MicroRNA and mRNA interactions coordinate the immune response in non-lethal heat stressed *Litopenaeus vannamei* against AHPND-causing *Vibrio parahaemolyticus*

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While *Vibrio parahaemolyticus* (VP_{AHPND}) has been identified as the cause of early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) in shrimp, mechanisms of host response remain unknown. Understanding these processes is important to improve farming practices because this understanding will help to develop methods to enhance shrimp immunity. Pre-treatment of shrimp with 5-minute chronic non-lethal heat stress (NLHS) for 7 days was found to significantly increase *L. vannamei* survival against VP_{AHPND} infection. To elucidate the mechanism involved, mRNA and miRNA expression profiles from the hemocyte of *L. vannamei* challenged with VP_{AHPND} after NLHS with corresponding control conditions were determined by RNA-Seq. A total of 2,664 mRNAs and 41 miRNAs were differentially expressed after the NLHS treatment and VP_{AHPND} challenge. A miRNA-mRNA regulatory network of differentially expressed miRNAs (DEMs) and differentially expressed genes (DEGs) was subsequently constructed and the interactions of DEMs in regulating the NLHS-induced immune-related pathways were identified. Transcriptomic data revealed that miRNA and mRNA interactions contribute to the modulation of NLHS-induced immune responses, such as the prophenoloxidase-activating system, hemocyte homeostasis, and antimicrobial peptide production, and these responses enhance VP_{AHPND} resistance in *L. vannamei*.

During its first outbreak, the devastating effect of early mortality syndrome (EMS) on the global shrimp industry was primarily caused by the lack of information regarding the disease and its causative agent. Later studies that were focused on mitigating this disease eventually identified the cause to be a toxin-harboring *Vibrio parahaemolyticus*, which causes Acute hepatopancreatic necrosis disease (AHPND) (VP_{AHPND}). This highlights how understanding disease etiology and a subsequent elucidation of host response can help to mitigate the effects of an outbreak. Understanding the functions of the shrimp immune system during disease progression is thus expected to create opportunities for the development of effective and efficient management strategies for VP_{AHPND} infection. Likewise, information regarding the molecular mechanisms of AHPND tolerance can lead to platforms for the development of AHPND-resistant shrimp through selective breeding using markers mined from transcriptome analyses at different rearing conditions.
It has been established that heat shock proteins (Hsps) and other immune-related genes in shrimp are up-regulated after infection with *Vibrio* spp. or white spot syndrome virus (WSSV). Non-lethal heat shock (NLHS) has also been shown to facilitate a tolerance or resistance to the pathogens through various molecular factors. For instance, the Hsp70 transcript is increased in the hepatopancreas of Chinese shrimp *Fenneropenaeus chinensis* after WSSV infection. Hsp70 and Hsp90 mRNAs are also up-regulated in the gills of black tiger shrimp *Penaeus monodon* upon *Vibrio harveyi* infection. In *L. vannamei*, *LvHsp60* and *LvHsp70* proteins are significantly up-regulated and expressed in the gills, hepatopancreas, and hemocytes after bacterial challenge. These findings from different shrimp species reveal the conserved functional role of Hsps in shrimp and also highlight some aspects of a potential resistance mechanism to pathogenic infection that is related to heat stress in shrimp, and perhaps in invertebrates in general.

MicroRNAs (miRNAs) are small non-coding RNA molecules that play an important role in RNA silencing and post-transcriptional regulation. The action of miRNAs begins when a mature miRNA is incorporated into the RNA-induced silencing complex (RISC), resulting in specific interactions with target mRNAs. The complementary target mRNA is degraded and thus, translationally repressed. Previous small RNA-Seq study has identified differentially expressed miRNAs from *L. vannamei* hemocytes upon *V. harveyi* infection. 222 shrimp miRNA target genes (involved in various biological functions) that encode protease inhibitors, apoptosis-related proteins, and heat shock proteins were predicted. Analysis of the expression of different miRNAs, in response to bacterial infections, indicates miRNAs are contributors in the host innate immune response, and thus help to illustrate the general role of miRNAs in immunity.

In this study, the functional roles of miRNAs in immunity and stress survival are further explored by using RNA-Seq to investigate the global expression of miRNA and miRNA populations in the hemocytes of *V. parahemolyticus*-infected shrimp that are pre-treated with NLHS. Several mRNAs and miRNAs were selected for expression analysis to verify the RNA-Seq data. The inferred relationships among the target genes and miRNAs will help to reveal important aspects of these molecules pertaining to AHNPND resistance or tolerance, providing valuable insights into the modulation of immune pathways by NLHS.

**Results**

**Effect of NLHS on shrimp survival upon *V. parahemolyticus* challenge.** Herein, we have confirmed that treating shrimp with NLHS prior to *V. parahemolyticus* infection results in a significantly higher survival rate, as previously demonstrated by Jungprung et al. This survival experiment was set-up by dividing shrimps into four groups of non-heat treatment control (NH), NLHS control (NLHS), *V. parahemolyticus* challenge (NH-VP), and NLHS plus *V. parahemolyticus* challenge (NLHS-VP) (Fig. 1). For the NLHS treatment group, shrimps were placed in tanks at 38°C for 5 min daily for 7 days and allowed to recover in 30°C tanks at ambient temperature, without any disturbance for 3 days. No mortality was observed until the end of the experiment in heat treatments without a VP *AHPND* challenge. In those with *V. parahemolyticus* challenge, the survival rate was increased in the group with NLHS. In particular, the survival rate of the NH-VP group (24.53%) is significantly lower than the NLHS-VP (58.33%) group based on a Log-rank test, which indicates a possible heat-induced tolerance to bacterial infection in the NLHS-VP group.

**Gene expression profiling in *L. vannamei* hemocytes under the NLHS condition.** The shrimp innate immune response upon NLHS treatment was analyzed in the hemocytes because it is the tissue in which the majority of immune reactions take place. Hemocytes from 30 individuals, each in the NH-VP and NLHS-VP groups, were collected at 0, 6, and 24 h post infection (hpi), pooled and used for cDNA library preparation. The experiments were completed as triplicates for a total of 18 cDNA libraries that were loaded into an Illumina Next-Seq 500 sequencer (Table S1). Raw sequence data from 18 normalized libraries were concatenated and analyzed. Average %Q30 and the sequence range were 81.975% and 30–151 bp, respectively. Total raw single pass reads for all the libraries amounted to 400,232,814 reads, which were reduced to 399,998,390 reads after additional adapter trimming, quality filtering and size selection (50–151 bp). Each library had an average number of filtered reads of 22,222,133. Sequencing reads were deposited in the Short Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) and can be accessed using accession numbers in Table S1.
All clean reads from mRNA libraries were concatenated and de novo assembled using Trinity, which generated 174,835 putative genes (unigenes) or 205,137 isotigs. The generated reference assembly has an N50 isotig length of 1,074 represented by various isotigs ranging from 201 bp to 22,966 bp (Table S2). The isotigs/transcripts were annotated by searching their sequences using BLAST against transcripts predicted from the available *L. vannamei* genome in NCBI Genbank, Swiss-Prot, GO, Cluster of Orthologous Groups (COG), and KEGG Pathway databases. A total of 47,401 (or 23.11%) sequences had significant hits (E-value $\leq 10$) to the Swiss-Prot database and the majority of these sequences were homologous to *Homo sapiens* (26.54%), *Mus musculus* (16.77%), and *Drosophila melanogaster* (13.55%) genes (Fig. 2A). BLAST2GO mapped 184,422 level 2 gene ontologies (Fig. 2B), while COGs classified 11,350 sequences into different categories (Fig. 2C). Searching against the KEGG database showed that 33,475 sequences were mapped to a KEGG orthology, but only 20,183 were grouped into the reference pathways. The metabolic pathways, biosynthesis of secondary metabolites and biosynthesis of antibiotics were among the top 20 KEGG pathways (Fig. 2D) represented in the transcriptome assembly. A protein BLAST was also completed using the predicted coding sequences from the Trinotate protocol and these annotations, along with other supplementary information such as the transmembrane regions are shown in Table S3.

Differentially expressed genes (DEGs) in NLHS-treated *L. vannamei* upon VP$_{AHPND}$ challenge. In this study, we aimed to study the effect of NLHS treatment on transcription in VP$_{AHPND}$-challenged shrimp. Therefore, only NH-VP-responsive genes and NLHS-VP-responsive genes were identified. Differentially expressed genes (DEGs) in NLHS-treated *L. vannamei* (upon VP$_{AHPND}$ challenge) were identified by pairwise comparisons among the relevant groups as shown in the volcano plots (Fig. S1). Three biological replicates were used in the experiments and differential gene expression is represented as fold change against a specific group (Table S4; Table S4_summary_fold-changes_mRNAs.xlsx). Between the VP$_{AHPND}$ challenged groups at 0 hpi (0 NH-VP) and 6 hpi (6 NH-VP), 792 genes were differentially expressed and 272 of these genes were significantly up-regulated (FDR $< 0.05$) in 6 NH-VP, whereas 520 genes were down-regulated in 6 NH-VP. The gene, identified as *L. vannamei* Relish small isoform gene (FJ416145), had the highest up-regulation (362-fold) in this particular comparison. Between the 0 NH-VP and VP$_{AHPND}$ challenged group at 24 hpi (24 NH-VP), 676 genes were differentially expressed; 224 and 452 genes were up- and down-regulated, respectively, in the 24 NH-VP group. The Relish small isoform gene was also up-regulated in this group (144-fold), together with *P. monodon* triosephosphate isomerase gene homolog (7.4-fold).

In the NLHS-treated shrimp challenged with VP$_{AHPND}$ at 0 hpi (0 NLHS-VP) vs 6 hpi (6 NLHS-VP) comparison, 522 genes were differentially expressed, and 262 of these were significantly up-regulated in the 6 NLHS-VP group, whereas 260 genes were down-regulated. The gene homolog of lipoprotein aminopeptidase was found to be 5.3-fold...
up-regulated in the 6 NLHS-VP group, 3.9-fold up-regulated in the 6 NH-VP group, and 3.7-fold up-regulated in the 24 NH-VP group. Between the 0 NLHS-VP and 24 NLHS-VP, 272 genes were up-regulated in the 24 NLHS-VP group, whereas 520 genes were down-regulated.

Sequence analysis of shrimp miRNAs. To supplement the information derived from the transcriptome, we also explored the global miRNA expression to obtain a glimpse of some regulators that are associated with the observed gene expression. To do this, we analyzed the miRNAs expressed in VP_{AHPND}-infected and NLHS-treated shrimp (mir\_NLHS-VP) by sequencing them at various sampling times (0, 6, and 24 h post infection; hpi). High-throughput sequencing generated 1,086,629 total raw reads in the 0 mir\_NLHS-VP, 879,272 in the 6 mir\_NLHS-VP, and 1,114,328 in the 24 mir\_NLHS-VP. The high-quality sequences that passed initial quality filters were 948,089, 771,799, and 956,249 reads, respectively.

Final filtering and analysis generated a majority of the non-redundant sequences 20–22 nucleotides (nt) in length (Fig. 3A). Searching the NCBI nucleotide database revealed that, on average, 25% of the sequences are most likely contaminating RNAs (Fig. 3B). After removal of these contaminating mRNA, rRNA, and tRNA homologs, the final counts of sequences were 78,000, which were mapped to miRBase 22.1 generating 77,415 sequences with hits. The percentages of matched mature miRNA sequences were 93.86%, 93.93%, and 94.19%, respectively, for the 0 mir\_NLHS-VP, 6 mir\_NLHS-VP, and 24 mir\_NLHS-VP libraries. Sequences with unknown identities and homologs were also listed. Of those, forty-one miRNA homologs were identified from the NLHS-VP experimental group (Table S5).

RT-qPCR validation of significant differentially expressed miRNAs (DEMs) and DEGs. In order to confirm the presence of the identified miRNAs and mRNAs, as well as to analyze the expression of interesting L. vannamei miRNAs and mRNAs in response to VP_{AHPND} infection under NLHS and control conditions, the expression profiles of 10 DEMs (lva-miR-7170-5p, lva-miR-2169-3p, lva-miR-184, lva-miR-92b-5p, lva-miR-317, lva-miR-92a-3p, lva-miR-4901, lva-miR-61, lva-miR-2898, and lva-miR-6090) and 8 DEGs (relish, lipoprotein receptor, dynamin, importin7, juvenile hormone epoxide hydroxylase 1; JHEH-1, DNAJ5, prophenoloxidase 1; PO1, and prophenoloxidase 2; PO2) that were identified from the sequencing data, were analyzed for their expression using RT-qPCR.

Under the NLHS-VP condition, Relish gene expression was significantly higher in all experimental groups than the respective controls. The dynamin gene was up-regulated 2-fold at 6 hpi and down-regulated 2-fold at 24 hpi. The lipoprotein receptor gene was up-regulated nearly 2-fold at 6 hpi. Importin7, JHEH-1, DNAJ5, PO1, and PO2 were significantly down-regulated 1.5- to 10-fold at 6 hpi and 24 hpi (Fig. 4). It should be noted, the expression pattern determined from the RNA-Seq data was similar to the expression pattern of selected DEGs determined from the RT-qPCR results.

Meanwhile, stem loop RT-qPCR analysis revealed that the expression levels of all 10 chosen DEMs were altered in shrimp hemocytes following NLHS treatment and VP_{AHPND} challenge by about 1.5- to 8-fold. For
the NH-VP condition, only some of these DEMs had significant changes in their expression levels. These were lva-miR-2898, lva-miR-2169-3p, lva-miR-7170-5p, and lva-miR-92b-5p, which were all up-regulated at 6 and 24 hpi by around 1.5- to 10-fold, respectively (Fig. 5). The expression of 7 of 10 selected miRNAs were significantly altered under the NLHS-VP condition, also consistent with the small RNA-Seq data.

Figure 4. Validation of RNA-Seq using RT-qPCR. Eight representative genes (relish, lipoprotein receptor, dynamin, importin7, juvenile hormone epoxide hydroxylase 1; JHEH-1, DNAJ5, prophenoloxidase 1; PO1, and prophenoloxidase 2; PO2) were evaluated for their expression in hemocytes of shrimp under the NLHS and NH conditions in response to VP_AHPND infection and are referred to as NLHS-VP and NH-VP, respectively. Total RNA from hemocytes of NLHS-VP and NH-VP *L. vannamei* at 0, 6, and 24 hpi was used for cDNA synthesis. The relative expression levels of eight genes were determined by RT-qPCR and normalized against EF-1α, the internal reference. The relative expression ratio was calculated using the \(2^{-\Delta\Delta CT}\) method. The experiments were completed using triplicates. The expression level was calculated relative to that of the normal shrimp under the NH condition at 0 h after the VP_AHPND challenge. The bar graphs are the data from RT-qPCR presented as means ± standard deviations and the triangles (▲) are data from the RNA-Seq. Asterisks indicate significant difference (\(P < 0.05\)) from the respective VPAHPND infected NH shrimp at 0 hpi.
Correlation of DEMs and DEGs of NLHS-treated shrimp in response to VPA_HPN discord. The DEGs of 3,980 NLHS-VP-responsive genes and 3,141 NH-VP-responsive genes were compared and used to construct a Venn diagram highlighting specific groups of DEGs (Fig. 6A). A grouping of 2,664 DEGs were considered to be the NLHS-VP-responsive genes, while another grouping of 1,825 DEGs were categorized as the NH-VP-responsive genes. Their intersection with 1,316 DEGs were expressed in response to VP_HPN discord whether or not the shrimp were treated with NLHS. In the future, it will be interesting to analyze the NLHS- and VP-responsive genes to broaden our knowledge regarding the shrimp immune response against NLHS and VP_HPN discord.

The DEGs of NLHS-VP analyzed in this study and of NH-VP identified in our previous work were analyzed to further identify miRNAs that regulate immune genes of NLHS-treated shrimp infected with VP_HPN discord. As with the expression profiles of the DEGs, a Venn diagram was also created to highlight specific groupings of DEGs between the libraries of NLHS-VP and NH-VP. Eighteen DEGs were specifically grouped into NLHS-VP-responsive miRNAs and two DEGs were added to a NH-VP-responsive miRNAs group (Fig. 6A).

The DEGs from the NLHS-VP-responsive miRNAs that group only with their corresponding target mRNAs from the sequencing dataset were analyzed using CUI-mir (in-house) and RNA-hybrid software. This analysis facilitated the identification and functionalizing of specific miRNA-mRNA interactions, which then served as a clue to the general regulatory mechanisms underlying the immune response of shrimp under the NLHS and VP_HPN discord. 1,833 DEM-DEG pairs with negative correlations were identified and included in a miRNA-target network (Table S6). Some of the biological functions that might be regulated by the NLHS-VP miRNAs were found to include: “Defense & Homeostasis”, “Energy & Metabolism”, “Cell cycle & DNA Synthesis/repair”, “Gene expression & Protein synthesis/degradation”, “Receptor”, “Signaling & Communication”, “Transporter”, “Hypothetical protein”, and “Unknown”. Several miRNAs such as lva-miR-7278-5p, lva-miR-6813-5p, lva-miR-745b, lva-miR902l-5p, lva-miR-502b-3p, and lva-miR-2898 had high degrees of connectivity and might play crucial roles in the regulatory network. Meanwhile, genes involved in “Defense & Homeostasis”, “Energy & Metabolism”, and “Cell cycle & DNA Synthesis/repair” were the most common miRNA targets (Fig. 6B).

In order to further characterize the identified biological pathways, an enrichment analysis specific for immune-related pathways was done for the identified DEGs of miRNA-mRNA pairs. Three pathways that changed significantly (P-value < 0.05) were “hemocyte homeostasis”, “prophenoloxidase system”, and “AMP production”. Given these immune pathways and the information on the canonical members of these pathways, we were able to find sequence homologs of these genes in our RNA-Seq dataset. We then used publicly available information on shrimp as well as other sequences in our transcriptome data to further annotate these homologs. This sequence information was used to look up gene expression patterns in the identified pathways using RT-qPCR.

Figure 7 shows the expression profiles of some canonical members of pathways identified in this study. It is of note that Fig. 7 presents the expression profile of PO1 and PO2 under the NLHS condition, which is also presented in Fig. 4, which reveals that PO1 and PO2 of the prophenoloxidase system are highly up-regulated from 1.5- to 8-fold, in the NLHS-VP group. The activation of prophenoloxidase system is a classical way of triggering an immune defense response to bacterial infection. This is supported by previous studies in shrimp, where PO activity was stimulated by heat shock (HS) and reduced by thermal stress (TS)14. The mechanism by which PO is activated involves the cleavage of proPO into active forms and is mediated by increased proPO gene expression15.

Discussion
The AHPND is known to be caused by VPA_HPN discord, which accumulates in the stomach of shrimp and secretes PirA/B toxins in the hepatopancreas14. The mechanism by which AHPND kills shrimp is currently unclear. Recent data demonstrates that the genes of Toll and IMD pathways, and their downstream antimicrobial peptides (AMPs) are suppressed in the stomach and in hemocytes, but are overexpressed in the hepatopancreas15. This implies that while the stomach and hepatopancreas are major VPA_HPN discord targets, the hemocytes, being the major immune organs of shrimp, may also provide informative clues regarding the immune mechanisms against disease14.

The previous observation that the non-lethal heat shock enhances the production of heat shock proteins and, subsequently, increases the expression of some immune-related genes resulting in enhanced immunity16, is of research interest because of its potential application in developing preventive strategies for diseases in shrimp. For instance, a short-term hyperthermic treatment that is suggested to reduce gill-associated virus replication in P. monodon, may prove to be a simple and effective prophylactic strategy17.

In this study, we found that NLHS improved the survival of VPA_HPN discord-infected L. vannamei (Fig. 1), revealing that the NLHS may indeed modulate immune factors to alleviate the mortality caused by AHPND. However, previous studies have shown that the tolerance and survival rate of L. vannamei after VPA_HPN discord infection is not influenced by Hsp70 accumulation or changes in immune-related proteins, such as proPO and hemocyanin14. This indicates that the mechanisms of resistance to VPA_HPN discord infection under the NLHS conditions remain unexplored and may (potentially) be explained by some undescribed immune proteins. Thus, we performed RNA-Seq and small RNA-Seq analyses of either NLHS-treated or NH control shrimp infected by VPA_HPN discord, to explore the genes, gene networks, and miRNAs that regulate these unknown immune mechanisms.
Figure 5. Relative expression analysis of miRNAs in response to VP_AHPND infection following the NLHS and NH treatments in L. vannamei hemocyte. Total small RNAs from hemocyte of VP_AHPND-infected L. vannamei under NH- and NLHS-treated conditions which are NH-VP and NLHS-VP, respectively, were used as templates for specific stem-loop first strand cDNA synthesis. Relative expression levels of 10 miRNAs (lva-miR-7170-5p, lva-miR-2169-3p, lva-miR-184, lva-miR-92b-5p, lva-miR-317, lva-miR-4901, lva-miR-92a-3p, lva-miR-61, lva-miR-2898, and lva-miR-6090) were determined by RT-qPCR and normalized against U6, the internal reference, at 0, 6, and 24 hpi. The bar graphs are data from RT-qPCR presented as means ± standard deviations and triangles (▲) are data from the small RNA-Seq. The results were derived from triplicate experiments. Asterisks indicate significant differences (P < 0.05) from the respective VP_AHPND-infected shrimp at 0 hpi.
Here, sequencing of mRNAs generated approximately 400 million (400 M) reads across 18 libraries for de novo assembly, which is 4-times and 6-times higher than those of recent transcriptome libraries from the hepatopancreas\textsuperscript{18} and stomach\textsuperscript{19}, respectively. These 18 libraries represent 3 biological replicates with an average of 22 M reads, each with 6 samples ensuring optimum statistical power to detect differentially expressed genes\textsuperscript{20}. We also assembled a higher number of 205,137 isotigs with higher 1,074 N\textsubscript{50} isotig length. Regarding the number of unigene sequences with COG and KEGG annotations, this study includes a higher number of hits compared to Rao, et al.\textsuperscript{18} However, the percentage of BLASTx hits of our reference assembly to the SWISS-Prot database and the *L. vannamei* genome-transcribed data was lower (23.11\% vs 26.13\%)\textsuperscript{18}. The percentage (26.89\%) of unknown contigs in our assembly may indicate that we mined more novel genes than previously described, a collection of interesting genes related to immune response and bacterial tolerance to characterize in the future.

Small RNA libraries prepared from the NLHS-treated and VP\textsubscript{AHPND}-infected *L. vannamei* hemocytes (NLHS-VP) generated 3 million reads across 3 libraries, which revealed 41 DEM homologs, and 27 up-regulated and 14 down-regulated miRNAs. Previously, there were 47 up-regulated and 36 down-regulated miRNA homologs identified in VP\textsubscript{AHPND} challenged *L. vannamei* hemocytes\textsuperscript{18}. In this study, eight miRNA homologs that included lva-miR-184, lva-let-7, lva-miR-9, lva-miR-305, lva-miR-71, lva-miR-2, lva-miR-274, and lva-miR-317 were found to be DEMs, similar to a previous study from 2018\textsuperscript{18}. Predicted target genes of NLHS-VP-responsive miRNA homologs such as caspase, c-type lectin, and Kazal-type serine proteinase inhibitor were similar to those of VP\textsubscript{AHPND}-responsive miRNAs identified in a previous study\textsuperscript{18}, confirming the roles of NLHS-VP-responsive miRNAs in the regulation of immune-related genes in shrimp.
The RNA-Seq data was useful in identifying genes associated with bacterial infection and NLHS response and in providing sequence data, which can be used to predict the interactions with miRNAs. Relish, a gene identified in the sequencing dataset as a NH-VP-responsive gene, was found to be highly expressed in both NH-VP and NLHS-VP groups based on RT-qPCR confirmation. This indicates that the RNA-seq dataset can correctly identify candidate genes that are of significant relevance to the experimental treatment. Nevertheless, it should be noted that the observed profiles from the RNA-seq dataset could only be detected during either heat stress or bacterial infection (but not both), as demonstrated by RT-qPCR. For example, the dynamin and lipoprotein receptor

**Figure 7.** Relative expression analysis of genes in the NLHS-VP modulated immune pathways. Expression level of genes in the prophenoloxidase system (PO1 and PO2), IMD pathway (IMD, IKKε, IKKβ, and Relish), Toll pathway (Toll1, Toll2, Toll3, MyD88, TRAF6, Pelle, Drosal, and Cactus), antimicrobial peptide (ALF AA-K, Penaedin 4a, and AMP type 2), and hemocyte homeostasis (TGase and Inhibitor of apoptosis protein) was determined by RT-qPCR in the hemocytes of VPAHPND-challenged *L. vannamei* at 0 hpi (NH 0 hpi) and of NLHS-treated *L. vannamei* challenged with VPAHPND at 0 hpi (H 0 hpi), 6 hpi (H 6 hpi), and 24 hpi (H 24 hpi). Relative expression ratios are calculated using EF-1α as the internal control. Relative expression level of each gene in hemocytes of NLHS-treated *L. vannamei* challenged with VPAHPND at each time point after infection was normalized to that of NH 0 hpi to determine the effect of both NLHS and VPAHPND challenge. The results were derived from triplicate experiments. Asterisks indicate significant differences at *P* < 0.05 from the NH 0 hpi. The expression profile of PO1 and PO2 under the NLHS condition was modified and re-presented from Fig. 4.
were predicted in the RNA-Seq dataset to be up-regulated genes in the NLHS-VP group. The RT-qPCR expression analysis confirmed this, but also demonstrated a similar expression profile in the NH-VP group. Likewise, the JHEH-1, importin-7, and DNAJ5 were predicted to be down-regulated genes in the NLHS-VP dataset and were again confirmed by RT-qPCR. However, these genes also showed the same down-regulated expression in the NH-VP group. The RNA-Seq data, therefore, needs validation by RT-qPCR to confirm the mechanisms of immune modulation that are specific for NLHS and for bacterial infection.

Gene expression data from the RNA-seq and RT-qPCR analyses also identified some genes that are up-regulated after bacterial infection when there is a prior NLHS applied to shrimp. A synergistic effect could be seen in the expression of some of these genes, e.g. lipoprotein receptor and dynamin, whose expression were not significantly changed in NH-VP, but then changed in the NLHS-VP group. This finding also supports that there is an immune modulation mechanism by NLHS prior to infection.

The RT-qPCR analysis of the expression of 10 selected miRNAs validated their predicted expression profiles in the RNA-Seq dataset (Fig. 5). Through the use of datasets containing DEMs and DEGs as well as miRNA-targeting information, we acquired different miRNA-mRNA pairs. Negative correlations were identified in the miRNA-mRNA pairs, which can be considered as evidence of miRNA targeting. In a previous study, a total of 407 miRNA-mRNA interaction sites were predicted from the VP AHPND-infected L. vannamei. Among these, 11 DEMs with regulatory roles on 37 DEGs related to immunological responses to VPAHPND infection were predicted. This previous target prediction of VPAHPND-responsive miRNAs showed that the VPAHPND-responsive miRNAs might regulate caspase in the apoptosis pathway, trypsin, Kazal-type serine proteinase inhibitor, c-type lectin, chitinase, and lectin. In this study, 18 DEMs were identified as unique NLHS-VP-responsive miRNAs, including lva-miR-61, lva-miR-3689c, lva-miR-6658-3p, lva-miR902l-5p, lva-miR8623c, lva-miR-184, lva-miR-184-3p, lva-miR-7373b-3p, lva-miR-502b-3p, lva-miR-9000, lva-miR-6813-5p, lva-miR-2898, lva-miR-70, lva-miR-279b-3p, lva-miR-7278-5p, lva-miR-7003-5p, lva-miR-745b, and lva-bantam. Among the interactions identified, 11 unique NLHS-VP-responsive miRNAs were predicted to regulate shrimp immune pathways that include hemocyte homeostasis, the prophenoloxidase system, and AMP production, which is proposed in Fig. 8. Currently, this and previous studies have identified novel and unique miRNA homologs in shrimp that are involved in the regulation of the immune system.

Within the interactome, the NLHS-VP-responsive miRNAs were mapped against DEGs. In this study, lva-miR-6813-5p, lva-miR-7278-5p, and lva-miR-745b were found to be down-regulated miRNAs. As previously demonstrated, several miRNAs can target the same genes. Interestingly, several up-regulated miRNAs such as lva-miR-61, lva-bantam, lva-miR-70, lva-miR-6813-5p, lva-miR-7278-5p, lva-miR-279b-3p, and lva-miR-745b were predicted to target caspase, which is a protease enzyme that plays essential roles in programmed cell death for most crustacean species. The down-regulation of caspase at 6 hpi, regulated by miRNAs upon NLHS and VPAHPND infection, suggests that a lower number hemocyte might undergo apoptosis.

In hemocyte homeostasis, caspase and transglutaminase (TGase) are two important proteins for most crustacean species. TGase transcript expression in NLHS-treated shrimp was up-regulated at 24 hpi upon VPAHPND challenge. This expression profile is expected because TGase is known to be involved in hemolymph
Non-lethal heat stress (NLHS) and bacterial challenge experiments. The VPAHPND inoculum was prepared by culturing bacteria overnight in 3 mL of tryptic soy broth (TSB) containing 1.5% NaCl at 30°C and 250 rpm. Then, the starter culture was transferred to 200 mL TSB with 1.5% NaCl and further incubated at 30°C and 250 rpm until the OD600 reached 2.0 (approximately 10⁷ CFU/mL).
After rearing at 30°C, the chronic non-lethal heat stress (NLHS) was applied to the shrimp. The shrimps were divided into four groups of 10 shrimps each. Two groups were NLHS-treated by placing the shrimp in tanks containing 10 L of sea water at 38°C for 5 min daily for 7 days, shrimp were given a 3-day recovery period in their respective rearing tanks. The other two groups were reared in the tank at the ambient temperature (30°C) as control groups of non-heat (NH) treatment. Shrimp were then challenged with VP$_{\text{AHPPD}}$, by immersion in tanks containing the bacterial inoculum at a final concentration of 1.5 × 10$^6$ CFU/mL. The uninfected control group was immersed in the TSB containing 1.5% NaCl. The shrimp survival was observed for 53 h. Experiment was completed in triplicates. Statistical analyses of the results were conducted using GraphPad Prism version 6. The infographic outlining all experimental groups is shown in Fig. S3.

**RNA extraction.** NLHS and bacterial challenge experiments were performed as described above and approximately 500 μL of hemolymph of VP$_{\text{AHPPD}}$-challenged NLHS-treated and VP$_{\text{AHPPD}}$-challenged NH control shrimp at 0, 6, and 24 h post infection (hpi) time points were drawn out from the ventral sinus using a sterile syringe pre-loaded with an equal volume of anticoagulant (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, and 9 mM EDTA, pH 5.6)$^{37}$. Hemocytes were immediately collected by centrifugation at 800 g for 10 min at 4°C and kept in liquid nitrogen. The hemocytes from 30 individuals were pooled and extracted for large and small RNA using the mirVana miRNA Isolation Kit (Ambion, Life Technologies) following the manufacturer’s protocol. These experiments were done using triplicates. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer chip, RNA 6000 Pico Kit and Small RNA Isolation Kit (Ambion, Life Technologies) following the manufacturer’s protocol. The mirVana miRNA Isolation Kit was used to isolate miRNAs from the hemocyte samples.

**RNA sequencing (RNA-Seq) and data analysis.** Six cDNA libraries that included 0 NLHS-VP, 6 NLHS-VP, 24 NLHS-VP, 0 NH-VP, 6 NH-VP, and 24 NH-VP with three biological replicates were prepared from 4 μg total RNA following the manufacturer’s protocol for TruSeq Stranded mRNA LT Sample Prep Kit (Illumina). Eighteen indexed libraries were normalized, pooled and then sequenced with a 1% PhiX spike-in control using the NextSeq 500 Mid Output v2 Sequencing Kit (Illumina) in a NextSeq 500 desktop sequencer (Illumina). Additional adapter trimming and quality control of raw reads was performed using the FastQ Toolkit available through the BaseSpace (Illumina) public app repository. High quality reads were assembled to form a reference assembly in Trinity v2.06 software$^{38}$. Transcript abundance was estimated using RSEM wrapped by scripts included in Trinity. Differentially expressed genes (DEGs) were detected using the edgeR software$^{39}$ and R program$^{40}$ for each treatment group and checked for sequence quality and correlation (Fig. S4). The DEGs were selected based on FDR < 0.05 and fold change > 2. Pairwise comparisons between relevant groups were analyzed using 3 biological replicates and expressed as the fold change against a specific group. Bootstraps and permutation resampling were set to a default (5000) as well as the largest genes size (5000). No correction was applied to FDR, but only FDRs lower than 0.05 were considered significant and listed in Table S4. The de novo assembled sequences to the *L. vannamei* transcripts predicted from sequences to the *L. vannamei* transcripts predicted from sequences to the NCBI genome data available in GenBank (https://www.ncbi.nlm.nih.gov/genome/?term=vannamei).

Gene ontology enrichment analysis for differentially expressed features was done using the Trinotate protocol (http://trinotate.github.io/), leveraging different scripts and software for functional annotation, such as BLASTx$^{41}$, PFAM$^{42}$, HMMER$^{43}$, SignalP$^{44}$, tmHMM$^{45}$, KEGG Orthology$^{46}$, GO$^{47}$, and eggNOG$^{48}$, and, then, running GO-Seq$^{49}$. Using the UniProt Retrieve/ID mapping tool (http://www.uniprot.org/uploadlists/), the UniProt accession numbers from the Trinotate protocol were mapped into the Entrez GeneIDs, which were then used in KOBAS 2.0$^{50}$ (http://kobas.cbi.pku.edu.cn/index.php) to map the KEGG Orthology or conduct the enrichment analysis. The subsequent KEGG Orthology was then used as input in the KEGG Mapper – Search Pathway tool (http://www.kegg.jp/kegg/tool/map_pathway1.html) for mapping to the reference KEGG pathways and determining the distribution$^{51–53}$. The BLAST2GO$^{54}$ was also used for some supplementary annotation. The Fasta tools, Trinity software$^{38,55}$, BLAST + 4.10.0+ and other supplementary tools from the Galaxy services of the National Center for Genome Analysis Support (https://galaxy.ncgas-trinity.indiana.edu/)$^{56,57}$ and the Galaxy Queensland (https://usegalaxy.org.au/)$^{58}$ were also used.

**Small RNA-Seq and data analysis.** The cDNA libraries from small RNA from VP$_{\text{AHPPD}}$-infected NLHS-treated shrimp hemocytes at 0, 6, and 24 hpi were constructed following the manufacturer’s instruction and the TruSeq Small RNA Library Preparation Kit (Illumina). Three indexed libraries were normalized, pooled, and sequenced with a PhiX control spiked at 7.5% using MiSeq Reagent Kits v2 (Illumina) in a MiSeq sequencer (Illumina). The 3′-, 3′-adapter trimming and quality control of raw reads were performed using tools in a Galaxy instance (https://usegalaxy.org/)$^{49}$. High quality small RNA sequences with lengths shorter than 18 nucleotides, and longer than 24 nucleotides, were removed. Homology search for contaminating RNA, such as mRNA, tRNA, and rRNA was conducted using BLASTn against the NCBI nucleotide and Rfam database. After discarding the contaminating RNA, the remaining sequences were searched against miRBase 22.1 (http://www.mirbase.org/) in order to identify known miRNA homologs. Based on the number of reads from 3 libraries cut off > 10, the miRNA homologs were selected for the differentially expressed miRNA (DEM) analysis. The specific procedures were as follows: (1) treatment and control groups were normalized to the same orders of magnitude. Formula: Normalized expression level = miRNA expression level/total expression level of the sample × normalized magnitude; (2) Normalized results were used to calculate the fold change and P-value$^{61}$.

**Quantitative real-time PCR analysis.** Several transcripts from the reference assembly were selected for quantitative real-time PCR analysis (RT-qPCR) to evaluate and confirm the differential expression profiles reported by RNA-seq analysis. The gene specific primers (Table S7) were designed by Primer3 as packaged in
Geneious R6 (Biomatters)\(^3\). Using 1 µg total RNA, the first strand cDNA synthesis was carried out in a reaction containing 1 mM dNTP, 10 units of RNase inhibitor, 0.5 µM oligo-dT (Promega), 1 × RevertAid buffer (BiotechRabbit), and 100 units of RevertUP Reverse Transcriptase (BiotechRabbit). The RT-qPCR reactions comprised 5- or 10-fold diluted cDNA template, 1 × QPCR Green Master Mix (LRox) (BiotechRabbit) and 0.5 or 0.25 µM forward and reverse primers, and were run in a MiniOpticon Real-time PCR system (Bio-Rad). The relative expression of each gene was determined and analyzed using EF-1α gene as an internal control gene. The expression levels from the zero-time point of non-heated shrimp samples upon VPAHPND infection (0 NH-VP) were used as a control group.

The miRNAs of interest consisting of lva-miR-7170-5p, lva-miR-2169-3p, lva-miR-184, lva-miR-92b-5p, lva-miR-317, lva-miR-92a-3p, lva-miR-4901, lva-miR-61, lva-miR-2898, and lva-miR-6090 were selected for expression analysis using stem-loop RT-qPCR. The pooled total small RNA samples from the VPAHPND-infected NLHS-treated (NLHS-VP) and control shrimp hemocytes (NH-VP) at 0, 6, and 24 h were prepared using the mirVana miRNA Isolation Kit (ThermoFisher Scientific). The extracted total small RNA was then used as a template for the first strand stem-loop cDNA synthesis using the stem-loop primers (Table S7) by RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific). The U6 gene expression was used as an internal control\(^4\). Stem-loop RT-qPCR was performed using the RT-qPCR reactions comprised of 2- or 5-fold diluted cDNA templates for each miRNA specific oligonucleotide primers (Table S7), and QPCR Green Master Mix (BiotechRabbit) in the MiniOpticon RealTime PCR System (Bio-Rad) under the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Relative expression was calculated and data were analyzed using paired-sample t-tests and are presented as means ± standard deviations\(^6\). The statistical significance was determined if P-values were <0.05. Experiment were performed in triplicates.

miRNA target prediction. The miRNA targets were identified by comparing the miRNA sequences with transcriptome data using CU-Mir software developed by our research group (http://shrimp-irn.org/mirtarget/index.php)\(^3\). The software searched for the sequences on mRNA that match (perfectly) or mismatch (by one nucleotide) the seed sequences (2–8 nucleotides from the 5’-end) of miRNA. The percent complementarity was calculated from the number of nucleotides that perfectly match the target mRNAs per total length of miRNA sequences. The percent total length complementarity cutoff was set at 55%. The RNAhybrid (http://bibiserv.tech.fak.uni-bielefeld.de/rnahybrid/) was also used to predict genes targeted by the miRNAs with the parameters of free energy <−15.0 kcal/mol\(^8\).

miRNA/mRNA interaction network analysis. In order to define all possible miRNA-mRNA interactions involved in a specific dataset of immune-related genes, only NLHS-VP-responsive DEGs and DEMs with negative correlations were grouped. NLHS-VP-responsive DEMs were used as queries to search for mRNA targets from NLHS-VP-responsive DEGs. Again, the miRNA/mRNA binding were predicted using RNAhybrid with the parameters of free energy <−15 kcal/mol. These target mRNAs were mapped against RNA-Seq data to determine their gene functions. Subsequently, the miRNAs/mRNA pairs involved in a specific dataset of immune-related genes were included in the integrated analysis of the NLHS-VP miRNA/mRNA network. The workflow of data integration approach used to build the shrimp NLHS-VP miRNA/mRNA network is shown in Fig. S5.

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Author contributions

Conceived and designed the experiments: K.S. Performed the experiments and analyzed the results: P.B., BA.M. and P.J. Supervised the experiments: K.S., A.T. Wrote the paper: K.S., P.B. and BA.M. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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