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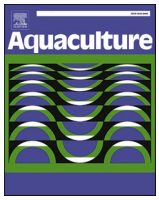

The gut microbiome and host molecular response of a grouper to acute and chronic heat stress

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ABSTRACT

Globally, aquaculture and fisheries play an important role in providing animal protein for human consumption. However, climate change poses a significant threat to these industries, with marine heatwaves becoming increasingly frequent and severe. Especially the effect of heat stress on the important symbiotic relationship of a host and the intestinal microbiome is understudied. In this study, we investigated the impact of short-term acute heat stress (3 days, AHS) and a longer-term marine heatwave (21 days, MHW) of +3 °C on the microbial community and molecular response of commercially and culturally important juvenile Malabar grouper.

Our findings indicate that both the AHS and MHW resulted in measurable effects on the fish, which are still detectable after a four-week recovery period. While the microbial richness in the stomach showed a decreasing trend post-heatwave, no significant increase in pathogenic *Vibrio* was observed. In contrast, the host transcriptome of the stomach, particularly in regard to the MHW, showed a response in the form of a downregulation of mitochondrial function and digestive processes. After recovery, these effects turn into an upregulation of tissue repair and extracellular matrix reorganisation. Contrastingly, the pyloric caeca showed minimal response to either treatment, suggesting maintenance of function during both AHS or an MHW.

These findings highlight the variable response of the Malabar grouper to AHS and an MHW. While some aspects reveal resilience, such as the absence of increased *Vibrio* in the stomach and the stability of both microbiome and host transcriptome in the pyloric caeca, other indicators suggest negative impacts. Overall, the results indicate that while the fish can cope with AHS and a strong MHWin certain aspects, others can pose challenges. Understanding these complex dynamics is crucial for assessing the full impact of climate change on both wild and cultured populations of economically and culturally important mesopredators.

1. Introduction

Aquaculture and fisheries account for ~17% of animal protein for human consumption worldwide (FAO, 2022). This figure is even higher for coastal and island nations, reaching up to 50% in several countries in Asia and Africa. These regions rely heavily on their fishery and aquaculture industries to ensure food security and economic prosperity; however, climate change and its associated effects pose a serious threat to these industries (Lee et al., 2023). While different climate change models predict varying future scenarios, some effects have already been observed now. Particularly concerning are marine heatwaves, which are increasing in magnitude, frequency, and length as climate change advances (Oliver et al., 2018). These heatwaves disproportionately affect areas highly dependent on marine fisheries and aquaculture for their livelihoods, impacting fish populations both in coral reefs and coastal aquaculture farms (Galappaththi et al., 2020; Morales-Nin et al., 2024). Coastal aquaculture and mariculture production systems are particularly vulnerable as they often rely on the ambient environment for their operation, making them vulnerable to changes to the ocean temperature and chemistry (Mugwanya et al., 2022).

Marine heatwaves can illicit both chronic and acute heat stress in fish, leading to suppressed immune responses, impaired metabolism, oxidative stress, and generally impaired fitness and growth of an organism (Islam et al., 2022). For example, increased temperatures can

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result in decreased performance, decreased mitochondrial energy transduction efficiency increasing the cost of growth (Little et al., 2020). This decreased performance and increased energetic cost can be further exacerbated by decreased nutrient uptake due to temperature induced disruptions on food digestion and absorption in the gastrointestinal tract (GIT) of fish (Volkoff and Rønnestad, 2020). These combined effects can lead to significant reduction in annual biomass and available protein. However, it is still unclear to what extent this heat stress can affect the microbial communities associated with their fish host. Investigating this is important because it has been well established that there is an association between aquaculture productivity and microbial diversity, as changes in bacterial microbiomes are implicated in animal performance and health, in disease prevention of both bacterial and viral origin, and in dysbiosis triggered by environmental stressors or diet choice (Infante-Villamil et al., 2021). These common aquaculture diseases, many of which are of bacterial origin, cost billions of dollars in losses every year (Lafferty et al., 2015). For example, the mortality caused by vibriosis, an illness caused by the ubiquitous bacterial genus *Vibrio*, is estimated to cause a mortality of 16.2% in Asian seabass (*Lates calcarifer*) cultured in Malaysia (Mohd Yazid et al., 2021).

Based on this, the development of management strategies towards preserving the microbial balance, including maintaining or increasing diversity in the host, is critical for the health of cultured aquatic animals and will be critical for the expansion of aquaculture. In any system, the microbial community can be influenced by many environmental factors (e.g., temperature, pH, salinity, turbidity, light, etc.) (Infante-Villamil et al., 2021). While the general body of knowledge of environment-host-microbiome interactions is growing, information on how increased temperatures, in the form of transient marine heatwaves or future predicted temperatures caused by climate change, affect the host microbiome is still limited. There are indications that increased temperatures not only negatively affect the host organism directly (e.g., through stress or metabolic changes), but can also increase the incidence rate of bacterial dysbiosis which then further leads to impacted health (Cascarano et al., 2021). These health impacts can range from nutrient deficiency (e.g., lack of Vitamin B_{12} produced by bacteria) and impaired digestion, to decreased protection against pathogenic bacteria like *Vibrio* spp. which then paves the pathway to full blown infections, and all potentially culminating in inflammatory, metabolic, and neurodegenerative diseases (Medina-Félix et al., 2023). The genus *Vibrio* is a cosmopolitan marine genus within the family *Vibrionaceae*, which contains both (facultative) pathogenic and probiotic species (Vandenberghe et al., 2003). So while the genus *Vibrio*, and as an extension the family *Vibrionaceae* as a whole, are often considered to be a biomarker for pathogenicity and dysbiosis, this is highly dependent on the individual bacteria involved and the environmental conditions (Brumfield et al., 2021; Hassan et al., 2021).

As a large and long-lived mesopredator, the Malabar grouper (*Epinephelus malabaricus*) provides important functional roles on coral reefs, such as regulating communities through the top down removal of smaller fish like damselfish (Pomacentridae) and wrasses (Labridae) (Boaden and Kingsford, 2015; Hempson et al., 2018). However, their reliance on structurally complex reef environments and slow growth rates make grouper susceptible to anthropogenic disturbances like climate change and reef degradation (Hempson et al., 2017). Moreover, of the coral reef fish studied to date, mesopredators are severely understudied, therefore limiting our understanding of how complex reef fish communities will respond to warming. This is partially due to groupers being more difficult to research due to their size and slow reproductive cycle, in contrast to smaller species which have short generation times and are easier to culture (e.g., damselfish (Veilleux et al., 2018) or anemonefish (Moore et al., 2024)) or other mesopredators with generation times of 1 to 2 years (e.g., snapper (McMahon et al., 2023) or coral trout (Johansen et al., 2015)). This is also evident in the lack of research on tropical aquaculture species as reviewed by (Islam et al., 2022), even though groupers are a commonly produced aquaculture species in Asia (Rimmer and Glamuzina, 2019).

The stomach of predatory fish mostly functions as food storage and is the location of mechanical and chemical pre-digestion (Egerton et al., 2018). In this regard, the stomach provides an important function to prepare for nutrient absorptions in the later parts of the gastrointestinal tract (GIT), which can be impaired during heat stress. In contrast, the pyloric caeca provide an environment with an increase surface for further digestion and also absorption of some nutrients like amino acids and fatty acids (Egerton et al., 2018). While an important mechanism, to date there is a lack of studies on the effect of heat stress on the molecular response in stomach and pyloric caeca, especially combining microbiome and transcriptomic measurements in long-lived mesopredators.

Nutrient absorption is an important factor in the growth and health of wild fish populations on coral reefs and in aquaculture production. Therefore, the aim of the present study was to investigate the effect of a short 3-day acute heat stress and a longer 21-day marine heatwave of +3 °C on the host molecular response and microbial community in juvenile Malabar grouper (*E. malabaricus*). Sampling was done immediately after both an AHS and the MHW treatments, as well as after four weeks of recovery at the control temperature, in order to assess possible long-lasting effects of the heatwave.

2. Materials and methods

2.1. Fish husbandry conditions

Epinephelus malabaricus juveniles were obtained from a single spawning at the Okinawa Prefectural Sea Farming Center, Motobu-cho, Okinawa, Japan. The experiment was carried out at the Okinawa Institute of Science and Technology Marine Science Station located in Onna, Okinawa, Japan. Sixty fish (~ 60 days old) were randomly distributed across 10 experimental tanks with six fish per tank. Tanks were initially kept at 28 °C in a flow through system for 30 days until the start of the experiment. The initial average weight and length of the fish was 3.4 g ± 0.9 and 50.7 mm ± 5.1, respectively, with no significant differences among treatment groups (ANOVA, p-value >0.05). Furthermore, length and weight measurements among the three treatment groups were not significant at any of the measured timepoints (ANOVA, p-value >0.05). At the first sampling point (after the end of the heat treatments), the average weight and length of the fish was 7.4 g ± 3.0 and 64.4 mm ± 9.8, respectively. At the second sampling point (after the recovery period) the average weight and length of the fish was 12.1 g ± 5.0 and 73.7 mm ± 11.3, respectively. All length measurements can be found in Suppl. Data S1 and Suppl. Fig. S1. Fish were fed ~5% body weight of formulated feed (Otohime EP4, Nisshin Feed Co.) each morning and evening throughout the whole experiment.

The experimental conditions were divided into control (28 °C), acute heat stress (AHS, 3 days of 31 °C), and marine heatwave (MHW, 21 days of 31 °C). The temperatures were chosen based on Moore et al. (2023, 2024). In brief, the control temperature of 28 °C is the average temperature of Okinawan Sea Surface Temperature in summer (Loya et al., 2001. The treatment temperature was set to 31 °C based on future ocean warming by IPCC SSP3–7.0 predictions (IPCC, 2021; Masson-Delmotte et al., in press) and present-day marine heatwaves (Wernberg et al., 2013). Furthermore, the heatwave treatment follows the classification of Hobday et al. (2016) in length (≥5 days) and no days below threshold. Based on length and intensity, this heatwave can be classified as a strong heatwave (Hobday et al., 2018).

At the start of the experiment, the ten tanks were split into the three treatment groups, four tanks at 28 °C (control), three tanks were raised gradually to 31 °C over 12 h (3-week MHW treatment), and the remaining three tanks were initially kept at 28 °C (3-day AHS treatment) (Fig. 1). The treatments were maintained for three weeks, and the temperature in the tanks for the AHS treatment was gradually raised to 31 °C (over 12 h) in the last three days. Fish were sampled at the end of the 3-week period; length and weight were recorded for all fish, and

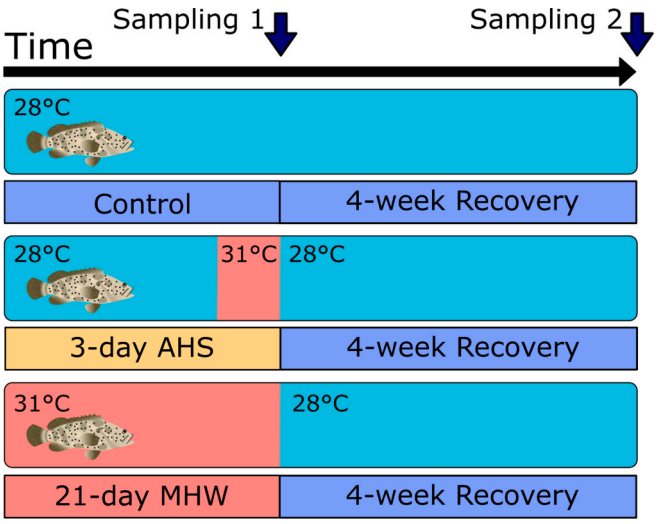

Fig. 1. Experimental design of 3-day acute heat stress and 21-day marine heatwave at +3 °C with arrows indicate times of sampling after the treatment period and after four weeks of recovery. (Grouper figure by Christine Thurber, Integration and Application Network, ian.umces.edu/media-library.)

eight fish per treatment were sacrificed to obtain tissues for further analysis (see below). Sampling was staggered over three days, each day sampling from all three conditions; therefore, the raising the temperature in the tanks of the acute treatment was also staggered across three days. After sampling, temperatures in all tanks were brought down to 28 °C over 12 h and fish were allowed to recover for four weeks, when the same sampling was repeated.

The experiment was conducted in accordance with the 2006 guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan and were approved by the Committee for Care and Use of Animals at the Okinawa Institute of Science and Technology, an AAALAC-accredited facility, under approval N°2019–252-3.

2.2. Dissections

During sampling, the fish were euthanized by cervical dislocation, and immediately dissected. Stomach and pyloric caeca (see Fig. 2) were preserved in RNAlater™ (ThermoFisher Scientific) immediately after dissection for tissue specific transcriptome sequencing and 16S rRNA gene bacterial diversity profiling.

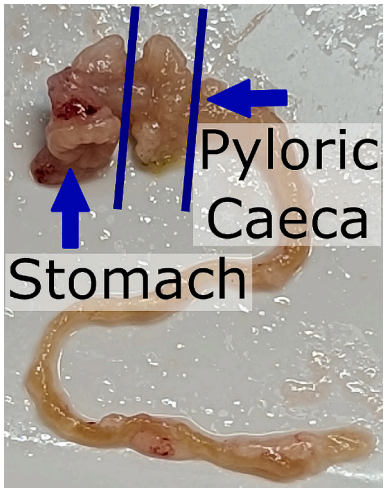

Fig. 2. Stomach and pyloric caeca tissue from juvenile Malabar grouper (*Epinephelus malabaricus*) sampled for this study.

2.3. DNA/RNA extractions

The tissues of five of the eight fish collected for each treatment were used for the extractions. DNA and RNA was co-extracted from fish stomachs and intestines using the ZymoBIOMICS™ DNA/RNA Miniprep Kit (Zymo, R2002) following the manufacturer's instructions, with minor modifications in the preparation step for homogenization. 1000 µL DNA/RNA shield was added along with 20–30 mg of fish tissue to ZR BashingBead Lysis Tubes (2.0 mm, Zymo Research, S6003). Bead beating was done using a MP Fast-prep bead beater for 1 min at 10 m/s to lyse the host tissue. Then the complete lysate was transferred to a ZR bead tube (0.1 mm & 0.5 mm, Zymo Research, S6012) for bacterial lysis in five rounds of bead beating. Each round consisted of 6.5 m/s for 1 min interspersed with 3 min cooling breaks on ice. Following the protocol, after centrifuge, 400 µL supernatant was taken for extracting DNA and RNA separately following the manufacturer's protocol. Extraction blank controls were extracted alongside each extraction batch. Zymo Bacterial Community Standard (75 µL, Zymo Research, D6300) was included as a positive control sample.

2.4. Library preparation and sequencing

For the microbial sequencing, the V3 and V4 hypervariable regions of the 16S rRNA gene were amplified using Illumina primers 341F: 5′ TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG and 805R: 5′ GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C (Klindworth et al., 2013). The mixture for the first stage Polymerase Chain Reaction (PCR) consisted of 12.5 µl Q5 High-Fidelity 2× Master Mix (NEB, M0492L), 0.25 µl of each primer at 20 µM, 10 µl nuclease free water, and 2 µl of template DNA. ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, D6306) was included as PCR positive control and water as a no-template negative control. Cycling conditions were as follows: (i) 98 °C for 30 s; (ii) 35 cycles at 98 °C for 10 s, 60 °C for 30 s and 72 °C for 30s; (iii) and a final elongation step at 72 °C for 2 min. DNA libraries were prepared following the Illumina 16S metagenomic sequencing protocol (Part #15044223 Rev. B). The remainder of the library preparation and sequencing was carried out at the Okinawa Institute of Science and Technology Sequencing Section (OIST, Japan) on an Illumina MiSeq platform using a V3, 600 cycle kit with paired end reads of 300 bp length.

For the transcriptomics analysis, the library preparation was also carried out at the OIST Sequencing Section using the NEBNext® rRNA Depletion Kit (Bacteria) (Cat #E7850) using both bacterial as well as grouper specific rRNA probes (Suppl. Table 1). Grouper specific probes were created using the NEBNext Custom RNA Depletion Design Tool (https://depletion-design.neb.com/). Sequencing was carried out with the pooled library split across two lanes of a Illumina NovaSeq 6000 S4 flow cell generating paired-end reads of 150 bp length.

2.5. Microbiome analysis

The quality measures of the raw data for the microbiome analysis was assessed using FastQC (Andrews, 2010) and summarised with MultiQC (Ewels et al., 2016) (Suppl. Material 1)The microbiome analysis was carried out in DADA2 (V1.24.0) (Callahan et al., 2016), following the recommended workflow. Quality and length trimming was done using the filterAndTrim function with the following parameters: truncLen = c(240, 240), trimLeft = c(20,21), maxN = 0, maxEE = c (3,4), truncQ = 2, rm.phix = TRUE. Chimeric sequences were removed using the consensus method. Taxonomic assignment of resulting ASVs was carried out using the SILVA database (V138.1) (Quast et al., 2012; Yilmaz et al., 2014) obtained from zenodo (DOI:https://doi.org/10.5281/zenodo.4587955). The final ASV table, metadata, and taxonomic assignment was then combined into a single object using phyloseq (V1.40.0) (McMurdie and Holmes, 2013) for further processing.

Filtering included removing any ASV with no taxonomic assignment at the phylum level (i.e., phylum = NA), removing ASV identified as Chloroplast at the order level (since they are of plant origin) and ASV identified as Mitochondria at family level (since they are of Eukaryotic origin). The final read tracking statistics across the DADA2 pipeline can be found in Suppl. Data S2, the sample metadata file in Suppl. Data S3, the final ASV table in Suppl. Data S4, and the taxonomic assignments in Suppl. Data S5. For the statistical analyses of the Richness and Evenness data, a Shapiro-Wilk normality test and a Levene's Test for Homogeneity of Variance were carried out in RStudio. This was then followed by a one-way Analysis of Variance (ANOVA). For Differential Abundance Analysis of the bacteria, the LinDA function implemented in the MicrobiomeStat package (Zhou et al., 2022) was used. Data was normalised using Total Sum Scaling where appropriate (McKnight et al., 2019).

2.6. Transcriptomic analysis

Raw read quality was assessed using FastQC (Andrews, 2010). Low quality bases and adaptor sequences were filtered using TrimGalore (V0.6.5) (Krueger, 2015) and cutadapt (V2.10) (Martin, 2011). Cleaned reads were mapped using STAR (V2.7.9a) (Dobin and Gingeras, 2015) with "–quantMode GeneCounts", using the previously published genome and annotation gene models (PRJNA798702, (Huerlimann et al., 2024)). Trimming, mapping, and quality control, with the exception of no kraken filtering being done, was carried out as described in Huerlimann et al. (Huerlimann et al., 2024) and github.com/R-Huerlimann/Malabar_grouper_genome. The quality control results of the quality filtering and read mapping were summarised using MultiQC (Ewels et al., 2016) and are available in the form of a html report (Suppl. Material 2 and 3 for filtering and mapping, respectively). The unstranded mapped reads were then loaded into Rstudio (V2022.02.4) (Racine, 2012) using R (V3.6.3) (R Core Team R, 2013). DESeq2 (V1.36.0) (Love et al., 2014) was used for general data analysis. A soft filtering was applied to remove genes that did not reach at least ten counts in five samples (e.g., dds[(rowSums(counts(dds) >= 10) >= 5),]). Lists of differentially expressed genes (alpha = 0.05, log2FoldChange = 0.58) were generated using the 28 °C treated fish at the end of the treatment period or at the end of the recovery period as reference for the respective treatments. GO-term enrichment was carried out on significantly up or down regulated genes (both up/down regulated together, as well as up or down regulated separately) using the *enricher* function from the R-package *clusterProfiler* (V4.10.0) (Wu et al., 2021; Yu et al., 2012) with a pvalueCutoff = 0.05 and pAdjustMethod = "fdr". GO-terms were taken from the genome annotation as per (Huerlimann et al., 2024) and KEGG pathways were annotated using KAAS (Moriya et al., 2007). GO-terms were only considered if they appeared in at least three genes, in addition to the normal adjusted *p*-value cut-off of 0.05. In general, GO-terms and KEGG pathways were used to complement the interpretation of the differentially expressed genes and also used for grouping purposes. Figures were plotted using ggplot2 (V3.4.1) (Wickham and Wickham, 2016), and the analysis made general use of the tidyverse package (V1.3.2) (Wickham et al., 2019). All significantly Differentially Expressed Genes, enriched GO-terms and enriched KEGG pathway results can be found in the Suppl. Tables S2-S36. Plots of the principal component analyses (based on significant genes as per LRT analysis) and Venn diagrams (significant genes based on contrasts of AHS and MHW against the respective control) for the two tissues can be found in Suppl. Fig. S2.

3. Results

3.1. General characteristics of the sequencing datasets

The 16S rRNA gene sequences were of high quality and resulted in 146,993 ± 7,727 raw reads (mean ± stdev) per sample, with 87.7% ± 3.2 (mean ± stdev) of the reads left after filtering and chimaera checking in DADA2 across 2,972 ASV. Further filtering of the ASV (removal of eukaryotic contamination and ASV with no taxonomic assignment at the phylum level) resulted in a total of 2,748 ASV used for the analysis. Concerning the positive controls, the microbial community cellular standard showed a similar bacterial community to the microbial community DNA standard (Suppl. Fig. S3 A). This indicates that the extraction efficiently lyses both gram positive and negative bacteria with minimal bias. Both DNA and cellular standards show a slightly different profile to the expected profile provided by Zymo (Suppl. Fig. S3 A). This indicates that there might be a slight bias introduced by the primer set; however, this bias will be equal across all samples and should not impact the analysis. The rarefaction curve shows that all samples are plateauing and have therefore been sequenced at an appropriate depth (Suppl. Fig. S3B). Lastly, there is an even distribution of reads across all samples (Suppl. Fig. S3C), and the read distribution across all ASV shows no concerning trends (Suppl. Fig. S3D).

The RNA-seq data was of high quality and resulted in approximately 111.0 M ± 12.9 (mean ± stdev) raw read pairs per sample before filtering. 100% of the reads in each sample passed filtering, retaining >99.5% of the base pairs. From these reads, 80.2% ± 2.4 (mean ± stdev) were uniquely mapped to the genome.

3.2. Microbiome

The microbial results were generally highly variable across individual fish. This is especially noticeable in the alpha-diversity measures, both in terms of Richness and Evenness, although some of the treatments show low variability (Fig. 3). As a general trend, the bacterial richness in the stomachs of fish that experienced either a AHS or prolonged MHW exhibited lower Richness right after the treatment and stayed lower even after four weeks of recovery (Fig. 3A). However, these trends are not statistically significant (ANOVA $F_{5,24} = 1.39$, p-value = 0.265). In contrast, the bacterial richness in the caecal tissue was relatively stable (Fig. 3B), with also no significant differences between treatments (ANOVA $F_{5,24} = 0.33$, p-value = 0.893). Similarly, there was no clear pattern or significant differences in the Evenness in stomach (Fig. 3C, ANOVA $F_{5,24} = 0.93$, p-value = 0.477) or intestine ((Fig. 3D, ANOVA $F_{5,24} = 1.99$, p-value = 0.116).

At the phylum level, the stomach bacterial communities were dominated by *Protobacteria*, with *Actinobacteria* and *Firmicutes* being next highest in abundance, followed by *Bacteroidota* and *Cyanobacteria* (Fig. 4A). As a general trend at the end of the treatment period, *Actinobacteria* and *Proteobacteria* were least abundant at the control temperature and most abundant after the MHW (Fig. 4A). In contrast, *Firmicutes* were most abundant in the control fish, and least abundant in the fish that underwent a MHW (Fig. 4A). However, due to the high variability, none of these trends were statistically significant. At the end of the 4-week recovery period, the most notable changes were the higher relative abundance of *Actinobacteria* and lower relative abundance of *Proteobacteria* in fish that had undergone AHS, and an increased number of *Cyanobacteria* in the control fish.

Like the alpha-diversity metrics, the results at the Genus level in the stomach samples showed high variability (Suppl. Fig. S4A). On average, *Vibrio* and *Erythrobacter* appeared to be more abundant in the stomach of fish that had undergone a MHW, at the expense of *Pseudomonas* and Burkholderia (Fig. 4B). However, this was mostly driven by two samples with a very high load of Vibrio and one sample with a high load of Erythrobacter (Suppl. Fig. S4A), and therefore not statistically significant. In contrast, fish at the end of the AHS had an increased relative abundance of *Burkholderia*, *Enterovibrio*, *Prausella* and *Pseudomonas* compared to the control. Finally, the control fish showed a higher abundance of *Vibrio* compared to the 3-day treatment, but again this was mainly limited to two samples with high abundance (Fig. 4B and Suppl. Fig. S4A). The recovery phase is marked by the appearance of *Endozoicomonas*, which is a cosmopolitan marine bacterium usually associated

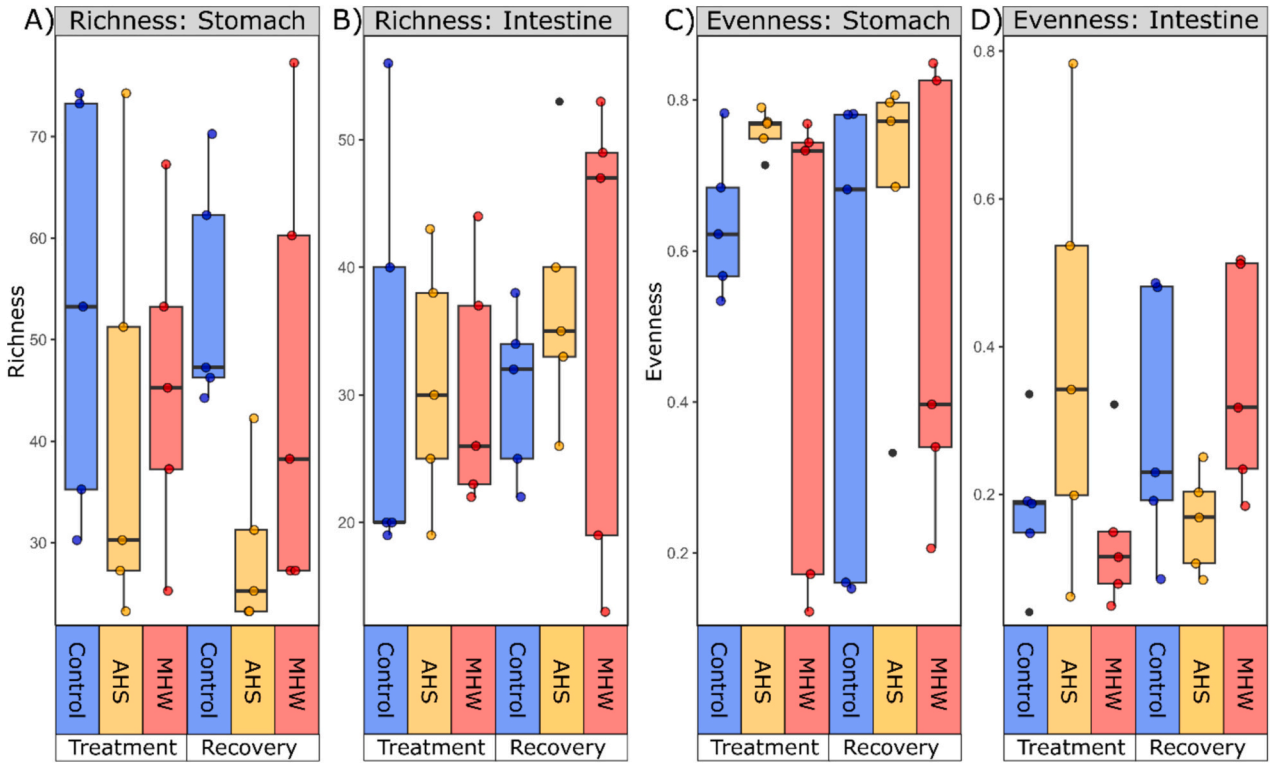

Fig. 3. Genus level 16S rRNA gene sequencing bacterial alpha diversity measures of A) stomach richness and B) pyloric caeca richness, C) stomach evenness, and d) pyloric caeca evenness. Boxplots show median as middle line, first and third quartiles as box, and data range as lines extending from the box. Coloured circles show individual datapoints, while outliers are shown as black circles. Abbreviations: AHS: 3-day acute heat stress. MHW: 21-day marine heatwave.

with corals (Fig. 4B). However, this was limited to two control fish, one AHS fish, and three MHW fish. Vibrio was high in abundance in a control fish (~90%) and one fish that had undergone the MHW (~45%).

At the phylum level, the pyloric caeca samples were dominated by *Proteobacteria* (>90%), with lower abundance of *Actinobacteria*, *Bacteroidota*, *Cyanobacteria*, and *Firmicutes* (Fig. 5A). At the genus level, and in contrast to the stomach, the pyloric caeca generally showed less variability between different fish, independent of the heat treatment or recovery (Suppl. Fig. S4B). One reason for this is that the caeca are dominated by *Vibrio* at the genus level, potentially providing more stability (Fig. 5B). However, one sample in the 3-day heat treatment is an exception, having only a very low abundance of *Vibrio*. Additionally, there were six samples with a lower *Vibrio* abundance of about 50%, consisting of one sample in the AHS group, two samples in the control recovery group, and three samples in the recovering fish that underwent a MHW (Suppl. Fig. S4B). Overall, other bacterial genera included *Burkholderia*, which was mostly present in the AHS treatment, and in all three treatments during recovery, *Catenococcus*, which was mostly present in the MHW treatment during recovery (primarily limited to one sample), and *Pseudomonas* which was present in all treatments, but especially in the AHS treatment after the heat treatment and the control and MHW groups after the recovery (Fig. 5B).

3.3. Transcriptome analysis: stomach

Looking at the stomach samples, only the MHW had a marked effect with 158 differentially expressed genes (DEGs), while the AHS treatment caused minimal changes in gene expression (6 DEGs) (Fig. 6A). After recovery at the control temperature (28 °C) for 4 weeks, fish that had undergone the MHW still exhibited 93 DEGs, while the number of DEGs of the fish in the AHS treatment increased to 49 (Fig. 6A). This indicates that while initially only the MHW exposure induced a significant reaction short-term, there were long term changes in gene expression independent of the length of the treatment. Looking at the principal component, there was no distinct separation between the treatments (PCA, Suppl. Fig. S2). However, the different treatments showed limited overlap in DEGs based on a pairwise comparison to the respective control groups (Venn diagram, Suppl. Fig. S2), indicating that the stomach tissue responded differently to the different treatments (as discussed below). Generally, the up- and down regulation was much higher in fish during the recovery period irrespective of whether they had undergone an AHS (average log2 fold change: up 1.1, down −3.0) or MHW (average log2 fold change: up 2.1, down −3.8). In contrast, the changes in gene expression were lower in fish at the end of the AHS (average log2 fold change: up 0.9, down −0.9) and MHW (average log2 fold change: up 1.0, down −0.9). This further supports the delayed effect of the heat stress on the stomach transcriptomic landscape.

The stomachs of fish that experienced the MHW only showed enrichment of GO-terms for downregulated genes, while the AHS fish showed no enriched GO-terms (Suppl. Tables S2- S7). Many key pathways tied to mitochondria appeared to be affected in fish undergoing the MHW. This can be seen in the downregulation of several enzymes in the tricarboxylic acid (TCA) cycle and beta oxidation, with pyruvate and fatty acid transport into the mitochondria also being affected.

Specific enzymes associated with mitochondrial biochemical pathways included pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 1 (*pdk1*, log2FC: −0.91), isocitrate dehydrogenase [NAD] subunit beta (*idh3b*, log2FC: −0.67), 3-ketoacyl-CoA thiolase (*acaa1*, log2FC: −0.77), very long chain specific acyl-CoA dehydrogenase (*acadvl*, log2FC: −0.74), and short-chain specific acyl-CoA dehydrogenase (*acads,* log2FC: −0.62). On the transport side, mitochondrial pyruvate carrier (*mpc*, log2FC: −1.07), and carnitine O-palmitoyltransferase (*cpt,* log2FC: −1.69) were downregulated. Lastly, the biogenesis of mitochondria might also be affected, with the downregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*pgc1a*, log2FC: −1.19), and the tightly linked AMPK pathway in the form of 5'-AMP-activated protein kinase subunits beta (*ampkb* log2FC: −0.63, although not significantly with padj = 0.066)

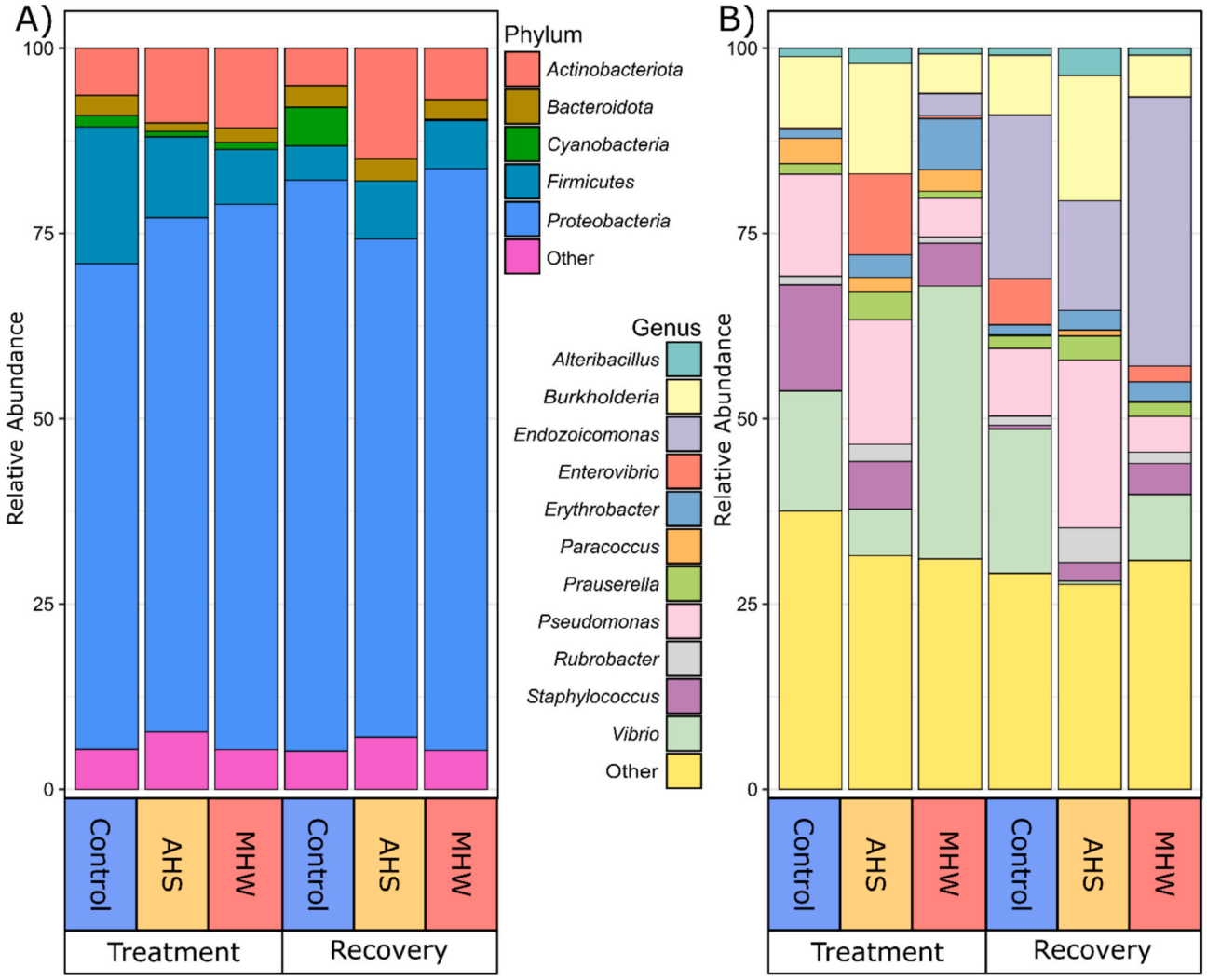

Fig. 4. Bacterial communities of the stomach of fish after a 3-day AHS or a 21-day MHW (+3 °C), and after recovery. Showing relative abundance of A) the top 5 phyla and B) the top 10 genera, with lower abundance taxa collapsed into "Other". Each treatment consists of five replicates. Abbreviations: AHS: acute heat stress. MHW: marine heatwave.

and gamma (*ampkg* log2FC: −0.60). Furthermore, digestive processes might also have been impaired, with the downregulation of genes involved in the production of gastric enzymes and pH regulation in the stomach, such as gastricsin-like protein (*pgc*, log2FC: −1.21), the histamine H2 receptor (*hrh2*, log2FC: −0.90), and carbonic anhydrase 6 (*ca6*, log2FC: −0.88). Lastly, there might be also a general impact on membrane integrity and homeostasis, with the downregulation of 1-acyl-sn-glycerol-3-phosphate acyltransferase alpha and beta (*agpat,* both log2FC: −0.68), and aquaporin (*aqp*, log2FC: −0.77).

Fish that had undergone the MHW, still showed many differentially regulated genes four weeks later. Even fish that underwent a AHS, now showed a higher number of upregulated genes. The results below mostly focus on the MHW results; however, similar broad changes were seen after a AHS, although to a lesser degree (Suppl. Tables S8-S17).

One group significantly downregulated genes included genes involved in innate immunity and inflammation responses and blood coagulation. The immune related genes included complement C3 (*c3*, log2FC: −4.4), complement factor I (*cfi*, log2FC: −3.62), complement factor H (*cfh*, log2FC: -4.99), CD59 glycoprotein (*cd59*, log2FC: −3.28), c-type lectin (*clec*, log2FC: −3.85), secreted phosphoprotein (*spp*, log2FC: −3.68), and NACHT, LRR and PYD domains-containing protein 3-like (*nlrp3*, log2FC: −0.66). This downregulation of several immune related genes compared to the control fish could indicate a reduced ability to prevent dysbiosis in the stomach in fish who have undergone either an AHS or an MHW. Furthermore, three genes involved in blood coagulation were also significantly downregulated: vitamin K-dependent protein C (*proc*, log2FC: −3.02), coagulation factor VII (*f7*, log2FC: −2.73), and heparin cofactor 2 (*serpind1*, log2FC: −2.19). Further indicating long-term changes in the stomach.

In contrast, significantly upregulated genes were related to tissue homeostasis, regulation of gastric acids, and extracellular matrix reorganisation. The two genes related to tissue homeostasis are aquaporin-8 (*aqp8*, log2FC: 4.20), and broad substrate specificity ATP-binding cassette transporter ABCG2-like (*abcg2*, log2FC: 2.82). Significantly upregulated genes related to the regulation of gastric acids included carbonic anhydrase 7 (*ca7*, log2FC: 4.20) and carbonic anhydrase 12 (*ca12*, log2FC: 3.60), solute carrier family 26 member 6 (*slc26a6*, log2FC: 3.47), and glucagon-like peptide 2 receptor (*glp-2r*, log2FC: 1.42). There is also evidence for extracellular matrix reorganisation, through the significant upregulation of two collagen genes (*col*, log2FC: 9.60 and 5.36) which are particularly abundant in the submucosa and muscularis externa layers of the stomach and contribute to the strength and contractility of the stomach wall, Lysyl oxidase homolog 4 (*loxl4*, log2FC: 3.25), matrix metalloproteinase-9 (*mmp9*, log2FC: 2.42), and integrin alpha-10 (*itga10*, log2FC: 1.55). Many of these genes are involved in collagen reorganisation and tissue repair, suggesting a

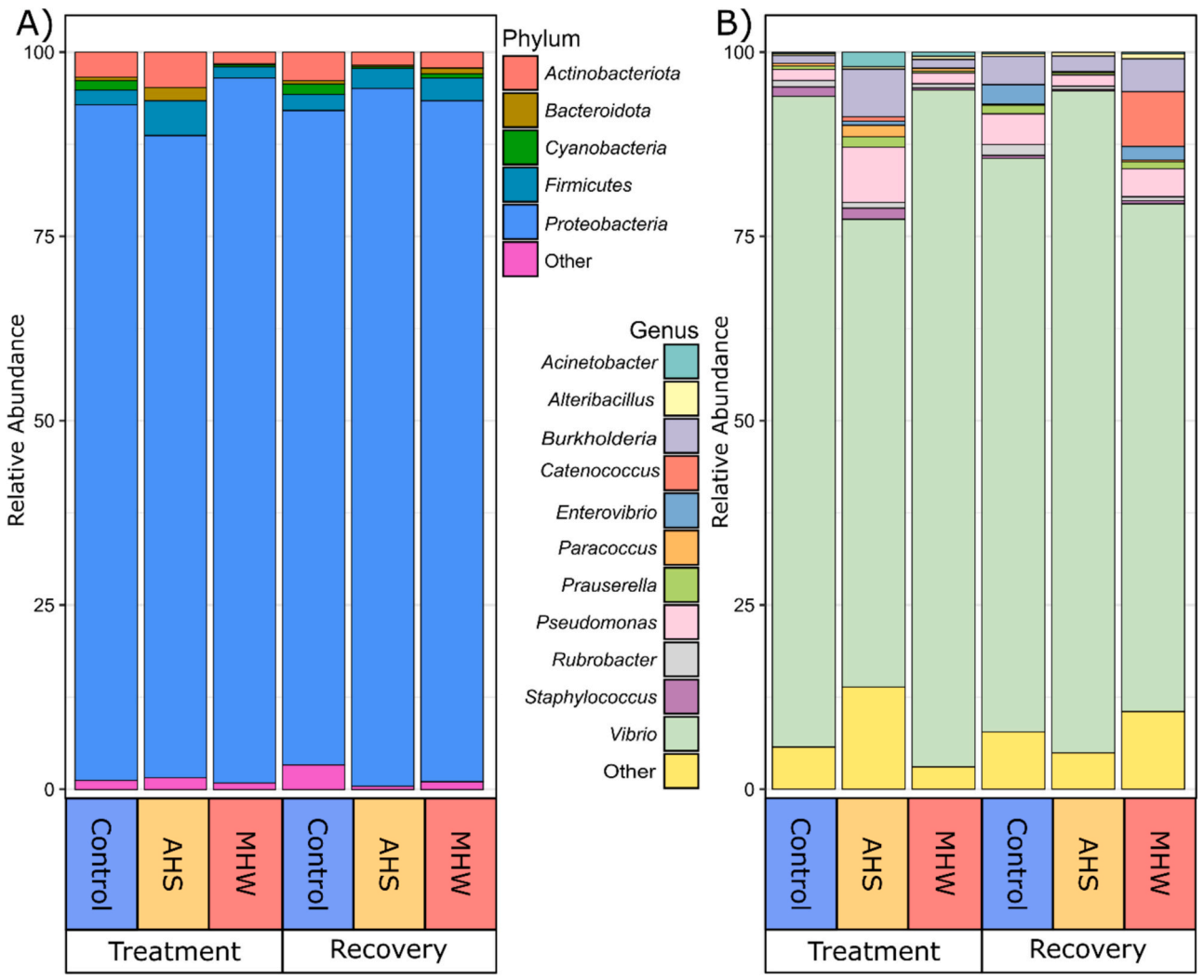

Fig. 5. Bacterial communities of the pyloric caeca of fish after a 3-day AHS or a 21-day MHW (+3 °C), and after recovery. Showing relative abundance of A) the top 5 phyla and B) the top 10 genera, with lower abundance taxa collapsed into "Other". Each treatment consists of five replicates. Abbreviations: AHS: acute heat stress. MHW: marine heatwave.

recovery from the heat stress damage. Other upregulated genes include intestinal fatty acid-binding protein (*fabp*, log2FC: 5.24), and homeobox protein CDX-1a (*cdx1a*, log2FC: 2.49).

3.4. Transcriptome analysis: pyloric caeca

In contrast to the stomach, the pyloric caeca samples only showed a weak response after the AHS (18 DEGs) or MHW (23 DEGs) (Fig. 6B, Suppl. Tables S18-S27). This suggests a stable environment even during heat stress, similar to the microbiome results described above. However, after recovery at the control temperature for 4 weeks, fish that had undergone the AHS showed a massively increased number of DEGs at 177, while the number of DEGs of the fish in the MHW treatment stayed low (14 DEGs) (Fig. 6B, Suppl. Tables S28-S36). Looking at the principal component, the AHS and MHW showed high variability at the end of the treatment period, while all three groups after the recovery period clustered more tightly but separated from the treatment samples (PCA, Suppl. Fig. S2). Similar to the stomach samples, the different treatments showed limited overlap in DEGs based on a pairwise comparison to the respective control groups (Venn diagram, Suppl. Fig. S2); however, as discussed in this section, the number of DEGs was low in most treatment groups.

While there were fewer differentially regulated genes or enriched GO-terms or KEGG-pathways compared to the stomach samples, there are a few that are worthwhile to highlight. There seems to be a general heat response though the expression of heat shock proteins (Hsp). Specifically, cytoplasmic Hsp70 (log2FC: 1.01) and Hsp90 (log2FC: 1.98) were significantly upregulated, irrespective of the length of the heat stress, while mitochondrial Hsp60 (log2FC: 0.59) was only upregulated after the MHW. Especially in the MHW treatment, there seems to be an increase in cellular maintenance with the upregulation of E3 ubiquitin-protein ligase RNF115 (*rnf115*, log2FC: 0.71), and pentraxin fusion protein-like (*pxn*, log2FC: 0.69). This can also be seen in the AHS with interferon-induced, double-stranded RNA-activated protein kinase-like protein (*eif2ak2*, log2FC: 0.92). And a down regulation of interleukin-1 beta-like (*il1b*) both after the AHS (log2FC: −2.04) and a MHW (log2FC: −2.29).

Even though the pyloric caeca of fish that have gone through a 3-day AHS showed 177 DEGs, a surprisingly low number of significantly enriched results were found in the GO-term (3 terms) and KEGG pathway (8 pathways, excluding human diseases) analyses.

Similar to the stomachs of fish recovering from the heat stress, c-type lectin (*ctl*, log2FC: −4.88) was heavily downregulated, which could result in an increased likelihood of dysbiosis. Furthermore, these fish might also struggle maintaining homoeostasis, with several genes related to ion transport, water movement, pH regulation, and general

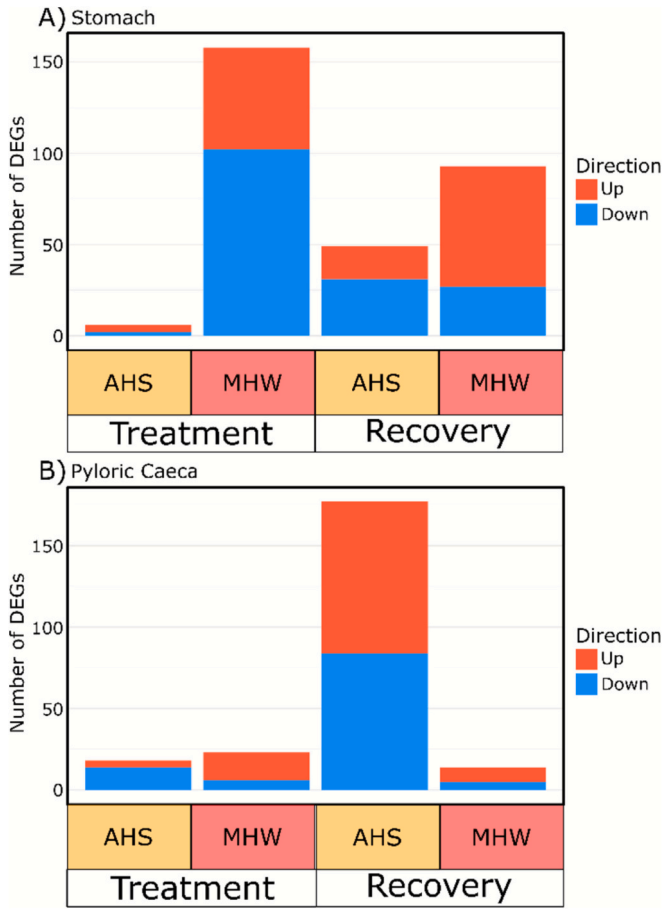

Fig. 6. Number of up and down regulated genes based on Wald test in DESeq2 contrasting fish who have undergone a 3-day AHS and a 21-day MHWs (+3 °C), and also after 4 weeks of recovery at the control temperature (28 °C) for stomach (A) and pyloric caeca (B) tissues. Abbreviations: AHS: 3-day acute heat stress. MHW: 3-week marine heatwave.

homeostasis functions being downregulated. This included aquaporin-10a (*aqp10a*, log2FC -0.66), solute carrier family 12 member 1 (*slc12a1*, log2FC -0.61) and solute carrier family 26 member 6 isoform X1 (*slc26a6*, log2FC -0.64), inositol-trisphosphate 3-kinase Cb (*itpkb*, log2FC -0.69), sodium/hydrogen exchanger 1 isoform X2 (*nhe1*, log2FC -0.69), and sodium-coupled monocarboxylate transporter 1 (*smct1*, log2FC -0.82). Other stress response and tissue repair related genes included glutathione peroxidase 3 (*gpx3*, log2FC -0.60), annexin A2-A-like (*anxa2*, log2FC -0.68), fibronectin 1a isoform X2 (*fn1*, log2FC -0.63), and acidic mammalian chitinase-like (*amcl*, log2FC: −0.69).

In contrast, a large number of genes involved in the regulation and formation of collagen in the extracellular matrix were upregulated, indicating an ongoing healing of the potential damage caused by the heat stress. This included collagen alpha-2(VI) chain-like (*col6a2*, log2FC 0.72), procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 isoform X2 (*plod2*, log2FC 0.61) and lysyl oxidase homolog 3 (*loxl3*, log2FC 0.60), prolyl 4-hydroxylase subunit alpha-2 isoform X1 (*p4ha2*, log2FC 0.66), lumican (*lum*, log2FC 0.83), and sestrin-3 isoform X1 (*sesn3*, log2FC 0.58).

The pyloric caeca of fish recovering from a MHW showed fewer DEGs. However, similar to the fish recovering from the AHS, downregulated genes included homeostasis (hepatocyte nuclear factor 4-alpha isoform X1, *hnf4a*, log2FC: −0.62), immune response (interferon regulatory factor 7, *irf7*, log2FC: −0.93) and wound healing (F-actin-uncapping protein LRRC16A-like isoform X1, log2FC: −0.76), while upregulated genes included extracellular matrix organisation (zinc finger protein 469, *znf469*, log2FC: 0.66), cell adhesion (adhesion G protein-coupled receptor B3 isoform X1, *adgrb3*, log2FC: 0.77), and signalling (ephrin type-B receptor 1-B isoform X2, *ephb6*, log2FC: 0.81; kelch-like protein 38, *klhl38*, log2FC: 1.11).

4. Discussion

Here we used microbial 16S rRNA gene data and host transcriptomic responses to investigate the effect of acute heat stress (AHS) or a strong marine heatwave (MHW) on the stomach and pyloric caeca of economically and culturally important Malabar grouper (*Epinephelus malabaricus*). Our analyses show there is not only an immediate effect of a MHW on the fitness of the fish, but that both AHS and MHW can cause changes that are still measurable after four weeks of recovery. Specifically, while only the MHW affected the transcriptomic landscape of the fish stomachs, we measured long-term changes in the transcriptome of both treatments still present after four weeks of recovery at the control temperature. Furthermore, the stomach of the fish exposed to the AHS or MHW showed lower bacterial richness on average, both just after the treatment period and after recovery period; however, this was just a trend and not statistically significant. In contrast to the stomach, the pyloric caeca showed a simpler microbiome composition, dominated by *Vibrio*, and also a more stable transcriptomic landscape.

The dominance of proteobacteria followed by *Firmicutes*, *Actinobacteria* and *Bacteroidota* in the stomach is similar to what other studies in fish have found (e.g., (Egerton et al., 2018; Gao et al., 2020; Llewellyn et al., 2014; Sullam et al., 2012). Especially the high relative abundance of proteobacteria appears to be something that separates marine fish from freshwater (Kim et al., 2021). This is in contrast to mammalian gastrointestinal tracts (GIT), which are generally dominated by *Firmicutes* and *Bacteroidota* (Egerton et al., 2018).

Generally, microbial richness has been linked to health for all organisms (Neish, 2009), which is also true for fish where microbial diversity has been linked to health and growth in aquacultured fish (Infante-Villamil et al., 2021). Furthermore, temperature seems to have a disruptive effect on the gut microbiome of animals in general (Sepulveda and Moeller, 2020). As in other animals, the reduction in alpha diversity has been linked to dysbiosis in fish (Xavier et al., 2024). The reduction in microbial diversity during heat stress and after the recovery period, while not significant, indicates that these fish may be experiencing negative effects. This may make these fish susceptible to dysbiosis in the long term. Additionally, while gastric microbiome studies on tropical reef fish and heatwaves are rare, these effects can be seen in different parts of the gastrointestinal tracts of greater amberjack (*Seriola dumerili*) (Sánchez-Cueto et al., 2023), Eastern striped grunter (*Pelates sexlineatus*) (Suzzi et al., 2023), Juvenile Milkfish (*Chanos chanos*) (Hassenrück et al., 2020), and Atlantic Salmon (*Salmo salar*) (Neuman et al., 2016); although there are also cases of increased richness with increased temperatures as found in Chinook salmon (*Oncorhynchus tshawytscha*) (Steiner et al., 2022). At the genus level, the large within treatment variability complicated the analysis. For example, while the stomachs of fish that experienced a MHW showed a much higher relative abundance of *Vibrio*, this was driven by two samples that were dominated by *Vibrio*. A similar sample dominated by *Vibrio* was also found in the control sample after the recovery period. There are two explanations for this high abundance of *Vibrio* in these samples. One explanation is that when sampling the stomach, parts of the caeca were sampled too. The other explanation is that these fish had some unknown condition that increased the amount of Vibrio, potentially invading the stomach from the caeca. Considering there was no marked overall increase in *Vibrio* spp. in the fish that underwent an AHS or MHW, other than these individual samples, this could indicate that these groupers are more resilient to pathogenic *Vibrio* infections at the tested conditions. This is in contrast to studies in other animals which showed an increase in *Vibrio* with heat stress, for example in clownfish (*Amphiprion ocellaris*) (Moore et al., 2024) and Pacific oyster (*Crassostrea gigas*) (Green et al., 2019). However, even though there was no natural increase in *Vibrio*,

there could still be an increased susceptibility Vibrioris induced mortality, as found in orange-spotted grouper (*Epinephelus coioides*) when challenged with *Vibrio* under temperature stress (Cheng et al., 2009). Interestingly, after the recovery period, several samples exhibited a high relative abundance of the genus *Endozoicomonas* irrespective of treatment. While this bacterium is cosmopolitan in the marine environment and generally associated with corals, it has also been found other organisms like poriferans, molluscs, annelids, and fish (Neave et al., 2016); however, the exact function remains unclear. While *Endozoicomonas* is generally considered as being a beneficial symbiont which is potentially involved in host-associated protein and carbohydrate transport and cycling (Neave et al., 2016), there are also indications that some species of this genus can be pathogenic (Qi et al., 2018). Therefore, further research on the occurrence and function of *Endozoicomonas* in the stomach of grouper is needed.

The pyloric caeca were dominated by a single ASV of *Vibrio*. This genus belongs to the family of *Vibrionaceae* and could be aiding in the digestion of food through the expression of enzymes like amylase, lipase, cellulose, and chitinase (Gatesoupe et al., 1997; Itoi et al., 2006; Sugita and Ito, 2006), as cited in (Diwan et al., 2022), which would explain their presence in the caeca of these Malabar groupers. Overall, there was no clear short- or long-term effect of the AHS or MHW on the microbial composition of the pyloric caeca. This is an indication that the simpler and *Vibrio* dominated microbiome of this tissue are more resilient to the marine elevated heat conditions tested in this experiment.

In contrast to the microbiome data, the host transcriptome of the stomach showed a strong response to the MHW. Furthermore, even after four weeks of recovery, a large number of genes were still upregulated. And while the AHS did not directly elicit a strong response, the number of DEGs increased drastically after recovery. This indicates that even a shorter, acute heat stress can have longer lasting effects. The affected biological processes were associated with energy production in mitochondria, digestion, and maintenance of homeostasis. The thermal limit of fish has previously been tied to the function of mitochondria (Bermejo-Nogales et al., 2014; Iftikar et al., 2014). For example, it has been shown that heat stress especially affects the function of mitochondria in tissues like heart (Iftikar and Hickey, 2013) and muscle (Banh et al., 2016), ranging from reduced protein activity (Hunter-Manseau et al., 2019) to membrane damage (Iftikar et al., 2014). *Pgc1α* is a potential key controlling step of mitochondrial biogenesis, reacting to various external triggers including thermal stress (Bermejo-Nogales et al., 2014; Wenz, 2013). However, while *pgc1α* was found to be highly upregulated during thermal stress in the liver of gilthead sea bream (*Sparus aurata*) (Bermejo-Nogales et al., 2014) and muscle of Rohu (*Labeo rohita*) (Kumar et al., 2023), it was found significantly downregulated in the stomach of heat stressed fish present study. Similarly, the tightly linked *ampkβ* was also downregulated (although only near significantly), indicating a wider down regulation of mitochondrial biogenesis in grouper stomach undergoing heat stress. Similarly, *cpt*, which encodes for a rate limiting enzyme in the fatty acid beta-oxidation, was also downregulated in the present study, while being upregulated in muscle of *Sparus aurata* (Bermejo-Nogales et al., 2014). This, together with other downregulated genes associated with mitochondrial function, tricarboxylic acid (TCA) cycle and beta-oxidation, could potentially be a mechanism to counter oxidative stress in the stomach during heat stress, limiting ATP production.

At the same time, the expression of proteins involved in digestion and the regulating acid production was also downregulated. Generally, water temperature affects the digestion efficiency through affecting the enzyme activity, with temperature outside the optimal range having a negative effect (Volkoff and Rønnestad, 2020). This was coupled with a downregulation of genes associated with membrane integrity and homeostasis (e.g., *agpat* and *aqp*). Together, this potentially indicates a disruption of the digestive processes and therefore nutrient uptake, coupled with potential damage to the digestive tract during the AHS or MHW. While research into the effect of heat stress on the digestive ability of fish is rare, there is some supporting evidence of physiological dysfunction from sturgeon (Yang et al., 2022), and oxidative stress and antioxidant response in flounder (Lu et al., 2016).

Further evidence for a potential disruption of the digestive functions during a AHS or MHW can be found in the differentially expressed genes in the stomach of fish after the recovery period. Many significantly upregulated genes were involved in tissue homeostasis and extracellular matrix reorganisation. For example *aqp8* is involved in fluid balance (Eissa and Wang, 2016) and *abcg2* is involved in protecting cells from dietary toxins (Luckenbach et al., 2014). Other upregulated genes involved in extracellular matrix reorganisation included ones that produce (*col*) and cross-link (*loxl4*) collagen, degrade damage collagen (*mmp9*), or are associated with cell adhesion (*itga10*). Collagen provides an essential function in tissue strength and contractility, which is vital in the proper function of the stomach. All this taken together, it can be hypothesised that these genes are upregulated to reinstate proper tissue homeostasis and function potentially lost during the heat stress. This effect is stronger in fish that have undergone a 3-week long heatwave, but the upregulation of related genes in fish that have undergone a AHS shows that this is already enough to potentially cause lasting damage.

The transcriptomic results of the pyloric caeca told a different story to the stomach. The heat stress itself, irrespective of being an AHS or MHW, had a minimal effect on the number of DEGs, with only 18 DEGs after three days and 23 DEGs after three weeks. Notable upregulated genes in all heat challenged fish included different heat shock proteins (*Hsp*) and inflammation (*IL-1β*), as well as genes involved in maintaining protein homeostasis (*RPLP0*) after the AHS and protein degradation and repair (*RNF115*) and immune response (*ptx*) after the MHW. In contrast to the stomach, the pyloric caeca are a much simpler environment. These results together with the minimal changes in the microbiome, indicate that the function of the pyloric caeca is maintained during a strong +3°C MHW of at least up to 21 days, and that the main effect occurs in the stomach of these fish.

Interestingly, after the 4-week recovery period, the pyloric caeca of fish that had undergone the AHS showed many DEGs, while the MHW treated fish showed only few DEGs. The lack of clear GO-term and KEGG pathway enrichment indicates that these changes do not follow a clear detectable theme; however, looking at overall genes, it appears that there is a general dysfunction in maintaining homeostasis. The downregulated genes include genes involved in water regulation (e.g., *aqp*) and ion transport (e.g., *slc12A1*), but also protection from oxidative damage (*gpx3*), and cell membrane repair (*anxa2-A*), among others. Similar to the stomachs during recovery, upregulated genes include ones that are involved in the regulation and formation of collagen and general extracellular matrix repair. It is not clear why only fish that had undergone a AHS show this response, while fish from the 3-week treatment showed minimal changes. It could be explained by the pyloric caeca undergoing a remodelling after a period of elevated temperature, which had already happened earlier in the fish that underwent the longer treatment. In any case, more research is needed to replicate this result and find a potential explanation for this phenomenon.

Concerning the variability shown in the data, the exact source is unclear. We know that our molecular techniques are working well since none of the quality control metrics show any areas of concern. For the transcriptomic data, the read quality is high as shown by the low number of discarded reads/bases and the high mapping rate. For the microbiome data, the quality of the reads is also high, the DNA extraction is unbiased as shown by the mock cellular and DNA standards, the sequencing depth is adequate as shown by the rarefaction curve and the number of reads per sample was very even. In terms of fish husbandry, the fish were randomly distributed across multiple replicated tanks with a single water source, and the environmental variables and feeding regime were kept the same in each tank. All fish were sourced from the same cohort from a local fish hatchery, also reducing genetic variability. This leaves individual biological variability between fish or other factors we have not considered. While the variability might reduce the power to see less

pronounced trends, the stronger trends shown in this research are still allowing a general assessment of the effect of AHS and MHW on parts of the gastric tract of Malabar grouper. Future studies will be able to build up on this work and strengthen the findings.

In conclusion, the present study is one of the few that look at both microbiome and host molecular responses in the GIT of a large, tropical, long-lived mesopredator of commercial importance to marine heatwaves. We showed that both short (3-day) acute heat stress and long (3-week) marine heatwaves can have varying effects on the stomach and pyloric caeca of malabar grouper, which can potentially extend into the recovery period and beyond. In terms of microbial communities, minimal changes were observed, with indications of heat stress lowering the bacterial richness in the stomach, but no increase in pathogenic *Vibrio* being observed. Concerning the host response, the MHW had an immediate effect on the molecular response in the stomach disrupting the function of the mitochondria and digestive processes; however, both stomach and pyloric caeca showed a long-term response tied to the repair of tissue damage. This indicates that while these fish can handle a +3°C heatwave, there may be long term repercussions. This has both implications for fish on the reef, as well as fish in aquaculture production, where future heatwaves can impair yield, reduce growth, or increase mortality. Future studies should follow these fish through a longer recovery period, investigating how long the effects of heatwaves will last in these fish and whether there are differences in growth or health outcomes after extended periods of time.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742141.

CRediT authorship contribution statement

Roger Huerlimann: Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shannon J McMahon:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Michael Izumiyama:** Writing – review & editing, Investigation. **Chengze Li:** Methodology, Investigation, Data curation. **Jeffrey Jolly:** Methodology, Investigation. **Erina Kawai:** Methodology, Investigation. **Timothy Ravasi:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Data availability

The data associated with this research can be found on ncbi under BioProject PRJNA1103930. The scripts will be deposited in Github before publication. The microbial sample metadata, ASV table and taxonomic assignment can be found in Suppl. Data S3, S4 and S5, respectively.

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