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ORIGINAL ARTICLE

Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection

Ana Lokmer[1,2] and Karl Mathias Wegner[1]
[1]*Helmholtz Centre for Polar and Marine Research, Alfred Wegener Institute, Coastal Ecology, Wadden Sea Station Sylt, List, Sylt, Germany and* [2]*GEOMAR Helmholtz Centre for Ocean Research Kiel, Evolutionary Ecology of Marine Fishes, Kiel, Germany*

Microbiota provide their hosts with a range of beneficial services, including defense from external pathogens. However, host-associated microbial communities themselves can act as a source of opportunistic pathogens depending on the environment. Marine poikilotherms and their microbiota are strongly influenced by temperature, but experimental studies exploring how temperature affects the interactions between both parties are rare. To assess the effects of temperature, temperature stress and infection on diversity, composition and dynamics of the hemolymph microbiota of Pacific oysters (*Crassostrea gigas*), we conducted an experiment in a fully-crossed, three-factorial design, in which the temperature acclimated oysters (8 or 22 °C) were exposed to temperature stress and to experimental challenge with a virulent *Vibrio* sp. strain. We monitored oyster survival and repeatedly collected hemolymph of dead and alive animals to determine the microbiome composition by 16s rRNA gene amplicon pyrosequencing. We found that the microbial dynamics and composition of communities in healthy animals (including infection survivors) were significantly affected by temperature and temperature stress, but not by infection. The response was mediated by changes in the incidence and abundance of operational taxonomic units (OTUs) and accompanied by little change at higher taxonomic levels, indicating dynamic stability of the hemolymph microbiome. Dead and moribund oysters, on the contrary, displayed signs of community structure disruption, characterized by very low diversity and proliferation of few OTUs. We can therefore link short-term responses of host-associated microbial communities to abiotic and biotic factors and assess the potential feedback between microbiota dynamics and host survival during disease.
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Introduction

Over the last couple of decades, it has become clear that microbiota are of vital importance for survival, homeostasis and development of animals (McFall-Ngai *et al.*, 2013). Tight relationships between hosts and their symbionts even inspired a hologenome theory of evolution (Rosenberg *et al.*, 2007), proposing a holobiont—a host together with the associated microorganisms—as the unit of selection. One prominent service that microbiota provide for their hosts is protection from pathogens (Kamada *et al.*, 2013). However, in compromised hosts or under (un)favorable environmental conditions, the symbionts themselves can act as opportunistic pathogens (Garnier *et al.*, 2007; Cerf-Bensussan and Gaboriau-Routhiau, 2010; Olson *et al.*, 2014). As disease has a large impact on population dynamics and evolution of affected organisms (Altizer *et al.*, 2003), it is important to understand how the environmental factors and stress affect the composition and function of microbiota and the outcome of host–microbe interactions.

Tissues of healthy marine invertebrates usually harbor species-rich microbial communities (Prieur *et al.*, 1990; Gomez-Gil *et al.*, 1998; Romero *et al.*, 2002; Gomez-Gil *et al.*, 2010; King *et al.*, 2012; Trabal *et al.*, 2012; Wegner *et al.*, 2013). This also applies to the hemolymph (Olafsen *et al.*, 1993; Garnier *et al.*, 2007, Wendling *et al.*, 2014)—the functional analog of blood in vertebrates (Bachere *et al.*, 2004). The presence of viable bacteria in the hemolymph of healthy oysters can influence the outcome of pathogen infections either by stimulating immunity or by competitive exclusion (Schmitt *et al.*, 2012); isolation of antimicrobial compounds of bacterial origin from oyster hemolymph has provided support for the latter hypothesis (Defer *et al.*, 2013). Yet oyster hemolymph microbiota have

Correspondence: A Lokmer, Helmholtz Centre for Polar and Marine Research, Alfred Wegener Institute, Coastal Ecology, Wadden Sea Station Sylt, Hafenstrasse 43, List, Sylt 25992, Germany.
E-mail: alokmer@awi.de
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rarely been studied and so far only by means of culture-dependent methods (Olafsen *et al.*, 1993; Garnier *et al.*, 2007; Wendling *et al.*, 2014). However, neither cultivation nor molecular fingerprinting methods provide realistic estimates of community diversity and composition (Pedros-Alio, 2006; Bent and Forney, 2008). Next-generation sequencing, although by no means free of biases (Fierer and Lennon, 2011; Sergeant *et al.*, 2012; Cai *et al.*, 2013), enables detailed characterization of microbial community composition and dynamics, including rare phylotypes (Huse *et al.*, 2008) that can act as a seed bank and mediate community response to environmental change (Caporaso *et al.*, 2012; Pedros-Alio, 2012; Sjostedt *et al.*, 2012). Recently, amplicon sequencing has been used to characterize oyster gut (King *et al.*, 2012; Trabal Fernandez *et al.*, 2013) and gill microbiomes (Wegner *et al.*, 2013), resulting in higher estimates of α-diversity and challenging the long-held view that the oyster microbiota are dominated mainly by pseudomonads and vibrios (Prieur *et al.*, 1990; Olafsen *et al.*, 1993; Garnier *et al.*, 2007).

Temperature is an important factor in shaping microbial communities in marine abiotic and biotic habitats (for example, Fuhrman *et al.*, 2008). Shifts from mutualist- to pathogen-dominated communities due to global warming have already been reported (Ritchie, 2006), as well as an increase in occurrence of infectious diseases (Altizer *et al.*, 2013). At lower latitudes, warming usually implies crossing the upper limits of thermal tolerance and is almost certain to have adverse effects on the affected organisms (Lafferty *et al.*, 2004; Fan *et al.*, 2013). The outcome in temperate regions can be much less predictable, depending on thermal optima and ranges of hosts and pathogens (Thomas and Blanford, 2003). For example, temperatures over 20 °C are necessary for oyster summer mortalities to occur (Samain *et al.*, 2007; Watermann *et al.*, 2008), but it is low temperatures (<14 °C) that promote development of brown ring disease in clams (Paillard *et al.*, 2004).

Many temperature-dependent disease outbreaks have been linked to various *Vibrio* species (Lacoste *et al.*, 2001; Lee *et al.*, 2001; Garnier *et al.*, 2007; Cervino *et al.*, 2008; Elston *et al.*, 2008) that are commonly isolated from healthy marine animals (Prieur *et al.*, 1990; Olafsen *et al.*, 1993; Gomez-Gil *et al.*, 1998; Iida *et al.*, 2000; Vega Thurber *et al.*, 2009; Trabal *et al.*, 2012; Zhao *et al.*, 2012). The effects of temperature on *Vibrio* spp. virulence have also been demonstrated experimentally (Kushmaro *et al.*, 1998; Wendling and Wegner, 2013), making this group a suitable candidate for experimentally exploring temperature-dependent host–pathogen interactions in the marine environment.

So far, most of the research on microbial dynamics in marine poikilotherms has been observational and focused on seasonal changes (Beleneva and Zhukova, 2009; Preheim *et al.*, 2011; Zurel *et al.*, 2011; Erwin *et al.*, 2012; Carlos *et al.*, 2013; Mahalaxmi *et al.*, 2013). However, seasonality does not equal temperature (Farcy *et al.*, 2007; Gilbert *et al.*, 2012) and experimental studies addressing the temperature-dependent short-term microbial dynamics, which may be highly relevant to development of disease, are mostly confined to corals and sponges (Vega Thurber *et al.*, 2009; Simister *et al.*, 2012; Fan *et al.*, 2013).

Although higher temperatures usually promote microbial growth including pathogens, acclimated eurythermic hosts may be well prepared to deal with them. *In vitro* experiments on oyster hemocytes revealed little change in immunocompetence over wide range of environmentally relevant temperatures (Ashton-Alcox and Ford, 1998; Gagnaire *et al.*, 2006). Temperature stress, on the other hand, severely compromised host defenses (Malham *et al.*, 2009), illustrating the need to examine temperature effects in broader context of animal condition and history. Mortalities observed in wild populations of marine poikilotherms are often due to complex interplay of multiple stressors, such as in the well-documented case of oyster summer mortalities, (for example, Samain *et al.*, 2007; Wendling and Wegner, 2013) or *Vibrio harveyi* infection in abalones (Travers *et al.*, 2008). Controlled experiments are therefore the only way to assess the importance and relative contributions of individual factors.

To examine how infection, temperature and temperature stress affect oyster survival and the composition and dynamics of hemolymph microbiota, we experimentally challenged Pacific oysters with a virulent *Vibrio* spp. strain and exposed them to different temperature treatments. We thus present experimental data describing the short-term microbial dynamics in response to abiotic and biotic stress in Pacific oysters. With the combination of the above experimental factors and a high temporal resolution of microbial community dynamics, we can now try to link changes in microbial communities to different stressors and host survival.

Materials and methods

Biological material

All oysters were collected in a Wadden Sea tidal flat in Königshafen, Germany (55° 1′ 44″ N, 8° 26′ 3″ E) and subsequently kept in flow-through aquaria in climate chambers set to either 22 °C (warm-acclimated) or 8 °C (cold-acclimated). To avoid temperature shock during the transfer to the laboratory, the warm-acclimated oysters were collected in late august 2010, while the cold-acclimated oysters of matching size were collected 10 days before the experiment (beginning of November 2010). Warm-acclimated oysters were fed three times a week using shellfish diet instant algae mix (Varicon Aqua Solutions Ltd., Malvern, UK).

For infection, we used the isolate *Vibrio* sp. D29w affiliated to *Vibrio orientalis/tubiashii* clade based on multilocus sequence typing sequencing (Thieltges *et al.*, 2013), which was obtained from hemolymph of the local oyster population. This isolate was shown to induce intermediate levels of mortality upon injection in adults at ambient temperatures (Thieltges *et al.*, 2013), but was highly virulent for larvae at elevated temperatures (Wendling *et al.*, 2014). We cultured bacteria overnight in 8 ml of soya-peptone medium at 25 °C and shaking at 240 r.p.m. Bacterial cells were then collected by centrifugation for 10 min at 5000 r.p.m., resuspended in fresh soya-peptone medium and adjusted to the concentration of 2×10^8 cells per ml.

Experimental design and hemolymph sampling

The experiment was designed to examine the effects of temperature, temperature stress and infection in a fully crossed three-factorial design for duration of 7 days. A total of 48 oysters (24 animals from each acclimation group) were kept singly in aerated 2.5 l glass jars with no flow-through, after being randomly assigned to experimental treatments. The oysters were either left at their acclimation temperature or stressed by non-abrupt transfer (together with seawater, allowing for gradual temp. equalization) to the opposite temperature, resulting in four temperature treatments: cold-acclimated (CC), warm-acclimated (WW), cold-stressed (WC) and warm-stressed (CW). Half of the oysters in each of the four groups were injected either with *Vibrio* sp. D29w strain or with the pure soya-peptone medium. Injections of 500 µl ($\sim 10^8$ bacteria) were applied into the adductor muscle with $23 \times 1/4$ gauge (dm 0.60×30 mm) needle introduced via a notch drilled on a ventral shell side. During the experiment, the oysters were kept individually in fully-aerated 2.5 jars filled with the filtered seawater (Supplementary File 1).

To examine the composition and dynamics of hemolymph microbiota, 200 µl of hemolymph was drawn from the adductor muscle just before the experiment and on the third, fifth and seventh day of the experiment. Samples were immediately stored on ice and transferred to −80 °C as soon as possible. The oysters were checked for survival during hemolymph collection. To have a proxy for the abundance of vibrios, 4 µl of hemolymph was streaked on vibrio-selective TCBS (thiosulfate-citrate-bile-salts-sucrose) agar immediately after sampling, the plates were grown at 25 °C, and colony forming units were counted after 24 h cultivation.

DNA extraction and amplicon sequencing

DNA was extracted from 174 whole hemolymph samples (Table 1; Supplementary File 1) with the Illustra TriplePrep kit (GE Healthcare Life Sciences, Hamburg, Germany) according to the manufacturer's protocol. DNA concentration and purity were checked with a Nanodrop ND-1000 spectrometer (peqlab, Erlangen, Germany) and all samples were adjusted to equal DNA concentration (5 ng µl^{-1}).

Table 1 Number of oysters and hemolymph samples (analyzed/collected) per treatment and sampling point

Treatment[a]	*Sampling point (day)*				*Oysters per treatment*	*Samples per treatment*
	0	*3*	*5*	*7*		
CC_0	6/6	6/6	6/6	6/6	6	24/24
CC_I	6/6	6/6[b]	6/6[b,c]	4/4[c]	6	22/22[b,c]
CW_0	6/6	6/6	6/6	6/6	6	24/24
CW_I	6/6	6/6[c]	1/1	1/1	6	14/14[c]
WC_0	6/6	5/6	5/6	6/6	6	22/24
WC_I	6/6	6/6	5/6[b]	6/6[c]	6	23/24[b,c]
WW_0	6/6	6/6[c]	3/5	4/5	6	19/22[c]
WW_I	6/6	6/6	4/4[b]	4/4[c]	6	20/20[b,c]
Total	48/48	47/48[b,c]	36/40[b,c]	37/38[c]	48	168/174[b,c]

Abbreviations: CC, cold acclimated; CW, warm stressed; WC, cold-stressed; WW, warm-acclimated.
[a]First letter denotes acclimation temperature, Second experimental temperature, followed by infection status.
[b]Moribund oysters included. Total moribund samples: 5/6.
[c]Dead oysters included. Total dead samples: 14/14.

Ribosomal 16S rDNA V1-V2 region was amplified with a barcoded universal bacterial 27F (CTATGCGC CTTGCCAGCCCGCTCAG-MID-TCAGAGTTTGATC MTGGCTCAG)-338R (CGTATCGCCTCCCTCGCGCC ATCAG-MID-TGCTGCCTCCCGTAGGAGT) primer pair. Forward primers were marked with barcodes MID02 and MID03 and the reverse primers with MID01-MID98 (excluding MID09 and MID12). Each individual PCR including negative controls was coded by a unique combination of forward and reverse MIDs (Binladen *et al.*, 2007; Wegner, 2009). In all, 20-µl PCRs contained 1.5 mM $MgCl_2$, 0.2 mM of each nucleotide, 0.2 µm of each primer, 0.5 unit of Taq polymerase (GoTaq Flexi DNAp; Promega, Mannheim, Germany) and 20 ng of DNA template. DNA was amplified using the following protocol: 1 min initial denaturation at 94 °C, 30 cycles of 40 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and final extension 2 min at 72 °C.

Quality of PCR products was checked on the QIAxcel system using a QIAxcel DNA Screening kit (Qiagen, Hilden, Germany). Equal amounts (10 µl) of each product were then pooled together and cleaned by ethanol precipitation. Pooled samples were finally adjusted to 150 ng µl^{-1} and sequenced at Roche GS-FLX 454 platform at the Institute of clinical molecular biology at the Christian-Albrechts-University Kiel, Germany.

Data analysis

Processing of raw sequence data. Raw reads were demultiplexed using modified Python scripts from the cogent package (Knight *et al.*, 2007). In short, original binary file (sff) was split into multiple sff files corresponding to individual samples allowing

for only perfect matches to both barcodes and primers and translated into sfftxt-files using Mothur (Schloss *et al.*, 2009). Quality control included denoising and chimera removal and was performed using the AmpliconNoise V1.23 pipeline (Quince *et al.*, 2011). Only flowgrams with a minimum length of 200 bp before the first noise signal were kept for further analysis. Initial cutoff value for removing sequencing noise was 0.01 and cluster size 60. No ambiguities were allowed and maximum homopolymer length was set to 7. PCR noise removal cutoff value was 0.08 with the cluster size of 30. Chimeras were identified with Perseus and sequences with probability higher than 50% of being chimeras were excluded from further analysis. A custom perl script was then used to trim primer sequences and create an input fasta file for further analysis.

Raw demultiplexed sequence data are available at European Nucleotide Archive under the study accession number PRJEB5702 (sample accession numbers ERR457899–ERR458074).

We used the QIIME pipeline (Caporaso *et al.*, 2010) to create operational taxonomic unit (OTU) tables and perform rarefaction, taxonomical composition assessment and phylogenetic diversity analyses. OTUs were picked with uclust (Edgar, 2010) at a 97% similarity threshold. Taxonomy was assigned with RDP classifier (Wang *et al.*, 2007) to the genus level, with 60% confidence using the 110 Greengenes taxonomy 12_10 (McDonald *et al.*, 2012) as a reference database. The sequences assigned to the genus *Vibrio* were then compared with 16S rDNA sequences obtained from cultured strains isolated from hemolymph of local oysters (courtesy of C Wendling) to identify OTU corresponding to the injected strain. We defined contaminants as OTUs with more than five reads in negative controls and removed them from further analysis. This threshold was chosen because negative control samples represented pools of several individual reactions and most sequences were represented with a single read. Sequences were aligned with mafft (Katoh *et al.*, 2002) and a phylogenetic tree was built using fasttree (Price *et al.*, 2010). We calculated rarefaction curves for Shannon, evenness, species richness and phylogenetic diversity (Faith, 1992) to assess the effects of sample sizes on these α-diversity metrics.

Statistical analyses

All other statistical analyses were performed in R (R Core Team, 2013). Host survival analysis was conducted using the survival package (Therneau, 2013). For bacterial communities, we analyzed relative species abundance, α- and β-diversity with the Vegan package (Oksanen *et al.*, 2013). Differences in α-diversity patterns between treatments were analyzed using non-parametric tests and linear mixed models (nlme package; Pinheiro *et al.*, 2013). To assess β-diversity, we calculated Bray-Curtis and weighted UniFrac distances (Hamady *et al.*, 2010) between the samples and analyzed them by non-metric multidimensional scaling (NMDS) and the *Adonis* implementation of Permanova (non-parametric permutational multivariate analysis of variance; Anderson, 2001). Results are reported for Bray-Curtis distances if not explicitly stated otherwise. We also determined how abundant or dominant ($\geqslant$1% of sample) community members contributed to explaining the variation between the treatments. To statistically examine taxonomical composition and identify phylotypes associated with individual treatments and conditions, we applied indicator species approach implemented in indicspecies package (Cáceres and Legendre, 2009).

Results

Oyster survival upon infection

Experimental challenge with *Vibrio* sp. D29w resulted in 54% mortality of infected oysters, as opposed to a single death event in the control treatment ($\chi^2(1) = 14.52$, $P < 0.001$, odds = 25.33 (3.10, 1195.27)). Survival analysis of infected oysters revealed significant differences between warm-stressed animals and all the other groups (Peto & Peto modification of the Gehan-Wilcoxon test, $\chi^2(3) = 9$, $P = 0.029$). Not only did the warm-stressed oysters experience the highest mortality, but they also died earlier, within the first 3 days of the experiment (Figure 1).

Hemolymph microbiome: general characteristics

Results of sequence data processing are shown in Supplementary Table 1. Singletons (OTUs represented with a single read in the data set) and the samples with fewer than 100 reads were excluded

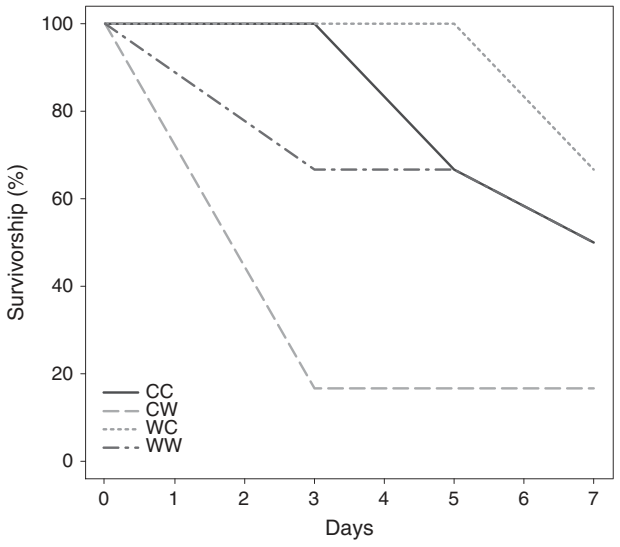

Figure 1 Survival curves for infected oysters. CC, cold acclimated; CW, warm stressed; WC, cold-stressed; WW, warm-acclimated.

from further analyses. It is noteworthy that four out of six low-coverage libraries came from two uninfected oysters (Table 1; Supplementary File 1), suggesting that low number of reads in these cases may reflect the true absence of bacteria.

Phyla Proteobacteria and Bacteroidetes encompassed the bulk of OTU diversity and abundance (Figures 2a and b; Supplementary Figure 1). Relative OTU abundances fitted well to Fisher's log-series distribution, both in the data set as a whole and in the individual samples (Kolmogorov–Smirnoff test, $D = 0.000–0.196$, $P > 0.881$, details not shown), indicating that few OTUs accounted for the majority of reads. The amount of variability in community composition explained by treatments was slightly higher when only abundant OTUs ($\geqslant 1\%$), and

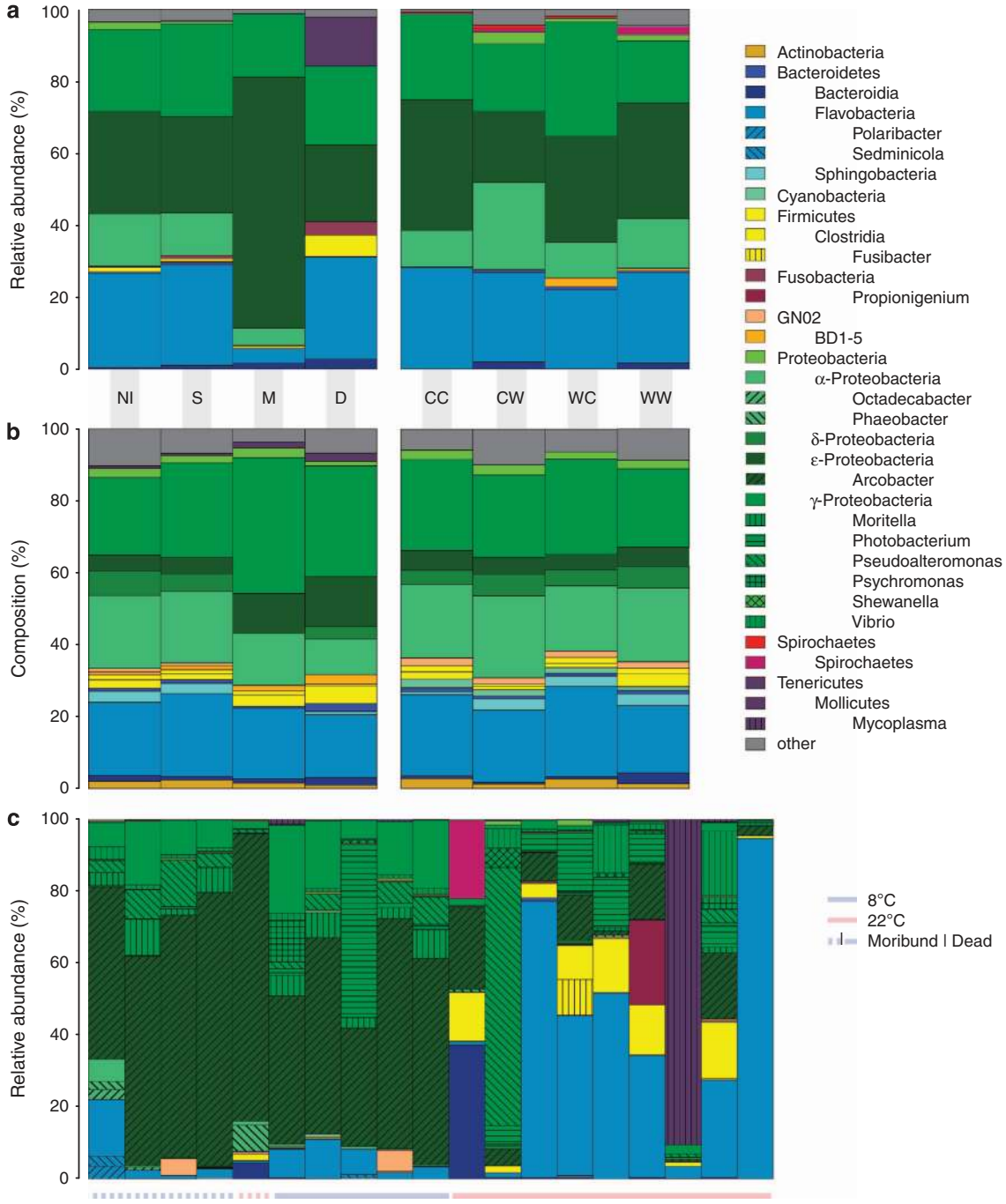

Figure 2 (**a**) Relative abundance and (**b**) taxonomical composition (number of OTUs per taxon) at the class level of hemolymph microbiomes (days 3–7) grouped by oyster condition (left, all oysters) and temperature treatments (right, alive oysters). (**c**) Relative abundance of genera in individual moribund and dead oysters. CC, cold acclimated; CW, warm stressed; WC, cold-stressed; WW, warm-acclimated; NI, non-infected; S, survivors; M, moribund; D, dead.

not the complete data set were considered (Supplementary Figure 2).

Rarefaction curves of α-diversity metrics show that Shannon's H and evenness—the indices we based our conclusions upon—were relatively stable even at the sampling depths of <100 reads. Furthermore, the relative differences between the treatments were fairly constant over a large range of sampling depths (Supplementary Figure 3). To assure negligible effects of sampling effort on α- and β-diversity analyses, we generated 10 random subsets of 100 reads per sample and calculated correlations between the α-diversity metrics (Spearman) and NMDS ordinations (Procrustes) based on subsets and the original data set. High correlation values indicated that our results were not influenced by differences in sampling depths of individual libraries (Supplementary Tables 2 and 3). The main analysis was thus based on the complete data set (excluding singletons) to keep the estimates of α-diversity realistic and to avoid information loss.

The transfer from the flow-through system to the experimental conditions was followed by significant drop in OTU diversity of alive oysters (39% OTUs were found only on day 0; Figure 3a) contributing to a clear distinction between pre-experimental and experimental communities (Supplementary Figure 4a). Loss of rare OTUs (mean relative abundance of OTUs present only on day 0 was 0.29%, quartiles: 0.14%, 0.36%) and increase in dominance were reflected in substantially lower Shannon's H (median: day $0 = 4.545$, days $3–7 = 3.059$, $W = 4931$, $P < 0.001$, $r = -0.718$) and evenness (median: day $0 = 0.899$, days $3–7 = 0.675$, $W = 4836$, $P < 0.001$, $r = -0.720$), regardless of treatment (Figure 3a; Supplementary Figure 5). Since pre-experimental communities could not be meaningfully assigned to the tested groups, they were omitted from the analyses concerning the effects of experimental treatments and oyster condition.

Hemolymph microbiome: effects of infection

To describe effects of infection on hemolymph microbiota, we grouped the infected oysters into three categories: surviving (S, alive at the next sampling point), moribund (M, dead at the following sampling point) and dead (D). We refer to control and surviving oysters together as healthy (H).

Experimental challenge barely affected α- (Table 2) and β-diversity (Table 3). Microbiomes from healthy oysters (H) formed largely overlapping clusters (Figure 4b), reflecting a comparatively small but significant effect of infection on β-diversity (Table 3). In contrast, moribund (M) and dead (D) oysters formed separate groups from healthy oysters in the NMDS plot (Figure 4a). M and D communities were characterized by proliferation of one or very few OTUs and therefore displayed very low α-diversity (Figure 3b). At 8 °C, the microbiomes of dead oysters (D) closely resembled those of moribund animals (M, Figure 2c), whereas we observed more variation in community composition and an increase in anaerobic bacteria soon after death at 22 °C.

In contrast to our expectation, the microbiomes of moribund oysters were dominated by the genus *Arcobacter* and not, as expected, by *Vibrio* spp. (Figure 2c; Supplementary File 2). Overall, the infected oysters harbored more strains from other genera with described pathogenic species, such as *Photobacterium* and *Shewanella* (Supplementary File 2).

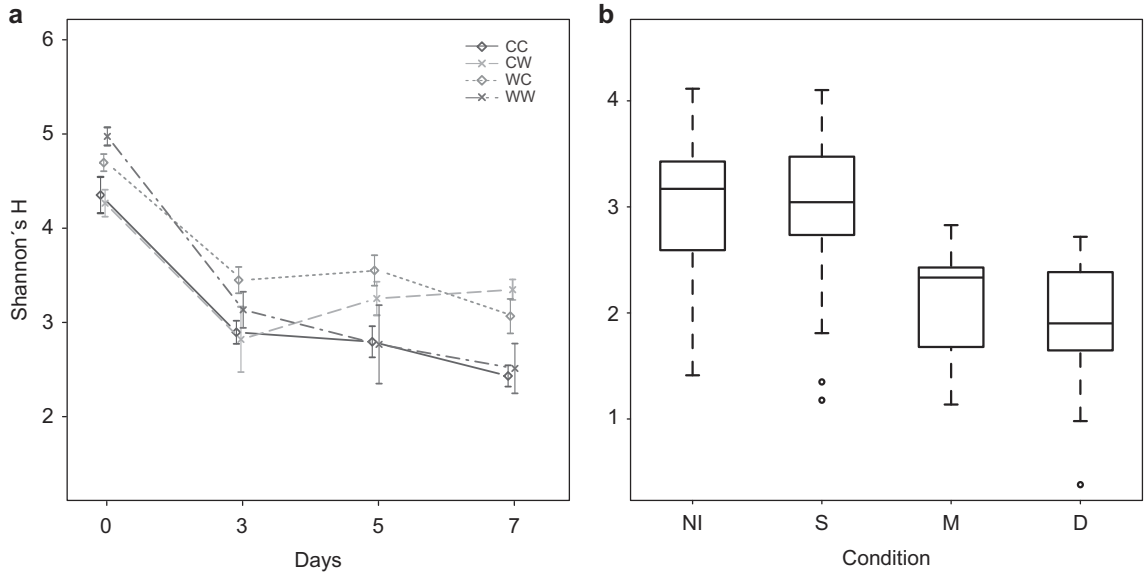

Figure 3 Alpha-diversity, expressed as Shannon's H, in response to (**a**) temperature treatments over time and (**b**) to oyster condition. Infection is not shown for clarity. Only alive oysters are shown in (**a**); day 0 samples are omitted from (**b**). Error bars represent standard error of mean. CC, cold acclimated; CW, warm stressed; WC, cold-stressed; WW, warm-acclimated; NI, non-infected; S, survivors; M, moribund; D, dead.

The indicator species analysis revealed that higher *Vibrio* sp. D29w abundances were associated with infection (IndVal = 0.769, $P = 0.017$), but not directly with mortality or disease (Supplementary File 2). Overall, the OTUs assigned to *Vibrionaceae* resp. genus *Vibrio* (including the injected strain) were common (present in 98.85%, resp. 92.26% of the samples), but not very abundant (5.1%, resp. 1.6% mean relative abundance). The presence of vibrios (other *Vibrionales* occasionally grow on TCBS agar, hence the taxonomically non-specific term) was lower (87.36%) when calculated from colony forming unit counts (Supplementary File 1). The discrepancy was most likely due to the low abundance coupled with the low volume of hemolymph plated. Unlike relative read abundances, higher colony forming unit counts were related to disease and death (generalized linear mixed model: $Z = -2.61$, $P = 0.009$ (moribund and dead) vs healthy parameter estimate: -0.249 ± 0.095, confidence interval (CI) = (-0.436, -0.062); Supplementary Table 4).

Hemolymph microbiome: effects of temperature and temperature stress

Before the experiment, Shannon's H (median: 8 °C = 4.228, 22 °C = 4.711, $W = 117$, $P < 0.001$, $r = -0.525$) and phylogenetic diversity indices (median: 8 °C = 19.697, 22 °C = 28.216, $W = 81$, $P < 0.001$, $r = -0.652$) were higher in warm-acclimated communities, with no difference in evenness between the groups (median: 8 °C = 0.653, 22 °C = 0.717, $W = 214$, $P < 0.128$). During the experiment, only the phylogenetic component (phylogenetic diversity) of α-diversity remained positively correlated with higher ambient temperature (Supplementary Figure 5; Supplementary Tables 5 and 6), indicating the presence of more rare, divergent phylotypes in warmer environment.

Both directions of temperature stress prevented a steady decrease in diversity and evenness that we observed in the acclimated communities (Figure 3a; Table 2; Supplementary Figure 5). The effect became more pronounced toward the end of the experiment, indicating its persistence and reflecting the gradual and lagged microbiome response to stress. We expected that the OTUs present or dominating the acclimated communities were already established in hemolymph before the experiment, thus getting a head start; and that, in contrast, the temperature stress would facilitate colonization by new species and promote growth of rare or dormant OTUs. However, we found little support for this hypothesis, as the proportion of the OTUs that were dominant in experimental communities and already present at the beginning did not significantly differ between the warm-acclimated and stressed oysters, although it was higher for cold-acclimated microbiomes (Supplementary Table 7).

Acclimation and experimental temperature, as well as their interaction, considerably affected the composition of hemolymph microbiota throughout the experiment (Table 3). Ordination by NMDS clearly separated the communities by temperature treatments (Figure 4b). Ordination and Permanova based on weighted UniFrac showed that little of phylogenetic β-diversity was attributable to the treatments (Supplementary Figures 2 and 4), reflecting similarity of the microbiome composition at above-OTU taxonomic levels. However, we found some indication for increase in potentially pathogenic genera in stressed oysters and at 22 °C in general (Supplementary File 2).

To estimate how experimental treatments affected microbial dynamics, we used the area of polygons connecting all samples from a single oyster in the NMDS plot as a proxy for temporal stability: the smaller the area, the more stable the community. Analysis of variance confirmed the visual impression (Figure 4b) that microbiomes in the cold environment were more stable compared with microbiomes from the warm environment ($F_{1,26} = 17.86$, $P < 0.001$). Stress and infection, on the other hand, had no significant effect on temporal

Table 2 Linear mixed model for treatment and condition effects on Shannon's H, day 0 samples excluded (AIC = 275.599, BIC = 320.717, logLik (d.f. = 17) = −120.800)

	Factors	*d.f.*	*F*[a]	*Significant contrasts*	*Estimate*	*s.e.*	*2.5% CI*	*97.5% CI*
Main effects	Acclimation temp.	1, 43	1.776					
	Experimental temp.	1, 43	2.547					
	Time	2, 62	0.735					
	Health status	2, 62	13.569***	Moribund and dead vs healthy	0.298	0.06	0.177	0.418
	Infection	1, 43	0.735					
Interaction terms	A. temp. × E. temp.	1, 43	14.280***	Stressed vs acclimated	−0.421	0.11	−0.646	−0.196
	A. temp. × Time	2, 62	1.407					
	E. temp. × Time	2, 62	4.954*	E. temp. on (day 3 vs days 5–7)	0.148	0.05	0.042	0.253
	A. temp. × E. temp. × Time	2, 62	3.361*	(Stress. vs acc.) on day 3 vs on days 5–7	−0.178	0.07	−0.321	−0.035
Random intercept	Oyster				0.113		0.005	2.671

Abbreviations: AIC, akaike information criterion; BIC, bayesian information criterion; CI, confidence interval; d.f., degrees of freedom.
[a]Significance levels: * ⩽ 0.05, *** < 0.001.

microbiome dynamics ($F_{1,26}=0.157$, $P=0.695$, $F_{1,26}=0.89$, $P=0.367$).

Discussion

Host–pathogen interactions have an important role in population dynamics and evolution of organisms. Although microbes inherently differ in their pathogenic potential, a disease usually arises from a complex interplay of multiple factors. Here, we show that temperature represents a notable determinant of microbial dynamics in oyster hemolymph, and propose that the lower temporal stability of microbiomes at higher temperatures may have contributed to the higher mortality of the heat-stressed hosts upon infection. We also show that a decrease in diversity and proliferation of opportunistic pathogens precede death, thus representing a good indicator of declining health.

Table 3 Adonis (Permanova) results for experimental communities (day 0 excluded) based on Bray-Curtis distances

	d.f.	*SS*	*MS*	*pseudoF*	$R^{2\,a,b}$
Main effects					
Acclimation temp.	1	3.308	3.308	12.152	0.076***
Experimental temp.	1	4.062	4.062	14.923	0.093***
Time	2	1.435	0.717	2.636	0.033***
Infection	1	0.853	0.853	3.133	0.020***
Health status	2	1.769	0.885	3.25	0.041***
Interaction terms					
A. temp. × E. temp.	1	1.154	1.154	4.239	0.026***
A. temp. × Time	2	0.729	0.365	1.34	0.017**
E. temp. × Time	2	1.034	0.517	1.899	0.024***
A. temp. × E. temp. × Time	2	0.672	0.336	1.235	0.015**
Residuals	105	28.579	0.272		0.656
Total	119	43.594			1

Abbreviations: MS, mean sum of squares; SS, sum of squares.
[a]Significance values based on 999 permutations.
[b]Significance levels: ** < 0.01, *** < 0.001.

Infection, microbiota and oyster health

Higher mortality at 22 °C can be partially attributed to faster growth of the injected strain (Supplementary Figure 6) and/or to temperature-dependent increase in expression of virulence factors (Kimes *et al.*, 2012). The effect of absolute temperature, however, cannot account for the difference in mortality between stressed and acclimated oysters. Wang *et al.* (2012) reported synergic effects of heat stress and infection on scallop survival and attributed it to energetic stress. A similar mechanism may have played a role for the oyster mortality, because only heat stress, but not cold stress has been shown to increase energy consumption in Pacific oysters (Bougrier *et al.*, 1995; Malham *et al.*, 2009).

Injection of the virulent *Vibrio* sp. strain clearly caused mortality, but was also associated with an increased number of cultivable vibrios in the oyster hemolymph. In contrast, we could not directly link incidence or abundance of the injected strain in the sequenced libraries to disease (Supplementary File 2). For once, we cannot discriminate between sequences from active and inactive cells that would not cause disease (Williams *et al.*, 2009). Moreover, *Vibrio* spp. can have significant influence on the host health, despite the low relative abundance (Vega Thurber *et al.*, 2009). Still, low abundance of the injected strain suggests that other vibrios could

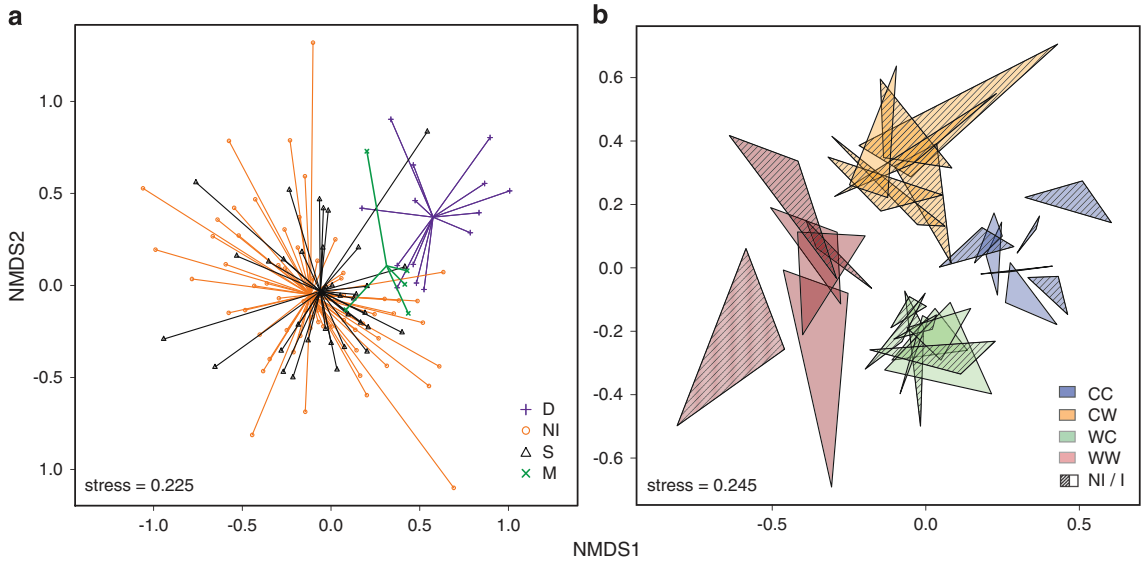

Figure 4 NMDS plots of Bray-Curtis distances between the microbial communities (day 0 excluded): (**a**) grouped by oyster condition and (**b**) showing temporal stability and effects of treatments (alive oysters only). Each triangle represents an oyster with vertices representing days 3, 5 and 7 after the onset of experiment. CC, cold acclimated; CW, warm stressed; WC, cold-stressed; WW, warm-acclimated; NI, non-infected; S, survivors; M, moribund; D, dead.

have contributed to the mortalities, as the exogenous bacteria can be cleared quickly from the hemolymph (Parisi *et al.*, 2008), while stimulating growth of inactive residents (Froelich and Oliver, 2013).

The abundance of *Arcobacter* spp. in moribund oysters also suggests a pronounced role of the indirect effects for the mortalities (Hauton *et al.*, 1997). *Arcobacter* spp. is often found in association with marine organisms—ranging from bottle-nosed dolphins (Lima *et al.*, 2012), marine seaweeds (Hollants *et al.*, 2011), crabs (Givens *et al.*, 2013), mussels (Collado *et al.*, 2009), abalones (Tanaka *et al.*, 2004) and oysters (Romero *et al.*, 2002). The ε-Proteobacteria are usually rare in coastal seawater and sediments (Campbell *et al.*, 2011; Gobet *et al.*, 2012), and they were rare in recent studies of oyster stomach (King *et al.*, 2012), gut (Trabal Fernandez *et al.*, 2013) and gill microbiota (Wegner *et al.*, 2013). Thus, the high abundance of *Arcobacter* spp. here could mean that these strains represent hemolymph-specific symbionts, which were not investigated in previous studies of oyster microbiota. *Arcobacter* spp. are often microaerophilic (Vandamme and Deley, 1991) and growth in the oysters could be facilitated by periodical valve closing (Sow *et al.*, 2011) or high variation in respiratory time activity (Bougrier *et al.*, 1998)—both of which may promote microaerophilic conditions. Dominance of *Arcobacter* spp. in moribund oysters, on the other hand, might have been a consequence of increased hypoxia due to disease-induced reduction of filtration activity (McHenery and Birkbeck, 1986). Nevertheless, the high abundance of *Arcobacter* spp. strains in moribund oysters, starved abalones (Tanaka *et al.*, 2004) and necrotic sponges (Fan *et al.*, 2013) may suggest their potential as opportunistic pathogens when occurring in high enough densities (Olson *et al.*, 2014). Dominance of *Arcobacter* spp. in unhealthy animals resulted in low microbial diversity, in contrast to the diverse microbiomes of infection survivors, which were barely distinguishable from controls (Figures 3b and 4b). This might reflect the crossing of a resilience threshold that a healthy community has against disturbance (Lozupone *et al.*, 2012). We cannot directly relate microbiome stability to disease resistance, but low diversity has repeatedly been found to coincide with impaired health in various animals (Garnier *et al.*, 2007; Chang *et al.*, 2008; Green and Barnes, 2010).

Microbial community dynamics in relation to infection and temperature stress

Warm temperatures can lead to higher heterogeneity in microbiome composition (Erwin *et al.*, 2012; Boutin *et al.*, 2013) and stress can favor shift toward more pathogen-dominated communities (Boutin *et al.*, 2013). Thus, the lower community stability at 22 °C (Figure 4b) and temperature- or stress-related increase in potentially pathogenic bacteria (Supplementary File 2) could have contributed to the observed mortality pattern. However, microbiomes often remain stable and pathogen-free even in stressful conditions (Erwin *et al.*, 2012; Simister *et al.*, 2012; Pita *et al.*, 2013; Wegner *et al.*, 2013), indicating their potential role in host acclimation (Rosenberg *et al.*, 2007). Phylogenetic similarity of the microbiomes suggests a common origin from the local seawater microbiota (Lozupone *et al.*, 2007). The drop in diversity following the transfer to the non-flow-through conditions was associated with a loss of rare and probably transient OTUs, underlining the importance of immigration for the assembly of the hemolymph microbiome. However, the persistence of relatively rare, presumably resident bacteria—such as *Vibrio* spp. (Pruzzo *et al.*, 2005)—contrasts the loss of transient OTUs (Romero *et al.*, 2002) and indicates that the hemolymph microbiome is not a simple result of filter-feeding lifestyle. While the individual stability indicates the importance of host genotype for the community assembly (Wegner *et al.*, 2013), the fine-scale differences between the treatments also illustrate acclimation potential of the hemolmyph microbiome. Multiple competing ecotypes usually coexist in bacterial populations (Cohan and Koeppel, 2008) and even the isolates with virtually identical 16s rRNA sequences are sometimes adapted to very different conditions (Cohan and Koeppel, 2008; Hall *et al.*, 2010), We cannot determine bacterial activity (Campbell *et al.*, 2011) nor function (Salles *et al.*, 2012) based on 16s rDNA sequences; moreover, our coverage is insufficient to capture very rare bacteria and thus estimate the full potential for community acclimation (Sjostedt *et al.*, 2012). Nevertheless, relatively high persistence of bacterial residents and the adjustments in fine-scale community composition following the environmental change could represent the microbiome's way to buffer the impact of environmental stress. Shifts in composition of the endogenous OTUs can thus prevent growth of external pathogens (Sjostedt *et al.*, 2012; Froelich and Oliver, 2013) and contribute to the maintenance of homeostasis.

The microbiome of warm-acclimated oysters might have been influenced by their extended time to acclimate to laboratory conditions. This bias would mainly influence day 0 samples, where we cannot discriminate between effects of temperature or acclimation. Since we can assume that cold acclimated oysters recovered from handling stress at the start of the experiment (Thompson *et al.*, 2012), we mainly focus on those results where oysters could uneqivocally be assigned to experimental treatments (days 3–7) for our interpretation to avoid any such bias. During those stages of the experiment, the quick shift of communities in response to experimental conditions, the strong effect fo experimental temperature (Supplementary Figures 2 and 3) and the similarity of microbiomes from surviving oysters in response to infection indicate that oyster history did not largely affect our results throughout the experiment.

Our detailed insight into short-term microbial dynamics of Pacific oyster hemolymph microbiota in response to environmental conditions and infection shows that temperature is indeed a master switch determining community structure and dynamics of oyster-associated microbiota. Microbial communities responded quickly to environmental change, but remained relatively stable within individuals for the duration of the experiment. Community disturbance by heat stress, coupled with host stress and faster bacterial growth at 22 °C may have acted in concert to cause high mortality rates. Stress can also amplify the direct immediate effects by giving rise to secondary opportunistic pathogens (for example, *Arcobacter*). Heat stress alone was not sufficient to cause mortality, showing that direct or indirect effects of pathogenic bacteria are necessary to induce mortality. To disentangle direct from indirect effects mediated by resident microbial communities further studies are needed for increasing the temporal resolution during the early phases of an infection to cover more bacterial communities associated with moribund oysters. The robustness of microbial communities against infection and plasticity in response to temperature suggest that the hemolymph microbiome can indeed have a vital role for host defense in changing environments.

Conflict of Interest

The authors declare no conflict of interest.

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