**Supplements to “Climate challenges for fish larvae: Interactive multi-stressor effects impair acclimation potential of Atlantic herring larvae” by Franke et al.**

**Supplementary information: Material and methods**

**Treatment abbreviations**

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)

High temperature rise and *V.* *anguillarum* exposure (H.Van).

**2.2 Hydrodynamic Baltic Sea model**

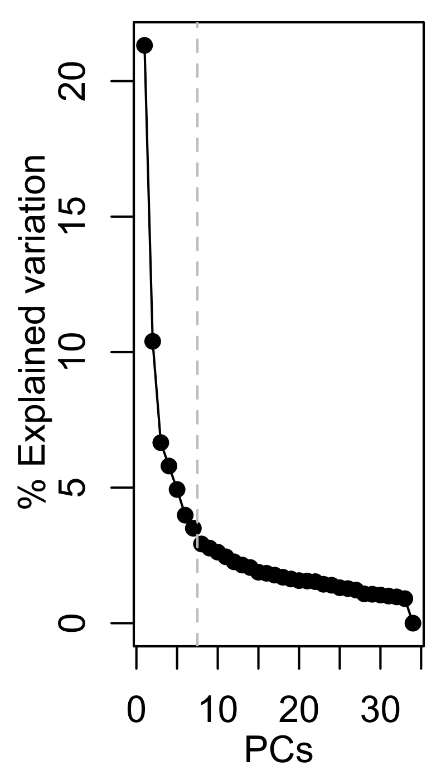
Two spring temperature scenarios were chosen corresponding to daily temperature and salinity data, obtained from a hydrodynamic coupled sea ice-ocean model of the entire Baltic Sea (BSIOM, Lehmann et al., 2014). The hydrodynamic model has a horizontal resolution of 2.5 km and vertical resolution of 3 m down to 100 m depth and 6 m from 100 to 250 m depth. The model is realistically forced using the ERA5 global re-analysis in the preliminary extension version back to 1950 (Bell et al., 2021), with a 3-hourly temporal and approx. 50 km spatial resolution, respectively. The atmospheric forcing data were interpolated on the model grid. They include surface air pressure, precipitation, cloudiness, and air- and dew point temperatures at 2 m height from sea surface. Wind speed and wind direction at 10 m height from sea surface were calculated from geostrophic winds with respect to different degrees of roughness on the open sea and off the coast (Bumke et al., 1998). BSIOM forcing functions, such as wind stress, radiation and heat fluxes were calculated according to Rudolph and Lehmann (2006). Additionally, river runoff was prescribed from a monthly mean runoff data set (Kronsell and Andersson, 2012). The performance and applicability of BSIOM has been demonstrated in a number of publications (e.g. Hinrichsen et al., 2016; Lennartz et al., 2014).

**2.3 RNA and DNA extraction**

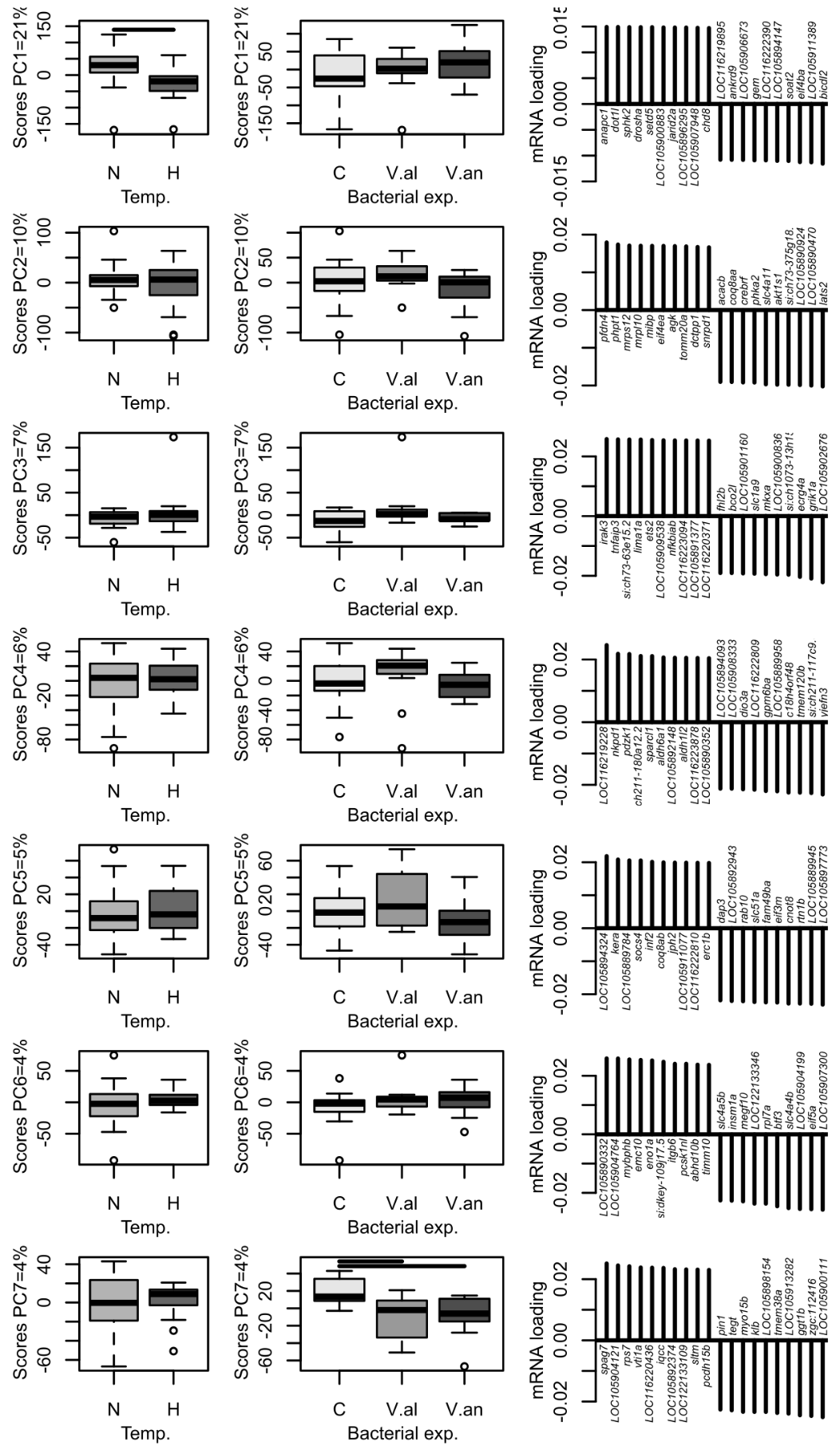
To extract the mRNA, miRNA and microbial DNA simultaneously from each larval and water sample, the Machery-Nagel ‘NucleoSpin® miRNA mini kit for miRNA and RNA purification’ was combined with the ‘NucleoSpin® RNA/DNA Buffer Set’. To homogenize the larval tissue and lyse bacterial walls, MP Biomedicals ‘Lysing Matrix A’ tubes were filled with 300 µL Buffer ML (step 1 ‘NucleoSpin® miRNA and RNA purification kit’). After photographing each larva, one larva was added per tube. Tubes were shaken for 2 min at 30 m/s in a bead mill (Qiagen TissueLyser II). From the water samples (1 ml), bacteria were harvested by centrifugation at 4000 g for 5 min. The supernatant water was discarded and the tubes were filled with ‘Lysing Matrix A’ and ‘300 µL Buffer ML’ and shaken for 2 min at 30 m/s in a bead mill. Subsequently, the standard protocol of the ‘NucleoSpin®miRNA and RNA purification kit’ was followed until step 4. Step 5 (desalt silica membrane) was not performed and instead replaced with the steps of the ‘NucleoSpin® RNA/DNA Buffer Set protocol’. Afterwards, the extraction was continued with step 6 of the miRNA and RNA purification kit. To measure the mRNA, miRNA and DNA of each sample, the Qubit® 2.0 fluorometer was used following the standard protocols of the Qubit™ assay kits ‘RNA HS’, ‘microRNA’ and ‘dsDNA HS’. Afterwards, samples were kept at -80 °C and finally shipped on dry ice to BGI (Hongkong) where mRNA-seq, miRNA-seq and 16S rRNA-seq were performed.

**2.5 mRNA-seq & analysis**

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 in the main text). From this model, the residuals were used to compute a PCA on scaled log-transformed expression value residuals. A scree plot was created and the first seven PCs were selected for downstream analysis (Fig. S1). Notably, PC3 appeared to be dominated by another outlier, which in this case was not removed to not further compromise sample size (Fig. S2). Per PC, t-tests and ANOVAs with subsequent TukeyHSD-tests were used to test for differences among temperature and bacterial exposure groups, respectively.



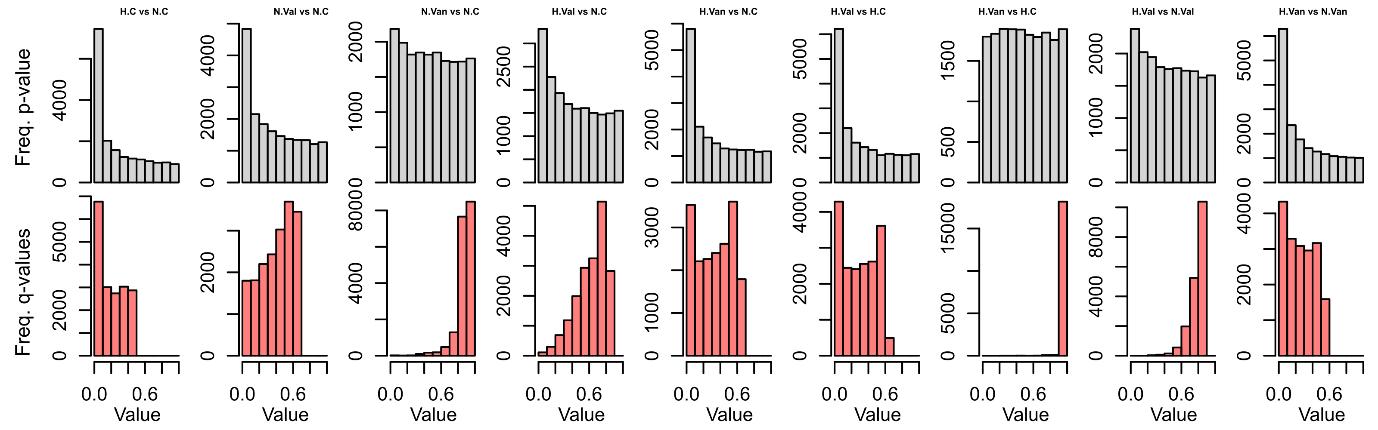
**Fig. S1:** Scree-plot of mRNA PCA. The vertical dashed line indicates the cut-off for the seven PCs considered in the study.



**Fig. S2**: PC scores and rotations of PCAs for expression values of mRNAs. Left side: Boxplots of the sample scores in the respective PC separated for temperature and bacterial exposure treatments. Lines above boxplots indicate statistical significance (p < 0.05). N = Normal temperature, H = High temperature, C = Control, V.an = *V. anguillarum*, V.al = *V. alginolyticus.* Right side: The genes with the ten most positive and negative loadings are shown.

**Pairwise comparisons mRNA**

To specifically compare mRNA expression between treatment groups, a mixed model was computed for each gene including both treatments and their interaction as independent factors. In total, nine pairwise comparisons were performed for each gene (Table 1 in the main text) using the glht() function (‘multcomp’ package v.1.4-25; Hothorn et al., 2023). Q-values were calculated using the q-value() function (‘qvalue’ package v.2.30; Storey et al., 2023) to correct for multiple testing after histograms of p-value distributions were inspected for uniform distributions (Fig. S3).



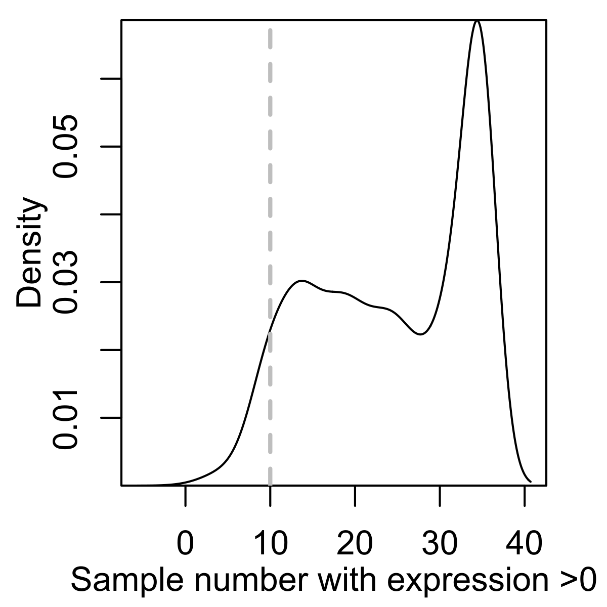
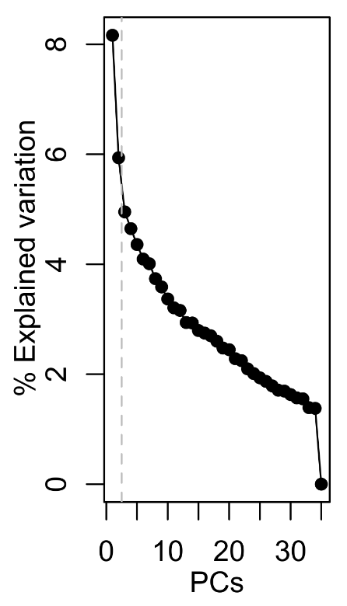
**Fig. S3**: Histograms of p-values and q-values of all 9 pairwise comparisons of mRNA expression

**2.6 miRNA-seq & analysis**

We chose a liberal approach to identify herring miRNAs and retained all that had a miRDeep2 score of larger than zero (miRdeep 2 default settings), leading to 1,626 miRNAs (probably including some redundancy). When inspecting them, it was noted that the mature sequence of 39 miRNAs was very simple, consisting of only two repeating bases (e.g., ‘ugug…’). Since they had high scores and a high confidence assigned by the software, they were kept in the analysis and revealed unique expression profiles. The quantifier module of the miRDeep2 package was used to calculate the miRNA expression. The expression data for 1,626 unique miRNAs of all 35 samples were imported into R. Counts were normalized across samples for all downstream analyses. Density plots of log-transformed counts were inspected per sample but no outliers were detected. Subsequently, for each miRNA, the number of samples, in which the respective miRNA was expressed, was calculated and miRNAs expressed in fewer than ten samples were not considered downstream (Fig. S4a), retaining 1,536 herring miRNAs. As miRNA prediction without further functional testing tends to be over-liberal (Pinzón et al., 2017), we also conducted a more conservative analyses using the mature sequences of 375 already described zebrafish (*Danio rerio*) miRNAs (see below, page 12 ff). The identified patterns of both analyses are highly congruent.

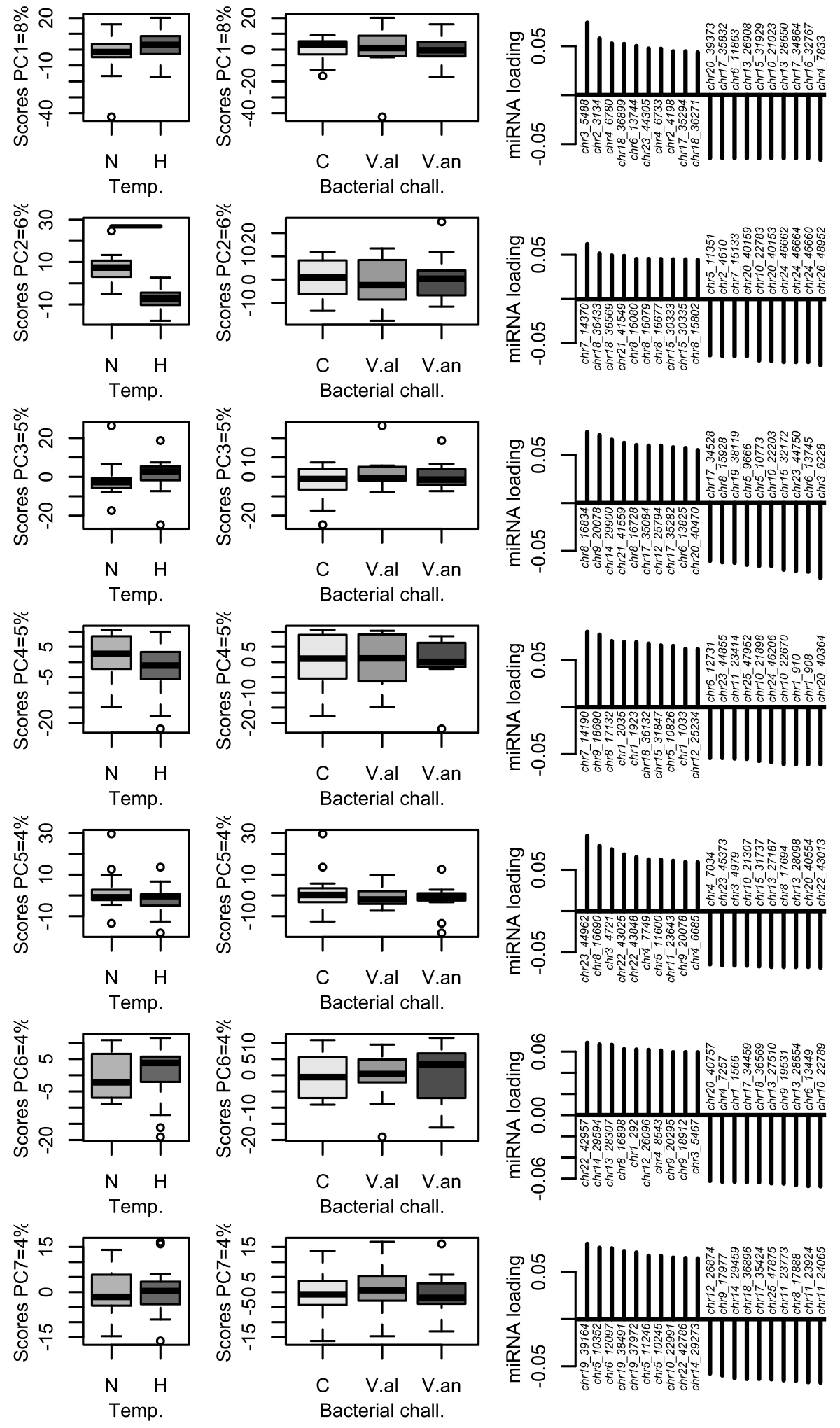
**miRNA PCA**

The PCA for the miRNA was conducted as described above for the mRNA data set. A density plot (Fig. S4a) and a scree plot were created (Fig. S4b) and the first two PCs were selected for downstream analysis (Fig. S5).

(a) (b)

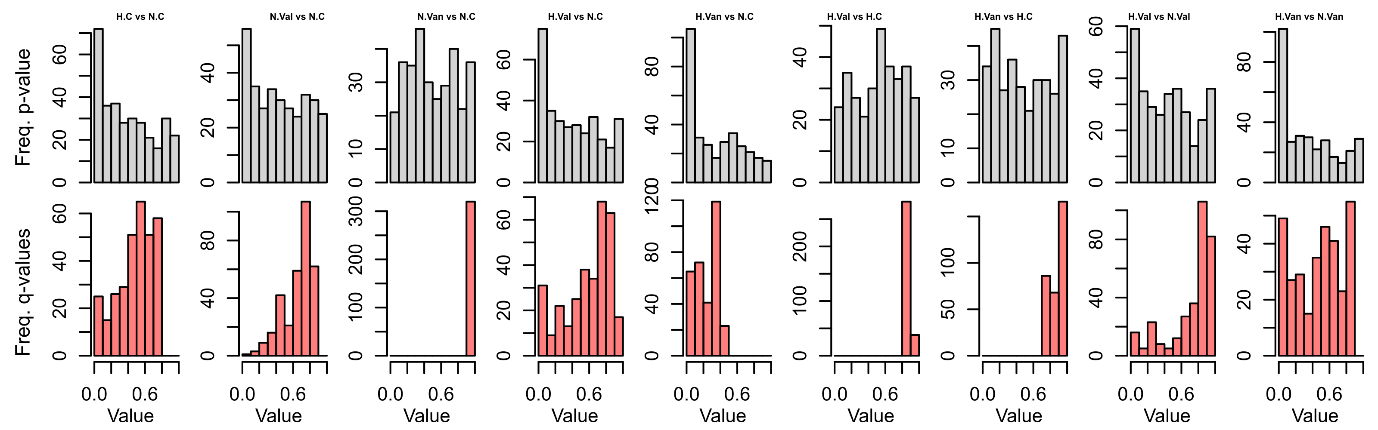
**Fig. S4:** (a) Density plot indicating number of samples with non-zero expression for all miRNAs. The dashed line indicates a liberal cutoff value, which retained most miRNAs in the analysis and only those expressed in less than ten samples were discarded. (b) Scree-plot of PCAs on herring miRNAs. The vertical dashed line indicates the cut-off for the two PCs considered in the study.



**Fig. S5:** PC scores and rotations of PCAs for expression values of miRNAs. Left side: Boxplots of the sample scores in the respective PC separated for temperature and bacterial exposure treatments. Lines above boxplots indicate statistical significance (p < 0.05). N = Normal temperature, H = High temperature, C = Control, V.an = *V. anguillarum*, V.al = *V. alginolyticus* Right side: The genes with the ten most positive and negative loadings are shown.

**Pairwise comparisons miRNA**

As for mRNAs, miRNA data were analyzed using a mixed model and subsequently the same pairwise comparisons were calculated (see above). Histograms of raw p-values were plotted to confirm that distributions were mostly uniform, afterwards q-values were computed to determine significance (Fig. S6).



**Fig. S6**: Histograms of p-values and q-values of all 9 pairwise comparisons for miRNA expression

**2.7 Microbiome sequencing and analysis**

PCR and sequencing were performed using the primers 338f (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) to amplify the V3-4 regions of the 16S rRNA gene. The reads were analyzed with QIIME2 version 2022.2 and DADA2 version 1.22.0. Briefly, paired reads were imported and quality checked before primer and quality trimming using the cutadapt plugin. After further quality checking, DADA2 was run via QIIME2 with recommended settings and forward and reverse truncation lengths of 274 and 268 bp, respectively. The resulting quality trimmed ASVs were classified against the SILVA 138 database using the sklearn method in QIIME2. Reads classified as chloroplast or mitochondrial origin were removed from the dataset.

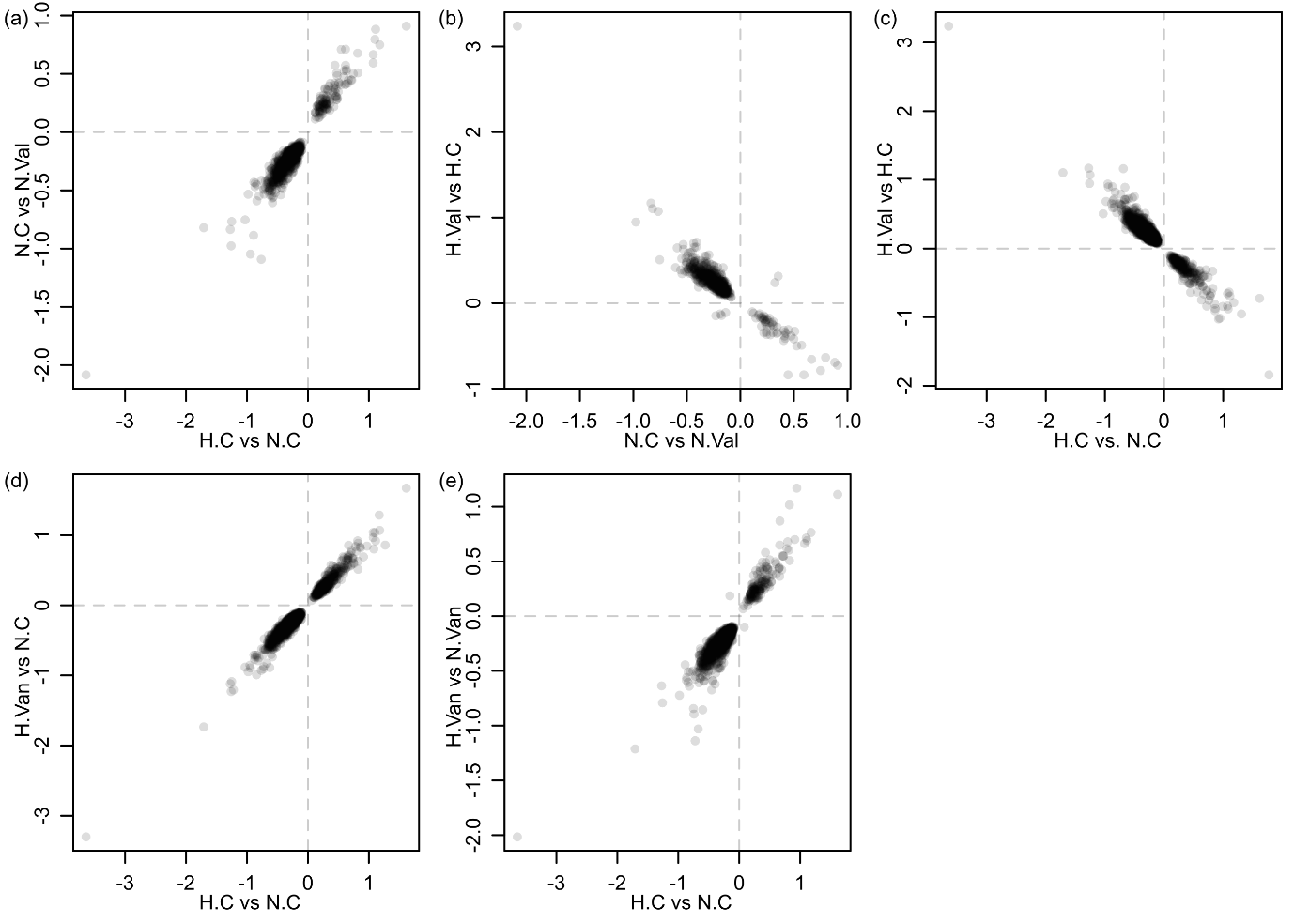
The count data of the microbiome samples and associated taxa information were imported into R and normalized per sample for total count number (i.e., subsequent analyses refer to relative counts). To identify the *Vibrio* *anguillarum* and *Vibrio alginolyticus* strains used for the bacterial exposures, mean count values per experimental group were investigated for all *Vibrio* taxa using a heatmap. The *V. anguillarum* culture used for the exposure consisted of one strain while the *V. alginolyticus* culture consisted of four *Vibrio* strains. To test if temperature had an effect on the strains, counts of the four *V. alginolyticus* strains were summed up per individual. Then, for both experimental *Vibrio* species separately, relative bacterial counts per individual were used as the dependent variable and temperature as the independent variable in a mixed model (see 2.4 main text).

**Supplementary information: Results**

## **3.1 Growth**

Larval lengths were normally distributed (Shapiro-Wilk-Test: n=35, W=0.982, p=0.808) and variance across groups was homogenous (Levene's Test: df=5, F=0.328, p=0.892).

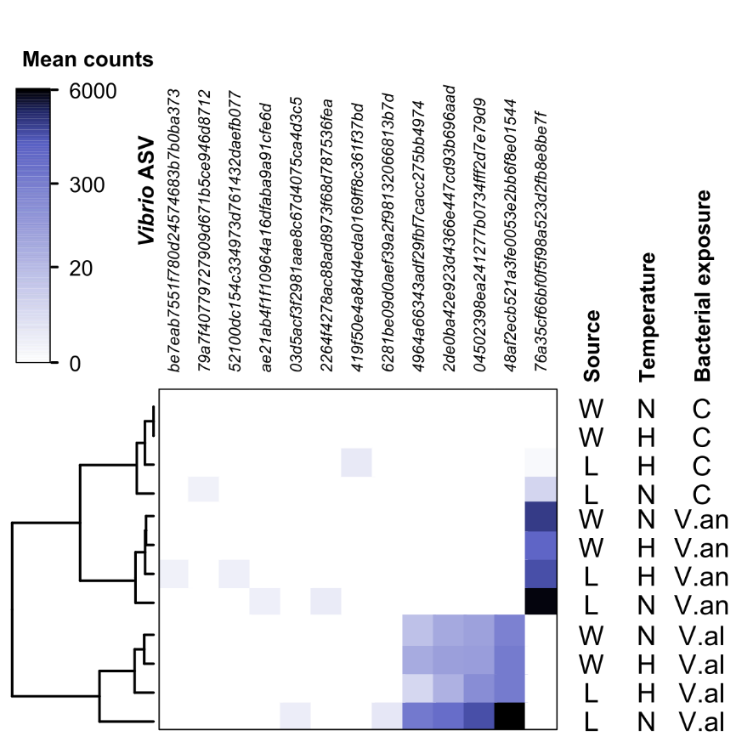
**3.3 mRNA transcriptome**

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**Fig. S7:** Scatterplots showing the direction (up- or downregulated) of DE mRNAs of two different comparisons based on model estimates. The number of DEGs which go into the same direction in both comparisons (i.e., both up- or both downregulated) is for (a) 941, (b) 624, (c) 2,099, (d) 2,072, and (e) 1,712.

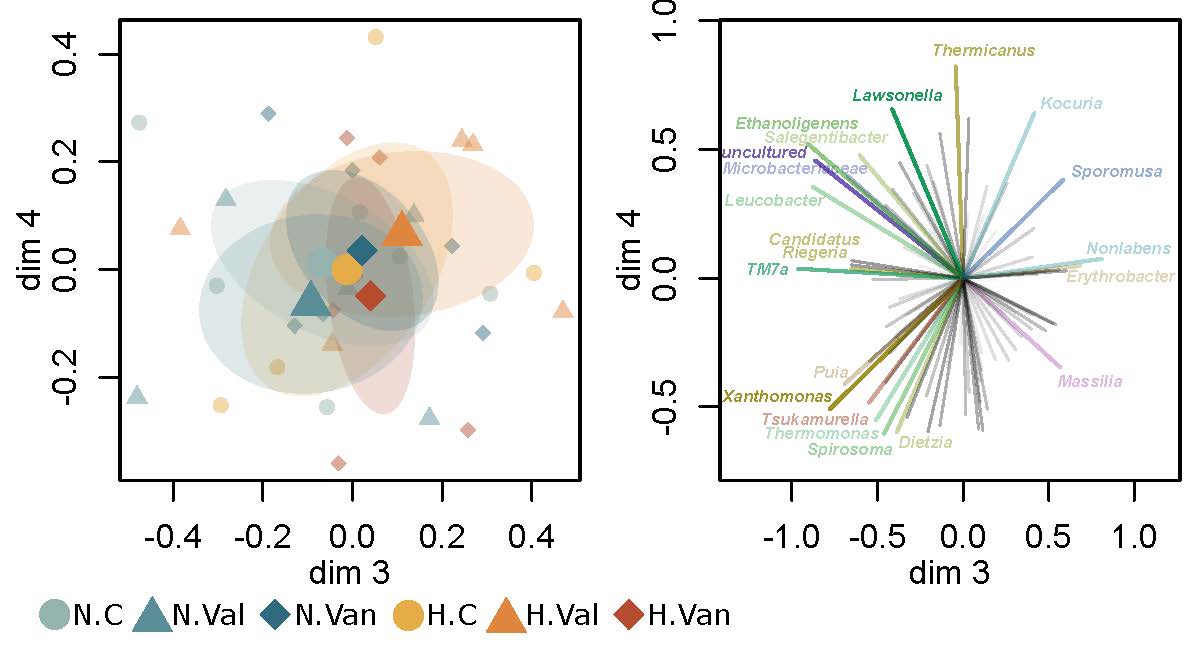
**3.5 Microbiome**

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).



**Fig. S8:** Heatmap of all *Vibrio* strains and experimental groups. For all strains assigned to the genus *Vibrio*, the mean value of relative counts per experimental group was calculated and clustered. ASVs are ordered from left to right with increasing mean expression. W = water sample, L = larval sample.

For the microbiome nMDS, dimensions 3 and 4 were also analyzed for differentiation (Fig. S9).

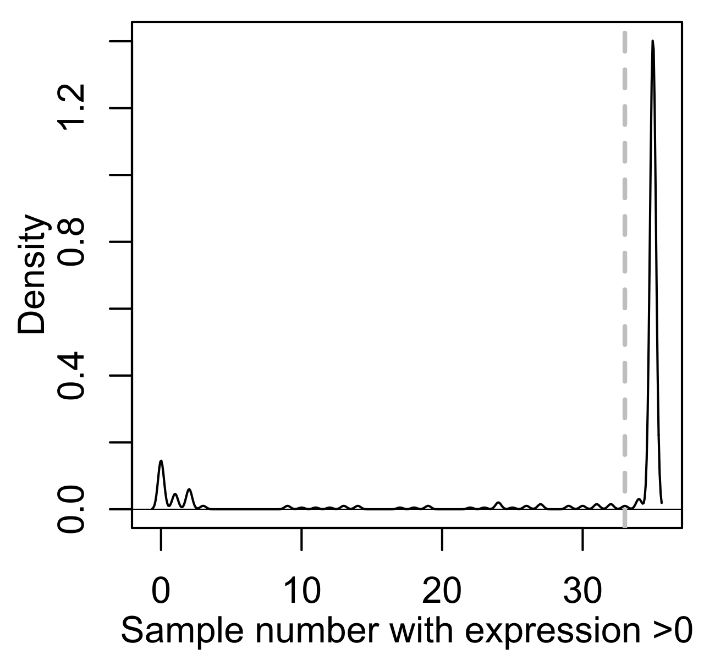
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**Fig. S9:** nMDS plots of the genus-level microbiome composition. Left side: Ordination plot of samples for nMDS dimension 3 and 4. Data ellipses reflect 0.3 CI. Large symbols reflect mean coordinates for each treatment. Blue color hues indicate the normal temperature groups (N), orange color hues indicate higher temperature groups (H). Van = *V. anguillarum*, Val = *V. alginolyticus*. Right side: Microbiome genera associated with dimension 3 and 4, the 20 most influential are labeled. Colors were assigned arbitrarily.

**Additional miRNA analysis using zebrafish miRNA sequences**

**Material and methods**

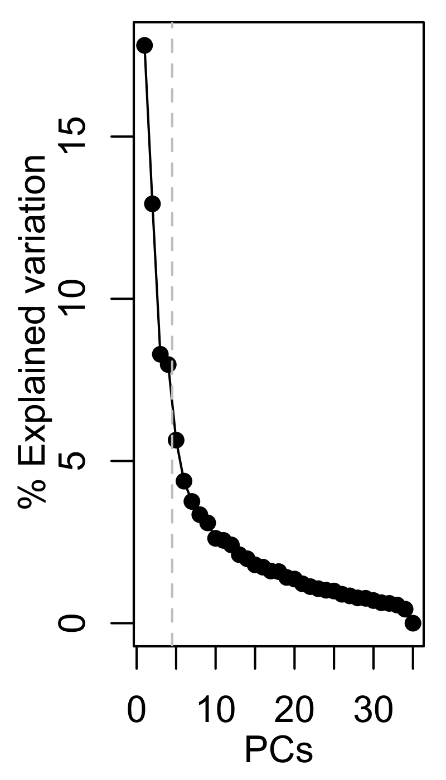
The quantifier module of the miRDeep2 package was used to calculate the miRNA expression of previously described miRNAs from *D. rerio*, yielding 375 miRNAs with reads assigned to them (suppl. data S6). Counts were normalized across samples for all downstream analyses. Density plots of log-transformed counts were inspected per sample but no outliers were detected. Subsequently, for each miRNA, the number of samples, in which the respective miRNA was expressed, was calculated and miRNAs expressed in fewer than 33 samples were not considered downstream (Fig. S10), retaining 321 zebrafish miRNAs.



**Fig. S10:** Density plots indicating number of samples with non-zero expression for all zebrafish miRNAs. The dashed line indicates a conservative cutoff value, which still retained most miRNAs in the analysis and only those expressed in less than 33 samples were discarded.

**PCA**

As for the mRNA data-set and the herring miRNA data set, a mixed model was computed for each zebrafish miRNA using the lme(…, method=’REML’) function to obtain start-beaker-corrected residuals of log-transformed counts for a PCA (see 2.4 main text). From this model, the residuals were used to compute a PCA on scaled log-transformed expression value residuals. A scree plot was created and the first six PCs were selected for downstream analysis (Fig. S11).



**Fig. S11:** Scree-plot of PCAs using zebrafish miRNA sequences. The vertical dashed line indicates the cut-off for the PCs considered in the study.

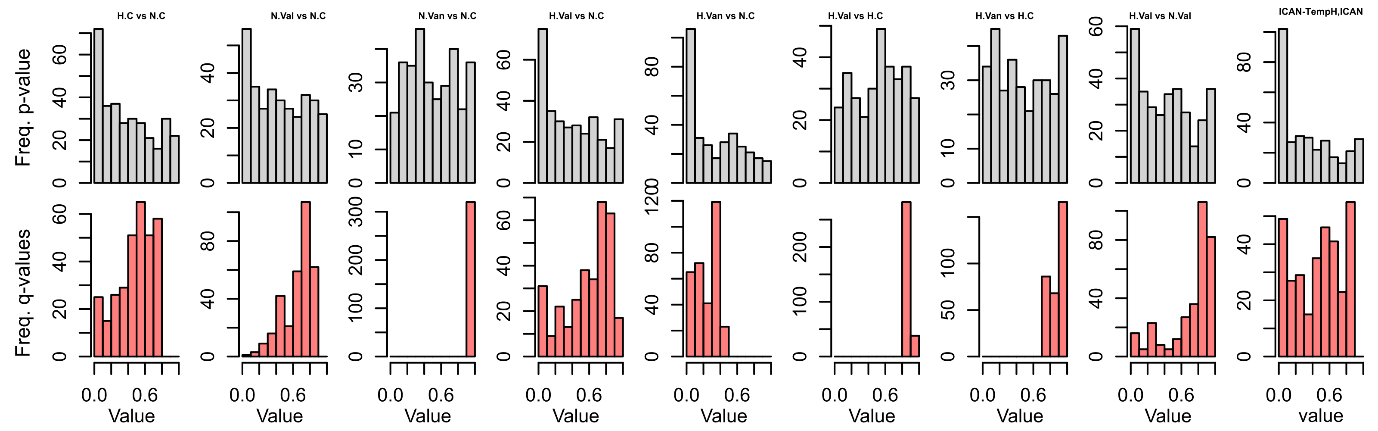
PC one to six were inspected for association with the temperature or bacterial exposure treatment using Welch ANOVAs and TukeyHSD tests, respectively. An association of PC2, PC3 and PC4 with temperature was found (t=3.706,2.409,-2.953; df=31.835,31.868,32.977; p=0.0008,0.022,0.0058; respectively) but none with the bacterial exposure treatment (Fig. S12). The fact that the temperature effect was significant on multiple PCs suggests that it interacted with other sources of variance such as the bacterial exposure.

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**Fig. S12:** PC scores and rotations of PCAs on expression values of zebrafish miRNAs. Left side: Boxplots of the sample scores in the respective PC separated by temperature or bacterial exposure treatment. Lines above boxplots indicate statistical significance (p < 0.05). N = Normal temperature, H = High temperature, C = Control, V.an = *V. anguillarum*, V.al = *V. alginolyticus*. Right side: The genes with the ten most positive and negative loadings are shown.

**Pairwise comparisons**

As for mRNAs, miRNA-wise mixed models were calculated and subsequently pairwise comparisons computed. Histograms of raw p-values were computed to confirm distributions were mostly uniform, after which q-values were computed to determine significance (Fig. S13).



**Fig. S13**: Histograms of p-values and q-values of all 9 pairwise comparisons of miRNA expression using zebrafish miRNA sequences.

As for herring miRNAs, mRNA targets were predicted for each miRNA using the miRanda program. For conserved and novel miRNAs separately, targets were filtered to have a binding energy smaller -20Kcal/Mol. Then, per DE miRNA identified in the pairwise comparisons, miRNA expression was correlated individually with expression values from all remaining target genes using Spearman correlations (one miRNA sample was again excluded for these correlations as one mRNA sample had been excluded previously). For each miRNA with typically multiple target genes, fdr corrections were performed as q-values could not be calculated when too few tests were performed (suppl. data S6). As with herring miRNAs, we noted that miRNAs with either positive or negative correlation coefficients dominated. To explore if a predominant negative or positive regulatory effect of individual miRNAs on its target mRNAs could be identified, Spearman correlations with all its predicted target mRNAs (ranging from one to 374 target genes) were computed for each miRNA separately. The mean of all significant correlation coefficients (raw p<0.05) per miRNA was calculated. As for the herring miRNA analysis, a random distribution of these mean correlation coefficients was produced.

**Results**

**Pairwise comparisons**

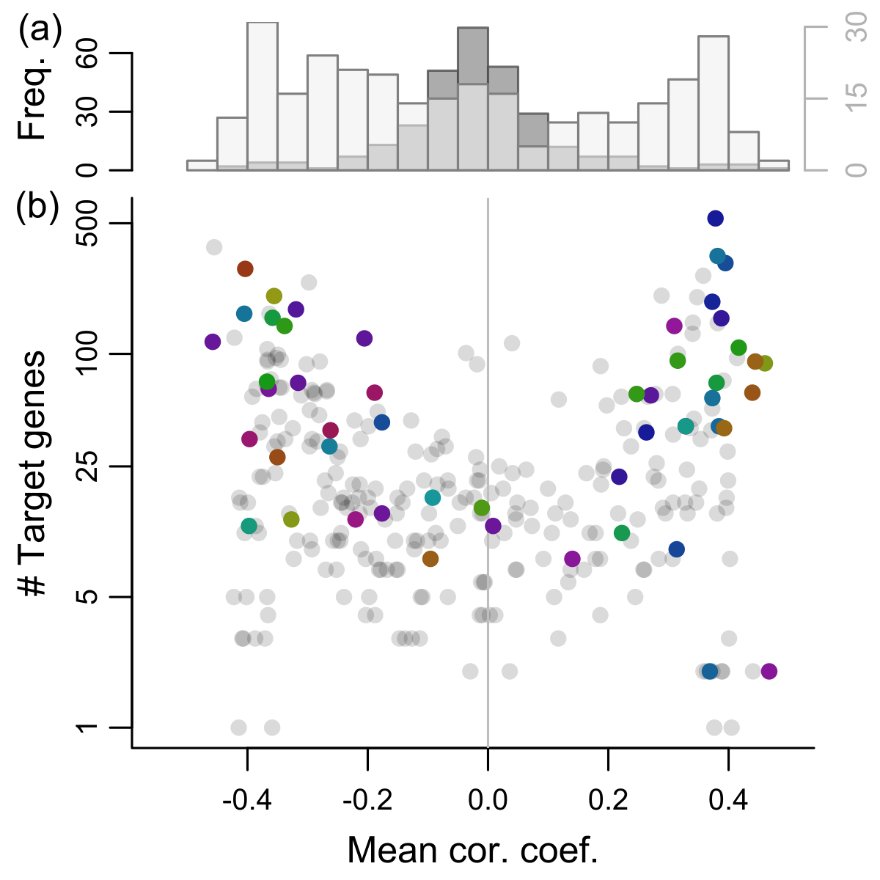
Across pairwise comparisons, DE miRNA were only detected in comparisons with a difference in temperature (Table S1). In total, 53 unique DE miRNA were identified, some of them appearing in different comparisons. For 31 of these 53 miRNAs one or multiple mRNA were predicted to be a regulatory target (q-value<0.05) and four DE miRNAs were found in all of the five comparisons with significant DE miRNA. One of these four was ‘*dre-miR-181a-2-3p’*, a zebrafish miRNA identical to the herring miRNA ‘*chr7\_14370’* that was identified to respond strongest to the temperature treatment in the main manuscript’s analysis. Furthermore, the two comparisons including a *V. anguillarum* treatment (H.Van vs N.C, H.Van vs N.Van) had higher numbers of DE miRNAs than their *V. alginolyticus* counterparts or the comparison of the pure temperature effect. However, as the *V. anguillarum* treatment by itself did not induce any DE miRNAs, it remains unclear if this observation is a coincidence or a more complex interaction of *V. anguillarum* with the high temperature treatment.

**Table S1:** Number of DE zebrafish miRNAs in pairwise comparisons and number of predicted target unique mRNAs (in parentheses non-unique numbers, as several miRNA DEGs may regulate the same mRNA).

|  |  |  |
| --- | --- | --- |
| **Pairwise comparisons** | **# DE zebrafish miRNA** | **# predicted target mRNAs** |
| H.C vs N.C | 17 | 1,133 (1,441) |
| N.Val vs N.C | 0 | 0 |
| N.Van vs N.C | 0 | 0 |
| H.Val vs N.C | 21 | 809 (1,134) |
| H.Van vs N.C | 27 | 1,110 (1,506) |
| H.Val vs H.C | 0 | 0 |
| H.Van vs H.C | 0 | 0 |
| H.Val vs N.Val | 16 | 825 (1,036) |
| H.Van vs N.Van | 31 | 1,237 (1,669) |

**miRNA target analysis**

The number of predicted target mRNAs is given in Table S1. As for herring miRNAs in the main manuscript, the biases towards directed regulatory effects were investigated by calculating a mean of all raw significant correlation coefficients (raw p<0.05) per zebrafish miRNA, ranging from one to 531 target genes (Fig. S14b). DE zebrafish miRNAs showed moderate to strong regulatory biases confirming the results given in the main text. The mean correlation coefficient distribution for all miRNAs and their target genes (with significant raw p-values) after filtering appears rather bimodal (Fig. S14a). This distribution pattern suggests that in our study miRNAs tend to predominantly regulate mRNA either negatively or positively, and rather rarely have mixed effects across their respective targets. And as for herring miRNAs, we determined negatively biased miRNAs to be more common than those with a positive regulatory bias.



**Fig. S14:** Mean of significant Spearman correlation coefficients for all identified zebrafish miRNA and all their predicted target mRNAs based on raw p-values < 0.05. (a) Histogram of mean correlation coefficients in light gray and expected unimodal distribution of correlation coefficients without regulation bias in dark gray. (b) Number of target genes per zebrafish miRNA plotted against the average correlation coefficients. DE miRNAs identified for comparisons in arbitrary colors, all other identified miRNAs in gray.

**References**

Bell, B., Hersbach, H., Simmons, A., Berrisford, P., Dahlgren, P., Horányi, A., Muñoz-Sabater, J., Nicolas, J., Radu, R., Schepers, D., Soci, C., Villaume, S., Bidlot, J. R., Haimberger, L., Woollen, J., Buontempo, C., and Thépaut, J. N. (2021). The ERA5 global reanalysis: Preliminary extension to 1950. *Quarterly Journal of the Royal Meteorological Society*, *147*(741), 4186–4227. https://doi.org/10.1002/QJ.4174

Hinrichsen, H. H., Lehmann, A., Petereit, C., Nissling, A., Ustups, D., Bergström, U., and Hüssy, K. (2016). Spawning areas of eastern Baltic cod revisited: Using hydrodynamic modelling to reveal spawning habitat suitability, egg survival probability, and connectivity patterns. *Progress in Oceanography*, *143*, 13–25. https://doi.org/10.1016/J.POCEAN.2016.02.004

Hothorn, T., Bretz; F, Westfall, P., Heiberger, R., Schuetzenmeister, A., and Scheibe, S. (2023). *Package multcomp. R package version v.1.4-25*. http://multcomp.r-forge.r-project.org

Kronsell, J., and Andersson, P. (2012). Total and regional runoff to the Baltic Sea. *HELCOM Baltic Sea Environment Fact Sheet*. http://www.helcom.fi/baltic-sea-trends/environment-fact- sheets

Lehmann, A., Hinrichsen, H. H., Getzlaff, K., and Myrberg, K. (2014). Quantifying the heterogeneity of hypoxic and anoxic areas in the Baltic Sea by a simplified coupled hydrodynamic-oxygen consumption model approach. *Journal of Marine Systems*, *134*, 20–28. https://doi.org/10.1016/J.JMARSYS.2014.02.012

Lennartz, S. T., Lehmann, A., Herrford, J., Malien, F., Hansen, H. P., Biester, H., and Bange, H. W. (2014). Long-term trends at the Boknis Eck time series station (Baltic Sea), 1957-2013: Does climate change counteract the decline in eutrophication? *Biogeosciences*, *11*(22), 6323–6339. https://doi.org/10.5194/BG-11-6323-2014

Pinzón, N., Li, B., Martinez, L., Sergeeva, A., Presumey, J., Apparailly, F., and Seitz, H. (2017). microRNA target prediction programs predict many false positives. *Genome Research*, *27*(2), 234–245. https://doi.org/10.1101/GR.205146.116

Rudolph, C., and Lehmann, A. (2006). A model measurements comparison of atmospheric forcing and surface fluxes of the Baltic Sea. *Oceanologia*, *48*, 333–380.

Storey, J., Bass, A., Dabney, A., and Robinson, D. (2023). *Package qvalue. R package version 2.30*. http://github.com/jdstorey/qvalue