boxes indicate significant differences between pCO_2 treatments for those selected lines as determined by Post-Hoc Tukey tests. Results were averaged over the factor "Temperature" because this factor was not significant.

Figure 5. Ordination plots of microbial microbiome in *S. glomerata* haemolymph using Constrained Analysis of Principal Coordinates (CAP) on Unifrac distances of samples for each oyster selected line. **Panel A** Shows the effects of elevated pCO_2 on the selected lines identified as significantly affected by PERMAOVA (selected lines D, E, G), the X-axis was constrained by CO_2 treatment. **Panel B** shows the effects of elevated temperature on the selected lines identified as significantly affected by PERMAOVA (selected lines D, E, I), the X-axis was constrained by temperature treatment. Polygons encircle temperature and pCO_2 treatments for each oyster selected line, indicated by colours and shapes of points. The percentages in parentheses represent the variability explained by significant axes.

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