



Stress response and silencing verification of heat shock proteins in *Dermatophagoides farinae* under temperature stress

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ABSTRACT

Dermatophagoides farinae is a major exogenous allergen. Its ability to tolerate adverse external temperatures makes it responsible for widespread occurrence of allergies. Heat shock protein (HSP), a recognized temperature stress response gene, but its role in *D. farinae* remained unclear. Here, we performed a comprehensive study. First, we found that 25 °C was the optimal temperature, and all mites died at 48 or –20 °C for 1 h (LT100). Thus, 41 °C (LT15), 43 °C (LT25), 45 °C (LT45), and –10 °C (LT25) were selected as stress temperatures to perform de novo RNA-seq. Then, 17 main genes of the 47 differentially expressed HSP, were detected by qRT-PCR. Temperature and time gradient versus expression magnitude histogram revealed that *HSP70*, *HSP83-1*, *HSP83-2*, and *HSP16-1* showed heat stress response only at 41–43 °C, while *HSC71* and *HSF* played a regulatory role under both heat and cold stress, particularly *HSF*, with strong intensity, long duration, and quick upregulation at recovery for 10–20 min. Finally, gene expression and *D. farinae* survival rates significantly decreased following RNAi. These findings indicated that HSPs conferred thermo-tolerance or cold-tolerance to *D. farinae*. In conclusion, this was the first meaningful exploration that confirmed HSP and HSF playing an important role in temperature resistance of *D. farinae*.

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1. Introduction

Dermatophagoides farinae (Hughes, 1961) is a species of small mites that is widely distributed among human habitats globally. As pests they not only destroy food products, such as flour, thereby causing huge economic losses, but also threaten human health as pathogens, causing allergic diseases such as allergic asthma, allergic rhinitis, and allergic dermatitis. *D. farinae* are poikilotherms: adults can tolerate 38 °C for 24 h [1]. *D. farinae* in carpets can survive for up to 3 h in sunlight [2]. At low temperatures, the survival rate of *D. farinae* decreases with decrease in temperature, but can still survive for some time at temperatures as low as –18 °C [3]. This strong adaptability and high tolerance to unfavorable external temperatures enables *D. farinae* to breed ubiquitously, thereby making it difficult to prevent and control allergic diseases they caused. However, the stress response mechanism of *D. farinae* resistance to environmental temperatures remains unclear.

Heat shock proteins (HSPs) are currently recognized as biological stress response genes and encode a series of stress response proteins widely distributed among various organisms. They are composed of several subfamilies, such as HSP110, HSP90, HSP70, HSP60, HSP40, and sHSP [4]. When organisms are subjected to stressful conditions, e.g., high temperatures, cold temperatures, hypoxia, starvation, trauma,

poisoning, and pathogens, HSP synthesis increases rapidly. They then participate in cell damage repair and heat tolerance processes, thus reducing peroxide damage, stabilizing cell structure, and maintaining normal physiological functions. HSPs have therefore been extensively studied in the field of biological stress. Although there have been reports on agricultural mites in the context of temperature stress, for example on Tetranychidae, *Panonychus*, Phytoseiidae, and Varroidae [5–15], little is known about medically important mites such as *D. farinae*, and no relevant literature can be found so far. In GenBank, the complete genome sequence of *D. farinae* is absent. Only three HSP sequences can be retrieved, namely *HSP70* (accession No: KC305502), *Der f 28* (accession No: KM009992), and *HSC70-5* (accession No: JQ001475). The scarcity of the HSP gene sequences has hampered the research on regulation of responses to temperature stress on *D. farinae*.

In recent years, a new generation of high-throughput transcriptome sequencing technology (RNA-seq) has provided the technical means for determining *D. farinae* HSP sequences and for comprehensive analysis of HSP expression changes under temperature stress. In this study, a series of experiments at various temperatures were conducted to examine the viability changes of *D. farinae*. Then, all differentially expressed HSPs under heat and cold stress were determined using de novo RNA-seq, and expression changes were confirmed by quantitative real-time PCR (qRT-PCR). The genes and functional characteristics of HSPs mainly involved in the temperature stress responses were screened and identified. Furthermore, the corresponding “temperature gradient versus

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expression magnitude" and "time gradient versus expression magnitude" histograms for each gene were determined to reveal expression change rule. Finally, RNA interference (RNAi) was used to silence key HSP genes, and expression and viability changes after gene silencing were examined; additionally, the temperature stress response function of HSPs found in *D. farinae* were verified. This study will be helpful in providing new insights for physical control and selection of target genes for molecular control of *D. farinae*.

2. Materials and methods

2.1. A series temperature survival experiments on *D. farinae*

D. farinae used for the experimentation were obtained from pure cultures maintained at 25 ± 1 °C and 70–75% relative humidity in our laboratory incubator and were collected by the vibrating sieve separation method. Adult female *D. farinae* with good vitality were chosen under a microscope, placed on a glass slide coated with Vaseline and covered with preservative film. They were then placed in hot water or ice salt baths to find the lethal temperatures in a preliminary experiment under heat and cold stress for 1 h, respectively. Temperatures of 48 and -20 °C were confirmed as lethal temperatures under heat and cold stress for 1 h, respectively, and 25 °C was selected as the normal control. A total of 27 temperature groups were then designed, ranging from 48 to -20 °C. The recovery condition was 25 ± 1 °C for 1 h. The vitality and the number of live *D. farinae* in each temperature treatment group was observed and recorded under the microscope, and the survival rates were calculated.

2.2. RNA-seq and functional annotation

2.2.1. RNA extraction

D. farinae were subjected to the normal control temperature of 25 °C, heat stress temperatures of 41 °C (lethal temperature of 15%, LT15), 43 °C (LT25), and 45 °C (LT45), and cold stress of -10 °C (LT25), for 1 h. After recovery at 25 ± 1 °C for 1 h, which was fixed based on previous studies [10,12,16], live adult female *D. farinae* were collected for total-RNA extraction according to instructions on the RNeasy Micro Kit (Qiagen, Germany).

2.2.2. RNA quality assessment

The RNA concentration and purity was assessed on a UV spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and qualified RNA samples were then sent to the Beijing Genomics Institute (BGI) (Wuhan, China) to evaluate RNA quality on an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). After that, the qualified samples were used for cDNA library construction.

2.2.3. cDNA library construction

Eukaryotic mRNA was enriched by means of magnetic beads carrying oligo (dT) and then digested with a disruption reagent at high temperature. The 1st and 2nd strand cDNAs were synthesized from the fragmented mRNA as the template and purified. The ends were blunted, a 3' terminal single nucleotide A (adenine) was added, and adapters were attached. cDNAs of the appropriate size were then separated by agarose gel electrophoresis and amplified by PCR. The constructed cDNA library was tested on the Agilent 2100 Bioanalyzer and an ABI StepOne Plus Real-Time PCR system. A suitable cDNA library was sequenced on the BGI seq 500 platform.

2.2.4. De novo assembly

Clean sequence reads were obtained using the SOAPnuker filter software of BGI after filtering out noise reads that were adaptor-polluted, had low quality, or contained >5% of unknown bases. Q20, Q30, GC%, and the proportion of clean reads were calculated, which were then assembled into transcripts in the Trinity software (v.2.0.6, parameter:-

min_contig_length150-CPU8-min_kmer_cov3-min_glue3-bfly_opts'-V5-edge-thr=0.1-stderr'). Ultimately, these were clustered using Tgicl to obtain the final Unigenes.

2.2.5. Functional annotation

BLAST (<http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi>, v2.2.23 with default parameters) was used to align the Unigenes with sequences in the following databases: non-redundant nucleotide (Nt, <ftp://ftp.ncbi.nlm.nih.gov/BLAST/db>), non-redundant protein (Nr, <ftp://ftp.ncbi.nlm.nih.gov/BLAST/db>), Clusters of Orthologous Groups (COG, <http://www.ncbi.nlm.nih.gov/KOG>), Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg>) and Swiss Protein (SwissProt, <http://ftp.ebi.ac.uk/pub/databases/swissprot>). BLAST2GO (<https://www.BLAST2go.com>, v2.5.0 with default parameters) was then performed with Nr annotations to retrieve the Gene Ontology (GO) annotations. Protein domains and motifs of the Unigenes were analyzed by means of InterProScan 5 (<https://code.google.com/p/interproscan/wiki/Introduction>, v5.11-51.0 with default parameters). The *E*-value $\leq 1.0E-5$ in BLAST was chosen as the threshold.

The raw data were subsequently submitted to the Sequence Read Archive (SRA) database in NCBI. The clean reads were mapped onto Unigenes using the Bowtie2 software (v2.2.5, parameter:-q-phred64-sensitive-dpad0-gbar99999999-mp1,1-np1-score-minL,0,-0.1-I1-X1000-no-Mixed-no-discordant-p1-k200, <http://bowtie-bio.sourceforge.net/Bowtie2/index.shtml>) and the gene expression of each sample was calculated in RSEM (v1.2.12, with default parameter, <http://deweylab.biostat.wisc.edu/RSEM>). The gene expression magnitude was calculated as fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed genes (DEGs) were identified by the PoissonDis algorithm and the $\log_2$ FoldChange ($\log_2$ FC) of expression differences between samples were calculated and compared. The screening criteria for DEGs were $|\log_2$ FC| ≥ 1 and false discovery rate (FDR) ≤ 0.001 .

2.3. Screening and analysis of DEGs for differentially expressed HSPs [1]

Annotated DEGs were screened to identify HSPs among them. Open reading frames (ORFs) were identified in the ORF Finder software (http://www.bioinformatics.org/sms2/orf_find.html). Through artificial Blast alignment, the HSPs gene sequences were determined based on coverage rates and similarities with the relative species. The quantities of Unigenes with the same ORF were added up. Neighbor joining (NJ) phylogenetic trees were constructed by the P-distance method with 1000 permutations in MEGA 5.0 software after multiple sequence alignment in Clustal X 1.8. These procedures helped in determining the genes and subfamilies of HSPs.

2.4. qRT-PCR detection

The highly expressed HSPs with FPKM ≥ 100 were regarded as the main stress response genes and were chosen for confirmation of expression using qRT-PCR. The residual genomic DNA was removed from total RNA using DNase I (Tiangen, Beijing, China). The treated RNA was employed as the template and was reverse-transcribed into 1st-strand cDNA according to the instructions for reverse transcriptase M-MLV (TaKaRa, Nijmegen, Shiga, Japan). These cDNA samples were then stored at -20 °C. qRT-PCR primers were designed in Primer Premier 5 software (Table 1). The specificity and accuracy of the amplification products were determined by general PCR amplification [17], recombination, cloning, and sequencing. α -tubulin was selected as the reference gene for qRT-PCR confirmation of the RNA-seq results [18]. The 20 μ L qRT-PCR reaction system consisted of 10 μ L SYBR Premix Ex Taq™ II (TaKaRa Bio), 0.5 μ L each primer (10 μ M), 1 μ L of cDNA, and 8 μ L nuclease-free water. The qRT-PCR experiments were performed on the ABI instruments (MX3005P) under the following thermal cycling conditions: 95 °C for 10 min; then 40 cycles of 95 °C for 15 s, 60 °C for

Table 1The qRT-PCR primers of main stress HSP genes in *D. farinae*.

Gene	Sequence (5'-3')	Amplicon	Gene	Sequence (5'-3')	Amplicon
<i>dnaJ</i> A1	F:GTGCTGAGAAAGCAATCAAAGAA R:CCATGATCACGTTTGTATCGA	103	<i>HSC71-2</i>	F:GGCTGCTGCCATTGCTTA R:TTCACTTCAAAGATACCGTCTC	129
<i>dnaJ</i> 1	F:CGAAGAAGGTCTTTCCGGT R:GTTTGTCTTGGATCGCCATG	108	<i>HSC71</i>	F:GCTGCTGCCATTGCTTA R:CTCGATAGTCAAGATGGAAC	110
<i>HSP16-1</i>	F:GCCTGATGATTTGATTGGAT R:CACGTTTGAATTGATTTCACG	125	<i>HSC71-3</i>	F:TGTATCAAGTGGTGTGGTG R:TAATCTACTTCTTACGGTTGGAC	103
<i>HSP16-2</i>	F:GCCAGGATGGTAAATTCGCA R:GGCTTGAAGTTGAAGATTGTGC	98	<i>HSC70-4</i>	F:CTCTTTCCTCGTCTCTGTAGC R:TGTCTTTGTCGCATCTTC	172
<i>HSP70</i>	F:GCCAAGAATCAAGTTTCCATG R:CTTCCAGATTCCGTTACAAC	138	<i>HSP83-1</i>	F:CGGCATCGGTATGACTAA R:CGGTAACCTTATCCGCCAC	167
<i>HSP70-3-1</i>	F:CATGCTGCTGTAACAGTTCAG R:CAATGGCAGCGCAGTAG	124	<i>HSP83-2</i>	F:GATCAATAATCTTGGTACTATCGCTAA R:GCCAGAATAGAACCGACACC	115
<i>HSP70-1</i>	F:TATGTAGCAGTTGCCCGGC R:AACACCCAATTACGTTGATTAC	111	<i>HSP90.Cdc37</i>	F:GAGCTAGAAAAACGTGAACAAGAGA R:CGTTGTTCAAGTCTTTCATCGG	158
<i>HSC71-1</i>	F:CAACGATGATGATTCGGGTAG R:CCATCGGGATAACGCATTTC	114	<i>HSF</i>	F:GGCTAATCAGGTGAACAACCTCC R:TTAGATGTTGTAGGTGAGGTGCT	162
<i>HSC70-5</i>	F:CGAACCATGTAAAGAAAGCTATCG R:TCGGGATTGACAGATTACTCG	153	<i>a tubulin</i>	F:CGTCTATCCAGCACCACAAG R:CAGATGTCGTAGATGGCTTCA	129

Amplicon: the length of amplicon (bp).

30 s, 72 °C for 30 s, and finally 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. This latter procedure was intended to generate a melting curve for determining the specificity of the amplification products. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method [19].

2.5. Bioinformatics analysis of key differentially expressed genes

The differentially expressed HSPs with baseline FPKM ≥ 500 , or baseline FPKM ≥ 100 and $\log_2 FC \geq 5$ in qRT-PCR were assumed to be key differentially expressed genes. The protein molecular weight and theoretical isoelectric point (PI) were predicted using ExPASy (https://web.expasy.org/compute_pi/). The relevant motifs and structural and functional domains were analyzed in the PROSITE software (<http://www.expasy.org/prosite>). The amino acid sequences were aligned in MEGA 5.0 with HSPs from *Drosophila melanogaster*, *Aedes aegypti* and *Tribolium castaneum* as the outgroups, for which full genome sequences were available.

2.6. Effect relationship of temperature gradient vs. expression magnitude

According to survival rates across a temperature gradient, 14 sets of temperatures from the gradient with approximate survival rates at high and low temperatures were selected as treatment temperatures for which qRT-PCR would be performed for confirmation. Three biological and technical replicates were set up for each group. The temperature gradient vs. expression quantity histogram was constructed with the temperature as the X-axis and $\log_2 FC$ of the relative expression level as the Y-axis, to reveal the patterns of key differentially expressed genes.

2.7. Effect relationship of time gradient vs. expression magnitude

In order to find out the expression magnitude changes of key HSP stress response genes during the recovery period, based on the effect relationship of temperature gradient vs. expression magnitude, six genes with $\log_2 FC \geq 2$ in qRT-PCR under temperature stress, *HSP70*, *HSC71*, *HSP83-1*, *HSP83-2*, *HSP16-1* and *HSF*, were selected to further study the effect relationship of time gradient vs. expression magnitude. The temperature at which the highest expression of each gene target was observed was chosen as the treatment temperature. The treatment was carried out for 1 h, followed by recovery at 25 °C for 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, and the resultant expression levels were detected by qRT-PCR. The histogram of time gradient vs. expression magnitude was drawn with recovery time as the X-axis and $\log_2 FC$ as the Y-axis.

2.8. Functional confirmation by RNAi

2.8.1. Double-stranded RNA (dsRNA) preparation

DsRNA primers containing a T7 promoter region (TAATACGACTC ACTATAGGG) in both the sense and antisense strand, were designed first (Table 2). DNA fragments were purified after PCR amplification and transcribed into dsRNA with the Transcript Aid T7 High Yield Transcription Kit (Thermo Scientific, UK). The transcription product was then purified by the phenol-chloroform method and dissolved in 20 μ L of diethyl pyrocarbonate (DEPC)-treated water. The quality of dsRNA was evaluated by 1% agarose gel electrophoresis.

2.8.2. RNAi

The functions of the representative key stress genes were verified by RNAi-based silencing. The target genes were screened according to the following criteria: FPKM ≥ 1000 and qRT-PCR $\log_2 FC \geq 2$, baseline FPKM ≥ 500 and qRT-PCR $\log_2 FC \geq 3$, or baseline FPKM ≥ 100 and qRT-PCR $\log_2 FC \geq 5$. RNAi was performed according to the procedure [20]. Specimens of 30 active adult *D. farinae* were placed into 1.5 mL RNase-free Eppendorf tubes, to which 20 μ L DEPC water, 1200 ng/ μ L non-target control *dslacZ*, or target gene-specific dsRNA were added. The tubes were then incubated at 4 °C for 24 h to induce RNAi, followed by washing three times with precooled DEPC-treated water. The mites were subjected to heat or cold shock at the temperature at which the expression of each target gene was the highest in order to detect the RNAi effect. The mites were subdivided into two groups. In one group, live *D. farinae* were used to extract RNA and perform qRT-PCR detection so that the gene silencing effect could be verified. In the other group, the survival rates of *D. farinae* were determined under a microscope to

Table 2The dsRNA primers in *D. farinae*.

Primers	Sequence (5'-3')	Length(position)	GC%
<i>dslacZ</i>	F: CGGTCCGCGATACAC R: AGTTCGCCAGTTAATAGTTTGC	321(2504-1635)	49
<i>dsHSP16-1</i>	F: GCCGACCTGCCAGGATG R: TTTCAATCGGTATAACACGTTCT	293(129-421)	38
<i>dsHSP70</i>	F: GTTGTAAAGCGAATCTGGAAG R: GAGAACAATTTTTTACCACC	309(192-439)	44
<i>dsHSP83-1</i>	F: TCATTGAAAGATTATGATCAGAA R: GCAATTCAAGACCTTCTTTGG	229(1308-1536)	34
<i>dsHSF</i>	F: CTGCCTTCCAAGCAGAAA R: CCAGATACAGCAGAATGTCG	290(1159-1448)	42
<i>dsHSC71</i>	F: GCAATAAACGTGCACTTCGT R: TTTAGGAATACGAGTGAGGC	272(764-1035)	46

verify the influence of the RNAi knockdown on viability. Each experiment was performed in triplicates.

2.9. Repeatability test and statistical analysis

Effect relationships of temperature gradient, time gradient and the RNAi experiment were set up with three biological and three technical duplicates to ensure reliability. The expression differences were evaluated by one-way ANOVA and Student's *t*-test in GraphPad Prism 6 (GraphPad Software Inc., USA). The survival rates were compared by the chi-square test in SPSS 18.0 software (SPSS Inc., Chicago, IL). Data with a *P* value < .05 were considered statistically significant.

3. Results

3.1. The experiments on *D. farinae* survival in a temperature gradient

Compared with the control group of 25 °C, the vitality of *D. farinae* strengthened after heat stress for 1 h, while it weakened under cold stress. However, the survival rates of *D. farinae* followed a symmetrical trend for the temperature increase and decrease (Fig. 1, Table S1). Temperatures groups 33, 25, and 5 °C showed survival rates of 100%. Mortality rates gradually increased when the temperature rose or fell outside this range. The mortality rates at 35 and 0 °C, 37 and −2 °C, 39 and −6 °C, 41 and −7 °C, 43 and −10 °C, and 45 and −15 °C were 3% ± 1%, 6% ± 1%, 13% ± 1%, 17% ± 1%, 26% ± 1%, and 45% ± 1%, respectively. In contrast, when the temperature reached 48 or −20 °C, the mortality rate reached 100%. To ensure that there were enough live *D. farinae* specimens to extract RNA, mites treated at 41, 43, 45, and −10 °C were chosen for RNA-seq analysis.

3.2. RNA extraction and RNA-seq

3.2.1. RNA extraction

The RNA concentrations of five samples ranged from 133 to 212 ng/μL and the absorbance A_{260}/A_{280} ratio ranged from 1.68 to 2.08 according to spectrophotometry. The Agilent 2100 detection results showed a smooth baseline and a distinct 18S rRNA peak (Fig. S1A–E), and the RNA integrity numbers (RINs) were above 6.5. This result indicated that the extracted RNA was intact and met RNA-seq requirements.

3.2.2. RNA-seq

More than 6G of raw data were produced on the BGI seq 500 platform (SRA: SRR9005248 and SRR9005249). The clean reads ratio, Q20, and Q30 were 85.95%, 95.17%, and 86.50% respectively, indicating good sequencing quality. The average length and N50 of Unigenes were 957 bp and 1756 bp (Table S2), respectively, indicating efficient assembly. A Venn diagram showed that 11,800 Unigenes were simultaneously annotated by NR, COG, KEGG, SwissProt, and Interpro (Fig. S2). The numbers of DEGs in four treatment groups (41, 43, 45, and −10 °C) were 10,031, 7376, 5687, and 3842 (Fig. S3), respectively. The numbers of Unigenes with FPKM > 100 in each group were 269, 233, 202, and 108, respectively.

3.3. Screening, alignment and analysis of DEGs for differentially expressed HSPs

A total of 163 Unigenes turned out to be annotated as HSPs. 88 Unigenes were differentially expressed, of which 63 had ORFs and were assigned to 47 HSP genes belonging to six major subfamilies. These subfamilies included HSP20 (3 genes), HSP60 (1), and HSP70 (16), HSP90 (6), HSF (2), and dnaJ (19 genes) (Fig. 2A, Table S3). Twenty-five Unigenes had FPKM ≥ 100 in any group and belonged to 17 HSP genes and five subfamilies consisting of HSP20, HSP70, HSP90, HSF, and dnaJ (Table 3). According to BLAST alignment, the similarity of HSP70 and HSC70-5 with the corresponding sequences of *D. farinae*

in GenBank was 100.00% and 99.65%, and the coverage rates were 100.00% and 81.00%, respectively. The similarities and coverage rates of the other 15 HSP genes with the corresponding sequences of *D. pteronyssinus* in GenBank ranged from 83.29% to 93.64% and from 96.00% to 100.00%, respectively. The phylogenetic trees showed that the each of the subfamilies clustered separately (Fig. 2B, Table S4). Among them, HSP70s involve the most mite species, including *D. farinae*, *D. pteronyssinus*, *Aleuroglyphus ovatus*, *Tetranychus urticae*, *T. cinnabarinus*, *Panonychus citri*, *Neoseiulus barkeri* and *N. cucurbitis*, followed by HSP90s, which involves *D. pteronyssinus*, *T. cinnabarinus* and *P. citri*. HSP20s, HSFs and dnaJs are clustered with *D. pteronyssinus*.

3.4. qRT-PCR verification of DEGs

qRT-PCR analysis of 17 differentially expressed HSPs with FPKM ≥ 100 uncovered seven types of response to temperature stress (Fig. 3, Table S5). HSP16-1, HSP16-2, HSP70, HSC71-3, HSP83-1 and HSP83-2 were upregulated under heat stress conditions of 41 and 43 °C and were downregulated under heat stress at 45 °C and cold stress at −10 °C. In particular, HSP83-1 and HSP70 were upregulated most significantly at 41 and 43 °C with $\log_2FC > 4$. HSP16-1 and HSP16-2 were downregulated most significantly at 45 °C with $\log_2FC < -7$. HSF, HSC71-1, and HSC71 were upregulated at all four temperatures of heat and cold stress, especially HSF, which was upregulated at 41 °C with $\log_2FC$ of 5.61. HSC71-2, HSC70-5, and dnaJ A1 were upregulated at 43 and −10 °C, while there were no significant changes at 41 and 45 °C. HSP70-1 and dnaJ1 were upregulated at 41 and 43 °C, but no significant changes were observed at 45 and −10 °C. HSP70-1 was upregulated more significantly at 43 °C than at 41 °C, whereas dnaJ was upregulated more significantly at 41 °C than at 43 °C. HSP90 Cdc37 was upregulated more significantly at 41 and 43 °C than at −10 °C, but there was no significant change at 45 °C. HSC70-4 was upregulated only at 41 °C, and downregulated at the other three temperatures. HSP70-3-1 was downregulated at all four temperatures.

3.5. Bioinformatics analysis of key differentially expressed HSP genes

Eight HSP genes in *D. farinae*, which had baseline FPKM ≥ 500, or baseline FPKM ≥ 100 and $\log_2FC \geq 5$ in qRT-PCR under temperature stress, were defined as key differentially expressed HSP gene. Their properties are shown in Table 4. The bioinformatics analysis of amino acid sequences indicated each key stress response genes family of *D. farinae* shared conserved motifs with the closely related species and displayed high amino acid sequence homology (Fig. S4). HSP70 of *D. farinae* contains three conserved signatures of the HSP70 subfamily, namely IDLGTTYSIDLGTTY, IFDLGGGTfdvSVL, and IvLvGGsTRIPklqK, basically consistent with the closely related species *A. ovatus*, *T. cinnabarinus* and *P. citri*. The C-terminal sequence is EEVN, and thus of HSP70 of *D. farinae* belongs to the cytoplasmic HSP70 group. HSC71 contains three conserved signatures of the HSP70 family, namely IDLGTTYs, IFDLGGGTfdvSIL, and IvLvGGsTRIPklqK, and the C-terminal sequence is EEVN, and therefore HSC71 also belongs to the cytoplasmic HSP70 group. HSP70-3-1 contains three conserved signatures of the HSP70 subfamily, namely IDLGTTYs, VFDLGGGTfdvSLL, and IvLvGGsTRIPklqQ. The C-terminal sequence is KDEL, and therefore HSP70-3-1 belongs to the endoplasmic-reticulum HSP70 group.

HSP83-1 and HSP83-2 of *D. farinae* both contain a conserved signature of the HSP90 subfamily: YsNKEIFLRE, shared conserved signature not only with the closely related species *Tyrophagus putrescentiae*, *P. citri*, *N. cucurbitis*, *T. cinnabarinus*, but also with outgroups *Drosophila melanogaster*, *Aedes aegypti*, and *Tribolium castaneum*. A conserved MEEVD sequence is present at the C terminus, and therefore HSP83-1 and HSP83-2 belong to the cytoplasmic HSP90 group.

HSP16-1 and HSP16-2 each contain a conserved signature of the sHSP subfamily, NWLSTLNSDIHMPTCQDGKFAmNFDVRNFDPNsVQVKL DGNLQVQAKQEKKADGHYEYREFVRHVTVPQNVQSDQLKCKMDKDG

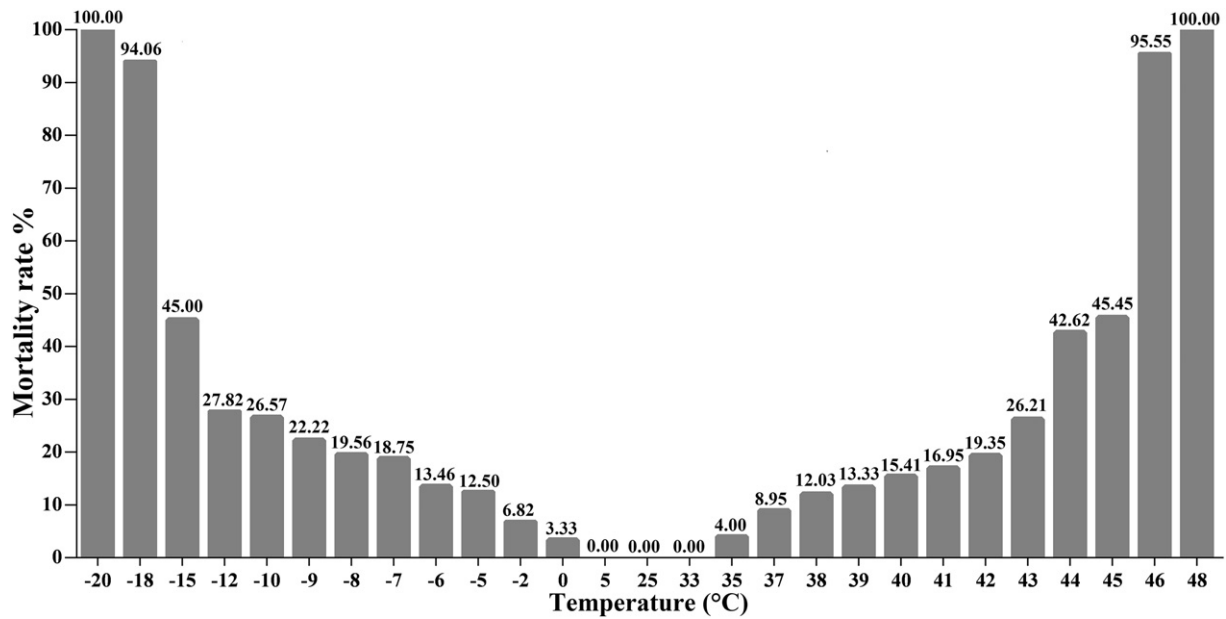


Fig. 1. Mortality rates of *D. farinae* at various stressful temperatures.

LRFEAPLKQIKNEPKERVIPIE and RTPLSRALPMETIEDGQRMFKIEIDLPLDFKPNVNVTVKKGEVKIDAKCEITDGSQKQFKINYSYSLPDDVDLDRVRSLLKTDGRLVIEAPLPALEAPKENREIPIKRE, respectively, share high amino acid sequence homology with *D. pteronyssinus*.

HSF contains an HSF-type DNA-binding domain signature: LplyFKHsNmASFIRQLNmYgFRKV, completely consistent with *D. pteronyssinus*.

3.6. The patterns of HSP expression change under gradient temperature stress

Based on the mortality rates of *D. farinae* in a series of stressful temperatures, the expression histogram of eight key differentially expressed HSP genes were plotted, and three kinds of change pattern

as a function of temperature were noted (Fig. 4, Table S6). Compared with the normal control group of 25 °C, *HSP16-1*, *HSP16-2*, *HSP70*, *HSP83-1*, and *HSP83-2* showed the same pattern of responses to temperatures, namely only upregulated under heat stress but downregulated under cold stress. The survival rate of *D. farinae* was 100% at 33 °C, but the gene expression was downregulated. As the temperature continued to rise, mortality and expression increased synchronously. *HSP16-1* was the first gene to be upregulated at 37 °C at a mortality rate of 6% ± 1%, followed by *HSP16-2* upregulation at 39 °C. When the temperature increased to 41 °C, the mortality rate rose to 17% ± 1%, and these five genes were upregulated to their highest levels. This was especially true of *HSP70*, *HSP83-1*, and *HSP16-1*, which were upregulated by 4.31 in log₂FC (19.84-fold, absolute expression quantity [FPKM] of 38,122.90), 3.96 (15.56-fold, absolute [FPKM] of 12,808.54), and 2.31

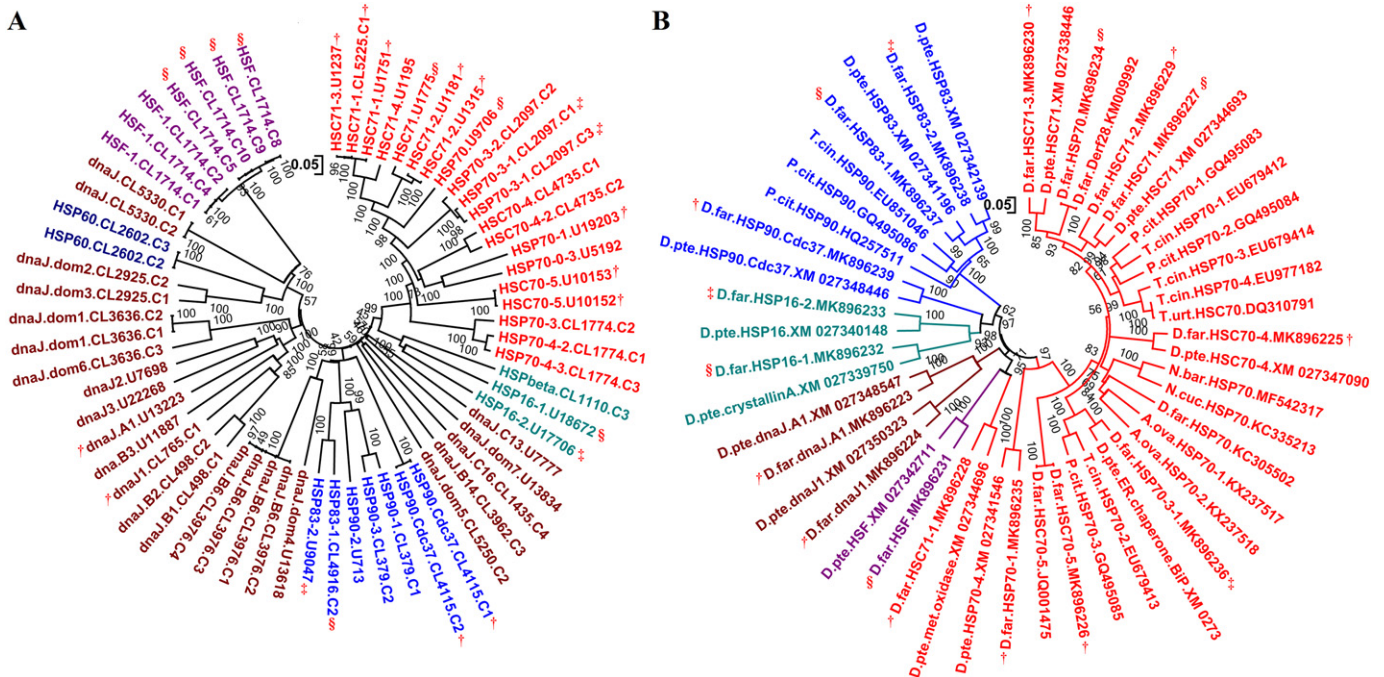


Fig. 2. The NJ tree of ORFs for (A) differentially expressed HSP genes and (B) main stress response HSP genes with the closely related species.

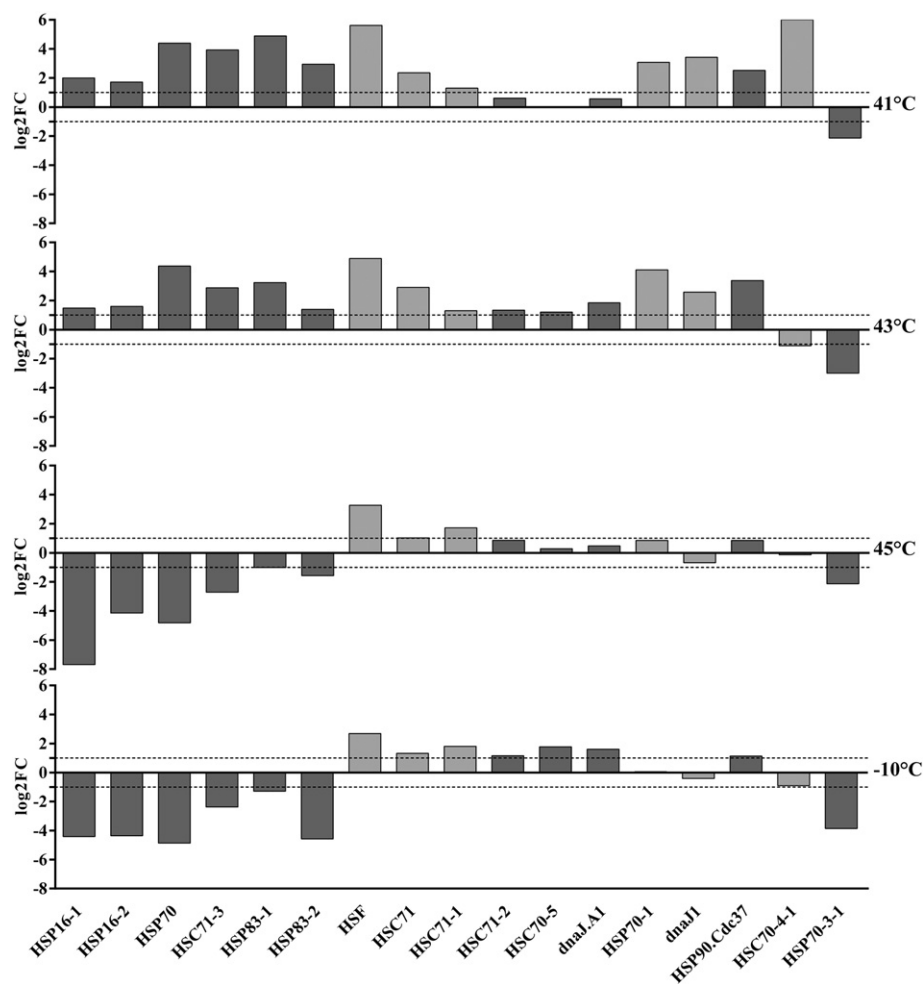


Fig. 3. The histogram of qRT-PCR results on the main stress response *HSP* genes.

(4.96-fold, absolute [FPKM] of 19,162.71), respectively. When the temperature rose to 43 °C and the mortality rate rose to $26\% \pm 1\%$, their expression began to decrease. When the mortality rate reached $45\% \pm 1\%$ at 45 °C, expression decreased abruptly. Expression of *HSP16-1* and *HSP16-2* decreased most notably, to $\log_2FC$ of -5.20 and -6.82 (0.03 and 0.01-fold, absolute [FPKM] of 105.13 and 10.49), respectively.

HSF and *HSC71* manifested another pattern of stress response, namely always being upregulated under heat and cold stress. Upregulation started at 33 or 5 °C, and *HSF* reached the highest expression at 41 °C under heat stress, and then *HSC71* expression reached its maximum at 43 °C. At these maxima, *HSF* and *HSC71* were upregulated 5.31 (39.67-fold, absolute [FPKM] of 5126.64) and 4.35 (20.39-fold,

Table 3

The annotated FPKM and $\log_2FC$ of main stress response *HSP* genes in *D. farinae*.

Gene	25 °C	41 °C		43 °C		45 °C		−10 °C	
	FPKM	FPKM	$\log_2FC$	FPKM	$\log_2FC$	FPKM	$\log_2FC$	FPKM	$\log_2FC$
<i>HSP16-1</i>	3864.36	16,101.54	2.06	6927.63	0.84	76.13	−5.67	139.04	−4.80
<i>HSP70</i>	1921.97	6198.56	1.69	5667.60	1.56	57.18	−5.07	174.45	−3.46
<i>HSP16-2</i>	1185.39	6697.18	2.50	3118.52	1.40	51.90	−4.51	111.70	−3.41
<i>HSC71</i>	831.39	2217.90	1.42	2492.00	1.58	1286.63	0.63	832.03	0.00
<i>HSP83-1</i>	823.04	6091.26	2.89	2775.53	1.75	87.05	−3.24	87.28	−3.24
<i>HSP70-3-1</i>	794.13	512.05	−0.63	824.51	0.05	192.80	−2.04	300.10	−1.40
<i>HSP83-2</i>	634.56	3759.06	2.57	1376.88	1.12	372.55	−0.77	291.25	−1.12
<i>HSC71-2</i>	383.90	1043.30	1.44	1502.52	1.97	183.93	−1.06	301.14	−0.35
<i>HSC71-1</i>	237.07	679.67	1.52	609.69	1.36	131.71	−0.85	162.75	−0.54
<i>HSC71-3</i>	236.35	581.63	1.30	725.80	1.62	85.93	−1.46	82.90	−1.51
<i>HSC70-5</i>	166.97	58.41	−1.52	128.53	−0.38	72.00	−1.21	103.96	−0.68
<i>HSF</i>	129.23	397.22	1.62	198.61	0.62	165.86	0.36	167.01	0.37
<i>HSP70-1</i>	123.52	1044.50	3.08	2132.90	4.11	222.64	0.85	127.88	0.05
<i>dnaJ1</i>	104.42	763.95	2.87	570.84	2.45	45.31	−1.20	61.12	−0.77
<i>dnaJ1A1</i>	61.76	155.76	1.33	141.14	1.19	57.04	−0.11	57.01	−0.12
<i>HSP90.Cdc37</i>	43.05	314.39	2.87	87.42	1.02	43.72	0.02	47.89	0.15
<i>HSC70-4</i>	5.41	161.55	4.90	1.85	−1.55	29.92	2.47	5.50	0.02

FPKM: fragments per kilobase of transcript per million mapped reads;
 $\log_2FC$: $\log_2$ FoldChange.

Table 4The properties of key stress response HSP amino acid sequences in *D. farinae*.

Gene	ORF (bp)	aa	MW (kDa)	PI	C-terminus sequence	Cell localization
<i>HSP70</i>	1965	654	72.6	5.81	EEVN	Cytoplasm
<i>HSP70-3-1</i>	2010	669	73.9	5.20	KDEL	ER
<i>HSC71</i>	1983	660	71.9	5.38	EEVD	Cytoplasm
<i>HSP83-1</i>	2193	730	84.7	4.94	MEEVD	Cytoplasm
<i>HSP83-2</i>	2157	718	82.8	5.01	MEEVD	Cytoplasm
<i>HSP16-1</i>	462	153	17.9	6.83	–	–
<i>HSP16-2</i>	369	122	14.0	4.94	–	–
<i>HSF</i>	1611	536	59.5	5.08	–	–

ORF: the length of ORF; aa: the number of amino acids; MW: molecular weight; PI: theoretical isoelectric point; ER: endoplasmic reticulum.

absolute [FPKM] of 16,954.51), respectively. *HSF* and *HSC71* both reached their highest expression at -10°C under cold stress and were upregulated 2.98 (7.89-fold, absolute [FPKM] of 1019.61) and 2.81 (7.01-fold, absolute [FPKM] of 5830.41), respectively. However, the up-regulation at 45 and -15°C was weaker.

HSP70-3-1 showed its own unique pattern of responses to temperatures: down-regulation under both heat and cold stress.

3.7. The patterns of HSP expression change under gradient recovery time

In view of the fact that the $\log_2\text{FC}$ of the highest expression level under gradient temperature of six genes *HSP70*, *HSC71*, *HSP16-1*, *HSP83-1*, *HSP83-2* and *HSF* were all >2 , they were defined as the key stress response genes and their effect relationship of time gradient vs. expression quantity was studied. The following four responses were observed under 41°C and 43°C heat stress (Fig. 5, Table S7). 1) After recovery for 10 min, *HSP70*, *HSC71*, *HSP16-1* and *HSP83-1* were significantly upregulated with $\log_2\text{FC}$ being 2.73, 2.44, 1.85 and 1.21 (6.63, 5.43, 3.60 and 2.31-fold), respectively. In addition, *HSP83-2* and *HSF* were upregulated after recovery for 20 min, and the $\log_2\text{FC}$ were 1.93 and 1.31 (3.81 and 2.48-fold), respectively. 2) After 40 min to 2 h recovery, they reached the highest up-regulation. *HSP16-1* and *HSP83-2* reached the highest up-regulation at 40 min, followed by *HSP70*, *HSC71* and *HSP83-1* at 1 h, and *HSF* reached its peak expression at 2 h.

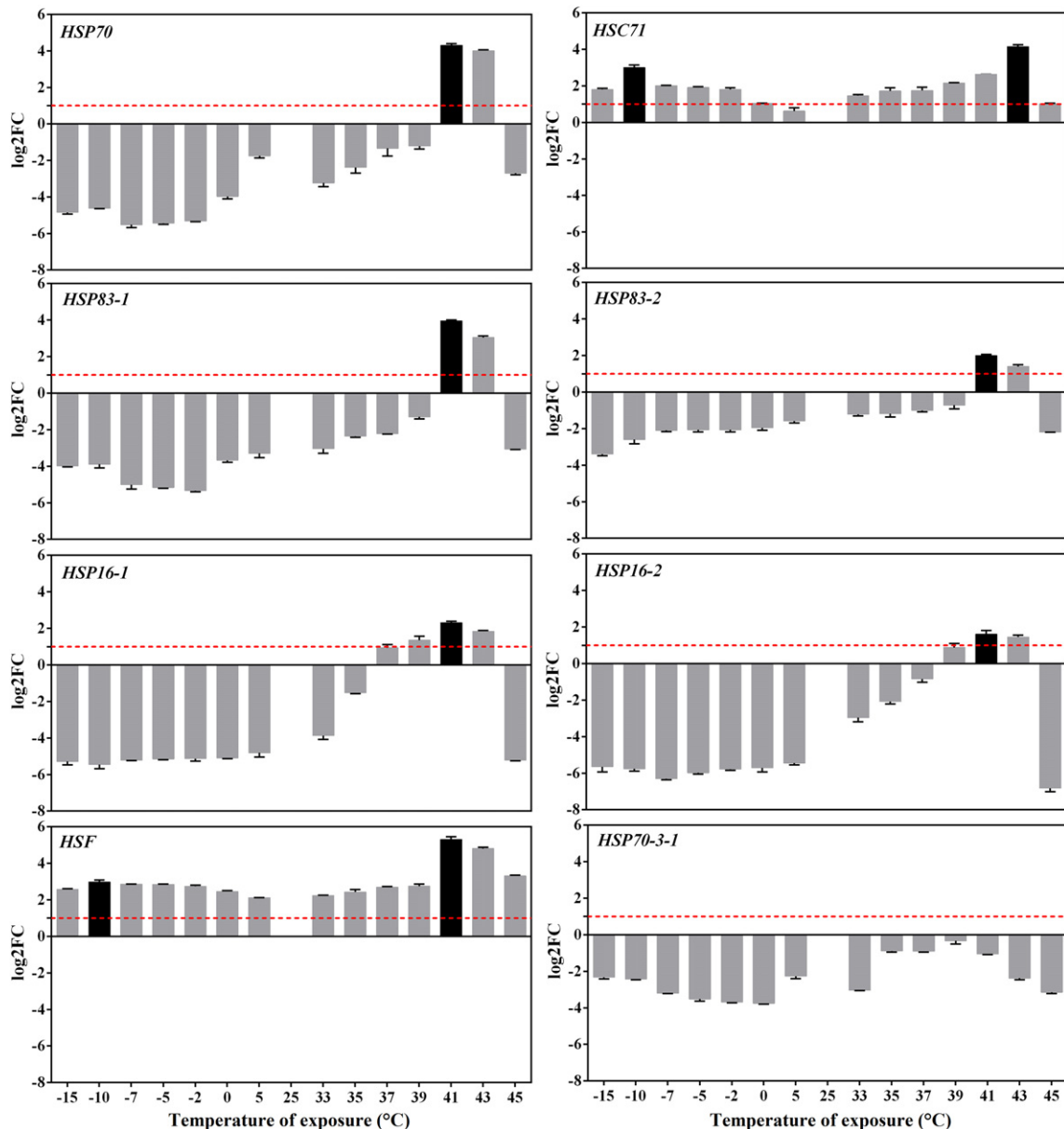


Fig. 4. The patterns of expression changes of HSPs vs. the temperature gradient.

3) The duration of stress response was between 12 h to 16 h, of which *HSP70* and *HSF* lasted for 16 h, and the other four HSP genes lasted for 12 h. 4) The comparative intensities of stress response were as follows: *HSF* > *HSC71* > *HSP70* > *HSP83-1* > *HSP83-2* > *HSP16-1*, $\log_2FC$ were 5.36, 4.17, 4.16, 3.46, 2.33 and 2.18 (41.07, 18.00, 17.88, 11.00, 5.02 and 4.53-fold), respectively.

Under cold stress at -10°C , only *HSF* and *HSC71* were upregulated. After recovery of 10 min, *HSF* and *HSC71* were up-regulated with $\log_2FC$ of 1.57 and 1.04 (2.97 and 2.04-fold), respectively, following which the expression gradually increased after 20 min recovery, and reached the highest expression at 16 h with $\log_2FC$ being 5.27 and 3.02 (38.59 and 8.11-fold), respectively. After that, the regulation effect decreased gradually, and the $\log_2FC$ was <1 after recovery for 28 h.

3.8. Confirmation of the participation of HSPs in the response to temperature stress by gene silencing

Based on the qRT-PCR results and temperature vs. expression magnitude histogram, there were five genes that met the criteria of baseline FPKM ≥ 1000 and qRT-PCR $\log_2FC \geq 2$, baseline FPKM ≥ 500 and qRT-PCR $\log_2FC \geq 3$, or baseline FPKM ≥ 100 and qRT-PCR $\log_2FC \geq 5$. These genes were *HSP16-1*, *HSP70*, *HSP83-1*, *HSC71*, and *HSF*. Among them, *HSP16-1*, *HSP70*, and *HSP83-1* were upregulated only under heat stress, and were most highly expressed at 41°C . *HSC71* and *HSF* were upregulated under heat stress and cold stress. *HSC71* was most highly expressed at 43°C and -10°C , whereas *HSF* at 41°C and -10°C . These genes were silenced separately, and their involvement in the response to temperature stress was thus confirmed.

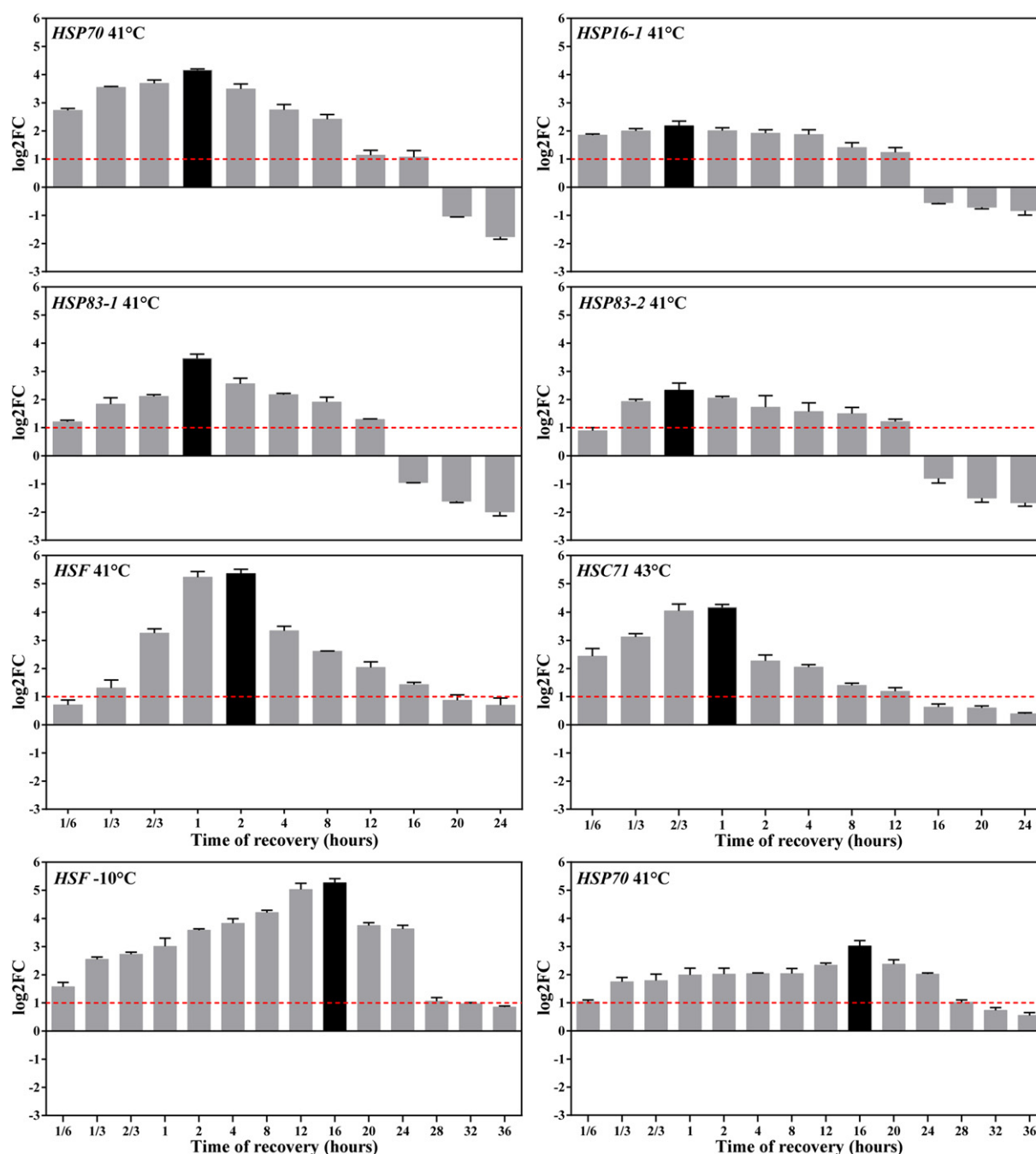


Fig. 5. The patterns of expression changes of HSPs vs. the recovery time gradient.

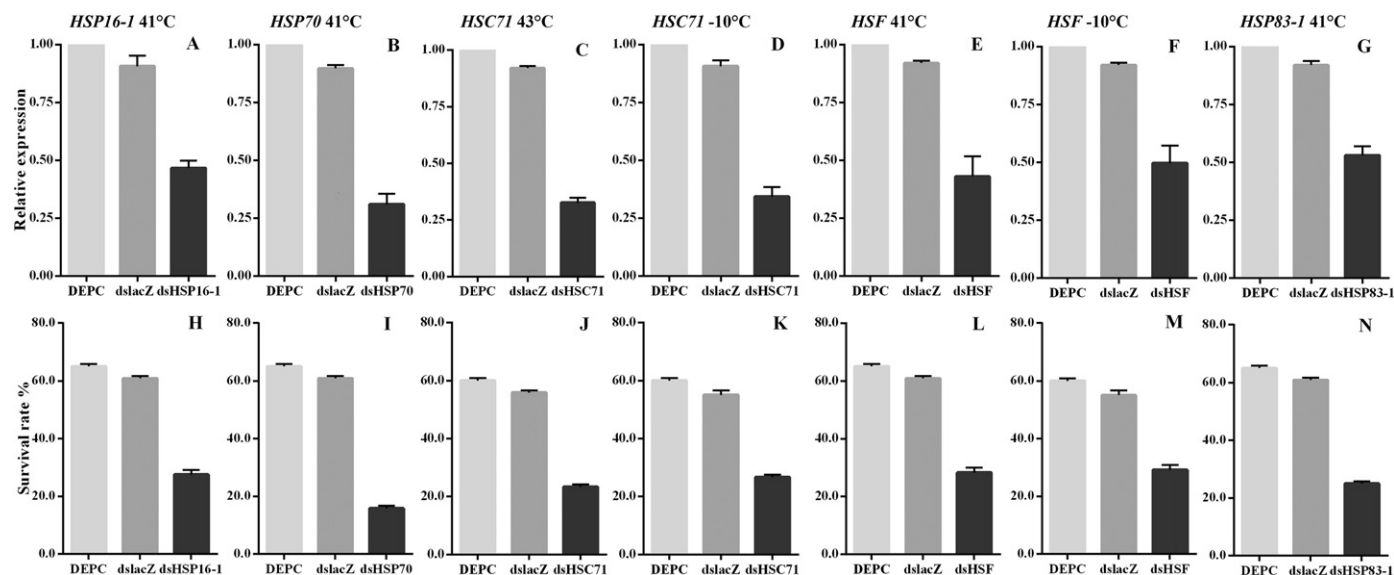


Fig. 6. Relative expression levels of HSPs (A–G) and survival rates of *D. farinae* after the RNAi-based knockdown of HSPs (H–N).

Fig. 6 shows that, compared with the blank control DEPC-treated water and the non-target control *dsLacZ*, the target genes of RNA oligos *dsHSP16-1* (Fig. 6A), *dsHSP70* (Fig. 6B), *dsHSC71* (Fig. 6C, D), *dsHSF* (Fig. 6E, F), *dsHSP83-1* (Fig. 6G), and were successfully downregulated by RNAi ($P < .0001$), and the corresponding survival rates of *D. farinae* (Fig. 6H–N) also significantly decreased (all $P < .0001$; Table S8). This finding indicated that the expression of *HSP16-1*, *HSP70*, and *HSC71* was silenced by RNAi, and thus the stress response ability of *D. farinae* diminished, the vitality of mites significantly weakened, while mortality of the mites significantly increased.

4. Discussion

HSPs are a class of ancient and highly conserved intracellular proteins. Although, in 1962, an Italian research team Ritossa et al., from the Laboratorio Internazionale di Genetica e Biofisica, Napoli, Italy, discovered the heat shock response (HSR) for the first time when they raised the culture temperature of *D. melanogaster* [21], by 1974, Swiss molecular biologists Tissières et al., isolated a new set of proteins via SDS-PAGE from *D. melanogaster* that participate in HSR and named them heat shock proteins [22]. In 1982, Adams et al., confirmed that both prokaryotic cells and eukaryotic cells can synthesize a class of proteins with similar function and biological activity when subjected to high temperatures [23]. Since then, an upsurge in the research on HSPs has gradually taken place internationally [24–26]. Subsequent studies revealed that hypoxia [27], heavy metals [28], viral infections [29], ultraviolet light [30], DNA damage, free radicals, and many other stimuli can cause an HSR and induce HSP synthesis.

Research on the HSR of mites had not been carried out until 2006 [5]. According to incomplete statistics, there are 12 reports on the HSR of mites in the literature. The stimuli include temperatures, drugs [7,11,13], and starvation [5], of which heat and cold stress are the most studied [6,8–14]. The high and low temperatures in those studies mainly are in the ranges of 30–45 °C and –8 to 20 °C, respectively. The main research method has been qRT-PCR. The expression levels of agricultural mites have been determined, including *A. ovatus*, *N. barkeri*, *P. citri*, *N. cucumeris*, *T. cinnabarinus*, and *T. urticae*. The target genes have mainly involved the four subfamilies: *HSP90*, *HSP70*, *HSP60*, and *HSP40*, especially *HSP70*. In general, these HSP genes have shown different degrees of upregulation at high temperatures but have not manifested a consistent pattern of expression changes at low temperatures. In addition, SDS-PAGE has been used for HSP analysis in *Varroa*: *HSP70* protein expression increased at 40 and 50 °C, especially at

40 °C [13]. However, the existing research on HSPs in mites has the following shortcomings: 1) There are obvious limitations of the detection methods. The existing reports have mainly been focused on qRT-PCR and SDS-PAGE. The limited number of *D. farinae* HSP gene sequences in GenBank has limited comprehensive research on all HSP family sequences by qRT-PCR. SDS-PAGE results involve some uncertainty because the proteins bands have been analyzed based on the standard marker; 2) The numbers of temperature groups have been small and have been arbitrarily defined by the researchers, thus lacking systematic experimental evaluation of survival rates in vitro; and 3) most studies lack silencing verification of the stress response function by RNAi.

In this context, the following breakthrough improvements were made in the experimental design of this study: 1) Firstly, from –20 to 48 °C, a total of 27 temperature groups were designed to discover the overall profile of the *D. farinae* viability changes under extremely high or low temperatures, thus providing a scientific basis for screening the treatment temperature of RNA-seq and constructing temperature gradient vs. gene expression histogram. 2) Because of the absence of the complete genome sequence of *D. farinae*, de novo RNA-seq was performed for the first time to determine all HSP sequences and screen all DEGs involved in temperature stress response. These findings not only enriched the molecular data on *D. farinae* HSPs in GenBank, but more importantly, provided candidate gene sequences for subsequent qRT-PCR confirmation of the DEGs. 3) The qRT-PCR method was used to verify the changes in expression of stress response genes (*HSPs*) under heat and cold stress and to confirm their stress response effects and characteristics. 4) The temperature gradient vs. expression magnitude histogram was built to clarify the relation between the HSP expression and survival rates. The temperature starting point, the highest point and the inactivation point in the regulation of the key *D. farinae* HSP genes were identified and the relationship of change rule of expression quantity with the survival rate was fully revealed. 5) Based on the temperature with the highest expression, the gradient time experiments were set to observe the change of HSPs expression from 10 min to 36 h recovery, and the time vs. expression quantity effect relationship and duration time change rule were deduced. 6) The RNAi method was employed to validate the observed correlation between gene silencing and mite viability changes. Finally, the stress response function of HSPs under temperature stress was confirmed at both the genetic level and the living body.

In this first exploratory study on the stress response function of HSPs under temperature stress in clinically important mites *D. farinae*, the following original results were obtained: A total of 163 Unigenes were

assigned to HSPs in five RNA-seq samples, among which 88 Unigenes were DEGs. These 88 HSP Unigenes were aligned to 47 genes. Seventeen HSP genes had FPKM >100 and were defined as the main stress response genes, including *HSP16-1*, *HSP16-2*, *HSP70*, *HSP70-1*, *HSP70-3-1*, *HSC70-4*, *HSC70-5*, *HSC71*, *HSC71-1*, *HSC71-2*, *HSC71-3*, *HSP83-1*, *HSP83-2*, *HSP90*, *Cdc37*, *dnaJ1*, *dnaJ A1*, and *HSF*. Eight HSP genes had baseline FPKM ≥ 500 or baseline FPKM ≥ 100 and $\log_2FC \geq 5$ in qRT-PCR analysis; they were defined as key stress response genes: *HSP16-1*, *HSP16-2*, *HSP70*, *HSP70-3-1*, *HSC71*, *HSP83-1*, *HSP83-2*, and *HSF*.

HSP16-1 and *HSP16-2* were the two important sHSP subfamily genes involved in the temperature stress response of *D. farinae* in this study. sHSP proteins can recognize and bind to incompletely folded or incorrectly assembled proteins to help peptides fold correctly, to ensure their proper transport, and to prevent aggregation [31]. Consequently, these functions protect cells from stressors and increase tolerance to stressful stimuli. In this study, based on the temperature gradient vs. gene expression histogram, it was found that *HSP16-1* and *HSP16-2* have the same expression pattern, i.e., they were upregulated under heat stress at 37–43 °C and downregulated under cold stress from 5 to –15 °C. Moreover, *HSP16-1* showed a greater expression change than *HSP16-2*: 1) higher baseline expression (3864.36 vs. 1185.39), 2) preferentially started upregulation (37 °C vs. 39 °C), 3) higher upregulation at 41 °C (2.31 vs. 1.61). Further, time gradient recovery experiment showed that when recovery for 40 min, *HSP16-1* reached the highest expression, and could last for 12 h. In addition, after RNAi knockdown of *HSP16-1*, the gene expression and the survival rate of mites decreased by 48.35% and 54.79%, respectively. Thus *HSP16-1* indeed performs a regulatory function under heat stress was confirmed. However, under cold stress, *HSP16-1* and *HSP16-2* were both downregulated, implying that they did not play regulatory roles under cold stress.

HSP83-1 and *HSP83-2*, belonging to the cytoplasmic HSP90 group, are two key stress response genes identified in this study. HSP90 is a regulatory enzyme that prevents irreversible protein aggregation under stressful conditions, thereby increasing cell tolerance to stress. The roles of *HSP83-1* and *HSP83-2* in *D. farinae* under temperature stress were basically the same as those of *HSP16-1* and *HSP16-2*. Although the change in their expression was greater under heat stress, upregulation started at a higher temperature and mainly indicated a participation in the response to stress within the narrow range from 41 to 43 °C. These findings are in agreement with the results of experiments carried out on *HSP90* gene of *N. cucumeris* [10] and *N. barkeri* [15], indicating that these two genes are involved only in the heat stress response.

HSP70 is the most studied stress response protein among HSPs and is widely distributed: it can be present in the cytoplasm, nucleus, endoplasmic reticulum, and Golgi apparatus. *HSP70* participates in many processes, such as growth and development, apoptosis, antioxidant defense, and synergistic immunity. As the most important molecular chaperone in the cell, it plays an important part in basic processes such as protein synthesis, correct folding, transmembrane transport, translocation, degradation, assembly, cytoskeleton dynamics, and nuclear-matrix stability. This study revealed that *HSP70s* are the gene family strongly involved in the temperature stress response in *D. farinae*. Nine genes were identified, including *HSP70*, *HSC71-3*, *HSP70-1*, *HSC70-4*, *HSC71*, *HSC71-1*, *HSC71-2*, *HSC70-5*, and *HSP70-3-1*. However, expression regulation patterns (changes in expression vs. temperature gradient) of the three key differentially expressed *HSP70* genes turned out to be different. We found that *HSP70* was the most potent HSP gene, especially for the response to heat stress. Its expression regulation pattern was similar to that of *HSP83*, but *HSP70*'s upregulation was greater. It ultimately reached the highest expression at 41 °C, with $\log_2FC$ up to 4.31 and absolute FPKM up to 40,296.59. The $\log_2FC$ of *HSP70* after 10 min recovery was 2.73, higher than that of *HSP83*, and the stress response time of 16 h was also longer than that of *HSP83* (12 h). Its upregulation later weakened at 43 °C and sharply switched to downregulation at 45 °C,

with the expression at only 0.17% of its highest expression level. Compared with the non-target RNAi control group, the tolerance of *D. farinae* at 41 °C decreased significantly, with relative expression decreasing from 0.90 to 0.31 (arbitrary units), and the survival rate decreasing from 60.83% to 15.83% after *HSP70* silencing. This result confirmed that *HSP70* indeed performs an important regulatory function in *D. farinae* during heat stress. Bioinformatics analysis indicated that the *HSP70* of *D. farinae* evaluated in this study belongs to the cytoplasmic HSP70 group and has the same regulatory effect as the *HSP70* of *P. citri* [8], namely participates only in the heat stress response.

Despite being a homolog of *HSP70s*, *HSC71* manifested a completely different pattern of expression regulation, apparently playing an important regulatory role in the responses to both heat and cold stress. Upregulation of *HSC71* gradually increased when the temperature became more extreme (as compared to 25 °C), and the highest upregulation under heat and cold stress was observed at 43 and –10 °C, respectively, and could sustain for 16 h and 28 h. At more extreme temperatures, *HSC71* expression weakened. This result was also verified by gene silencing. The expression regulation of *HSC71* has a pattern similar to that of *HSC71-1* of *D. farinae* obtained in this study, *HSP70-1* and *HSP70-3* of *T. cinnabarinus* [6], and *HSP70-2* of *A. ovatus* [14], reported in the literature. However, *HSC71-3* showed an expression pattern different from that of *HSC71* and yielded similar responses to temperature changes in comparison with *HSP70*, but with weaker intensity.

Heat shock factors (HSFs) are upstream regulators of HSPs and have the ability to protect cells from protein misfolding, inflammation and environmental damage. When the cell mounts a temperature stress response, HSF first dissociates from HSPs, releasing an free inactive HSF monomer. The free monomers are then polymerized into a trimer and phosphorylated. They are transferred from the cytoplasm to the nucleus, binding to a heat shock element to initiate transcription and increase HSP expression. A large amount of HSP has a cytoprotective effect, enhancing the cell's ability to resist damage. It in turn binds to HSF to inhibit its further activation, thereby performing negative feedback regulation of the HSR [32]. Notably, we determined, for the first time, that *HSF* plays a very important regulatory role in *D. farinae* under heat and cold stress. The regulatory function of *HSF* was found to be similar to that of *HSC71*, namely upregulating under both heat and cold stress. At gradient temperature, it was the stress response gene to preferentially start upregulation with temperature changes (at 33 °C or 5 °C), and was expressed at a sustained high level when temperature increased and decreased. Under the heat stress, the expression of *HSF* reached its peak at 41 °C ($\log_2FC$ of 5.31, 39.67-fold), which was higher than that of *HSC71* (20.39-fold) and *HSP70* (19.84-fold). However, the regulation intensity of *HSF* under cold stress was lower than that of heat stress. The expression of *HSF* reached the peak at –10 °C ($\log_2FC$ of 2.98, 7.88-fold), which was higher than that of *HSC71* (7.01-fold). The stress response time of *HSF* under temperature stress was just the opposite with the regulation intensity. Under –10 °C cold stress for 1 h, the upregulation could be detected after recovery for 10 min, and reached the peak at 16 h, and the $\log_2FC$ decreased to <1 after recovery for 32 h. Under 41 °C heat stress, the upregulation could be detected after recovery for 20 min, and reached the peak at 2 h, and the $\log_2FC$ decreased to <1 after recovery for 20 h. It can be seen that the stress response function of *HSF* was not only significantly greater than *HSP70*, *HSP83* and *HSP16*, but also greater than that of *HSC71*.

In summary, this study showed for the first time that multiple HSP genes play a key role in the temperature stress response of *D. farinae*. Different gene subfamilies have different regulatory functions under heat and cold stress. Many genes such as *HSP16-1*, *HSP16-2*, *HSP83-1*, *HSP83-2*, *HSP70*, *HSC71* and *HSF* are involved in heat stress response, while only *HSF* and *HSC71* are involved in cold stress response. These results imply that there are other gene or gene families should be involved in cold stress response of *D. farinae*, which needs to be confirmed by further research.

5. Conclusion

This study was the first systematic exploration on the stress response function of HSP under temperature stress in *D. farinae*. The following pioneering conclusions were derived: 1) It was confirmed that 17 differentially expressed genes in the HSP family were mainly involved in temperature stress response of *D. farinae*, and six genes played a key role—namely, *HSF*, *HSC71*, *HSP70*, *HSP83-1*, *HSP83-2* and *HSP16-1*. 2) The regulation patterns of these key genes were different. *HSP70*, *HSP83-1*, *HSP83-2* and *HSP16-1* were only involved in heat stress response, but not in cold stress, while *HSF* and *HSC71* played a regulatory role in both heat and cold stress. 3) It was found that *HSF* had the strongest regulatory effect for the first time; it not only started upregulation earliest, but also had the largest regulation intensity and the longest duration. In addition, the upregulation of HSP key stress response genes could be detected at recovery for 10 min after temperature stress for 1 h. The results of this study not only help further promote the study of *D. farinae* at a molecular level, but also provide technical support for exploring the regulatory role of HSP under temperature stress in important medical mites such as *Sarcoptes scabiei* and *Demodex*.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2019.12.014>.

Declaration of competing interest

The authors do not have any commercial or other association that might pose a conflict of interest in this publication.

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#Hdog. far. HSP70. MS96234	SPS	KT-LAAAP	GIDGLTYTC	WQVPMQSV	LIANDQGHRT	TSYTPVND	2	TEHLIDGAK	WQVPMQSVRT	IPDAKHLGR	REPSSVSCV	MUNFPMVS	DSKPLVEV	[160]
#H1a.o. cas. HSP70-1. A0227714	MS	AT												
#H1a.o. cas. HSP70-2. A0227715		G												
#Hsd. nag. HSP70aA. ACJ647193														
#Hro. mol. HSP70. AAA28540														
#Htri. cas. HSP70. NP_001164098	MG	T												
*#Hder. far. HSC71. MS96227		AK	T											
#Hder. pto. HSC71. XP_027200494		AK	T											
#Htet. cin. HSP70-4. A4C720664		SE	T											
#Htet. cin. HSP70-3. AC060404		SE	T											
#Hpan. cit. HSP70-2. A0M63424		SA	G	C										
#Hpan. cit. HSP70-1. A0M63423		MSGR	A											
#Htet. cin. HSP70-1. AC060422		AK	A											
#Hra. ooc. HSP70-1. APM05688		MA	A											
#Hnoo. bar. HSP70. AXZ94644		VG	G											
#Hnoo. cec. HSP70. AQ050609		VG	G											
*#Hder. far. HSP70-3-1. MS96236	MMNNNN	IRSPWMMPL	LYLALTPVN	Y-LAKDQ	Q-QDVTG									
#Hra. ooc. HSP70-2. A0M63425		ML	LLAS-VLQ	LVANSLASD										
#Htet. cin. HSP70-2. A0M62932		MT	LPVALLITL	LYLVITYSR	E-VGSA									
#Hpan. cit. HSP70-3. A0M63425	MPFRSGSGK	VLPILITGL	LYTPVSSAK	EKDSKSDQ	MDQSGVT									
#Htet. cin. HSP70-2. AC060423	MPFRYSKSG	VMPILITGL	LYTPVSSAK	EKADAKADA	D-DVTG									
#Htri. cas. HSC70-3. XP_026200986	MK	LIALGALCL	VLS-VYARD	D-KKX	E-DVTG									

[illegible][illegible]

*Hdor. fte. HSP70. M8969234	ITYAGWVWT LIDRNTYITTA IQQVITTTTA INQAPVITQT YGEBRAMEKD NMRGTFITQI GIPAPRVPV QIVTDFVFOA NGILNVAUD KSTGRQWMTI ITNDEGLSKS ADITMFWQFA BYHEDROED RERIAKQAL RATAPELST	[600]
*Halle. ooc. HSP70. V. AQD27714	V.....A.....S.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....P.....F.....K.....E.....D.....D.....T.....R.....T.....S.....I.....S.....I.....S.....	[600]
*Halle. ooc. HSP70. V. AQD27715	V.....L.....I.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....P.....F.....K.....E.....D.....D.....T.....R.....T.....S.....I.....S.....I.....S.....	[600]
*Hadv. aag. HSP70a. ACJ64193	V.....K.....E.....S.....R.....C.....I.....S.....G.....S.....F.....P.....R.....Q.....Q.....S.....T.....M.....K.....E.....K.....K.....K.....Q.....Q.....Q.....R.....D.....S.....K.....E.....K.....E.....S.....S.....R.....G.....C.....G.....S.....	[600]
*Hdro. mol. HSP70. AAP28840	V.....K.....E.....C.....R.....C.....S.....G.....S.....A.....S.....S.....L.....K.....E.....M.....K.....K.....K.....Q.....Q.....Q.....E.....D.....R.....N.....K.....A.....E.....K.....Q.....V.....T.....S.....R.....A.....S.....H.....V.....L.....N.....Q.....A.....	[600]
*Htri. ooc. HSP70. NP_001164098	V.....S.....K.....I.....V.....E.....A.....R.....C.....S.....S.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Hdor. fte. HSC71. M8968227	V.....P.....E.....A.....R.....C.....S.....S.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Hdor. pte. HSC71. XP_002704494	V.....P.....E.....A.....R.....C.....S.....S.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Htet. cin. HSP70. A. ACJ78064	V.....A.....T.....S.....I.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Htet. cin. HSP70. S. AC0604504	V.....A.....T.....S.....I.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Hpan. cit. HSP70. 2. ADN83424	V.....A.....T.....S.....I.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Hpan. cit. HSP70. 1. ADN83423	V.....P.....M.....G.....S.....I.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Htet. cin. HSP70. 1. AC060442	V.....P.....M.....G.....S.....I.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Htra. ooc. HSC70. 1. APN05688	V.....S.....V.....K.....E.....S.....T.....I.....S.....G.....L.....L.....K.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Hho. bar. HSP70. AIZ29464	V.....L.....S.....V.....R.....P.....F.....P.....V.....L.....A.....I.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Hho. cnc. HSP70. AOG06059	V.....L.....S.....V.....R.....P.....F.....P.....V.....L.....A.....I.....E.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Hdor. fte. HSP70-9. 1. M8969236	V.....L.....K.....P.....V.....K.....S.....I.....S.....A.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Htra. ooc. HSP70. 2. ACJ64194	V.....L.....K.....P.....V.....K.....S.....I.....S.....A.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Htra. ooc. HSP70. 2. ANH9232	V.....L.....K.....P.....V.....K.....S.....I.....S.....A.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Hpan. cit. HSP70. 3. ADN83425	V.....L.....K.....P.....V.....K.....S.....I.....S.....A.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Htet. cin. HSP70. 3. AC060423	V.....L.....K.....P.....V.....K.....S.....I.....S.....A.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]
*Htri. ooc. HSP70. 3. XP_008200968	V.....L.....K.....P.....V.....K.....S.....I.....S.....A.....S.....T.....I.....L.....H.....Q.....Q.....E.....S.....E.....R.....L.....A.....D.....R.....E.....K.....E.....D.....S.....G.....S.....V.....N.....V.....Q.....	[600]

[illegible]

#HD06.far.HSP33.1.MSR92328	MPENQO	—	RIETFAFQA	ELIAGLSLILI	NYTYSKEIIF	LEHLSINSSD	ALDKRZTSE	IPDSIANCIE	DYFIKILIPNK	DEKILITIDY	GIMGKALIK	NULGTIARSG	TKAFMEALGA	GADISIMQEG	GVGYFVATLY	ADRYTYSKEH	[160]
#HD06.far.2.MSR92328		—						T.T.	LGRLQ	L.R.							[160]
#Tyr.put.HSP90.AJ301840		Q	—	V			A	T.T.	SRV		R.R.						[160]
#Pan.cit.HSP90.ADM3426		PMWATN	EDA				T	A	LDSSG	L	V					R.V.T.	[160]
#tet.cin.HSP90.ACF76907		TMSEVB	—	P			L	T	LDSSG	BL	V					R.V.T.	[160]
#Pan.cit.HSP90.ADG20111		PMWQ	—	SV				T	MRSSG	SLS	R	D	EN			R	[160]
#Hso.cou.HSP90.AG060610							A	T.A	MDAG	BLT	R	DT	EN			R	[160]
#Hso.cou.HSP93.YF01648767							AA		T	LDSSG	BL					S	[160]
#Hdro.maj.HSP93.NF.00161962							AA		T	LDSSG	BL					T	[160]

Table S1 Mortality rates of *D. farinae* at various stressful temperatures

Group	Tem (°C)	Dead/ total	Mortality %	Tem (°C)	Dead/ total	Mortality %
control	25	0/100	0.00			
Low tem	5	0/108	0.00	-9	36/108	22.22
	0	4/120	3.33	-10	36/134	26.57
	-2	9/132	6.82	-12	37/133	27.82
	-5	15/120	12.50	-15	54/120	45.00
	-6	14/104	13.46	-18	111/118	94.06
	-7	30/160	18.75	-20	135/135	100.00
	-8	30/144	19.56			
High tem	33	0/108	0.00	42	24/124	19.35
	35	4/100	4.00	43	27/103	26.21
	37	12/134	8.95	44	52/122	42.62
	38	13/108	12.03	45	85/187	45.45
	39	16/120	13.33	46	129/135	95.55
	40	22/142	15.41	48	135/135	100.00
	41	20/118	16.95			

Tem: temperature;

Dead/total: the ratio of the number of dead mites and total mites.

Table S2 The quality index of RNA-seq sample for *D. farinae* under temperature stress

samples	Data (G)	Q20%	Q30%	Clean Reads %	No. of Unigenes	Mean length	N50
25 °C	6.65	97.64	90.45	95.44	27,748	1140	2,114
41 °C	6.51	95.17	86.50	85.95	33,206	957	1,756
43 °C	6.67	98.00	91.80	95.63	29,663	1033	1,851
45 °C	6.57	95.07	86.26	86.04	30,745	1084	2,069
-10 °C	6.63	97.75	90.78	95.12	26,265	1129	2,051

Table S3 The sequence information of 47 HSP genes of *D. farinae* in Fig. 2A

annotation information of <i>D. farinae</i>			Blast alignment			HSPs confirmation in <i>D. farinae</i>		
Gene ID	ORF	baseline FPKM	Gene of closely related species	accession No.	C%	I%	Gene	accession No.
U1237	1929	236.35	D.ptc.heat shock cognate 71 kDa protein	XM_027338446	100	89.44	<i>HSC71-3'</i>	MK896230
CL5225.C1;U1751	1224	237.07	D.ptc.methanethiol oxidase	XM_027344696	100	87.70	<i>HSC71-1'</i>	MK896228
U1195	1980	89.63	D.ptc.heat shock cognate 71 kDa protein-like	XM_027338446	94	86.29	<i>HSC71-4</i>	MN046872
U1775	1983	831.39	D.ptc.heat shock cognate 71 kDa protein	XM_027344693	100	88.32	<i>HSC71'</i>	MK896227
U1315,U1181	1980	383.90	D.ptc.heat shock cognate 71 kDa protein	XM_027344693	100	86.09	<i>HSC71-2'</i>	MK896229
U9706	1965	1921.97	D.far.Der f 28	KM009992	100	100.00	<i>HSP70'</i>	MK896234
CL2097.C2	1995	64.73	D.ptc.endoplasmic reticulum chaperone BiP-like	XM_027340753	93	91.11	<i>HSP70-3-2</i>	MN046873
CL2097.C1,3	2010	794.13	D.ptc.endoplasmic reticulum chaperone BiP	XM_027340753	97	90.45	<i>HSP70-3-1'</i>	MK896236
CL4735.C1	1962	5.41	D.ptc.heat shock 70 kDa protein cognate 4	XM_027347090	96	93.64	<i>HSC70-4'</i>	MK896225
CL4735.C2	1962	3.67	D.ptc.heat shock 70 kDa protein cognate 4-like	XM_027347090	95	92.94	<i>HSC70-4-2</i>	MN046874
U19203	1959	123.52	D.ptc.heat shock 70 kDa protein 4	XM_027341546	99	87.71	<i>HSP70-1'</i>	MK896235
U5192	1899	1.16	D.ptc.NAD-specific glutamate dehydrogenase-like	XM_027344572	41	96.03	<i>HSP70-0-3</i>	MN046875
U10152,U10153	2073	166.97	D.far.heat shock protein cognate 5	JQ001475	81	99.65	<i>HSP70-5'</i>	MK896226
CL1774.C2	2094	1.96	D.ptc.heat shock 70 kDa protein-like	XM_027347006	99	98.42	<i>HSP70-3</i>	MN046876
CL1774.C1	2100	1.10	D.ptc.heat shock 70 kDa protein-like	XM_027347006	99	86.75	<i>HSP70-4-2</i>	MN046877
CL1774.C3	2079	0.58	D.ptc.heat shock 70 kDa protein-like	XM_027347006	99	90.22	<i>HSP70-4-3</i>	MN046878
CL1110.C3	939	2.15	D.ptc.heat shock protein beta-1-like	XM_027339362	91	84.80	<i>HSPbeta</i>	MN046849
U18672	462	3864.36	D.ptc.alpha-crystallin A chain	XM_027339750	100	84.63	<i>HSP16-1'</i>	MK896232
U17706	369	1185.39	D.ptc.heat shock protein Hsp-16.1	XM_027340148	98	83.29	<i>HSP16-2'</i>	MK896233
U7777	6447	11.57	D.ptc.dnaJ homolog subfamily C member 13-like	XM_027347481	100	90.44	<i>dnaJ.C13</i>	MN046853
U13834	1359	0.01	No significant similarity found	/	/	/	<i>dnaJ.dom7</i>	MN046854
CL1435.C4	2406	17.54	D.ptc.dnaJ homolog subfamily C member 16-like	XM_027338742	100	88.96	<i>dnaJ.C16</i>	MN046855
CL3962.C3	1164	67.26	D.ptc.dnaJ homolog subfamily B member 14-like	XM_027347483	99	90.82	<i>dnaJ.B14</i>	MN046856
CL5250.C2	942	0.13	No significant similarity found	/	/	/	<i>dnaJ.dom5</i>	MN046857
CL4115.C1,2	1116	43.05	D.ptc.hsp90 co-chaperone Cdc37	XM_027348446	96	87.31	<i>HSP90.Cdc37'</i>	MK896239
CL379.C1	2118	1.99	D.ptc.heat shock protein 90-like	XM_027346304	100	90.51	<i>HSP90-1</i>	MN046850
CL379.C2	2118	37.24	D.ptc.heat shock protein 90-like	XM_027346304	100	98.35	<i>HSP90-3</i>	MN046851
U713	2103	0.50	D.ptc.heat shock protein 90-like	XM_027349667	61	97.00	<i>HSP90-2</i>	MN046852
CL4916.C2	2193	823.04	D.ptc.heat shock protein 83	XM_027341196	99	87.67	<i>HSP83-1'</i>	MK896237
U9047	2157	634.56	D.ptc.heat shock protein 83	XM_027342139	100	91.62	<i>HSP83-2'</i>	MK896238
U13618	351	0.18	No significant similarity found	/	/	/	<i>dnaJ.dom4</i>	MN046858
CL3976.C1, 2, 3,4	951	25.54	D.ptc.E3 ubiquitin-protein ligase ubr3-like	XM_027338059	95	86.50	<i>dnaJ.B6</i>	MN046859
CL498.C1	1062	30.15	D.ptc.dnaJ homolog subfamily B member 1	XM_027346959	99	99.15	<i>dnaJ.B1</i>	MN046860
CL498.C2	1059	0.45	D.ptc.dnaJ homolog subfamily B member 1-like	XM_027346959	100	84.51	<i>dnaJ.B2</i>	MN046861
CL765.C1	1038	104.42	D.ptc.dnaJ protein homolog 1	XM_027350323	100	85.11	<i>dnaJ1'</i>	MK896224
U11887	726	3.73	D.ptc.dnaJ homolog subfamily B member 1-like	XM_027346959	21	75.86	<i>dnaJ.B3</i>	MN046862
U13223	1227	61.76	D.ptc.dnaJ homolog subfamily A member 1	XM_027348547	100	89.33	<i>dnaJ.A1'</i>	MK896223
U22268	1269	0.17	D.ptc.chaperone protein dnaJ 3-like	XM_027349640	82	96.67	<i>dnaJ3</i>	MN046863
U7698	1230	0.53	D.ptc.dnaJ protein homolog 2-like	XM_027337805	100	98.86	<i>dnaJ2</i>	MN046864
CL3636.C3	1653	0.21	No significant similarity found	/	/	/	<i>dnaJ.dom6</i>	MN046865
CL3636.C1, 2	1701	0.43	No significant similarity found	/	/	/	<i>dnaJ.dom1</i>	MN046866
CL2925.C1	771	0.64	No significant similarity found	/	/	/	<i>dnaJ.dom3</i>	MN046867
CL2925.C2	915	0.49	No significant similarity found	/	/	/	<i>dnaJ.dom2</i>	MN046868
CL2602.C2, 3	1773	61.03	D.ptc.heat shock protein 60A-like	XM_027341724	99	91.46	<i>HSP60</i>	MN046870
CL5330.C1, 2	1155	10.07	D.ptc.uncharacterized	XM_027345633	96	77.75	<i>dnaJ</i>	MN046869
CL1714.C5,8,9,10	1668	129.23	D.ptc.heat shock factor protein	XM_027342711	100	86.59	<i>HSE'</i>	MK896231
CL1714.C1,2,4	1653	38.56	D.ptc.heat shock factor protein-like	XM_027342709	100	86.81	<i>HSE-1</i>	MN046871

C%: Coverage Rate %; I%: identity %;

D.ptc.: *Dermatophagoides pteronyssinus*; D.far.: *Dermatophagoides farinae*;

¹: HSPs gene were performed qRT-PCR.

²: HSPs gene were performed temperature gradient versus expression magnitude curves plotting.

³: HSPs gene were performed RNAi.

Table S4 The related sequence information of HSPs in Fig. 2B

Subfamily	Gene	mites	accession No.	Gene	mites	accession No.
HSP70s	<i>HSC71</i> [†]	<i>D. far</i>	MK896227	<i>HSC71-2</i> [†]	<i>D. far</i>	MK896229
	<i>HSC71</i>	<i>D. pte</i>	XM_027344693	<i>HSC71-3</i> [†]	<i>D. far</i>	MK896230
	<i>HSC71</i>	<i>D. pte</i>	XM_027338446	<i>HSP70</i> [§]	<i>D. far</i>	MK896234
	<i>Derf28</i>	<i>D. far</i>	KM009992	<i>HSP70-1</i>	<i>P. cit</i>	GQ495083
	<i>HSP70-1</i>	<i>T. cin</i>	EU679412	<i>HSP70-2</i>	<i>P. cit</i>	GQ495084
	<i>HSP70-3</i>	<i>T. cin</i>	EU679414	<i>HSP70-4</i>	<i>T. cin</i>	EU977182
	<i>HSC70</i>	<i>T. urt</i>	DQ310791	<i>HSP70</i>	<i>N. bar</i>	MF542317
	<i>HSP70</i>	<i>N. cuc</i>	KC335213	<i>HSP70</i>	<i>D. far</i>	KC305502
	<i>HSP70-1</i>	<i>A. ova</i>	KX237517	<i>HSP70-2</i>	<i>A. ova</i>	KX237518
	<i>HSC70-4</i> [†]	<i>D. far</i>	MK896225	<i>HSC70-4</i>	<i>D. pte</i>	XM_027347090
	<i>HSP70-3-1</i> [‡]	<i>D. far</i>	MK896236	<i>ER.chaperone.BiP</i>	<i>D. pte</i>	XM_027340753
	<i>HSP70-2</i>	<i>T. cin</i>	EU679413	<i>HSP70-3</i>	<i>P. cit</i>	GQ495085
	<i>HSC70-5</i> [†]	<i>D. far</i>	MK896226	<i>HSC70-5</i>	<i>D. far</i>	JQ001475
	<i>HSP70-1</i> [†]	<i>D. far</i>	MK896235	<i>HSP70-4</i>	<i>D. pte</i>	XM_027341546
	<i>met.oxidase</i>	<i>D. pte</i>	XM_027344696	<i>HSC71-1</i> [†]	<i>D. far</i>	MK896228
HSFs	<i>HSF</i> [§]	<i>D. far</i>	MK896231	<i>HSF</i>	<i>D. pte</i>	XM_027342711
dnaJs	<i>dnaJ1</i> [†]	<i>D. far</i>	MK896224	<i>dnaJ1</i>	<i>D. pte</i>	XM_027350323
	<i>dnaJ.A1</i> [†]	<i>D. far</i>	MK896223	<i>dnaJ.A1</i>	<i>D. pte</i>	XM_027348547
HSP20s	<i>crystallinA</i>	<i>D. pte</i>	XM_027339750	<i>HSP16-1</i> [§]	<i>D. far</i>	MK896232
	<i>HSP16</i>	<i>D. pte</i>	XM_027340148	<i>HSP16-2</i> [‡]	<i>D. far</i>	MK896233
HSP90s	<i>HSP90.Cdc37</i>	<i>D. pte</i>	XM_027348446	<i>HSP90.Cdc37</i> [†]	<i>D. far</i>	MK896239
	<i>HSP83-1</i> [§]	<i>D. far</i>	MK896237	<i>HSP83</i>	<i>D. pte</i>	XM_027341196
	<i>HSP83-2</i> [‡]	<i>D. far</i>	MK896238	<i>HSP83</i>	<i>D. pte</i>	XM_027342139
	<i>HSP90</i>	<i>P. cit</i>	GQ495086	<i>HSP90</i>	<i>T. cin</i>	EU851046
	<i>HSP90</i>	<i>P. cit</i>	HQ257511			

D. far: *Dermatophagoides farinae*; *D. pte*: *Dermatophagoides pteronyssinus*; *A. ova*: *Aleuroglyphus ovatus*; *N. bar*: *Neoseiulus barkeri*; *T. cin*: *Tetranychus cinnabarinus*; *N. cuc*: *Neoseiulus cucumeris*; *T. cin*: *Tetranychus cinnabarinus*; *T. urt*: *Tetranychus urticae*; *P. cit*: *Panonychus citri*.

[†]: HSPs gene were performed qRT-PCR.

[‡]: HSPs gene were performed temperature gradient versus expression magnitude curves plotting.

[§]: HSPs gene were performed RNAi.

Table S5 log₂Foldchange and absolute expression quantity of main stress response HSP genes in *D. farinae*

Gene	25 °C			41 °C			43 °C			45 °C			-10 °C		
	FPKM	log ₂ FC	FC	FPKM	log ₂ FC	FC	FPKM	log ₂ FC	FC	FPKM	log ₂ FC	FC	FPKM	log ₂ FC	FC
<i>HSP16-1</i>	3864.36	2.00	4.00	15457.44	1.48	2.79	10779.58	-7.69	0.00	18.71	-4.41	0.05	181.78		
<i>HSP16-2</i>	1185.39	1.71	3.27	3878.13	1.60	3.03	3593.43	-4.14	0.06	67.24	-4.36	0.05	57.73		
<i>HSP70</i>	1921.97	4.39	20.97	40296.59	4.37	20.68	39741.81	-4.82	0.04	68.04	-4.86	0.03	66.18		
<i>HSC71-3</i>	236.35	3.93	15.24	3602.50	2.88	7.36	1739.89	-2.71	0.15	36.12	-2.37	0.19	45.72		
<i>HSP83-1</i>	823.04	4.89	29.65	24403.81	3.23	9.38	7722.32	-1.01	0.50	408.68	-1.28	0.41	338.92		
<i>HSP83-2</i>	634.56	2.95	7.73	4903.56	1.40	2.64	1674.61	-1.57	0.34	213.73	-4.57	0.04	26.72		
<i>HSF</i>	129.23	5.61	48.84	6311.63	4.89	29.65	3831.78	3.27	9.65	1246.61	2.69	6.45	833.94		
<i>HSC71-1</i>	237.07	1.30	2.46	583.73	1.30	2.46	583.73	1.72	3.29	780.99	1.81	3.51	831.27		
<i>HSC71</i>	831.39	2.35	5.10	4238.63	2.90	7.46	6205.71	1.03	2.04	1697.72	1.32	2.50	2075.70		
<i>HSC71-2</i>	383.90	0.61	1.53	585.93	1.34	2.53	971.85	0.86	1.82	696.79	1.16	2.23	857.85		
<i>HSC70-5</i>	166.97	0.03	1.02	170.48	1.20	2.30	383.60	0.28	1.21	202.73	1.77	3.41	569.46		
<i>dnaJA1</i>	61.76	0.56	1.47	91.05	1.84	3.58	221.11	0.47	1.39	85.54	1.61	3.05	188.52		
<i>HSP70-1</i>	123.52	3.08	8.46	1044.50	4.11	17.27	2132.90	0.85	1.80	222.64	0.05	1.04	127.88		
<i>dnaJ1</i>	104.42	3.42	10.70	1117.65	2.58	5.98	624.37	-0.68	0.62	65.18	-0.41	0.75	78.59		
<i>HSP90.Cdc37</i>	43.05	2.52	5.74	246.93	3.37	10.34	445.09	0.85	1.80	77.60	1.13	2.19	94.22		
<i>HSC70-4</i>	5.41	6.00	66.26	358.45	-1.11	0.46	2.51	-0.14	0.91	4.91	-0.91	0.53	2.88		
<i>HSP70-3-1</i>	794.13	-2.13	0.23	181.43	-2.99	0.13	99.96	-2.13	0.23	181.43	-3.86	0.07	54.69		

log₂FC: log2FoldChange; FC: FoldChange

Table S6 The expression of HSPs in the temperature gradient vs. expression magnitude histogram (Mean± SD)

Tem(°C)	HSP16-1	HSP16-2	HSP70	HSP83-1	HSP83-2	HSF	HSC71	HSP70-3-1
-15	-5.28±0.19	-5.64±0.28	-4.84±0.09	-3.98±0.05	-3.39±0.09	2.57±0.04	1.80±0.06	-2.33±0.09
-10	-5.45±0.22	-5.75±0.13	-4.61±0.02	-3.88±0.20	-2.59±0.24	2.98±0.11	2.81±0.14	-2.43±0.04
-7	-5.21±0.02	-6.30±0.06	-5.51±0.17	-5.00±0.25	-2.09±0.07	2.86±0.01	2.01±0.02	-3.20±0.02
-5	-5.14±0.04	-5.97±0.08	-5.43±0.07	-5.16±0.04	-2.09±0.02	2.84±0.02	1.91±0.04	-3.52±0.12
-2	-5.12±0.14	-5.77±0.06	-5.31±0.03	-5.33±0.06	-2.06±0.11	2.74±0.07	1.80±0.10	-3.69±0.31
0	-5.08±0.04	-5.70±0.23	-3.97±0.13	-3.67±0.10	-1.95±0.13	2.46±0.05	1.04±0.01	-3.76±0.04
5	-4.80±0.24	-5.44±0.10	-1.75±0.11	-3.29±0.24	-1.58±0.10	2.11±0.02	0.62±0.18	-2.27±0.14
25	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
33	-3.86±0.21	-2.95±0.24	-3.23±0.21	-3.03±0.26	-1.21±0.09	2.24±0.02	1.46±0.07	-3.04±0.01
35	-1.52±0.04	-2.07±0.13	-2.38±0.31	-2.35±0.06	-1.18±0.18	2.43±0.14	1.72±0.18	-0.89±0.06
37	0.94±0.18	-0.84±0.18	-1.33±0.43	-2.21±0.22	-1.00±0.08	2.70±0.03	1.74±0.19	-0.90±0.05
39	1.36±0.22	0.88±0.22	-1.21±0.16	-1.29±0.12	-0.71±0.20	2.75±0.12	2.16±0.02	-0.33±0.18
41	2.31±0.08	1.61±0.20	4.31±0.09	3.96±0.05	2.00±0.07	5.31±0.15	2.64±0.02	-1.05±0.03
43	1.84±0.05	1.45±0.11	4.01±0.05	3.05±0.08	1.20±0.10	4.81±0.07	4.35±0.11	-2.38±0.08
45	-5.20±0.04	-6.82±0.19	-2.70±0.08	-3.05±0.03	-2.18±0.14	3.31±0.04	1.01±0.04	-3.15±0.07

Mean relative expression ± SD following temperature stress for 1 h and 25 °C recovery for 1 h from qRT-PCR analysis.

Tem: Temperature;

The statistical differences were observed in the expression level between control group of 25 °C and each temperature group (Student's *t*-test, all *P* < 0.001).

Table S7 The expression of HSPs in the time gradient vs. expression magnitude histogram (Mean± SD)

Time of recovery (h)	<i>HSP70</i> 41 °C	<i>HSP16-1</i> 41 °C	<i>HSP83-1</i> 41 °C	<i>HSP83-2</i> 41 °C	<i>HSF</i> 41 °C	<i>HSC71</i> 43 °C	<i>HSF</i> -10 °C	<i>HSC71</i> -10 °C
1/6	2.73±0.07	1.85±0.04	1.21±0.05	0.89±0.11	0.71±0.17	2.44±0.27	1.57±0.16	1.04±0.06
1/3	3.55±0.03	2.00±0.08	1.84±0.22	1.93±0.08	1.31±0.28	3.12±0.12	2.55±0.08	1.75±0.15
2/3	3.69±0.12	2.18±0.17	2.11±0.06	2.33±0.25	3.26±0.15	4.05±0.24	2.73±0.07	1.79±0.23
1	4.16±0.04	2.01±0.10	3.46±0.15	2.05±0.06	5.23±0.20	4.17±0.11	3.01±0.29	1.99±0.24
2	3.49±0.18	1.92±0.12	2.56±0.20	1.73±0.41	5.36±0.15	2.27±0.21	3.58±0.05	2.02±0.21
4	2.75±0.19	1.87±0.17	2.17±0.05	1.57±0.31	3.34±0.16	2.05±0.09	3.83±0.16	2.04±0.02
8	2.42±0.16	1.41±0.17	1.91±0.17	1.50±0.22	2.61±0.01	1.40±0.08	4.21±0.08	2.04±0.18
12	1.14±0.17	1.24±0.17	1.29±0.02	1.22±0.08	2.04±0.20	1.19±0.13	5.03±0.22	2.34±0.07
16	1.07±0.23	-0.55±0.03	-0.95±0.10	-0.80±0.17	1.43±0.08	0.63±0.11	5.27±0.14	3.02±0.19
20	-1.03±0.03	-0.71±0.06	-1.61±0.05	-1.50±0.15	0.87±0.19	0.60±0.07	3.75±0.10	2.37±0.16
24	-1.76±0.09	-0.83±0.16	-1.99±0.14	-1.67±0.12	0.70±0.25	0.39±0.04	3.63±0.13	2.02±0.04
28							1.06±0.13	1.02±0.08
32							0.97±0.04	0.73±0.10
36							0.85±0.04	0.55±0.10

Table S8 The relative expression of HSP and survival rate of *D. farinae* after RNAi (Mean± SD)

Target gene	Tem (°C)	Before RNAi	Relative expression of HSP				Survival rate % of <i>D. farinae</i>			
			DEPC	<i>dsIacZ</i>	target	<i>P</i> value	DEPC	<i>dsIacZ</i>	target	<i>P</i> value
<i>HSP16-1</i>	41	2.31	1.00±0.00	0.91±0.05	0.47±0.03	0.0002	65.00(78/120)	60.83(73/120)	27.50(33/120)	0.000
<i>HSP70</i>	41	4.31	1.00±0.00	0.90±0.02	0.31±0.05	<0.0001	65.00(78/120)	60.83(73/120)	15.83(19/120)	0.000
<i>HSP83-1</i>	41	3.96	1.00±0.00	0.92±0.02	0.53±0.04	0.0001	65.00(78/120)	60.83(73/120)	25.00(30/120)	0.000
<i>HSF</i>	41	5.31	1.00±0.00	0.92±0.01	0.43±0.08	0.0006	65.00(78/120)	60.83(73/120)	28.33(34/120)	0.000
<i>HSC71</i>	43	4.35	1.00±0.00	0.92±0.01	0.33±0.02	<0.0001	60.00(72/120)	55.83(67/120)	23.33(28/120)	0.000
<i>HSF</i>	-10	2.98	1.00±0.00	0.92±0.01	0.50±0.08	0.0006	60.00(72/120)	55.00(66/120)	29.16(35/120)	0.000
<i>HSC71</i>	-10	2.81	1.00±0.00	0.91±0.03	0.34±0.04	<0.0001	60.00(72/120)	55.00(66/120)	26.67(32/120)	0.000

Tem: Temperature;

Before RNAi: log₂FC of the relative expression of HSPs before RNAi.*P* value: the statistical difference between the relative expression of HSP; the statistical difference between survival rate % of *D. farinae*.