Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Climate challenges for fish larvae: Interactive multi-stressor effects impair acclimation potential of Atlantic herring larvae

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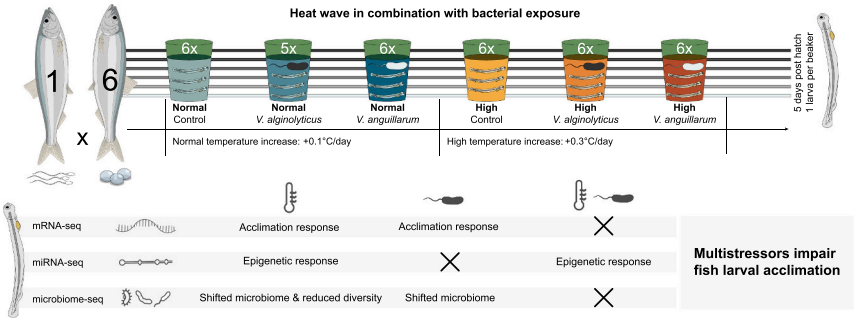
HIGHLIGHTS

- Effects of multiple climate change-related stressors on Atlantic herring larvae were experimentally investigated.
- A heat wave (HW) and bacterial exposure (BE) independently induce a similar acclimation response in gene expression.
- A combination of both stressors impairs this acclimation response, potentially causing cellular damage.
- The HW alone or combined with BE alter the epigenetic response via changed miRNA expression.
- HW and BE independently alter the herring microbiota, the HW alone reduces larval microbial richness & diversity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Olga Pantos

Keywords:
Heat wave
Ocean warming
Bacterial exposure
Vibrio
Transcriptomics
Epigenetics
Microbiota

ABSTRACT

Fish early life stages are particularly vulnerable and heavily affected by changing environmental factors. The interactive effects of multiple climate change-related stressors on fish larvae remain, however, largely underexplored. As rising temperatures can increase the abundance and virulence of bacteria, we investigated the combination of a spring heat wave and bacterial exposure on the development of Atlantic herring larvae (*Clupea harengus*). Eggs and larvae of Western Baltic Spring-spawners were reared at a normal and high temperature ramp and exposed to *Vibrio alginolyticus* and *V. anguillarum*, respectively. Subsequently, mRNA and miRNA transcriptomes, microbiota composition, growth and survival were assessed. Both high temperature and *V. alginolyticus* exposure induced a major downregulation of gene expression likely impeding larval cell proliferation. In contrast, interactive effects of elevated temperature and *V. alginolyticus* resulted in minimal gene expression changes, indicating an impaired plastic response, which may cause cellular damage reducing survival in later larval stages. The heat wave alone or in combination with *V. alginolyticus* induced a notable shift in

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https://doi.org/10.1016/j.scitotenv.2024.175659
Received 7 February 2024; Received in revised form 15 August 2024; Accepted 18 August 2024

miRNA expression leading to the down- but also upregulation of predicted target genes. Moreover, both increased temperature and the *Vibrio* exposures significantly altered the larval microbiota composition, with warming reducing microbial richness and diversity. The outcomes of this study highlight the high sensitivity of herring early life stages towards multiple climate change-related stressors. Our results indicate that interactive effects of rapidly changing environmental factors may exceed the larval stress threshold impairing essential acclimation responses, which may contribute to the ongoing recruitment decline of Western Baltic Spring-Spawning herring.

1. Introduction

The adverse impact of climate change on marine life is particularly high on fish early life stages, potentially leading to reduced recruitment, deteriorated ecosystem functioning and decreased food security (Baag and Mandal, 2022; Cheung et al., 2021; Franke et al., 2024). The main environmental factors influencing fish larval survival include temperature, oxygen and diseases (Blaxter, 1992; Vadstein et al., 2013; Wu, 2009). Ocean warming can alter the larval expression of genes, cause epigenetic modifications and shift the gut microbiota towards communities dominated by pathogenic bacteria such as *Vibrio alginolyticus* (Moore et al., 2023; Moore et al., 2024). Infections with (opportunistic) pathogens are a serious threat for fish larvae as their immunocompetence is still subject to maturation (Magnadottír et al., 2005; Zapata et al., 2006). Accordingly, herring larvae have been shown to be susceptible towards *Vibrio* spp. infections (Mohammed et al., 2020) and larvae from numerous fish species suffer from disease and high mortality caused by *V. anguillarum* and *V. alginolyticus* among others (Manchanayake et al., 2023; Rønneseth et al., 2017). This negative effect is enhanced by rising temperatures, which promote the growth and virulence of infectious agents (Baker-Austin et al., 2013; Vezzulli et al., 2013). However, most climate change studies on fish early life stages have focused on the effects of single abiotic stressors such as ocean warming and acidification (e.g., Franke and Clemmesen, 2011; Moore et al., 2024; Moyano et al., 2020) while multi-stressor experiments remain scarce (e.g. Bignami et al., 2017; Cominassi et al., 2019; Joly et al., 2023; Leo et al., 2018; Poirier et al., 2017; Sswat et al., 2018). Consequently, it is largely unknown to which extent interacting effects of multiple environmental stressors threaten marine fish (Gunderson et al., 2016; Huang et al., 2021; Rummer and Illing, 2022) hindering predictions for sustainable fisheries management and marine food security.

Atlantic herring (*Clupea harengus*) is the fourth most landed fish worldwide and a key species in Northern hemisphere marine ecosystems as it supports the energy flux from lower to higher trophic levels (FAO, 2022; Lindegren et al., 2011; Poiesz et al., 2020). Hence, it is vital to understand the species' response to climate change effects. Over the last decades an alarming decrease in the reproductive success of Western Baltic Spring-Spawning (WBSS) herring has been observed in line with a low recruitment and spawning stock biomass (Ory et al., 2024; Polte et al., 2021). It has been stressed that this negative trend is not caused by a single driver but rather by several interacting environmental factors, however, our understanding of these interactions is severely limited (Moyano et al., 2022). As the switch from endogenous to exogenous feeding represents the most critical life history phase (Hjort, 1914), the ontogenetic period before first feeding (i.e., until yolk-sac absorption) represents a major bottleneck in WBSS herring (Polte et al., 2014). Thus, profound insights into the phenotypic plasticity and acclimation potential of this early life stage are needed to advance our knowledge of critical biological processes involved in herring recruitment and to develop indicators of vulnerability to environmental change (Huang et al., 2022; Jonsson and Jonsson, 2019; Moyano et al., 2022; Polte et al., 2014; Vagner et al., 2019). Transcriptome-wide gene expression analysis (mRNA-Seq) is a powerful tool that can provide essential information on biological pathways and processes such as early development, stress responses and immunity (Beemelmanns et al., 2021a; Cao et al., 2021; Connon et al., 2018; Moore et al., 2024). Moreover, epigenetic gene expression regulation, e.g., via DNA methylation (Beemelmanns et al., 2021b) or microRNAs (miRNAs) respond to environmental alterations in fish including herring larvae (Cao et al., 2023; Heinrichs-Caldas et al., 2023; Kho et al., 2024). Hence, epigenetic mechanisms can shape the larval acclimation potential to climate change substantially (Anastasiadi et al., 2017; Bizuayehu et al., 2015). The microbiota, another main modulator of host gene expression, shapes the maturation of the larval immune system and plays a critical role in larval health (Sehnal et al., 2021).

As the development of fish larvae is mostly controlled by temperature, and ocean warming can facilitate fatal *Vibrio* outbreaks, we aimed to elucidate how herring larvae cope with these two interacting climate change-related stressors. We hypothesized that herring larvae exposed to potentially pathogenic bacteria in addition to a heat wave exhibit an additive or even synergistic stress response reflected in their transcriptome and microbial community structure. WBSS herring eggs and larvae were reared at two different temperature ramps and exposed to two ubiquitous *Vibrio* species in a controlled laboratory experiment. 5 days post hatch, potential interactive effects on larval mRNA and miRNA transcriptomes, microbiota composition, growth and survival were assessed. We discuss the larval regulatory response and acclimation potential towards the two stressors and their interaction on the molecular and microbial level.

2. Materials and methods

2.1. Ethics statement

The experiment was conducted at GEOMAR Helmholtz Centre for Ocean Research Kiel (Germany) in accordance with national animal welfare regulations. Realization and analysis of the experiment align with the ARRIVE guidelines.

2.2. Fish collection and experimental design

To investigate the potentially interactive effects of ocean warming and bacterial exposure on the larval development of Atlantic herring (*Clupea harengus,* WBSS), we ran a controlled laboratory experiment with the following 6 treatments (replicated 6 times each; Fig. 1): Normal temperature rise without bacterial exposure (control group N.C), high temperature rise without bacterial exposure (H.C), normal temperature rise and *Vibrio alginolyticus* exposure (N.Val), high temperature rise and *V. alginolyticus* exposure (H.Val), normal temperature rise and *Vibrio anguillarum* exposure (N.Van) and high temperature rise and *V. anguillarum* exposure (H.Van).

Baltic surface water temperature profiles up to 8 m depth (obtained from the hydrodynamic coupled sea ice-ocean model BSIOM; Lehmann et al., 2014; see suppl. text), revealed that the average water temperature increase in March and April from 1950 to 2020 was 0.1 °C/day, which was chosen as the 'normal temperature' ramp for the experiment. In some years, however, an exceptionally high temperature rise of 0.3 °C/day was observed and chosen as the 'high temperature' ramp reflecting a heat wave projected to occur more frequently in the future (IPCC, 2019).

Ripe female and male herring were caught by a fisherman in the Kiel

Canal and brought on ice to GEOMAR on 14 April 2021. The eggs of 6 females (total length 22.5 to 24.5 cm) were strip-spawned on 2 plastic slides per female, one for each temperature treatment. All eggs were fertilized with the sperm of one male (total length 26 cm; Fig. 1a/b). The water temperature for sperm activation and fertilization was 8.0 °C and the salinity 7 PSU. Then, one slide per female was put into a 800 ml glass beaker (painted dark green on the outside). Every beaker was supplied with aeration for oxygenation and water circulation to prevent fungal infections (Fig. 1c). The eggs were visually inspected daily during water exchange. Fertilization rates were high whilst fungal infections remained rare (the few infected eggs were removed). After hatch, aeration was removed to avoid disturbing the larvae. Daily water change (using water with the respective temperatures) was 100% before hatch and 66% after hatch. The salinity was kept at 7 PSU (salinity-reduced Baltic Sea water), water was 5 µm-filtered and UV-treated, the photoperiod was 14L: 10D. The oxygen levels (always above 88%) and temperatures were monitored daily in every beaker (WTW Oxi3310).

To rear the herring eggs at the two temperature scenarios, one glass beaker per female was assigned to a 'normal temperature' (N) and the second to a 'high temperature' (H) plastic box (60x40x20 cm; Fig. 1d). The daily temperature increase was 0.1°C for N and 0.3°C for H (both start temperatures 8°C). The plastic boxes contained tap water and were each equipped with a heater, a circulation pump and a temperature controller (H-Tronic TS 125, accurate to 0.1 °C). The experiment took place in a temperature-controlled room. To guarantee that the water temperatures in the plastic boxes (and, hence, in the beakers) did not exceed the intended temperatures, the boxes were placed in bigger tubs filled with water kept at 6°C using a cooler. The experimental setup was specifically designed to enable a precise temperature increase. At the end of the experiment, N reached 9.9 °C and H 13.1 °C.

The peak hatch occurred 14 (H) and 16 (N) days after fertilization. 1 day post hatch (dph), larvae from every beaker were randomly distributed over three new beakers (one each for control, *Vibrio alginolyticus* and *Vibrio anguillarum* exposure). The only exception was one N beaker, where not enough larvae had hatched, leading to a missing replicate in the *V. alginolyticus* exposure (Fig. 1d). The new 35 beakers with 24 to 50 larvae each (mean±SE: 45±1.41) were randomly distributed according to their respective experimental temperature and the daily normal and high temperature ramps were continued.

At 2 dph, when the mouth of the larvae was already open, *Vibrio* bacteria were added to the 'bacterial exposure' treatments. Bacteria of the genus *Vibrio* are of opportunistic nature and ubiquitous to marine and brackish environments (Le Roux et al., 2015). We used a *V. alginolyticus* strain ('K01M1'; Wendling et al., 2017) and a *V. anguillarum* strain ('87-9-116') obtained from the Laboratory of Aquaculture and Artemia Reference Center (Ghent, Belgium). Both strains were preserved in 25% glycerol at −80°C. Bacteria were cultured in medium 101 (7 PSU) overnight at 22°C. In the morning, 1 ml of the bacterial cultures was transferred into 24 ml medium 101 and cultured for another 4 hours at 22 °C. Bacteria were centrifuged at 4000 g for 10 min, washed twice in filtered and autoclaved Baltic Sea water (7 PSU) and added to the rearing water of the respective treatment groups at a final density of 10^7 CFU mL^{-1}. Hence, per female and temperature treatment, one beaker was treated with *V. alginolyticus*, one with *V. anguillarum* and one with filtered and autoclaved Baltic Sea water as a control, resulting in six treatments (see Fig. 1). 24h after the exposure (3 dph) the water was exchanged in all beakers. Mortality was monitored daily and dead larvae were removed when detected (suppl. data 1). When the yolk sac was absorbed, 3 days after *Vibrio* exposure (5 dph), one larva per beaker was randomly chosen, killed (with an overdose of 0.04% MS222) and preserved in RNAlater at -80°C for further analyses. Additionally, water samples from three beakers each per treatment were stored at -80°C as controls for microbiome analysis. Before nucleic acid extraction, the thawed larvae were individually photographed (Nikon DS-Fi3 camera connected to Nikon SMZ18 stereomicroscope). Larval body lengths were determined with UTHSCSA Image Tool (v.3.0; suppl. data 2).

2.3. RNA and DNA extraction

To extract the mRNA, miRNA and microbial DNA simultaneously from each sample, the Machery-Nagel 'NucleoSpin® miRNA mini kit for miRNA and RNA purification' was combined with the 'NucleoSpin® RNA/DNA Buffer Set' and MP Biomedicals 'Lysing Matrix A' to homogenize the larval tissue and lyse bacterial walls (see suppl. text). RNA and DNA samples were kept at -80 °C before shipped on dry ice to BGI (Hongkong) for mRNA-seq, miRNA-seq and 16S rRNA-seq (Fig. 1e).

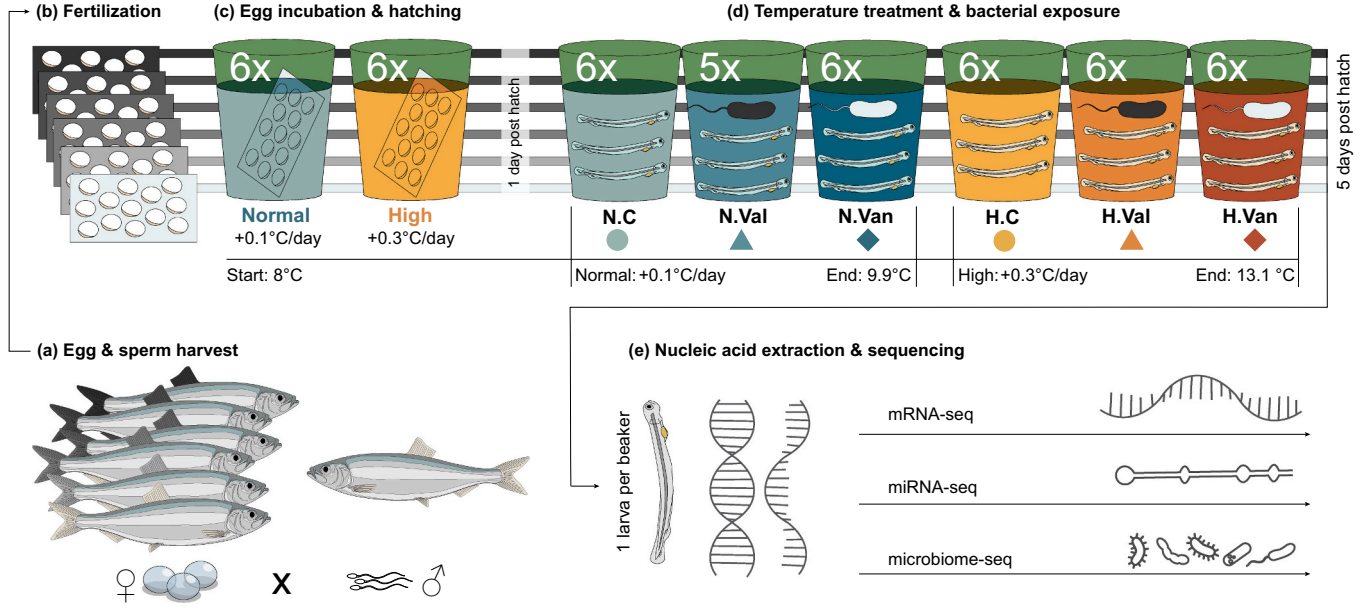

Fig. 1. Graphic overview of the experimental design. The course of the experiment is described from (a) to (e). N = Normal temperature, H = High temperature, Val = *V. alginolyticus*, Van = *V. anguillarum*. For further details, see material and methods.

2.4. Statistical analyses

For all statistical analyses, R version 4.3.0 was used (R Core Team, 2023). Each beaker used at the start of the experiment (start-beaker) contained larvae from one female, which were distributed over three new beakers for the bacterial exposure (see 2.2). The dependence of these three new beakers was considered using a mixed model with temperature and bacterial exposure as fixed factors and the start-beaker as a random factor (allowing for divergent intercepts between start-beakers and, thus, also for females) using the lme() function (method-='REML', 'nlme' package v.3.1; Pinheiro et al., 2023). p-values were calculated using the Anova(..., type="III") function ('car' package v.3.1-2; Fox et al., 2023). To analyze larval growth data, normality and homogeneity of variances were confirmed (Shapiro-Wilk test and Levene's test) before using the mixed model (see suppl. text). Survival between treatments was analyzed using a mixed effects Cox model (package 'survival' v.3.5-5; 'coxme' v2.2-18.1; Therneau, 2022, 2023).

2.5. mRNA-seq & analysis

Larval mRNAs of 35 samples were sequenced by BGI (Hongkong) in 150 bp paired end reads using the DNBseq technology. A total of 1,965 M reads was obtained, with an average of 56 M reads per sample (min: 50 M, max: 58 M). Quality trimming/filtering, mapping and read counting was performed with the nf-core RNASeq pipeline version 3.5 (Ewels et al., 2020). Briefly, the quality of reads was evaluated with FastQC, reads were trimmed with FastP (Chen et al., 2018) and mapped to the NCBI Herring RefSeq genome assembly (accession GCF_900700415.2) (Pettersson et al., 2019) using STAR (Dobin et al., 2013). The resulting bam files were processed with Salmon (Patro et al., 2017) to obtain gene-level counts. RNA-seq TPM data of 35 samples split over 28,171 genes were imported into R (suppl. data 3). tRNAs were removed and, after initial exploration using PCA analysis, one outlier (N.C female 4) was excluded (n=34). Genes expressed in five or fewer samples were excluded. Visual inspection of mean gene expression histograms confirmed overall sample homogeneity. Finally, genes with an average expression of $\leq$1 across all samples were excluded, leading to 18,485 genes considered in downstream analyses. mRNA-seq data were used to conduct a PCA with log-transformed (log(expr.+1)) gene expression as the dependent variable and random intercepts according to the start-beaker to correct for a potential start-beaker effect (see 2.4; suppl. data 3). From this model, the residuals were used to compute a PCA on scaled log-transformed expression value residuals (suppl. text; Fig. S1&2). To specifically compare mRNA expression between treatment groups, a mixed model (see 2.4) was computed for each gene including both treatments and their interaction as independent factors (suppl. text; Fig. S3). Differentially expressed (DE) mRNAs found in pairwise comparisons with a meaningful number of DEGs (i.e., >50 DEGs; Table 1) were further tested for GO enrichment for biological processes using the function gost() (package 'gprofiler2' v.0.2.2; Peterson et al., 2020; suppl. data 4), after a custom reference gmt-file with assigned GO categories for all genes was created. Additionally, the 'gSCS'-method was used to correct for multiple testing, α was kept at 0.05 and as background all retained 18,485 genes were chosen. For the presentation of enriched GO terms, very broad terms with more than 250 annotated genes were excluded as they are often less indicative for specific processes.

2.6. miRNA-seq & analysis

Larval miRNAs of 35 samples were sequenced by BGI (Hongkong) using the DNBseq technology to yield 1,014 M 50 bp single end reads. After removal of low-quality reads, adaptors and other contaminants, 897 M reads remained with an average of 25.6 M per sample (min: 23.8 M, max: 28.1 M). To identify miRNAs, we used the miRDeep2 pipeline (Friedländer et al., 2008). Briefly, the mapper module was used to map raw reads against the herring genome and produce collapsed reads and mapping files. These files, in addition to the herring genome sequence and known *Danio rerio* miRNA sequences from the miRBbase database were processed by the main miRDeep2 program to detect miRNAs (suppl. data 5&6). The identified overall patterns of both analyses are highly congruent (see suppl. text). Hence, we only report the herring miRNA analysis in the main text. miRNA expression was calculated (quantifier module), data were imported into R and counts normalized across samples for downstream analyses (suppl. text; Fig. S4a). A PCA was conducted as described for mRNA and a mixed model was used for pairwise comparisons (see 2.5; Fig. S4b, 5, 6). To link miRNA and mRNA expression, a target prediction for each miRNA with a binding energy smaller -20Kcal/Mol was conducted. For this, miRanda version 3.3a (Enright et al., 2003) was used to compare the miRNAs against all 3' UTR sequences extracted from the herring genome via the corresponding annotation file and a maximum of 553 target genes were predicted for individual miRNAs. Then, per DE miRNA identified in the pairwise comparisons, the miRNA expression was correlated individually with the expression values of all its target mRNAs using Spearman correlations (one sample was excluded since it was excluded from the mRNA analysis, see 2.5). For each DE miRNA with multiple target mRNAs, fdr corrections were performed as q-values could not be calculated when too few tests were performed (suppl. data 7). To illustrate the overall mean of predicted regulatory interactions, the mean of all significant Spearman correlation coefficients over all miRNAs (raw $p<0.05$) was calculated. While inspecting correlation coefficients of predicted miRNA-mRNA pairs, we noted that for many miRNAs either positive or negative correlation coefficients dominated. Therefore, we explored if regulatory effects of individual miRNAs on their target mRNAs were indeed predominantly negative or positive. For each miRNA, Spearman correlation coefficients for every target mRNA (raw $p<0.05$) were computed and afterwards averaged. Additionally, a random distribution of such mean correlation coefficients was calculated. Here, instead of using the significant correlation coefficients of the predicted target genes per miRNA, the same number of randomly chosen target genes (i.e., genes that showed significant correlations with any miRNA) was used and their correlation coefficients were averaged and plotted.

2.7. Microbiome sequencing & analysis

PCR and sequencing of 16S rRNA reads were performed by BGI (Hongkong). Amplification failed for one H.C larval sample and one H.Val larval sample, leading to 33 microbiome samples. The primers 338f (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify the V3-4 regions of the 16S rRNA gene. The reads were analyzed with QIIME2 version 2022.2, DADA2 version 1.22.0 and the SILVA 138 reference database (see suppl. text). The count data of the microbiome samples and associated taxa information were imported into R and normalized per sample for total count number (i.e., subsequent analyses refer to relative counts; suppl. data 8). The effects of temperature and bacterial exposure on the larval microbiome were investigated by removing the experimental *Vibrio* strains from the data set (see suppl. text), normalization and summing up strains of the same taxonomic genus resulting in 300 genera. After removing genera only present in single individuals (non-informative), a Bray-Curtis dissimilarity matrix on 144 genera was computed and the betadisper() function ('vegan' package v.2.6-4; Oksanen et al., 2012) in conjunction with the anova() function showed similar variance among experimental groups ($df=5$, $F=1.0393$, $p=0.415$). A PERMANOVA was calculated with the dissimilarity matrix as dependent variable matrix and temperature, bacterial exposure and their interaction as independent variables using the adonis2() function ('vegan' package). To reflect that samples were not independent, the start-beaker was used for the 'strata' parameter. To illustrate microbiome genera responding to the experimental treatments, nMDS analyses were calculated using the metaMDS() function ('vegan' package; setting: try=50, trymax=50) with different numbers

of allowed dimensions (k=1 to 6). Ordinations were evaluated based on stress levels and Shepard diagrams, using the stressplot() function ('vegan' package). Four dimensions were deemed sufficient, despite the analysis only suggesting weak ties among groups (stress=0.146; and, thus, conclusions should be drawn carefully) and nMDS dimensions were rotated according to the bacterial exposure treatment using the MDSrotate() function ('vegan' package). Larval microbial community compositions were explored across treatment groups by calculating the median normalized count per treatment group and genus and dividing these by the sum of all genera medians of the respective treatment group, an approach that prevents outlier individuals with exceptionally high or low counts for a genus having too much weight. Richness, Shannon evenness and Shannon diversity were calculated ('microbiome' package v1.23.1; Lahti and Shetty, 2023) using a mixed model (see 2.4).

3. Results

3.1. Growth

The mean standard length of all larvae was 6.66 mm (± 0.08 mm SE). Larval standard length was neither influenced by temperature, bacterial exposure nor their interaction (ANOVA: $\chi2$=0.128, 0.286, 1.129; df=1, 2, 2; p=0.721, 0.867, 0.569).

3.2. Survival

Survival was monitored until the end of the experiment (3 days after the bacterial exposure). The mixed effects Cox model (events: n=89, 1597; penalized log-likelihood: $\chi2$=40.5, df=9.96, p<0.0001) revealed that neither temperature (z=0.9, p=0.37) nor bacterial exposure had an effect (*V. alginolyticus*: z=1.42, p=0.16; *V. anguillarum*: z=1.29, p=0.20) on survival. Overall, the mortality was approximately 17% across all treatments.

3.3. mRNA transcriptome

3.3.1. mRNA PCA analysis

Temperature treatment affected the larval gene expression substantially (see PCA Fig. 2a; suppl. data 3). PC1 scores, which represent approx. 21% of the variation found in the mRNA transcriptome data set, are significantly different between the temperature treatments (t=2.322, df=29.22, p=0.027). Additionally, the scores of PC7, reflecting approx. 4% of total variation, show a significant differentiation between control samples and those exposed to bacteria (ANOVA: F=5.991, df=2, p=0.0063; TukeyHSD: C-V.al: p=0.0103, C-V.an: p=0.0194 & V.al-V.an: p=0.9438).

Table 1
Pairwise comparisons of treatments and their respective number of DE mRNAs (in parentheses number of up- and downregulated genes) and miRNAs and the number of predicted unique target mRNAs, i.e., if different DE miRNAs had the same target mRNA, the repeating targets were not counted again (and in parentheses the total number of predicted target mRNAs, both fdr corrected).

Pairwise comparisons	# DE mRNAs	# DE miRNAs	# predicted target mRNAs
H.C vs N.C	4,826 (840/3986)	12	541 (817)
N.Val vs N.C	1,048 (148/900)	2	0
N.Van vs N.C	12 (10/2)	0	0
H.Val vs N.C	37 (5/32)	54	540 (816)
H.Van vs N.C	2,264 (507/1757)	6	336 (593)
H.Val vs H.C	2,922 (2425/497)	14	0
H.Van vs H.C	0	0	0
H.Val vs N.Val	1 (1/0)	15	266 (268)
H.Van vs N.Van	2,402(253/2149)	9	372 (392)

3.3.2. Differential gene expression and GO enrichment

DE genes between treatment groups are presented in Table 1 (and suppl. data 3). The temperature alone (H.C vs N.C) had the strongest effect on larval gene expression resulting in 4,826 DEGs (corresponding to >26% of all analyzed genes) from which 82.6% were downregulated (Fig. 3). At normal temperature, the *V. alginolyticus* exposure led to 1,048 DEGs (N.Val vs N.C) from which 85.9% were downregulated. 941 of these DEGs are shared with the temperature treatment and without exception the gene expression responses to the *V. alginolyticus* exposure and to increased temperature are in the same direction (Fig. S7). When control larvae reared at a normal temperature (N.C) were compared to those exposed to *V. alginolyticus* at a higher temperature (H.Val), only 37 DEGs were identified. In contrast, at the higher temperature 2,922 DEGs were identified comparing *V. alginolyticus* exposed larvae to control larvae (H.Val vs H.C). 2,099 of these DEGs were also among the 4,826 DEGs responding to the heat treatment but the direction of response was inverse for all of them. Similarly, from the 2,922 DEGs responding to *V. alginolyticus* at a high temperature (H.Val vs H.C), 624 overlap with DEGs found in *V. alginolyticus* exposed larvae at a normal temperature (N.Val vs N.C) and all but five of them showed an inverse response direction (Fig. S7).

Upon *V. anguillarum* exposure, transcriptional responses remained much more subtle: at the normal temperature, only 12 DEGs were identified (N.Van vs N.C) and at the higher temperature no response was detected (0 DEGs for H.Van vs. H.C). The 2,264 DEGs found for H.Van vs N.C are almost all found in the pure temperature effect. 2,072 DEGs overlap with N.C vs H.C and all of them show a change in the same direction. Similarly, the 2,402 DEGs observed in H.Van vs N.Van overlap substantially with those observed in the pure temperature effect (1,712 overlapping DEGs) and show regulatory change in the same direction (except for two of them).

We identified 195 enriched GO terms for the comparison H.C vs N.C, 175 for N.Val vs N.C, 157 for H.Van vs N.C, 135 for H.Val vs H.C and 88 for H.Van vs N.Van. The dominating and almost exclusive theme for all the comparisons was cell proliferation (Fig. 4). Some of the most significant and more specific GO terms (≤250 annotated genes) found in the analyzed comparisons include 'DNA replication', 'protein folding', 'chaperone-mediated protein folding', 'mRNA transport', 'mitotic nuclear division', and 'double-strand break repair'. The enriched GO terms 'chaperone-mediated protein folding' and 'protein folding' motivated an in-depth examination of heat-shock proteins (HSPs) revealing that most HSPs were downregulated in H.C and N.Val (both vs N.C). Overall, the most downregulated genes in H.C and N.Val (both vs N.C) were two apolipoprotein A-IV (apoA-IV) paralogs while procathepsin L-like (in both groups) and trypsin-2-like (only H.C vs N.C) were highly upregulated. The muscle protein troponin I was highly downregulated in both H.C and N.Val vs N.C. Immunity-related GO terms were not enriched. However, since the herring larvae were only 5 dph, we were interested in the ontogenetic onset of immune genes and found mRNA of interleukins, IL-receptors, complement components, antimicrobial peptides, lysozyme but also nuclear factors of activated T cells, B cell receptors, immunoglobulins and MHC I (suppl. data 3).

3.4. miRNA transcriptome

3.4.1. miRNA PCA analysis

The miRNA PCA analysis showed that the temperature treatment affected the larval miRNA expression (Fig. 2b; suppl. data 5). PC2 scores, which represent approx. 6% of the total variation, are significantly different between the temperature treatments (t=7.5, df=31.861, p<0.0001). No significant difference between control groups and *Vibrio* exposed was found on considered PCs.

3.4.2. Differential miRNA expression and target mRNA prediction

An increase in temperature alone resulted in 12 DE miRNA (H.C vs N.C), which are predicted to regulate 541 different mRNAs (called target

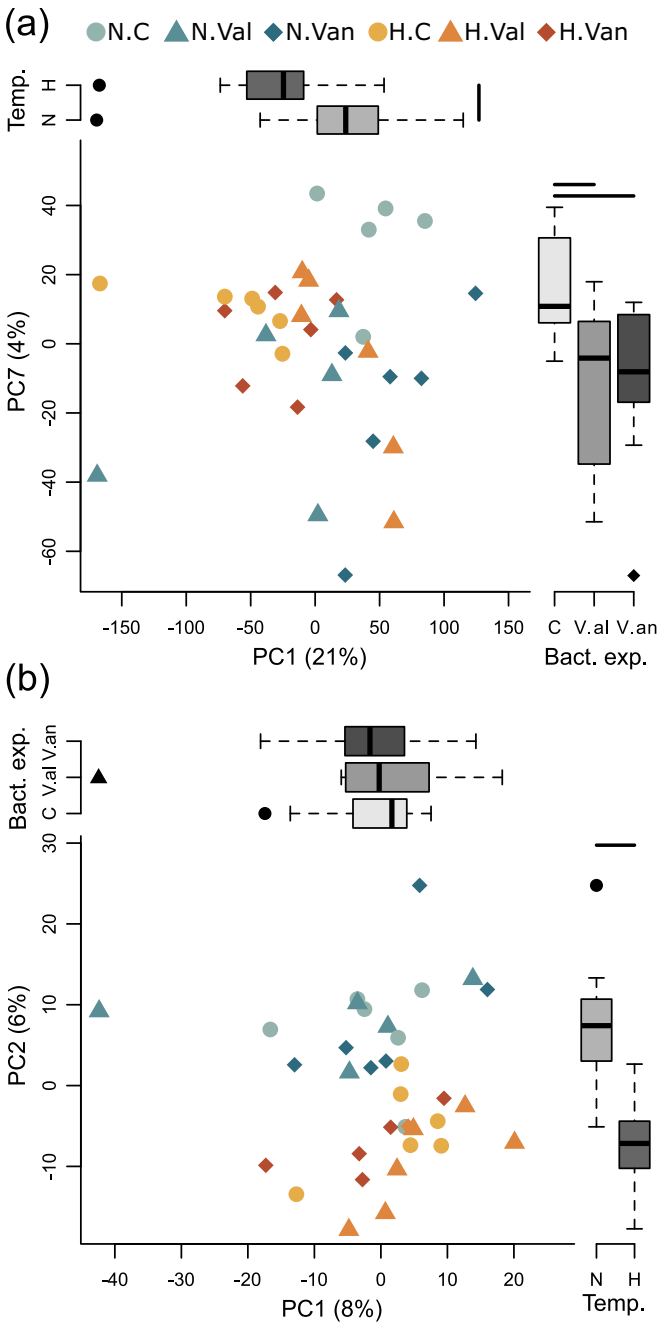

Fig. 2. PCA scores of (a) PC1 and PC7 for mRNA expression and (b) PC1 and PC2 for miRNA expression. Blue color hues indicate the normal temperature groups (N), orange color hues indicate higher temperature groups (H). Val = *V. alginolyticus*, Van = *V. anguillarum*. Bars indicate significant score differences between groups.

mRNAs hereafter). In response to the *V. alginolyticus* exposure at normal temperatures (N.Val vs N.C), only two miRNAs were differentially expressed and none responded to the *V. anguillarum* exposure (N.Van vs N.C and H.Van vs H.C). In contrast, the combination of higher temperature and *V. alginolyticus* exposure led to the highest number of 54 DE miRNA (H.Val vs N.C) with 540 different target mRNAs. In regard to the bacterial exposures, miRNA expression responded more pronounced (i) to *V. alginolyticus* than *V. anguillarum* and (ii) in combination with the higher temperature. For results for all comparisons, see Table 1 and suppl. data 5. Strikingly, for the 14 DE miRNAs found in H.Val vs H.C, no target mRNAs were predicted (after fdr correction). Hence, we explored them in more detail and noticed that the majority (12) was of simple and

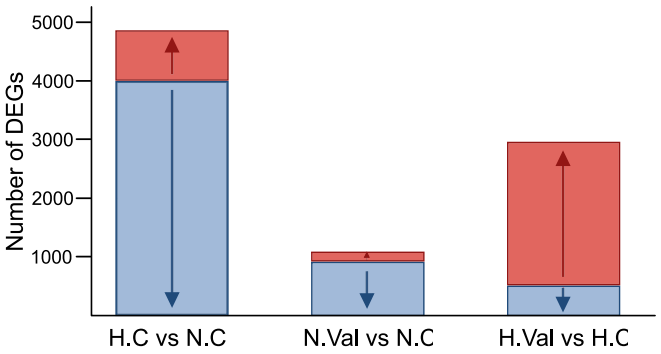

Fig. 3. Number of DEGs for comparisons with >50 DEGs. Upregulated genes in red, downregulated genes in blue. *V. anguillarum* data not shown as observed effects were attributed to the temperature treatment.

highly repetitive nature. Such miRNAs were only detected in one other comparison: H.Val vs N.C (39 out of 54 miRNAs) making them unique to larvae reared at the higher temperature and exposed to *V. alginolyticus* (group H.Val). To gauge their potential function, GO enrichment analysis was performed with their 777 unique target mRNAs identified before fdr correction and the GO term 'instar larval or pupal development' was significantly enriched. Across all comparisons, a total number of 79 unique DE miRNA were identified but only 17 of them were predicted to regulate a target mRNA. The number of fdr-corrected target mRNAs per DE miRNA varied between one and 256 (Fig 5c). In total, 586 unique target mRNAs were identified. Notably, miRNA '*chr7_14370*' (identical to zebrafish miRNA '*dre-miR-181a-2-3p*'; see suppl. text) was the only miRNA appearing in as many as five comparisons, was strongly and consistently upregulated in normal temperature samples, and had the highest number of target mRNAs (256). Moreover, we calculated a mean correlation coefficient (MCC) for each miRNA (raw p-values < 0.05) and all their respective target mRNAs and found moderate to strong regulatory biases, i.e., miRNAs regulate their target mRNAs either negatively or positively but rarely have bidirectional effects on their targets (Fig. 5a; suppl. data 7). Hence, the MCC distribution appears rather uniform (or even bimodal; Fig. 5a, in light gray), with negatively biased miRNAs being more common than positively biased miRNAs, which contrasts the normally distributed MCCs expected if the direction of regulatory interaction would be arbitrary (Fig. 5a, in dark gray). Across all predicted miRNA-mRNA correlations, coefficients were on average negative (mean ρ=-0.103). MiRNAs with particularly pronounced positive or negative MCCs also appear to have more target genes than miRNAs with MCCs closer to zero and DE miRNAs predominantly have MCCs distinct from zero (Fig. 5b, c).

3.5. Microbiome

The *V. anguillarum* culture used for the experiment consisted of only one strain while the *V. alginolyticus* culture consisted of four *V. alginolyticus* strains. The used *V. anguillarum* strain did not appear in the *V. alginolyticus* treatment and vice versa (Fig. S8). For both *Vibrio* species, increased temperature negatively affected relative counts significantly (*V. alginolyticus*: coef=-6285, χ^2=296.44, df=1, p<0.0001; *V. anguillarum*: coef=-3776, χ^2=8.56, df=1, p=0.0034). After experimental *Vibrio* counts were removed from the microbiome, the PERMANOVA revealed that both factors temperature and bacterial exposure affected the microbiome composition while no interactive effects were identified (Table 2).

Based on our nMDS analysis, dimension 1 reflects a separation of the control larvae and larvae exposed to *Vibrio* (suppl. data 8). While control larvae (in circles) have on average positive values (but also appear most scattered), the larvae exposed to bacteria (triangles and diamonds) have

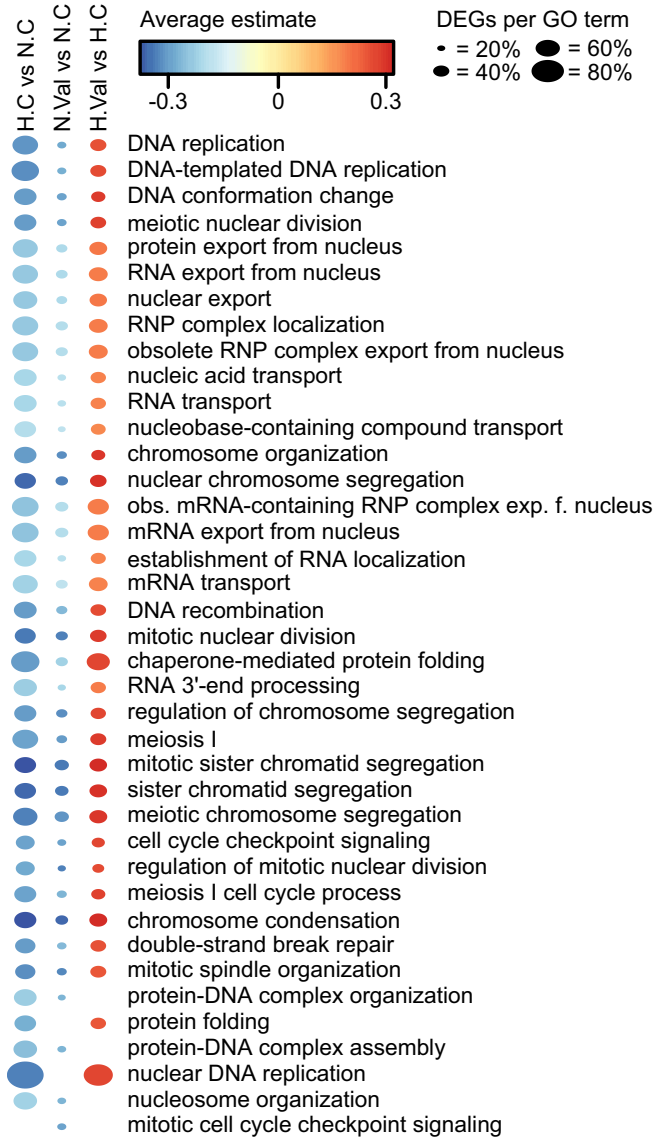

Fig. 4. Enriched GO terms for biological processes for treatment comparisons with >50 DEGs. The size of the ellipse indicates the percentage of enriched genes in the respective term. The color indicates if enriched genes were on average upregulated (red) or downregulated (blue) according to mixed model estimates. *V. anguillarum* data are not shown as observed effects were attributed to the temperature treatment. RNP = ribonucleoprotein. (Only GO terms with ≤250 annotated genes were considered as broader terms are less specific and, thus, less indicative. For all identified GO terms, see suppl. data 4).

on average negative values (Fig 6a). Dimension 2 rather reflects a temperature effect. Here, more positive values are on average associated with the higher temperature (dark colors) and more negative values with the normal temperature groups (light colors). Microbiota genera associated with the control treatment include *Klebsiella, Saccharimonadales* and *Friedmanniella* while *Ralstonia* and *Caulobacter* are associated with the *Vibrio* exposures (Fig. 6b; suppl. data 8).

The five most dominant bacterial genera found in all treatment groups include *Pseudomonas, Shewanella, Flavobacterium, Burkholderia-Caballeronia-Paraburkholderia* and *Pseudoalteromonas,* together making up over 67% of all larval microbial communities (Fig. 7). The increased temperature reduced the species richness (χ^2=5.39, df=1, p=0.0202) and Shannon diversity (χ^2=6.72, df=1, p=0.0095), while the Pielou's evenness was not affected (χ^2=3.34, df=1, p=0.0678). Bacterial exposure and the interaction of both factors did not affect the indices.

4. Discussion

The primary objective of this study was to determine how multiple climate change stressors, i.e., a combination of a heat wave and bacterial exposure, influence Atlantic herring larvae. Our results show that both the simulated spring heat wave and the bacterial exposure significantly influenced the larval mRNA and miRNA expression as well as the microbiota, even though the herring larvae were sampled at only 5 dph (at the end of their yolk-sac phase). Although larval growth and survival were not affected, potential effects could have occurred at a later larval stage as the larvae were only sampled 3 days after *Vibrio* exposure. For example, a significant effect on survival was only observed 6 days after *Vibrio* infection in another herring larvae study (Poirier et al., 2017).

The larval mRNA expression profiles were highly affected by the temperature, the bacterial exposure and their interaction. The comparisons between different treatment groups showed that temperature alone had the strongest effect on the overall larval gene expression resulting in 4,826 DEGs (H.C vs N.C), while the *V. alginolyticus* exposure (at normal temperature) led to 1,048 DEGs (N.Val vs N.C). In both H.C and N.Val (each compared to N.C) over 80% of the DEGs were downregulated, indicating a stress-induced transcriptional attenuation. Unraveling the mechanistic details of such transcriptional lockdowns is still in its infancy, however, reduced mRNA and protein production during stress may spare the proteostasis machinery, allowing cells to allocate resources to control stress-induced damages (Sawarkar, 2022). For example, transcriptome analysis revealed a major downregulation of genes (over 70%) upon thermal stress in the muscle of adult *Squalius torgalensis* (Jesus et al., 2016). In our study, the 195 (H.C vs N.C) and 175 (N.Val vs N.C) enriched GO terms for biological processes are almost exclusively involved in cell proliferation (e.g., 'DNA replication', 'protein folding', 'mRNA transport', 'mitotic nuclear division' and 'cell cycle checkpoint signaling'). Hence, the high temperature and the *V. alginolyticus* exposure at a normal temperature both may exert an inhibitory effect on the development of herring yolk-sac larvae. The same effect was found in zebrafish larvae where heat stress downregulated genes involved in, e.g., DNA replication and mitosis (Long et al., 2012). In heat-stressed clownfish larvae (stage 3) and golden pompano larvae, approximately 60% of the DEGs were downregulated (Han et al., 2021; Moore et al., 2023). GO terms associated with mRNA processing, translation and mitotic cell cycle were enriched in clownfish (Moore et al., 2023) while different metabolic processes were enriched in golden pompano (Han et al., 2021).

Since the GO term 'chaperone-mediated protein folding' was enriched in both groups (H.C/N.Val vs N.C) and additionally the term 'protein folding' in group H.C vs N.C, we had a closer look at the expression of HSPs. HSPs are highly conserved proteins usually functioning as chaperons during folding of new proteins and refolding of damaged proteins and are, thus, induced upon proteotoxic stress (Lang et al., 2021) as shown in many fish studies including herring flexion larvae (e.g., Beemelmanns et al., 2021c; Deane and Woo, 2011; Joly et al., 2023; Moore et al., 2024). However, in our study most differentially expressed HSPs were downregulated in both groups (H.C/N.Val vs N.C) and the same was observed in heat-stressed clownfish and golden pompano larvae (Han et al., 2021; Moore et al., 2023). Overall, this may have negative effects on the fish larval development since HSPs are not only essential for the stress response but generally play a crucial role in cellular proliferation and differentiation in embryonic and larval stages (Marvin et al., 2008; Pederzoli and Mola, 2016; Tokunaga et al., 2022). In stage 2 clownfish larvae, five of ten investigated HSPs were up- and five downregulated at 31°C compared to 28°C. In contrast, in stage 3 larvae all ten HSPs were downregulated at 31°C (Moore et al., 2023). Hence, there are several possible explanations for the observed downregulation in our study: (i) elevated HSP synthesis can be followed by suppressed HSP expression as sufficient HSPs are already available (Logan and Buckley, 2015), (ii) under high stress, the larval ability to express sufficient HSPs may be inhibited in critical (energy-demanding)

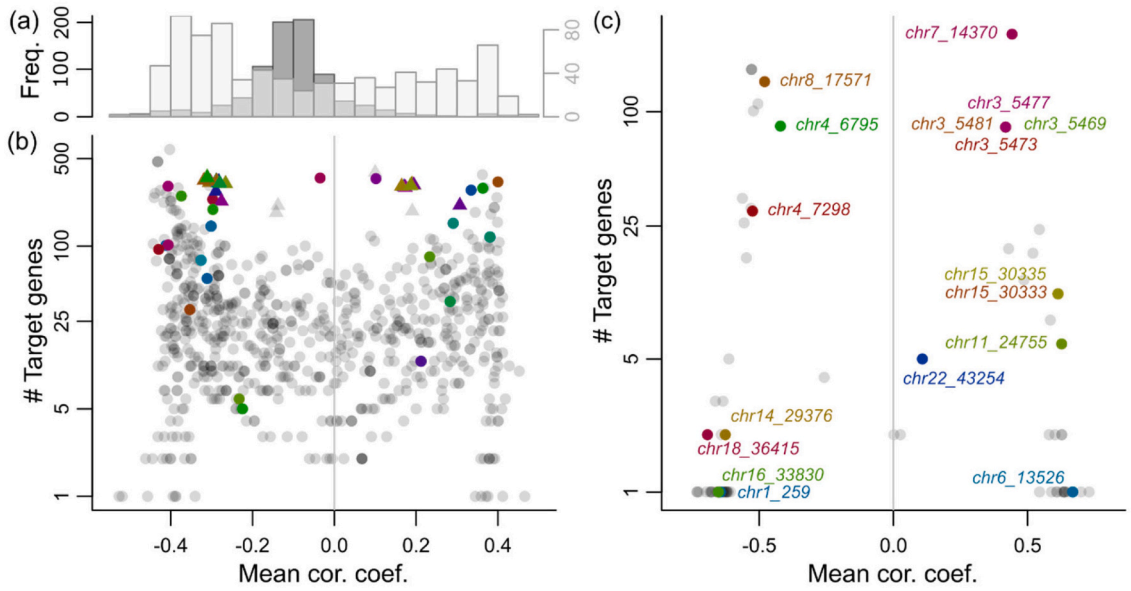

Fig. 5. Mean correlation coefficients (MCCs) for all identified miRNA and their predicted target mRNAs based on raw p-values < 0.05. (a) Histogram of MCCs found in this study (light gray) and expected unimodal distribution without regulation bias (dark gray). (b) Number of target genes per miRNA plotted against the MCCs. DE miRNAs identified for comparisons in arbitrary colors, all other identified miRNAs in gray. Triangles indicate repetitive miRNAs. (c) MCCs per miRNA and all its predicted mRNA targets (fdr-corrected).

Table 2
PERMANOVA results using microbiome relative counts as a dependent variable (experimental *Vibrio* ASVs were excluded).

Treatments	Df	Sum of Squares	R^2	F	Pr(>F)
Temperature	1	0.2181	0.05482	1.8825	0.0299
Bact. exposure	2	0.4657	0.11707	2.0101	0.0038
Temp. x Bact. exp.	2	0.1666	0.04187	0.719	0.7771
Residuals	27	3.1279	0.78624		
Total	32	3.9783	1		

developmental stages (Parsell and Lindquist, 1993), (iii) it may be a simple consequence of the observed cell cycle arrest (the inhibition of cell proliferation results in less HSPs in the stressed groups compared to the control group).

The most downregulated genes in H.C and N.Val (both vs N.C) were two apoA-IV paralogs. ApoA-IV is involved in lipid absorption and cellular lipid import and export facilitating the transport of lipids from yolk cells to the fish embryo and larva (Otis et al., 2015). A lower apoA-IV expression suggests diminished yolk lipid intake and is linked to reduced larval growth, development (especially nervous system and eyes) and activity as yolk-sac lipids serve as a primary energy source (Conceição et al., 1993). A differential expression of genes associated with the lipid metabolism was also observed in larval Tibetan naked carp upon acute heat stress (Zhang et al., 2017). In contrast, the proteases procathepsin L-like (in both groups) and trypsin-2-like (only H.C vs N.C) were highly upregulated, which goes in line with the observation that (heat) stress is associated with protein denaturation and proteolysis (Evans and Hofmann, 2012). In golden pompano larvae, e.g., heat stress led to DEGs enriched in pathways related to protein and fat digestion and absorption (Han et al., 2021). In fish early life stages, misfolded proteins, protein denaturation and proteolysis are especially disadvantageous since high rates of protein biosynthesis are essential for somitogenesis (Meekan et al., 2006). Moreover, the muscle protein troponin I, a central regulatory protein of muscle contraction (Darias et al., 2008), was downregulated in both groups (H.C/N.Val vs N.C), which may reduce swimming activity and, thus, the ability to hunt prey.

Altogether, high temperature larvae and *V. alginolyticus* exposed larvae (at a normal temperature) rather displayed a general stress response than a specific heat-shock or immune response as indicated by a 90 % overlap of DEGs and the same expression direction in both groups (H.C/N.Val vs N.C). The observed cellular stress response (CSR) is a universal defense reaction minimizing and repairing macromolecule damage regardless of the kind of stress causing the damage (Kültz, 2005, 2020a, 2020b). This compensation strategy reduces the level of irreversible cellular damage by sensing membrane lipid, protein and DNA damage, but also impairs cell growth and proliferation, and lowers the immune response (Kültz, 2005; Petitjean et al., 2019). For example, the observed reduction of DNA replication decreases the risk of DNA damage, permitting Atlantic herring larvae to cope with different stressors associated with ongoing climate change. The energy saved upon the CSR can be allocated towards macromolecular stabilization and repair (Kültz, 2005; Sawarkar, 2022). In our experiment, larvae were sampled already at 5 dph. During the first weeks post hatch, proper somitogenesis for successful swimming and hunting as well as growth to avoid predation are key prerequisites for the survival of fish larvae. The observed reduction in cell proliferation may ultimately be traded-off with reduced larval growth, immunity and survival at a later life stage and/or when experiencing an additional stressor (Alfonso et al., 2021; Kim et al., 2017). Hence, transcriptome analyses of feeding larvae reared under realistic climate change scenarios with a natural prey density are needed to estimate the real extent of sensitivity towards warmer temperatures observed in our study and in an Atlantic herring larvae mesocosm experiment (Sswat et al., 2018).

An exposure to *V. alginolyticus* in larvae reared at high temperatures (H.Val vs H.C) resulted in 2,922 DEGs. In contrast to the massive downregulation of gene expression upon temperature stress or *Vibrio* exposure alone, we observed an overall upregulation of genes in H.Val compared to H.C. Indeed, all of the downregulated DEGs in H.C vs N.C were upregulated in H.Val vs H.C and vice versa. Strikingly, the overall expression of genes in H.Val was not higher than the gene expression in N.C (resulting in almost no DEGs in H.Val vs N.C). At first glance, this seems counterintuitive, as both the high temperature and *V. alginolyticus* at normal temperature (H.C/N.Val vs N.C) had a strong inhibitory effect on the larval gene expression. In case of exposure to multiple stressors, we expected an additive or even synergistic stress response, i.e., an even stronger downregulation caused by the interaction of high temperature and *V. alginolyticus* exposure in H.Val vs N.C. However, the interacting

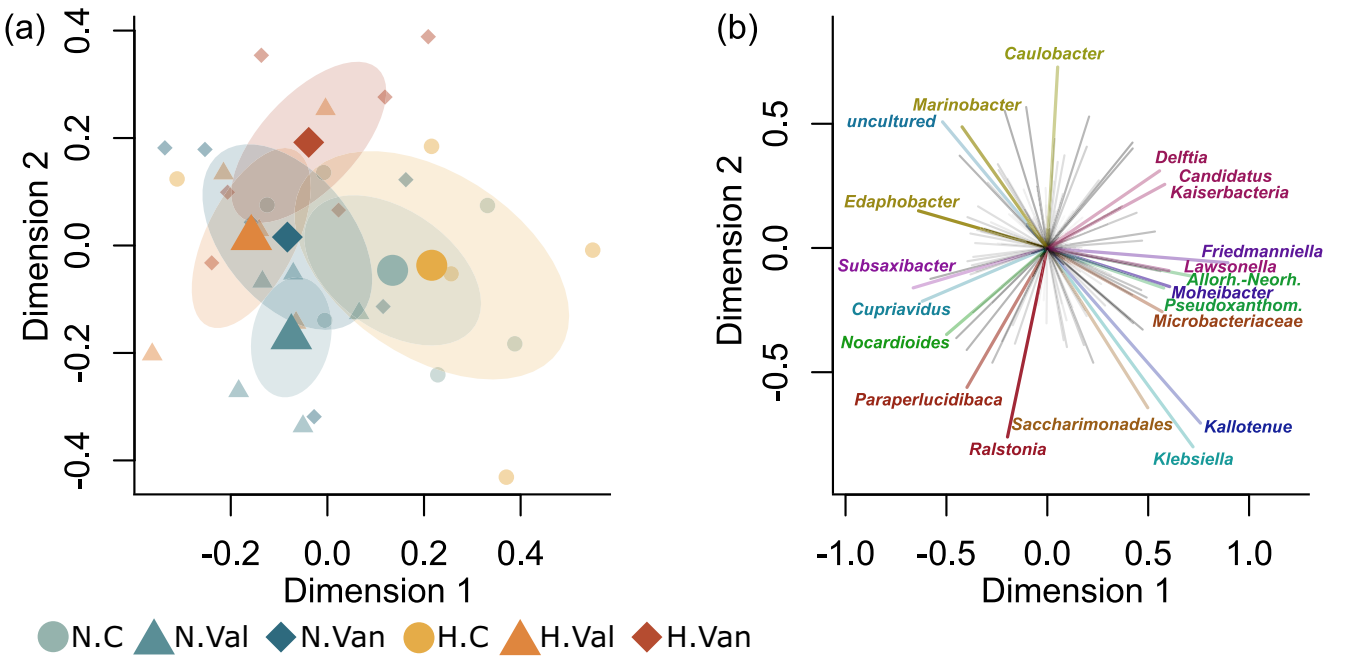

Fig. 6. nMDS plots of genus-level microbiome composition. (a) Ordination plot of samples for nMDS dimension 1 and 2. Data ellipses reflect 0.3 CI. Large symbols reflect mean coordinates for each treatment. Blue color hues indicate the normal temperature groups (N), orange color hues indicate higher temperature groups (H). Val = *V. alginolyticus*, Van = *V. anguillarum*. (b) Microbiome genera associated with dimension 1 and 2, the 20 most influential are labeled. Colors are assigned arbitrarily.

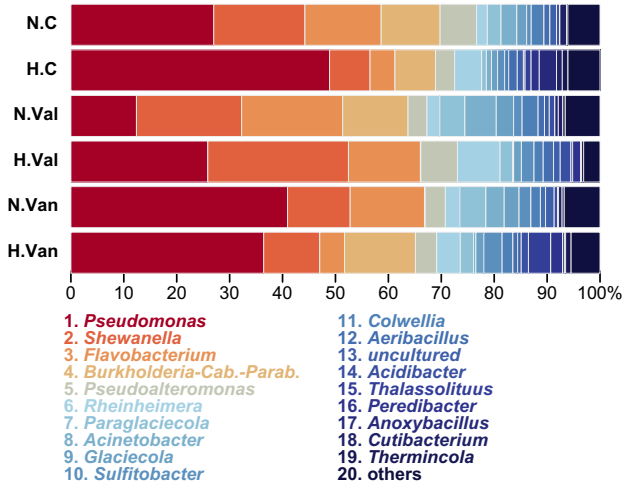

Fig. 7. Larval bacterial communities per treatment on the genus level (based on the medians of the normalized counts to correct for disproportional effects of outliers).

stressors, high temperature and *V. alginolyticus* exposure, induced an antagonistic effect indicating an absence of the observed acclimation responses to the single stressors. Apparently, herring larvae lack a cellular stress response when a certain stress threshold is exceeded, suggesting a loss of plastic responses. The inability to react to the combination of increased temperature and *V. alginolyticus* exposure may cause a high level of irreversible cellular damage, potentially reducing long term survival and fitness (Petitjean et al., 2019).

Despite the strong effect of *V. alginolyticus* on larval gene expression at both temperatures (N.Val vs N.C and H.Val vs H.C), immunity-related GO terms were not enriched. Early marine fish larvae mainly rely on their innate immune response and maternally-transferred immune molecules while their adaptive immune system is still maturing (Magnadottír, 2006; Swain and Nayak, 2009). While mRNA of innate immune genes but also nuclear factors of activated T cells, B cell receptors, immunoglobulins and MHC I was found in the herring larvae, no induction of immune gene expression was identified upon bacterial exposure. This lack of an immunological activation might indicate that: (i) maternally-transferred immune molecules were sufficient for the larval protection, (ii) the time window of upregulation of immune genes, which starts in between hours after bacterial exposure, might have been missed since the larvae were sampled 3 days post exposure (Bugg et al., 2021; Nasrullah et al., 2021), (iii) the strong CSR (N.Val vs N.C) impaired the expression of immune genes due to a resource allocation conflict.

In contrast to the strong influence of *V. alginolyticus* on Atlantic herring larval gene expression, *V. anguillarum* exposure only resulted in very few (N.Van vs N.C) or no (H.Van vs. H.C) DEGs. The DEGs identified in H.Van compared to N.C or N.Van were mostly attributable to the temperature effect, as most of them overlap with H.C vs N.C and show the same direction of differential expression. The lack of a *V. anguillarum* effect on larval gene expression suggests a low virulence for herring larvae even though the strain has been shown to be virulent for several fish species (Rønneseth et al., 2017).

Pairwise comparisons of larval miRNA expression suggest a significant response to an elevated temperature (e.g., '*chr7_14370*') but to a smaller extent than the mRNA expression. MiRNAs are evolutionarily conserved noncoding short RNA molecules that post-transcriptionally regulate gene expression by binding to their target mRNAs, inducing inhibition but also activation of translation (Cao et al., 2023; Fabian et al., 2010; Xiong et al., 2019). The 12 miRNAs that were differentially expressed between the two experimental temperature treatments are predicted to regulate the expression of a substantial number of genes (H.C vs N.C: 541 regulated mRNAs). A temperature effect on the miRNA regulation has been previously reported in other teleost early life stages and is presumably evolutionary conserved as it also appeared in our additional analysis considering only zebrafish miRNAs (Campos et al., 2014; Qiang et al., 2017; Zhang et al., 2017).

In contrast to the temperature impact on miRNA expression in

herring larvae, less pronounced changes were identified in *Vibrio* exposed larvae. Nonetheless, the *V. alginolyticus* exposure led to 14 DE miRNAs at the higher temperature (H.Val vs H.C) and to 2 DE miRNAs in N.Val vs N.C. Interestingly, these are not known from zebrafish suggesting that the miRNAs responding to the *V. alginolyticus* exposure might be evolutionarily less conserved than those involved in the temperature response. However, the cross-talk between host and microbiota may partly occur via miRNA uptake and release by bacteria (influencing the response to bacterial infections), hence, different host microbiomes can lead to diverging miRNA profiles (Williams et al., 2017).

The interaction of temperature and *V. alginolyticus* exposure (H.Val vs N.C) led to the highest number of 54 DE miRNAs. However, most of them (39) were of highly repetitive nature, as were 12 out of 14 in H.Val vs H.C, making them unique to the group H.Val. While erroneous predictions of these highly repetitive miRNAs might initially be suspected, their validity and distinct functionality is indicated by their exclusive expression in group H.Val and the enrichment of target genes (raw p-values < 0.05) in the GO term 'instar larval or pupal development' (however, after fdr correction, no target genes were retained). We hypothesize that the exclusive occurrence of repetitive miRNAs in group H.Val is connected to the observed loss of phenotypic plasticity in this group. Since miRNAs can serve as powerful biomarkers, e.g., for disease progression, growth and development (Eirin-Lopez and Putnam, 2019; Sarshar et al., 2020; Zhou et al., 2023), repetitive miRNAs might be good candidate biomarkers for reduced acclimation potential in fish early life stages.

Most miRNAs could unambiguously be assigned to having either a negative or a positive correlation with their target genes but only rarely both. To our knowledge we are the first to show this pattern on a transcriptome-wide scale. Consequently, a sizable number of miRNAs positively regulated their target genes, which contradicts the traditional understanding of miRNAs being only a negative regulator of mRNA stability and, thus, translation (Xiong et al., 2019). However, miRNA-mediated upregulation of target gene expression and translational activation have been reported before and may positively control global protein synthesis (Fabian et al., 2010; Ørom et al., 2008). Moreover, miRNAs have the potential to regulate translation in a cell cycle determined manner by activating translation during cell cycle arrest (and repressing it in cycling/proliferating cells) (Vasudevan et al., 2007, 2008). Hence, the observed miRNA-induced upregulation of target gene expression seems to play a role in the response of herring larvae to elevated temperature. While our data also suggest a role of miRNAs in response to bacterial infection, the link to gene expression remains less clear as no target genes were predicted (after fdr correction) for the bacterial exposures.

In our study, the higher temperature and the *Vibrio* exposures both had a significant effect on the larval microbiota. Thus, our results are in line with the observation that environmental biotic and abiotic factors, such as temperature and (opportunistic) pathogens, are driving fish microbial communities, which play a critical role in their health and nutrition (Moore et al., 2024; Morshed and Lee, 2023; Vestrum et al., 2020). Our results demonstrate that environmental stressors can change the composition of the larval microbiota rather quickly as the samples were taken only 3 days after the *Vibrio* exposures (corresponding to 5 dph). Bacterial genera associated with the *Vibrio*-exposed larvae included, e.g., Ralstonia and Caulobacter, which were both observed in diseased fish in other studies (Kim et al., 2023; Tran et al., 2018). The most abundant bacterial genera (making up over 50% of the larval microbial communities) were *Pseudomonas, Shewanella* and *Flavobacterium* – genera commonly found in fish and their early life stages (Bakke et al., 2015; Egerton et al., 2018; Hansen et al., 1992). By increasing the temperature, the proportion of *Pseudomonas* increased while *Shewanella* and *Flavobacterium* decreased. Interestingly, the *V. anguillarum* exposure also increased *Pseudomonas*, while the *V. alginolyticus* exposure decreased *Pseudomonas*. Changes in the relative abundance of those genera were also found in other studies upon environmental change and infection (e.g., Dehler et al., 2017; Zhang et al., 2018). While in most treatments *Pseudomonas* was the most abundant genus, *Shewanella* was the most abundant genera in the *V. alginolyticus* exposed larvae. Many members of the genus *Shewanella* are pathogenic, however, the observed increase is mainly attributed to *S. denitrificans* while pathogenic *Shewanella* species were not detected using our analysis approach (16S rRNA V3-V4 region). A closer look at the microbial diversity of the herring larvae revealed that the species richness and Shannon diversity are the highest in the normal temperature treatment (N.C). A healthy microbiota is usually characterized by high species richness and diversity while temperature stress can lead to a destabilization of the microbiota (Legrand et al., 2020; Mougin and Joyce, 2023). A lowered microbial richness and diversity (as found at the higher rearing temperature) can, thus, be disadvantageous for the development of fish larvae (Vadstein et al., 2013). A disturbance in microbiome homeostasis (i.e., dysbiosis) is associated with corresponding shifts in microbial community functionality, influencing the host's physiological parameters, including immune functions, and is directly correlated with disease resistance (Mougin and Joyce, 2023). In fish early life stages, this is especially problematic since a healthy microbiota is required for the maturation of the larval immune system (Franke et al., 2017). Hence, future heat waves, which have been projected to increase in frequency and intensity, have the potential to negatively affect the disease resistance of herring larvae through dysbiosis.

5. Conclusion

The primary objective of this study was to determine whether multiple climate change-related stressors, i.e., a combination of a heat wave and bacterial exposure, elicit an additive or even synergistic stress response in Atlantic herring larvae. Our study demonstrates high sensitivity in herring larvae to both increased temperature and *V. alginolyticus* infection, resulting in pronounced transcriptional downregulations likely reducing cell proliferation. The observed cellular stress response could facilitate acclimation by minimizing macromolecule damage. However, at this specific developmental stage – when yolk reserves are depleted – a continued cell cycle arrest may disrupt organogenesis and myogenesis, and increase the risk of starvation. The combination of elevated temperature and *V. alginolyticus* compared to the normal temperature (H.Val vs N.C) showed minimal gene expression changes. Hence, in contrast to our hypothesis, herring larvae exhibit an antagonistic stress response when exposed to bacteria during a heat wave. This indicates a lack of phenotypic plasticity upon multi-stressor exposure, which could lead to irreversible cellular damage, heightened vulnerability and reduced survival in later larval stages. We also noted a significant shift in miRNA expression and associated target genes in response to warming alone or in combination with *V. alginolyticus* exposure. When both factors were combined, a unique class of highly repetitive miRNA dominated. Additionally, both the temperature increase and the *Vibrio* exposures significantly affected the larval microbiome. Warmer temperatures lowered the microbial richness and diversity, potentially leading to dysbiosis and decreased larval health, although larval survival and growth were not significantly affected. In summary, our key findings include a pronounced transcriptional attenuation during the simulated heat wave but a lack of larval acclimation upon additional bacterial exposure. This prompts further research on the interactive effects of multiple environmental stressors on herring recruitment. The investigation of feeding larvae under realistic climate change scenarios needs specific attention. As gene expression changes may not manifest at the translational level, proteomics may be a valuable tool to assess larval condition. Future studies could help to unravel the factors behind the continuously decreasing recruitment of Western Baltic Spring-Spawning herring and, therefore, provide essential information for effective management strategies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.175659.

CRediT authorship contribution statement

Andrea Franke: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Till Bayer:** Data curation, Formal analysis, Resources, Validation, Writing – original draft, Writing – review & editing. **Catriona Clemmesen:** Conceptualization, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Fabian Wendt:** Investigation, Methodology. **Andreas Lehmann:** Formal analysis, Methodology, Writing – original draft. **Olivia Roth:** Conceptualization, Validation, Writing – original draft, Writing – review & editing. **Ralf F. Schneider:** Data curation, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Sequence data have been submitted to NCBI (BioProject ID PRJNA1065610) and miRNA data to FigShare (10.6084/m9.figshare.25029191). All other data have been uploaded as supplementary files.

Acknowledgments

The authors would like to thank three anonymous reviewers for taking the time and effort necessary to review the manuscript. We are very grateful for the constructive criticism which helped us to improve the quality of the manuscript. We would also like to thank the editor for the swift handling of the manuscript and the friendly communication. AF acknowledges support by the HIFMB, a collaboration between the Alfred-Wegener-Institute, Helmholtz-Center for Polar and Marine Research and the Carl-von-Ossietzky University Oldenburg, initially funded by the Ministry of Science and Culture of Lower Saxony (MWK) and the Volkswagen Foundation through the 'Niedersächsisches Vorab' grant program (ZN3285). OR was supported by the European Research Council under the European Union's Horizon 2020 Research and Innovation Program (Project: MALEPREG, 755659). The authors thank Prof Gabriele Gerlach for outstanding project support and Prof Reusch for granting access to GEOMAR facilities. We are grateful to Silke Mareike Marten, Josefine Karnatz, Julia Stefanschitz, Felix Mittermeier, Diana Gill and Svend Mees for their excellent support and Patrick Polte and Arseny Dubin for their valuable advice. The authors also thank Susanne Landis (scienstration.com) for the smooth and productive process of designing the graphical abstract and Fig. 1.

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