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INVERTEBRATE MICROBIOLOGY

Simulated Marine Heat Wave Alters Abundance and Structure of *Vibrio* Populations Associated with the Pacific Oyster Resulting in a Mass Mortality Event

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Abstract

Marine heat waves are predicted to become more frequent and intense due to anthropogenically induced climate change, which will impact global production of seafood. Links between rising seawater temperature and disease have been documented for many aquaculture species, including the Pacific oyster *Crassostrea gigas*. The oyster harbours a diverse microbial community that may act as a source of opportunistic pathogens during temperature stress. We rapidly raised the seawater temperature from 20 °C to 25 °C resulting in an oyster mortality rate of 77.4%. Under the same temperature conditions and with the addition of antibiotics, the mortality rate was only 4.3%, strongly indicating a role for bacteria in temperature-induced mortality. 16S rRNA amplicon sequencing revealed a change in the oyster microbiome when the temperature was increased to 25 °C, with a notable increase in the proportion of *Vibrio* sequences. This pattern was confirmed by qPCR, which revealed heat stress increased the abundance of *Vibrio harveyi* and *Vibrio fortis* by 324-fold and 10-fold, respectively. Our findings indicate that heat stress-induced mortality of *C. gigas* coincides with an increase in the abundance of putative bacterial pathogens in the oyster microbiome and highlights the negative consequences of marine heat waves on food production from aquaculture.

Keywords *Crassostrea* · *Vibrio harveyi* · Marine heat wave · Temperature stress · Disease event

Introduction

Extreme climatic events, such as heat waves, are becoming more frequent, intense and persistent due to the anthropogenic climate change, but their economic and ecological impacts are poorly understood, particularly in marine systems [1, 2]. Marine heat waves are defined as "discrete prolonged anomalously warm water events" [3] and can be caused by a combination of atmospheric and oceanographic processes [4, 5]. Well-known marine heat waves have occurred in the Mediterranean Sea [6], Western Australia [7], in the northwest Atlantic [8] and in the northeast Pacific [9, 10]. Ecological and economical impacts of these heat waves include fish kills and range expansion of marine fauna (Western Australia, [7]), benthic habitat loss (Mediterranean Sea, [11]) and harmful algal blooms prompting fishery closures (northeast Pacific, [12]).

Heat waves and rising seawater temperatures have also been linked to increased disease incidence in marine ecosystems [reviewed by 13]. In southeastern Australia, atmospheric and marine heat waves have coincided with several new disease events of farmed Pacific oysters (*Crassostrea gigas*) [14–16]. In January 2013 during an unprecedented atmospheric heat wave, where *C. gigas* inhabiting the intertidal zone would have experienced air temperatures > 40 °C during

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low tide (www.bom.gov.au), oyster farmers in the Hawkesbury River (New South Wales, Australia) experienced their first mass mortality event caused by Ostreid herpesvirus [15]. In January 2016, the first occurrence of Ostreid herpesvirus-derived mortality occurred in Tasmania [17], during the longest and most intense marine heat wave ever recorded in the region [16]. During this period, the ocean off the Tasmanian coastline reached 2.9 °C above mean climatology [16]. Notably, Ostreid herpesvirus is not the only cause of *C. gigas* mortalities in southeastern Australia. From January to June 2013 and November to January 2014, mass mortalities of cultivated *C. gigas* were reported in the Port Stephens estuary (New South Wales, Australia) [14]. No known aetiological agent was isolated from these disease events in Port Stephens. However, environmental data indicated that mortality coincided with periods of high temperature [14]. In synthesis, a pattern of mass mortality associated with heat stress is a reoccurring problem wherever *C. gigas* are farmed around the world [18–20].

There are a number of potential mechanisms for increased *C. gigas* mortality and disease susceptibility under higher temperatures, including effects on host physiology [20–22], and increases in the occurrence and virulence of potential pathogens [23]. *C. gigas* are known to survive a broad range of temperatures, but the thermal optimum for this species is predicted to be < 23 °C [24–29]. Abundant literature underlines the negative impacts of temperatures above 20–25 °C on *C. gigas* feeding activity (filtration rate), while showing respiration continues to exponentially increase over 30 °C [27, 24, 25]. *C. gigas* experiencing thermal conditions above ~ 21 °C are likely to be physiologically stressed due to reduced aerobic scope and a mismatch between energy acquisition and expenditure [27, 24]. It has been hypothesised that results in physiological trade-offs that divert energy from essential processes, such as immunity towards maintenance [30].

Heat waves may also exacerbate disease outbreaks in marine ecosystems by changing the virulence of pathogens [31]. For example, bacteria belonging to the *Vibrio* genus that can cause disease in oysters [reviewed by 32] have a preference for warm water conditions [33]. Elevated seawater temperature not only causes an increase in the growth rate and abundance of *Vibrio* species within coastal microbial communities [34, 35] but can also directly influence the expression of their virulence factors [36, 23, 37]. For instance, *Vibrio coralliilyticus* is a temperature-dependent pathogen of larval *C. gigas* [38, 39], for which numerous virulence factors involved in motility, host degradation, secretion, antimicrobial resistance, and transcriptional regulation are upregulated at higher temperatures (27 °C versus 24 °C) [23].

To date, our understanding of heat stress on oyster health has largely been derived from laboratory-based experiments that injected *C. gigas* with pathogens, such as Ostreid herpesvirus [40] and *Vibrio* species [41, 22]. These experimental challenges have typically used unrealistic doses of the pathogen, and intramuscular injection avoids natural barriers of immunity [42]. Here, we investigated how heat stress impacts the health and microbiome of *C. gigas* using an experiment designed to replicate the effect of a marine heat wave event. An antibiotic treatment was also included to disentangle the impacts of elevated temperature on *C. gigas* physiology and the pathogenicity of the microbial community associated with the oyster. Our results demonstrate that heat stress increases the abundance of putative pathogen(s) (*Vibrio* spp.) in the oyster microbiome, and these changes coincided with mortality of *C. gigas*.

Material and Methods

Simulated Marine Heat Wave

Triploid *Crassostrea gigas* (spat, shell length 6 mm) were collected from a Pacific oyster farm located at Oyster Cove (New South Wales, Australia) on the 9th of January 2017. *C. gigas* were deliberately collected prior to an atmospheric heat wave (10th to 14th of January) that affected large parts of New South Wales [43] to ensure the oyster's physiology and bacterial community were consistent between our experiment and mortalities that naturally occur in the field. The nearest weather station at Williamstown (station 061078) set a new temperature record on the morning of the 14th of January, with a minimum daily air temperature of 26.1 °C [43]. This extreme heat wave was forecasted by the heat wave Service of the Australian Bureau of Meteorology (www.bom.gov.au/australia/heatwave). The farm at Oyster Cove experienced high mortality of *C. gigas* spat during this period of time, which they attributed to the heat wave event.

C. gigas were transported from Oyster Cove to the Sydney Institute of Marine Science in an air-conditioned vehicle (< 3.5 h). Upon immediate arrival at the laboratory, four groups of *C. gigas* were exposed to a seawater matrix that differed in temperature (20 ± 1 °C versus 25 ± 1 °C) and concentration of penicillin-streptomycin. Each treatment consisted of three replicate glass tanks. Each tank held 25 *C. gigas* individuals within 500 ml of seawater. Three tanks at each temperature were treated daily with 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (Sigma #P4333). Each day, tanks received a 100% seawater change to avoid the accumulation of bacterial exo-toxins. Seawater was 5 μm filtered and UV sterilised. Oysters were fed daily with live microalgae (*Isochrysis galbana*, 10^8 cells). The *I. galbana* culture was routinely plated on thiosulfate citrate bile salts sucrose (TCBS) agar to confirm absence of culturable *Vibrio* species.

Oyster mortality was assessed each day, with dead *C. gigas* removed from tanks and frozen at – 80 °C for subsequent DNA extraction. Three live *C. gigas* were sampled from each

tank on day 0, 3, 4, 5 and 6. Each *C. gigas* was shucked using a sterile scalpel blade, and the oyster soft tissue was placed in an individual 2 ml sterile tube for storage at – 80 °C.

Nucleic Acid Extraction

Genomic DNA and total RNA were co-extracted from individual oysters. The whole oyster (soft tissue) was homogenised in lysis buffer using a bead mill (Qiagen TissueLyser II) and ceramic beads. Homogenised tissue was briefly centrifuged (14,000 *g* × 1 min) and split into two samples for nucleic acid extraction. DNA was purified using the Isolate II Genomic DNA Kit (Bioline), and RNA was purified using TriReagent® LS (Sigma #T3934). Total RNA was reverse transcribed using a Tetro cDNA synthesis kit (Bioline #BIO-65043) using random hexamers.

Quantitative PCR of the 16S rRNA Gene and OsHV-1

Absolute quantification of the bacterial 16S rRNA gene was performed using a TaqMan® assay adapted from Yu et al. [44]. PCR reaction volume was 10 μl and contained SensiFAST™ Probe Mix (Bioline #), and the BAC338F (5′-ACTCC TACGG GAGGC AG), BAC516F Probe (5′-6FAM-TGCCA GCAGC CGCGG TAATA C-TAMRA) and BAC805R (5′-GACTA CCAGG GTATC TAATC C) primers. Absolute quantification of the *Vibrio* 16S rRNA gene was performed using SensiFAST™ SYBR® No-ROX (Bioline) and 16S rRNA *Vibrio*-specific primers, Vib1-F (5′-GGCGT AAAGC GCATG CAGGT) and Vib2_R (5′-GAAAT TCTAC CCCCC TACAG) [35, 45]. The abundance of the 16S rRNA gene in oyster samples was estimated from a serial curve generated from *Vibrio harveyi* 16S rRNA amplicon cloned into the pCR4-TOPO vector (Thermo Scientific Inc.).

DNA from *C. gigas* samples (including dead oysters) was tested for the presence of OsHV-1 using quantitative PCR according to Pepin et al. [46]. All qPCR assays were performed in duplicate, and the reaction volumes were 10 μl containing SensiFAST™ SYBR® No-ROX (Bioline), C9 (5′-GAGGG AAATT TGCGA GAGAA), C10 (5′-ATCAC CGGCA GACGT AGG) and 50 ng of DNA. The qPCR assay included positive and negative samples.

16S rRNA Gene Sequencing

High-throughput sequencing of the V3-V4 region of the 16S rRNA gene was used to characterise the *C. gigas* microbiome. Equimolar amounts of DNA were combined from three replicate *C. gigas* from each tank to generate 15 pooled samples. This represented a pooled sample from each tank on day 4. Pooled DNA samples were PCR amplified using the 341F (5′-CCTAY GGGRB GCASC AG) and 806R (5′-GGACT ACNNG GGTAT CTAAT) primers, with indexing (Illumina, Nextera® XT Index Kit) and pair-end sequencing performed using the Illumina MiSeq protocols and sequencing platform (Australian Genome Research Facility (AGRF)). To account for possible contamination, a blank sample (milliQ water) was subjected to PCR amplification and sequencing. Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) with the study accession number SRP126703 under Bioproject number PRJNA421986.

Bacterial 16S rRNA reads were analysed as outlined in https://github.com/timkahlke/ampli-tool. Briefly, paired-end DNA sequences were joined using FLASH [47] and subsequently trimmed using mothur [48] (Parameters: maxhomop = 6, maxambig = 0, minlength = 441, maxlength = 466). The resulting fragments were clustered into operational taxonomic units (OTUs), and chimeric sequences were identified using vsearch [49] and the Silva v128 database. To assign taxonomy, QIIME Version 1.9.1 [50] was used with the uclust algorithm against the Silva v128 database. Sequences were then rarefied to the same sequencing depth (118,000 reads) to remove the effect of sampling effort upon analysis. Similarity matrices of the 16S rRNA gene sequencing data were prepared using Bray-Curtis distance and analysed with PRIMER V6 + PERMANOVA add-on (PRIMER-E Ltd). SIMPER analysis was used to identify operational taxonomic units (OTUs) contributing most to the dissimilarity between treatments.

Bacterial Isolation and Species-Specific TaqMan® Assays

Bacteria were recovered from live and dead *C. gigas* by plating a serial dilution of homogenised oyster tissue on tryptic soy agar supplemented with 2% NaCl (TSA). Plates were incubated for 48 h at 20 °C. Ten single colonies of the dominant morphotypes were picked and re-isolated in pure culture on fresh TSA. Pure isolates were identified by PCR amplifying and sequencing the 16S rRNA and gyrase B subunit genes [51–53] using a high fidelity polymerase (Accuzyme™, Bioline) and universal primer pairs 27F (5′-AGAGT TTGAT CCTGG CTCAG), 1492R (5′-GTTAC CTTGT TACGA CTT) and Up1E (5′-GAAGT CATCA TGACC GTTCT GCAYG CNGGN GGNAA RTTYR A), UP2AR (5′-AGCA G GGTAC GGATG TGCGA GCCRT CNACR TCNGC RTCNG YCAT). Sequences were aligned with selected reference 16S rRNA and gyrase B subunit sequences from GenBank using the ClustalW algorithim in Mega v 6.0, and phylogenetic trees were constructed using the neighbourhood-joining distance method [54].

Quantitative PCR primer and probe sets were designed using the GyrB partial gene sequences for the *Vibrio* isolates putatively assigned to be *V. harveyi* (2017-PS03 and 2017-PS05) and *Vibrio fortis* (2017-PS02). Primer and probe sequences targeting the *V. harveyi* isolates are Vhf (5′-AAGTA

TCAGG CGGTC TAC), Vhp (5′-6FAM-TTCTG ACTAT CCACC GCGGC GGT-TAMRA) and Vhr (5′-CAATT ACTGC TAGTG GC). Primer and probe sequences for the *V. fortis* isolate are Vff (5′-AGCAG GTTAC TCTTA CTATC), Vfp (5′-6FAM-GTG AAA CTG ACA AAA CGG GTA CAG AG-TAMRA) and Vfr (5′-GAATT CGGTG TTAGA GAACG). Specificity and amplification efficiency of each primer and probe set were verified by testing against a panel of DNA isolated from bacteria isolated from *C. gigas* (Table 1). The abundance of these *Vibrio* species in oyster samples was estimated from a serial curve generated from a *gyrB* subunit cloned into the pCR4-TOPO vector (Thermo Scientific Inc.).

Immune Gene Expression

The *C. gigas* immunological response was compared between heat stressed and control treatments by quantifying the mRNA expression of ten oyster immune genes by Reverse Transcriptase quantitative PCR (RT-qPCR). These ten genes represent a heat shock protein (*HSP68*), immune-signalling proteins (*Rel*, *IL17*, *TNF*) and antimicrobial peptides (*Laccase*, *Mpeg*, *Cg-DefH*, *Cg-DefM Cg-BigDef1*, *EcSOD*). Primer sequences are outline in [55]. The PCR reaction volume was 8 μl and contained SensiFast™ SYBR No-ROX master mix (Bioline), 100 nM of each specific primer and 20 ng of cDNA in a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD) using an initial denaturation (95 °C, 2 min) followed by 40 cycles of denaturation (95 °C, 5 s) and hybridization-elongation (60 °C, 30 s). A subsequent melting temperature curve of the amplicon was performed. EF1α was used as the internal reference for normalising *C. gigas* gene expression [56]. Data was analysed using the univariate general linear model (GLM) with post hoc Tukey's HSD test in IBM SPSS Statistics version 20.0.0.2.

Results

Heat Stress Affects Oyster Survival

The simulated marine heat wave had a significant effect on *C. gigas* survival (Fig. 1). Cumulative mortality of *C. gigas* in the heat stress treatment (25 °C) was 77.4 ± 10.7%, with the mortality starting on day 2 and continuing to day 6. The rate of mortality was highest between 3 and 5 days after the start of the experiment. The remaining (live) *C. gigas* in the heat stress treatment were sampled on day 6 when the experiment was terminated. In contrast, cumulative mortality of *C. gigas* in the normal temperature treatment (20 °C) was only 3.4 ± 5.9% after 6 days. Addition of penicillin-streptomycin caused a significant reduction in mortality of *C. gigas* in the heat stress

Table 1 Specificity of the quantitative PCR assays to a range of bacterial strains isolated from *Crassostrea gigas*. Primers and probes outline in the method section were designed to target *Vibrio harveyi* (strain 2017-PS03) and *V. fortis* (strain 2017-PS02). The GenBank accession numbers for partial nucleotide gene sequences for 16S rRNA and gyrase subunit B for each bacterial isolate are provided. Strain IDs beginning with an asterisk (*) were isolated in this study

Isolate			GenBank accession		qPCR results (±)	
Strain ID	Putative species ID	Vibrio clade	16S rRNA	Gyrase subunit B	*V. harveyi*	*V. fortis*
*2017-PS01	*Vibrio antiquarius*	*Harveyi* clade	MG693188	MG712842	–	–
*2017-PS02	*Vibrio fortis*	*Splendidus* clade	MG693189	MG712843	–	+
*2017-PS03	*Vibrio harveyi*	*Harveyi* clade	MG693190	MG712844	+	–
*2017-PS04	*Alteromonas* sp.		MG693191	MG712845	–	–
*2017-PS05	*Vibrio harveyi*	*Harveyi* clade	MG693192	MG712846	+	–
*2017-PS06	*Vibrio diabolicus*	*Harveyi* clade	MG693193	MG712847	–	–
*2017-PS07	*Vibrio coralliilyticus*	*Coralliilyticus* clade	MG693194	MG712848	–	–
*2017-PS08	*Vibrio coralliilyticus*	*Coralliilyticus* clade	MG693195	MG712849	–	–
*2017-PS09	*Vibrio harveyi*	*Harveyi* clade	MG693196	MG712850	+	–
*2017-PS10	*Pseudoalteromonas* sp.		MG693197	MG712851	–	–
2015-GR29	*Vibrio alginolyticus*	*Harveyi* clade	MG693198		–	–
2015-GR48	*Vibrio harveyi*	*Harveyi* clade	MG693199		+	–
2015-GR56	*Pseudoalteromonas* sp.		MG693200		–	–
2015-GR61	*Photobacterium* sp.		MG693201		–	–
2015-GR98	*Vibrio crassostreae*	*Splendidus* clade	MG693202		–	–
2015-GR100	*Pseudoalteromonas* sp.		MG693203		–	–

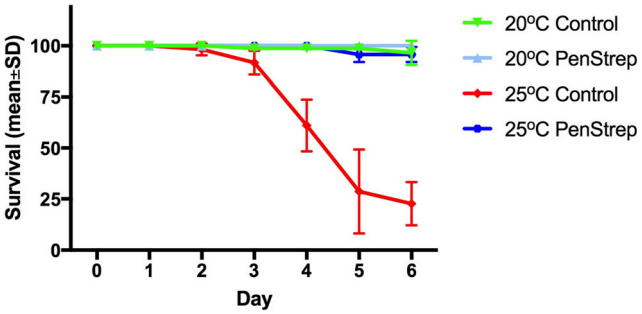

Fig. 1 Cumulative mortality (mean ± SD) of *Crassostrea gigas* in the heat stress (25 °C) and control groups (20 °C), with or without the addition of penicillin-streptomycin (PenStrep). Each group consisted of three replicate tanks. Cumulative mortality accounted for three oysters removed (sampled) from each tank on day 3, 4, 5 and 6

treatment with a cumulative mortality of only 4.3 ± 3.7% observed after 6 days (Fig. 1).

Heat Stress Is Associated with Increase Abundance of Total Bacteria and *Vibrio*

The low levels of oyster mortality in the penicillin-streptomycin treatment suggest bacteria played a key role in the mortality experienced in the heat stress treatment. Changes in the abundance of total bacteria and total *Vibrio* species were assessed using qPCR targeting the 16S rRNA gene. In the heat stress treatment, the abundance of the bacterial 16S rRNA gene increased from 2.5×10^7 copies ng $^{-1}$ of DNA on day 0 to a peak of 1.1×10^8 copies ng^{-1} DNA on days 4 and 5 (Fig. 2a). Likewise, the mean abundance of *Vibrio* species-specific 16S rRNA gene increased from 2.8×10^6 copies ng^{-1} DNA on day 0 to a peak of 3.6×10^7 copies ng^{-1} DNA on day 4 (Fig. 2b). In the normal temperature and penicillin-streptomycin treatments, the concentration of bacteria and *Vibrio* 16S rRNA gene in *C. gigas* tissue was stable at 10^7 and 10^6 copies ng^{-1} DNA, respectively. OsHV-1 viral DNA was not detected in any of the *C. gigas* samples tested in this study using an established qPCR assay for OsHV-1 (and OsHV-1 microvariant) [46].

Heat Stress Changes the Composition of the Oyster's Bacterial Community

To identify shifts in the *C. gigas* microbiome occurring in response to heat stress, we sequenced the hypervariable V3-V4 region of the 16S rRNA gene. Microbial community composition was significantly different between treatments (PERMANOVA, Pseudo-$F_{4,14} = 5.1206$, $p = 0.001$), with the bacterial community in heat stress samples 57.9% and 50.3% dissimilar to day 0 and 20 °C groups, respectively (SIMPER analysis). In addition, PCO analysis revealed the bacterial communities associated with heat stress clustered separately to day 0 and 20 °C groups (Fig. 3). Vector overlay ($r > 0.9$) showed the bacterial communities within the heat-stressed *C. gigas* possessed a different suite of dominant operational taxonomic units (OTU), in particular a *Vibrio* sp. (OTU_1) and an *Arcobacter* sp. (OTU_750).

Taxonomic classification revealed the bacterial community associated with *C. gigas* at day 0 was dominated by the *Rhodobacteraceae* (55.4 ± 6.2%), *Erythrobacteraceae* (10.5 ± 1.1%), *Flavobacteriaceae* (9.2 ± 1.7%) and *Vibrionaceae* (3.5 ± 2.3%). The relative proportion of 16S rRNA gene sequences is provided as mean ± standard deviation. During the course of the experiment, the bacterial community in the 20 °C treatment shifted slightly, with an increase in the relative proportion of *Flavobacteriaceae* (18.0 ± 6.3%), *Alteromonadaceae* (13.6 ± 0.9%) and *Vibrionaceae* (10.4 ± 1.5%) and a decrease in relative proportion of *Rhodobacteraceae* (20.5 ± 2.8%). These shifts are indicative of an experimental effect. However, the heat stress treatment (25 °C) caused a substantially greater shift in bacterial assemblage structure, with a large increase in the relative proportion of *Vibrionaceae* (56.6 ± 18.7%) and a concurrent decrease in the proportion of *Rhodobacteraceae* (6.4 ± 5.78%) and *Flavobacteriaceae* (3.4 ± 2.5%). In contrast, the bacterial communities associated with the penicillin-streptomycin treatments remained dominated by *Rhodobacteraceae* and *Flavobacteriaceae*.

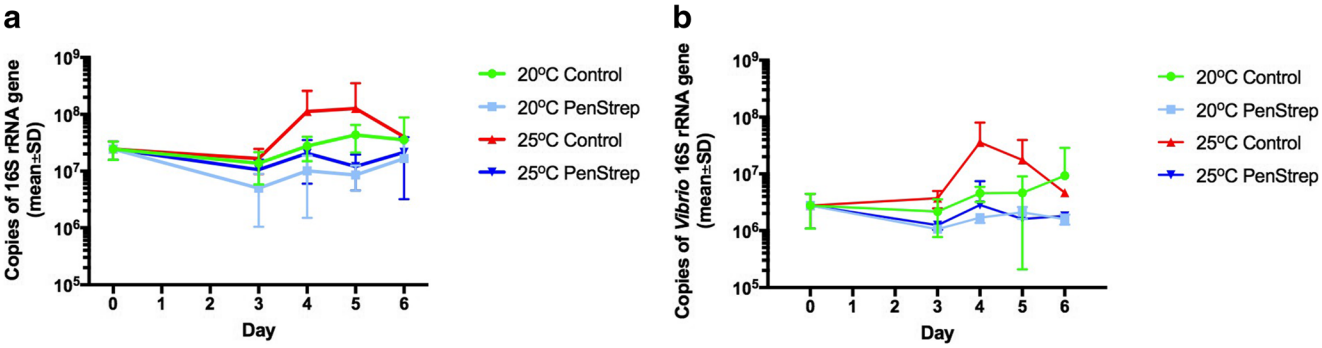

Fig. 2 Quantitative PCR assays were used to quantify the abundance of (**a**) total bacteria and (**b**) total *Vibrio* 16S rRNA gene in *Crassostrea gigas* tissue (copies of 16S rRNA gene.ng of total DNA; mean ± standard deviation). Treatments consisted of heat stress (25 °C) and control groups (20 °C), with or without the addition of penicillin-streptomycin (PenStrep). The dynamic range of the qPCR assays was 10^{10} to 10^3 copies of the16S rRNA gene

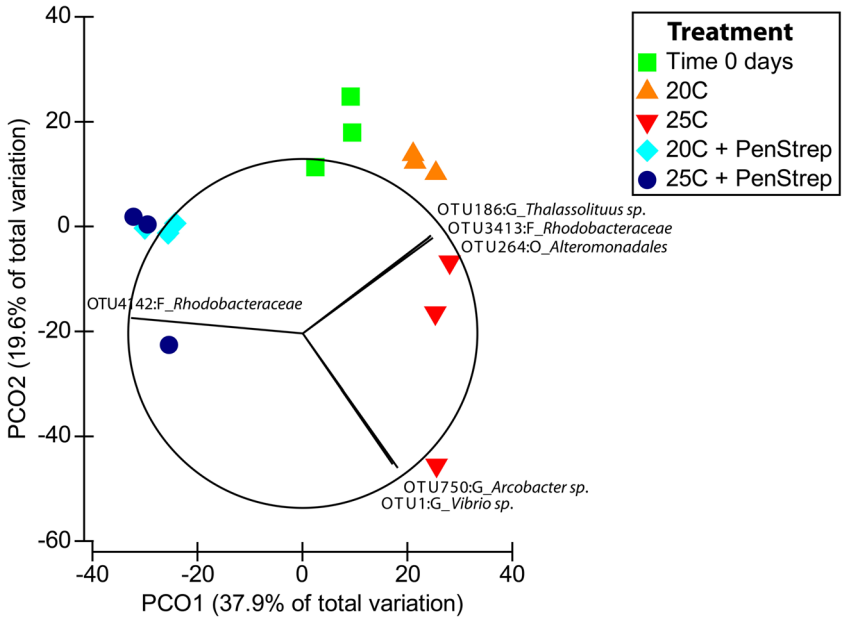

Fig. 3 Principal coordinate analysis plot based on a Bray-Curtis distance matrix calculated from the square-root transformed OTU abundance data of the bacterial community (V3-V4 region of the 16S rRNA gene) of *Crassostrea gigas* in the heat stressed (25 °C) and control treatments (20 °C) at day 4, with or without the addition of penicillin-streptomycin (PenStrep). Vector overlay ($r > 0.9$) showed the bacterial communities from heat-stressed *C. gigas* possess a different suite of dominant operational taxonomic units (OTU), in particular a *Vibrio* sp. (OTU_1) and an *Arcobacter* sp. (OTU_750)

SIMPER analysis identified OTU_1 (*Vibrio* sp.) as being the OTU that contributed the most to the dissimilarity in the bacterial community between the heat stress and control groups (20 °C and day 0 samples). The relative proportion of OTU_1 in the heat stress, 20 °C and day 0 samples was 40.5 ± 15.4%, 3.6 ± 3.4% and 0.7 ± 0.5%, respectively (Fig. 4). The relative proportion of OTU_1 in the penicillin-streptomycin treatments ranged from 0.0 to only 2.2%.

Heat Stress Changes the Abundance of *Vibrio harveyi*

A limitation of 16S rRNA gene sequencing is the technique has low phylogenetic power at the species level and poor discriminatory power for some genera, in particular *Vibrionaceae* [53]. In an attempt to identify the *Vibrio* sp. (OTU_1) that displayed marked increases in relative abundance in the heat stress treatment, homogenised *C. gigas* was plated on TSA, and 10 representative colonies were subcultured and characterised by sequencing the 16S rRNA and GyrB subunit genes. Species designation for the isolates was putatively assigned based on phylogenetic comparisons of the 16S rRNA and GyrB subunit genes (Supplementary Fig. 1). Details about the strains isolated and GenBank accession numbers are provided in Tables 1 and 2. Eight *Vibrio* strains were isolated, and several of these isolates had 16S rRNA gene sequences that matched (≥ 99% nucleotide identity) with OTUs identified in the SIMPER analysis as key drivers of differences between the heat stress treatment and control microbial assemblages (Table 2). In particular, *Vibrio harveyi* isolates (2017-PS03 and 2017-PS05) had 100% nucleotide identity to OTU_1. The *Vibrio fortis* isolate (2017-PS02) had 99.5% nucleotide identity to OTU_2.

The *gyrB* sequences of the bacterial isolates putatively identified to be *V. harveyi* (2017-PS03 and 2017-PS05) and

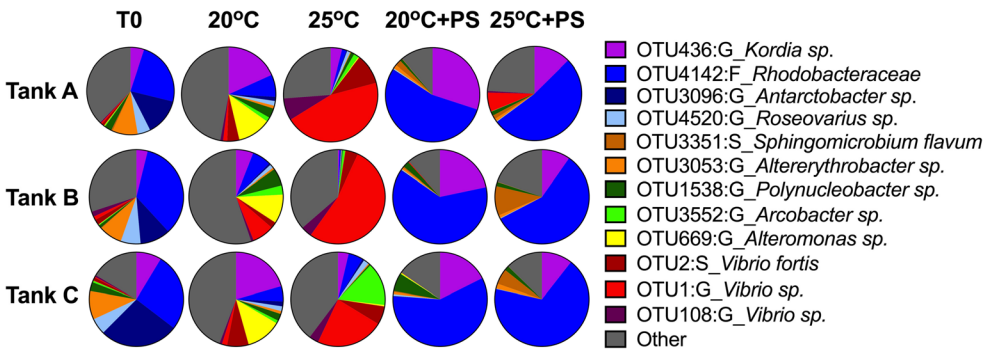

Fig. 4 Differences in the dominant operational taxonomic units (OTUs). The matrix shows the top twelve OTUs in each tank at the beginning of the experiment (T0) and in the heat stressed (25 °C) and control treatments (20 °C) at day 4, with or without the addition of penicillin-streptomycin (PS). The V3-V4 region of the 16S rRNA gene was sequenced from a pool of *C. gigas* tissue ($N = 3$) from each tank

Table 2 Taxonomic classification of bacterial isolates from *Crassostrea gigas* based on sequencing the 16S rRNA and gyrase subunit B genes. Top BlastN match (nucleotide identity) is provided for each bacterial isolate. Significant matches between bacterial isolate and dominant OTUs (identity) are also provided

Isolate ID	Condition	16S rRNA gene [GenBank #] (identity)	Gyrase B subunit [GenBank #] (identity)	OTU match	Identity (%)
2017-PS01	25C-Mort.	*Vibrio antiquarius* [MH044597] (99%)	*Vibrio alginolyticus* [CP001805] (97%)	OTU712	97.8
2017-PS02	25C-Mort.	*Vibrio fortis [KU197914] (99%)*	*Vibrio splendidus [JQ698508] (90%)*	OTU2	99.5
2017-PS03	25C-Mort.	*Vibrio harveyi [KY229855] (100%)*	*Vibrio harveyi [JQ698506] (98%)*	OTU1	100
2017-PS04	25C-Mort.	*Alteromonas mediterranea* [CP018029] (100%)	*A. mediterranea* [CP001103] (99%)	OTU3	99.5
2017-PS05	25C-Mort.	*Vibrio harveyi [KY229811] (100%)*	*Vibrio harveyi [JQ698506] (99%)*	OTU1	100
2017-PS06	Time 0	*Vibrio diabolicus* [CP014134] (100%)	*Vibrio splendidus* [JQ698508] (90%)	OTU712	97.5
2017-PS07	Time 0	*Vibrio coralliilyticus* [KX904710] (100%)	*Vibrio coralliilyticus* [CP016556] (96%)	OTU33	99.5
2017-PS08	Time 0	*Vibrio coralliilyticus* [CP009617] (99%)	*Vibrio* sp. GM4 [AY795846] (98%)	OTU1692	99
2017-PS09	25C-Live	*Vibrio harveyi* [KY229855] (99%)	*Vibrio harveyi* [JQ698506] (99%)	OTU570	98.5
2017-PS10	25C-Live	*Pseudoalteromonas* sp. [KF758689] (99%)	*P. undina* [AF007284] (88%)	OTU4	97.9

V. fortis (2017-PS02) were used for designing qPCR primers and probes. The specificity of these TaqMan® assays were verified against a panel of gram-negative bacteria isolated from *C. gigas* (Table 1). These TaqMan® assays were used to assess changes in the abundance of *V. harveyi* and *V. fortis*. On day 0, the average copy number of *gyrB* from *V. harveyi* was 4.1×10^3 copies ng DNA^{-1}. During the mortality event on day 4, the abundance of *gyrB* from *V. harveyi* and *V. fortis* was 324-fold and 10-fold higher within the heat-stressed *C. gigas* tissue (Fig. 5a and b).

Immunological Response of *Crassostrea gigas*

To determine whether heat stress causes immunosuppression in *C. gigas*, we quantified the expression of ten immune genes by RT-qPCR. Eight of these immune genes were upregulated in heat-stressed *C. gigas* (two-way ANOVA, $p < 0.05$). The expression of a defensin (*Cg-DefM*) peaked on day 3, whereas the highest expression of a heat shock protein (*HSP68*), immune-signalling proteins (*Rel*, *IL17*, *TNF*) and antimicrobial peptides (*Laccase*, *Mpeg*, *Cg-DefH*) occurred on day 4

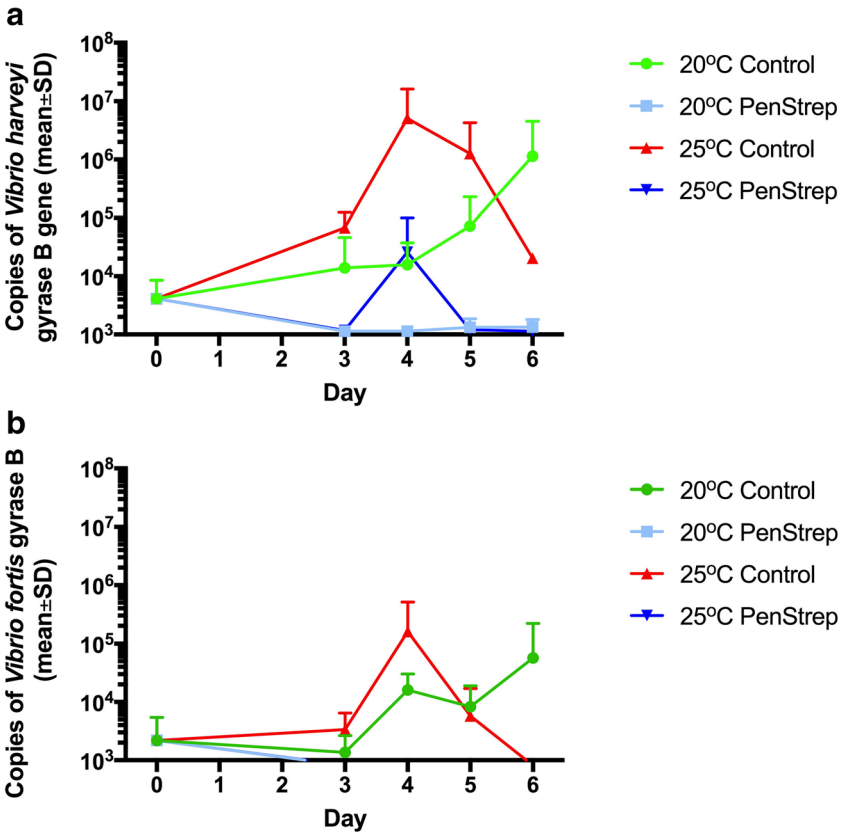

Fig. 5 TaqMan® PCR assays were used to quantify the abundance of (**a**) *Vibrio harveyi* and (**b**) *V. fortis* in *Crassostrea gigas* tissue by targeting the gyrase B subunit gene (copies of gyrase B subunit gene.ng of total DNA; mean ± standard deviation). Treatments consisted of heat stress (25 °C) and control groups (20 °C), with or without the addition of penicillin-streptomycin (PenStrep)

(Supplementary Fig. 2). Extracellular superoxide dismutase (*EcSOD*) and big defensin (*Cg-BigDef1*) were not differentially expressed during the experiment ($p > 0.05$).

Discussion

The results of this study indicate that a shift in the microbiome of *Crassostrea gigas* may have played an important role in oyster mortality during a stimulated marine heat wave. The total mortality of *C. gigas* exposed to heat stress was 77.4%, which occurred in concert with clear shifts in the bacterial community associated with *C. gigas*, whereby there was an increase in the abundance of putative pathogens belonging to the bacterial families of *Vibrionaceae* and *Campylobacteraceae*. The likely involvement of these bacteria in the mortality event was confirmed by the low-levels of mortality observed in an antibiotic-exposed treatment that experienced the same temperature regime. Specifically, the relative proportion of 16S rRNA gene sequences for three *Vibrio* OTUs and an *Arcobacter* OTU was more abundant in heat-stressed *C. gigas* (Fig. 4). In addition, qPCR data identified the abundance of *V. harveyi* and *V. fortis* to be 324-fold and 10-fold higher in *C. gigas* exposed to heat stress, respectively. These observations are highly relevant to the aquaculture industry, which is now the fastest food producing sector in the world [57]. *C. gigas* is one of the most important global aquaculture species [58]; however, the predicted increase in the frequency and intensity of marine heat waves due to anthropogenic climate change [1] may have a significant impact on global oyster production. Our data provides compelling evidence that the oyster's natural bacterial community can act as a source of opportunistic pathogens during heat stress events.

Our research builds upon previous studies investigating the role of opportunistic bacterial pathogens causing episodes of mortality of *C. gigas* during the water summer months [59–61, 22, 41, 62, 63]. The majority of these studies have been observational and reported seasonal changes to the oyster's bacterial community [59, 60, 63]. However, seasonality does not equal temperature [41, 64, 65]. Seasonality has an impact on many environmental and biological parameters that may alter the oyster's bacterial community. These include physiological stresses associated with host reproductive effort [20, 21] and changes in the quality and quantity of food [66]. Experimental studies investigating the role of temperature on the development of oyster disease have typically inoculated oysters with *Vibrio* pathogens via intramuscular injection [22, 41, 56], which circumvents natural barriers of immunity [42]. Our study avoided many of these pitfalls. Until this study, scientific efforts to simulate "summer mortality" in the laboratory had been unsuccessful [19, 56]. Our approach was to collect *C. gigas* immediately prior to a heat wave [43] to ensure variables, such as the oyster's metabolic rate and microbiome were consistent between our experiment and mass mortality events that naturally occur in the field [67, 14]. We did not inoculate oysters with bacterial pathogens, but instead used an antibiotic treatment to disentangle the effect of elevated seawater temperature and altered bacterial community on oyster health and survival. We also used triploid oysters, which have three sets of chromosomes, to circumvent the confounding factor of physiological stress associated with the oyster's reproduction and spawning. Triploid oysters have vastly reduced gonadogenesis [68].

The 16S rRNA gene-sequencing showed that heat stress increased the relative proportion of bacterial groups with close homology to known *C. gigas* pathogens, such as members of the *Vibrio* and *Arcobacter* genera [41, 32, 59]. The *Vibrio* genus comprises a diverse group of largely marine and estuarine bacteria that often occur in close association with marine plants and animals, where they act as mutualistic symbionts or pathogens [34]. Evidence is emerging that rising seawater temperatures associated with anthropogenic climate change are increasing the frequency of *Vibrio*-related infections [69]. The genus *Arcobacter* belongs to the family *Campylocateraceae* [70]. *Arcobacter* grows well under aerobic or microaerobic conditions [70] and has been described as a spoilage organism in many types of seafood, including *C. gigas* [71]. The bacterial community of diseased *C. gigas* can be dominated by *Arcobacter* [41]. While some strains of *Arcobacter* are known to be human pathogens [72], the pathogenic potential of *Arcobacter* towards *C. gigas* remains unexplored.

We identified the dominant *Vibrio* strains associated with heat-stressed *C. gigas* by isolating ten pure cultures of bacteria and putatively assigning their taxonomy based on phylogenetic analysis of their 16S rRNA and GyrB subunit gene sequences. In total, eight of the ten pure isolates belonged to the *Vibrio* genus, and they clustered with *V. harveyi*, *V. antiquarius* (*Harveyi* clade), *V. diabolicus* (*Harveyi* clade), *V. fortis* (*Splendidus* clade) and *V. coralliilyticus* (Supplementary Fig. 1). Although classification of *Vibrio* based on the 16S rRNA and *gyrB* gene sequences remains problematic [53], we view our taxonomic designations to be robust based on the consensus between our phylogenetic trees. *Vibrio* bacteria belonging to the *Harveyi* clade, *Splendidus* clade or to the species *V. coralliilyticus* are commonly reported in association with mortality events of *C. gigas* [32, 59]. Our bacterial isolates of *V. harveyi* and *V. fortis* had 16S rRNA gene sequences with ≥ 99.5% nucleotide identity to the dominant OTUs in heat-stressed *C. gigas* samples. Next, we developed qPCR assays to track changes in the abundance of these two *Vibrio* species. During peak mortality on day 4, the abundance of *V. harveyi* and *V. fortis* was 324-fold and 10-fold higher in *C. gigas* exposed to heat stress, respectively. These changes to the bacterial community indicate that specific *Vibrio* species, in this case *V. harveyi* and *V. fortis*, can proliferate and dominate the microbial community of *C. gigas*

during acute heat stress. However, our data cannot distinguish if *V. harveyi* and *V. fortis* are pathogenic, or whether they cooperate or act independently to cause disease. Experimental challenge trials using these isolates are required to answer this question. Intriguing, experimental infections of *C. gigas* using a bacterial inoculum comprising a mix of *V. harveyi*, *V. alginolyticus*, *V. splendidus* and *V. crassostreae*, which had been isolated during a disease outbreak in Port Stephens, Australia, during January 2014, could induce > 50% mortality within 72 h postinoculation [14]. Of the four *Vibrio* spp. used in the inoculum, *V. harveyi* was the most dominant organism re-isolated from the hemolymph of moribund oysters [14].

Having shown that heat stress coincides with an increase in *V. harveyi* and *V. fortis*, we next considered whether the origin of these putative pathogens was the oyster's natural bacterial community or an external environmental source, such as the daily seawater change or addition of microalgae. The microalgae fed to oysters are unlikely to be a source of these putative pathogens because the cultures are confirmed to be free of culturable *Vibrio* species. Despite filtration and UV sterilisation, the seawater used during the experiment was collected from Sydney Harbour and may have been the source of these putative pathogens, but we consider this scenario to be unlikely. The 16S amplicon sequencing identified *V. harveyi* (OTU_1) and *V. fortis* (OTU_2) in all samples from day 0 (Fig. 4), indicating these *Vibrio* strains, or highly related strains, were present in the *C. gigas* population from Port Stephens.

The immune system of *C. gigas* in the heat stress treatment was reactive to the mortality event by upregulating genes involved in immune-signalling pathways and antimicrobial peptides. Maximum expression for the majority of these immune genes coincided with peak abundance of *V. harveyi* and *V. fortis* in *C. gigas* tissue (Fig. 5). These immune genes were chosen from previous studies investigating the immune response of *C. gigas* to vibriosis [56, 73, 74]. In the current study, expression of big defensin (*Cg-BigDef1*) was not induced during the mortality event. This result, based on a single gene, does not indicate that acute heat stress at 25 °C caused the *C. gigas* immune response to be compromised. Indeed, the *Cg-BigDef1* gene is not present in the genomes of all *C. gigas* [73, 75], and no correlation has been found between transcription level of *Cg-BigDef1* and capacity of oysters to survive inoculation with virulent *V. tasmaniensis* [75]. Our immune gene data indicates that *C. gigas* were able to sense microbial invasion and respond by upregulating the expression of cytokines and antimicrobial peptides. Thus, acute heat stress treatment at 25 °C does not appear to compromise the immune response of *C. gigas*. Instead, our results are consistent with a previous study that found heat stress causes a rapid proliferation of opportunistic pathogens, and their abundance in *C. gigas* tissue exceeds the capacity of the host's immune system resulting in mortality [22]. These shifts in the bacterial community may be a direct effect of elevated temperature on the growth rate of *Vibrio* species [34, 35], or alternatively the elevated temperature may influence the virulence of oyster-associated *Vibrio* species [23, 37]. *V. harveyi* also causes disease in the marine gastropod, *Haliotis tuberculata* [76, 77]. Pathogenicity of *V. harveyi* to *H. tuberculata* is also temperature-dependent with a difference of only 1 °C having a significant impact on mortalities [76]. *V. harveyi* invades the tissues of *H. tuberculata* during the summer spawning period, when energy reserves are limited and the immune system of the host is partially depressed [77].

Conclusion

Our findings indicate that a marine heat wave has the potential to cause mass mortality of *C. gigas* by causing specific members of the oyster's bacterial community to proliferate and potentially overwhelm the oyster's immunological capacity. Importantly, these microbial shifts involve an increase in the abundance of *Vibrio* belonging to the *Harveyi* and *Splendidus* clades, which are known oyster pathogens [32]. Our research builds upon previous studies using cultured isolates [41, 22], to highlight that the diverse microbiome of *C. gigas* harbours putative pathogens that can rise to prominence during periods of environmental stress, such as a marine heat wave. Considering the global importance of *C. gigas* as an aquaculture species, this information is essential for understanding how anthropogenically induced climate change will impact future food production by aquaculture.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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