

Heat Shock Factor Regulation of Antimicrobial Peptides Expression Suggests a Conserved Defense Mechanism Induced by Febrile Temperature in Arthropods

Bang Xiao, Shihan Chen, Yue Wang, Xuzheng Liao, Jianguo He , Chaozheng Li 

School of Marine Sciences, Sun Yat-sen University, State Key Laboratory of Biocontrol /Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Guangzhou, China • Guangdong Provincial Key Laboratory of Marine Resources and Coastal Engineering/ Guangdong Provincial Key Laboratory for Aquatic Economic Animals, School of Life Sciences, Sun Yat-sen University, Guangzhou, China • China-ASEAN Belt and Road Joint Laboratory on Mariculture Technology, Guangzhou, China

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Abstract

Temperature is a critical factor influencing the outbreak and progression of viral diseases in organisms. Febrile temperatures have been shown to enhance immune competence and reduce viral replication in various species. However, the underlying mechanisms remain largely unknown. In this study, we investigate the molecular mechanisms by which elevated temperatures confer resistance to viral infections, focusing on the role of heat shock factor 1 (HSF1) in regulating antimicrobial effectors rather than the traditional target genes molecular chaperones. Using shrimp *Litopenaeus vannamei* as a model, we demonstrate that febrile temperatures induce HSF1, which in turn upregulates antimicrobial peptides (AMPs) that target viral envelope proteins and inhibit viral replication. Importantly, this is the first to show that HSF1 directly binds to the heat shock elements (HSE) motifs of AMPs both in shrimp and *Drosophila*, suggesting this may be a conserved regulatory mechanism in arthropods. Additionally, our findings highlight the role of HSF1 beyond the classical heat shock response, revealing its critical function in modulating innate immunity. These insights provide new avenues for managing viral infections in aquaculture and other settings by leveraging environmental temperature control.

eLife Assessment

This study reports an **important** finding on the mechanism underlying the enhancement of anti-viral immune responses by febrile temperatures, especially the role of the conserved heat-shock factor, HSF-1. The data provide **compelling** support for the authors' model wherein increased temperature in the shrimp *Litopenaeus vannamei* activates HSF1, which in turn enhances anti-viral response via up-regulation of the nSWD protein and antibacterial peptides. The work, which will be of interest to virologists, immunologists, and cell biologists, would benefit from more discussion of the function and roles of HSF-1 at 25°C vs. 32°C.

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1. Introduction

The proliferation of pathogens and the outbreak of diseases are influenced by various factors, including transmission vectors, host species, and environmental conditions (Marcogliese, 2008). Among these, temperature is one of the most critical environmental factors, significantly impacting disease occurrence and prevalence (Hou et al., 2023). Temperature can directly affect the immune response, metabolism, feeding rate, oxygen consumption, growth, and survival of animals (Deldicq et al., 2021). Aquatic animals such as fish, shrimp, crabs, and shellfish are ectothermic, and their susceptibility to diseases is directly related to temperature (Marcogliese, 2008). In fishes, Koi herpesvirus (KHV) can cause massive mortality in carp and koi carp at temperatures between 23–28 °C, while temperatures above 30 °C typically do not result in mortality (Yuasa et al., 2008). In crustaceans, high temperatures (32–33 °C) have been demonstrated to inhibit white spot syndrome virus (WSSV) replication and reduce mortality in infected shrimp (Du et al., 2006; Guan et al., 2003; Jiravanichpaisal et al., 2004). Notably, many ectothermic animals can respond to exogenous pathogenic infections by seeking warmer external environments (Covert and Reynolds, 1977; Louis et al., 1986). When infected, some ectothermic animals such as fish, insects, and crustaceans elevate their body temperature by moving to warmer areas (Bundey et al., 2003; Rakhshaninejad et al., 2023; Rakus et al., 2017). This behavioral fever, similar to fever responses in higher organisms, can help hosts suppress or overcome infections (Hasday et al., 2014), although the mechanisms underlying this heat-induced antimicrobial immunity remain unclear.

The heat shock response (HSR), also known as the heat stress response, serves as a bridge linking elevated temperatures with increased immunity (Evans et al., 2015). The HSR induced by temperature increase primarily involves the expression of heat shock proteins (HSPs) and other related factors regulated by heat shock transcription factors (HSFs) (Barna et al., 2018). Under non-stress conditions, HSF monomers are retained in the cytoplasm in a complex with HSP90 or the cytosolic chaperonin TCP1 ring complex (TRiC) (Neef et al., 2014; Zou et al., 1998). During the stress response, HSF dissociates from the complex, trimerizes, and converts into a DNA-binding conformation (Zheng et al., 2016). Activated HSF induces gene transcription through regulatory upstream promoter elements known as heat shock elements (HSEs) (Hahn et al., 2004). In the face of thermal or pathogenic stress, the heat shock response functions as a highly conserved cellular mechanism across various organisms (Anckar and Sistonen, 2011). Research suggests that the HSF-mediated heat shock response plays a crucial role in the regulation of the innate immune network through a combination of direct gene regulation, interaction with immune signaling pathways, and the broader cellular stress response (Morimoto, 2011). In

Drosophila, HSF-deficient flies, lacking a heat shock response, exhibit high sensitivity to viral infection (Merkling et al., 2015 [↗](#)), indicating that the heat shock response is a crucial component of innate antiviral immunity.

Heat shock factors (HSFs) are primarily recognized as inducible transcriptional regulators of genes encoding molecular chaperones and other stress proteins (Akerfelt et al., 2010 [↗](#)). Interestingly, members of the HSF family also participate in various cellular processes, including apoptosis, the unfolded protein response (UPR), oxidative stress, multidrug resistance, and pathogenic infection (Ankar and Sistonen, 2011 [↗](#)). Consequently, the repertoire of HSF targets has expanded well beyond traditional heat shock genes (Barna et al., 2018 [↗](#)). Recent research has illuminated the interplay between the heat shock response (HSR) and the innate immune system, revealing that HSFs can regulate innate immune responses (Akerfelt et al., 2007 [↗](#)). For instance, HSF1, the primary HSF, can modulate the expression of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6, thereby influencing the inflammatory response (Barna et al., 2018 [↗](#)). Additionally, HSF1 has been shown to interact with and influence the activity of the NF- κ B and MAPK pathways, thus altering the cellular environment to modulate the innate immune response (Chen et al., 2010 [↗](#)). These unexpected observations indicate complexity of HSF function. At present, however, the mechanisms by which the HSF family affects innate immunity are still poorly understood.

To explore the underlying mechanisms of the febrile temperature-inducible HSF in host antiviral immunity, the shrimp *Litopenaeus vannamei* was challenged with WSSV, and the thermal regulation of host immunity was characterized. The results indicated that febrile temperature-inducible HSF1 confers virus resistance by regulating the expression of antimicrobial peptides (AMPs) in *L. vannamei*. Additionally, it was revealed that high temperature (30 °C) restricts the replication of *Drosophila* C virus (DCV) in an invertebrate model system (*Drosophila* S2 cells) also mediated by the HSF1-AMPs axis. Together, these results suggest that heat shock factor regulation of AMPs expression is a conserved defense mechanism induced by febrile temperature in arthropods.

2. Results

2.1 Induction of Heat Shock Factor 1 in Response to WSSV at High Temperature (32 °C)

Studies have demonstrated that high temperatures (above 30 °C) markedly reduce mortality in shrimp infected with WSSV, while temperatures ranging from 22 to 30 °C favor higher levels of WSSV replication and associated mortality (Lin et al., 2011 [↗](#); Rahman et al., 2007 [↗](#)). To uncover the molecular mechanisms by which high temperature restricts WSSV infection, RNA-seq was performed to identify genes responsive to high temperature, particularly those encoding potential transcriptional regulators. Two shrimp groups, Group TW and Group W, were cultured at 25 °C. Group W comprised shrimp injected with WSSV and maintained at 25 °C continuously. In contrast, Group TW was subjected to a temperature increase to 32 °C at 24 hours post-injection (hpi). Gill samples were collected for analysis 12 hours post-temperature rise (hptr) and subjected to Illumina sequencing (Fig. 1A [↗](#)). As shown in Fig. 1B [↗](#) and 1C [↗](#), 10 of the 1285 high temperature-induced genes were predicted to encode transcription factors.

Special attention has been paid to heat shock factor 1 (HSF1), the master regulator of the heat shock response, which upon activation induces not only classical heat shock genes but also a broad array of genes beyond the scope of molecular chaperones (Barna et al., 2018 [↗](#)). To validate the high-throughput sequencing results, the expression profile of these transcription factors was confirmed by qPCR, showing a significant increase in LvHSF1 expression (Supplementary Fig. 1 [↗](#)). These results suggest that LvHSF1 may play a key role in enhancing shrimp resistance to WSSV at elevated temperatures (32 °C).

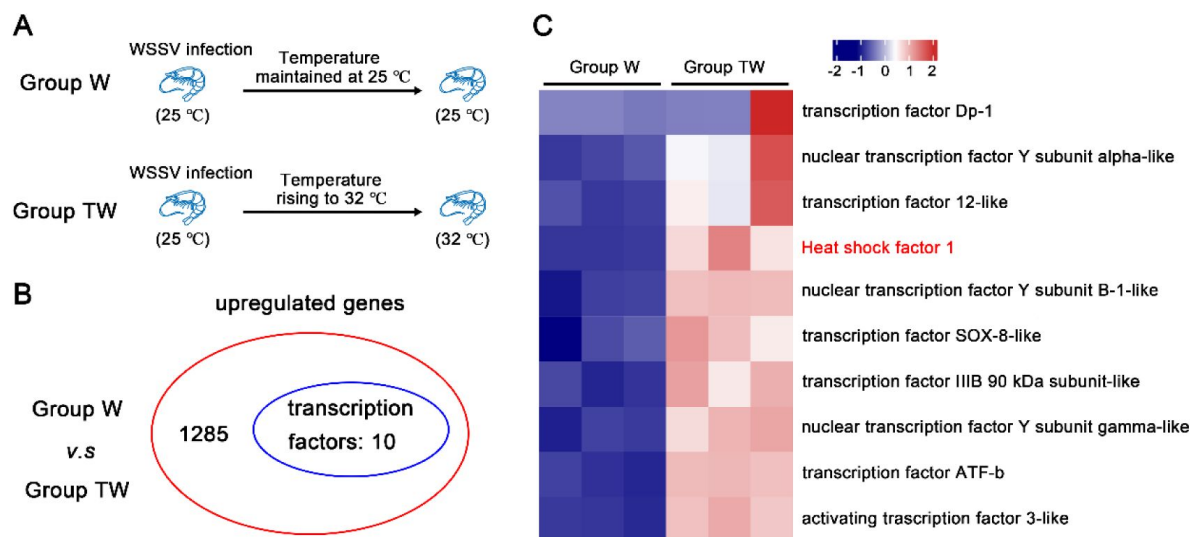


Figure 1.

Identification of HSF1 as a key factor induced by high temperature.

(A) Diagram illustration of the experimental setup for transcriptome analysis. Group W was injected with WSSV and maintained continuously at 25 °C. In contrast, Group TW, also injected with WSSV, had their culture temperature increased to 32°C at 24 hours post-injection (hpi). **(B)** Identification of high temperature-induced genes by RNA-Seq. RNA sequencing was performed 12 hours post-temperature increase. Gill samples from 8 shrimp in each group were collected for Illumina sequencing (NCBI SRA database accession number PRJNA1050424). **(C)** Heatmap showing the differential expression of upregulated genes encoding potential transcription factors. The heatmap displays the fragments per kilobase per transcript per million mapped reads (FPKM) values from three biological replicates, shown after logarithmic transformation.

2.2 Expression Profiles of LvHSF1 in Shrimp Under Varied Temperature Conditions and WSSV Challenge

To determine that LvHSF1 is related to high temperature and WSSV infection, its expression level was measured under high temperature and after WSSV infection. Tissue distribution analysis showed that LvHSF1 was expressed in all examined tissues at room temperature (25 °C) and increased rapidly in all tested tissues at high temperature (32 °C; **Fig. 2A**). Additionally, the protein expression of LvHSF1 was significantly up-regulated in hemocytes, gills, and intestines when exposed to 32 °C (**Fig. 2B**). Further investigation revealed LvHSF1's role in the immune response to Poly (I:C) and WSSV at 25 °C. Following exposure to Poly (I:C), LvHSF1 expression was upregulated at 12 h (2.43-fold), 24 h (2.60-fold), and 36 h (4.33-fold) in hemocytes (**Fig. 2C**); slightly upregulated at 4 h (1.97-fold) and downregulated at 12 h (0.64-fold) in gills (**Fig. 2D**); and slightly downregulated at 4 h (0.41-fold) and upregulated at 8 h (1.15-fold) in intestines (**Fig. 2E**). In response to WSSV infection, LvHSF1 expression in hemocytes was dramatically induced, peaking at 8 hours (6.04-fold; **Fig. 2F**). In the gills, LvHSF1 expression initially increased at 8 hours (2.10-fold) and significantly again at 72 hours (2.80-fold; **Fig. 2G**). In the intestines, a marked induction of LvHSF1 was observed at 8 hours (2.23-fold; **Fig. 2H**). These results suggest that LvHSF1 is actively involved in the response to both elevated temperatures and viral (WSSV) infection.

2.3 Role of LvHSF1 in Restricting WSSV at Low (25 °C) and High (32 °C) Temperatures

To investigate LvHSF1's role in shrimp during WSSV infection, RNA interference (RNAi) was employed *in vivo*. Two dsLvHSF1 constructs were synthesized: dsLvHSF1-1 (targeting 443-1001 bp) and dsLvHSF1-2 (targeting 1365-1854 bp). Analysis revealed a significant reduction in both transcription and protein levels of LvHSF1 in hemocytes (**Figs. 3A**, **3a**) and gills (**Figs. 3B**, **3b**), with dsLvHSF1-1 showing greater silencing efficiency. Consequently, dsLvHSF1-1 was selected for subsequent knockdown experiments. After LvHSF1 knockdown, shrimp were challenged with WSSV, and viral titers were quantified using absolute quantitative PCR. Shrimp with silenced LvHSF1 exhibited increased viral loads in hemocytes (**Fig. 3C**) and gills (**Fig. 3D**) at low temperature (25 °C). Additionally, transcription levels of VP28, one of the major structural proteins of WSSV, were higher in the LvHSF1-silenced group than in the GFP-silenced control in hemocytes (**Fig. 3E**) and gills (**Fig. 3F**). Similarly, the levels of VP28 protein were significantly elevated in the HSF1-silenced group (**Figs. 3G**, **3g**). However, no significant difference in shrimp survival rates was observed between LvHSF1-silenced shrimp and GFP-silenced shrimp at low temperature (25 °C) ($p = 0.8657$; **Fig. 3H**).

Next, LvHSF1's role in shrimp during WSSV infection at high temperature (32 °C) was further determined. Notably, the survival rate of LvHSF1-silenced shrimp infected with WSSV was significantly lower than the control group at high temperature (32 °C) ($p < 0.001$; **Fig. 4A**). Shrimp with silenced LvHSF1 exhibited increased viral loads in gills (**Fig. 4B**) and muscle (**Fig. 4C**) at high temperature (32 °C). Additionally, VP28 protein levels were significantly elevated in the LvHSF1-silenced group in gills at high temperature (32 °C; **Figs. 4D**, **4d**). These data suggest that LvHSF1 plays a crucial role in antiviral immunity against WSSV infection especially at high (32 °C) temperatures.

2.4 nSWD as a Potential Antiviral Effector Regulated by LvHSF1

To determine how temperature-induced LvHSF1 restricts WSSV infection, RNA-seq was performed to identify target genes regulated by LvHSF1. Candidates whose expression was inhibited by LvHSF1 knockdown after WSSV infection at high temperature (32 °C) were screened. As shown in

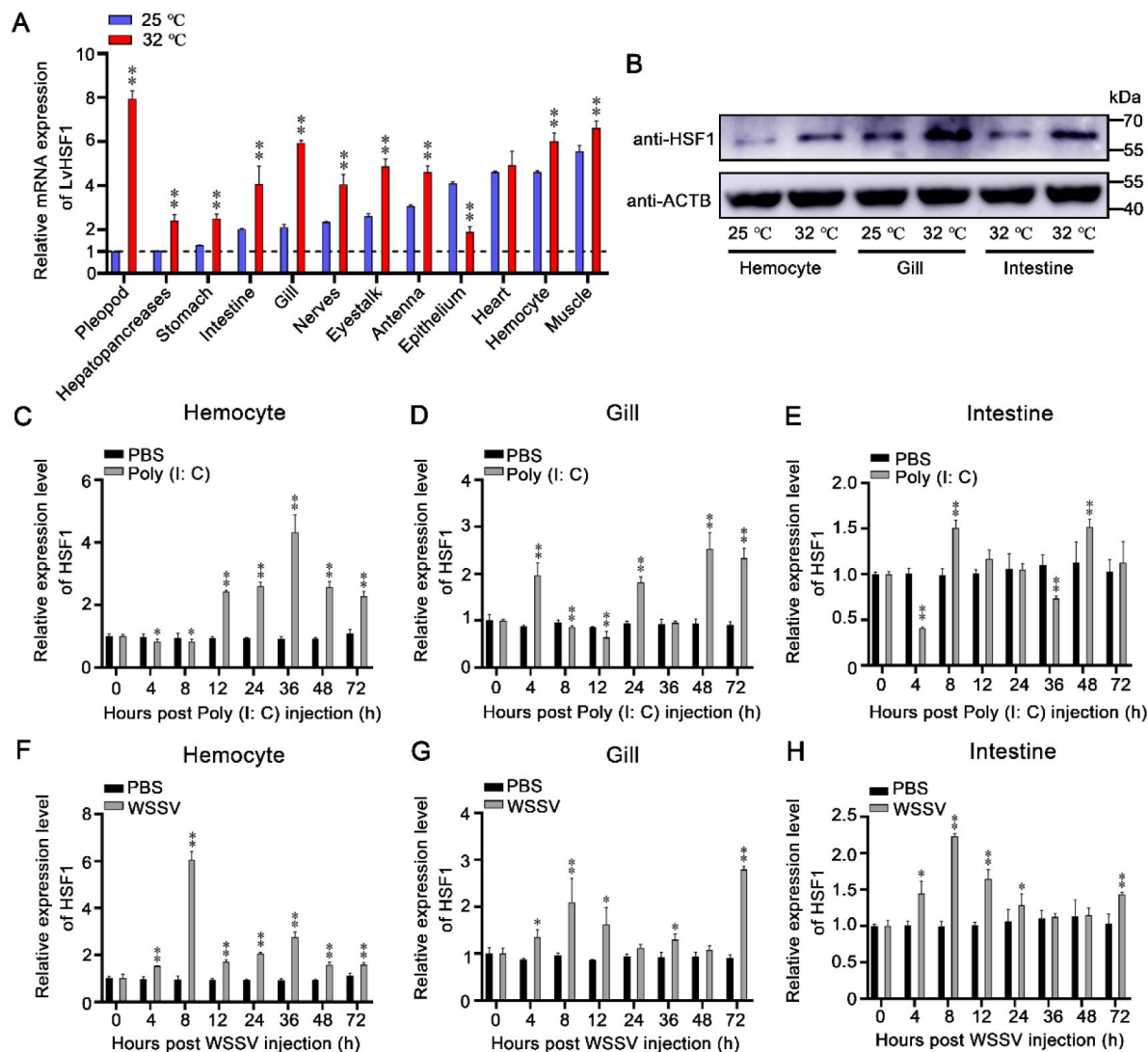


Figure 2.

Upregulation of LvHSF1 in shrimp challenged by WSSV at both low (25 °C) and high (32 °C) temperatures.

(A) Transcriptional expression of LvHSF1 in different tissues of healthy shrimp subjected to low (25 °C) and high (32 °C) temperatures. The pleopod tissue at 25 °C serves as a reference, with its expression level set to 1.0. Expression values were normalized against EF-1 α and β -Actin using the Livak ($2^{-\Delta\Delta CT}$) method. Data represent the mean $\pm$ SD from triplicate assays. (B) Protein levels of HSF1 at 25 °C and 32 °C detected by western blotting. (C-E) Expression profiles of LvHSF1 after Poly (I: C) injection in hemocytes (C), gills (D) and intestines (E). Expression levels were measured by qPCR, normalized against EF-1 α and β -Actin using the Livak ($2^{-\Delta\Delta CT}$) method, and presented as mean $\pm$ SD from triplicate assays. (F-H) Expression profiles of LvHSF1 after WSSV injection in hemocytes (F), gills (G) and intestines (H). Expression levels were measured by qPCR, normalized against EF-1 α and β -Actin using the Livak ($2^{-\Delta\Delta CT}$) method, and presented as mean $\pm$ SD from triplicate assays. Statistical significance was calculated using the Student's *t*-test (***p* < 0.01, **p* < 0.05). All experiments were representative of three biological replicates and yielded similar results.

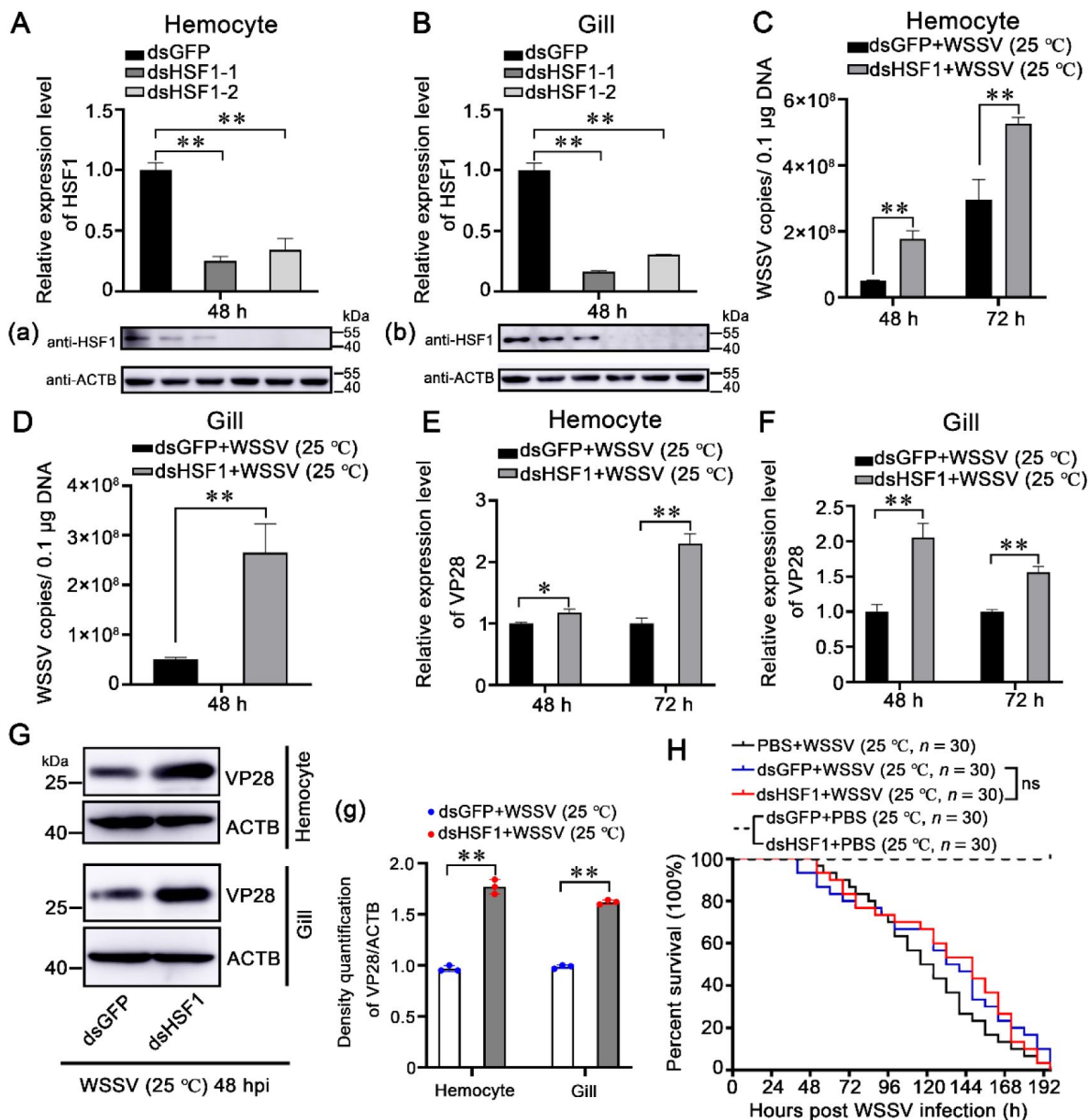


Figure 3.

Role of HSF1 in restricting WSSV replication in shrimp at low (25 °C) temperatures.

(A, B) Silencing Efficiency of dsRNA-LvHSF1. The efficacy of two dsRNA-LvHSF1 constructs (dsLvHSF1-1 and dsLvHSF1-2) was assessed by qPCR in hemocytes (A) and gills (B). Additionally, protein levels of LvHSF1 were evaluated by western blotting in both tissues (panels a and b). Expression values were normalized against EF-1 α and β -Actin using the Livak ($2^{-\Delta\Delta CT}$) method. Data represent the mean $\pm$ SD from triplicate assays. (C, D) Quantification of WSSV copies. The quantity of WSSV copies in hemocytes (C) and gills (D) at low temperature (25 °C) post-WSSV infection was determined using absolute quantitative PCR. (E, F) Transcriptional expression of the VP28 gene. The transcriptional expression of the VP28 gene was analyzed by qPCR in hemocytes (E) and gills (F) at low temperature (25 °C) following WSSV infection. (G) Expression of WSSV VP28 protein. Expression of WSSV VP28 protein was detected by western blotting in hemocytes and gills at low temperature (25 °C) after WSSV infection. Quantification and statistical analysis of three independent repeats were performed using ImageJ (panel g). (H) Survival rates of WSSV-infected shrimp post-LvHSF1 knockdown. The survival rates of WSSV-infected shrimp post-LvHSF1 knockdown were monitored at low temperature (25 °C), with recordings made every 4 hours. Statistical analysis was performed using the Kaplan-Meier plot (log-rank χ^2 test). All experiments were conducted with three biological replicates, consistently yielding similar results.

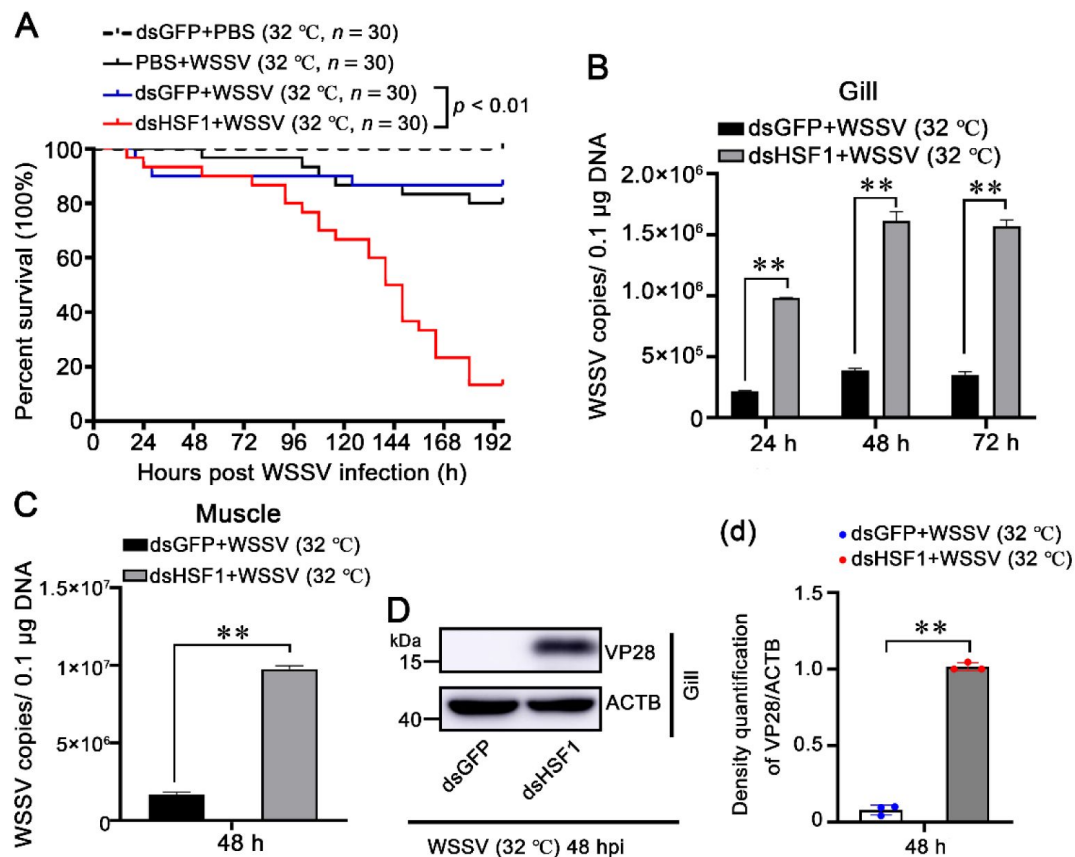


Figure 4.

Role of HSF1 in restricting WSSV replication in shrimp at high (32 °C) temperatures.

(A) Survival rates of WSSV-infected shrimp post-LvHSF1 knockdown. The survival rates of WSSV-infected shrimp post-LvHSF1 knockdown were monitored at high temperature (32 °C), with recordings made every 4 hours. Statistical analysis was performed using the Kaplan-Meier plot (log-rank χ^2 test). **(B, C)** Quantification of WSSV copies. The quantity of WSSV copies in gills (B) and muscles (C) at high temperature (32 °C) post-WSSV infection was determined using absolute quantitative PCR. **(D)** Expression of WSSV VP28 protein. Expression of WSSV VP28 protein was detected by western blotting in gills at high temperature (32 °C) after WSSV infection. Quantification and statistical analysis of three independent repeats were performed using ImageJ (panel d). Statistical significance was calculated using the Student's *t*-test (** $p < 0.01$). All experiments were conducted with three biological replicates, consistently yielding similar results.

Fig. 5A and **5B**, 8 of the 954 dsHSF1-suppressed genes were predicted to encode effector factors. These differentially expressed genes (DEGs) included immunity-effector molecules, such as antimicrobial peptides and cytokines. Among the 8 candidate genes, particular attention was paid to the newly identified single whey acidic protein domain-containing protein, designated nSWD. The nSWD belongs to type III crustins, which are crustacean cationic cysteine-rich antimicrobial peptides containing one or two whey acidic protein (WAP) domains at the carboxyl terminus and mainly exert antimicrobial activities. The detailed regulatory mechanism of nSWD against WSSV was unclear. To validate the high-throughput sequencing results, the expression level of nSWD and effector molecules were confirmed by qPCR, showing a significant reduction in hemocytes and gills after LvHSF1-knockdown (**Fig. 5C**; **Supplementary Fig. 2**). These results suggest that nSWD may play a pivotal role against WSSV infection regulated by LvHSF1.

To determine whether LvHSF1 regulates the expression of nSWD *in vitro*, a dual-luciferase reporter assay was performed in *Drosophila* S2 cells. The promoter region of nSWD was cloned by genome walking, containing two putative conserved HSE motifs located at approximately -1371 to -1359 (HSE-1) and -1035 to -1023 (HSE-2; Supplementary data). The dual-luciferase reporter assay showed that the promoter of nSWD could be regulated by ectopic expression of LvHSF1 in a dose-dependent manner (**Figs. 5D** and **5d**). Three reporter plasmids containing mutant promoter regions of nSWD with one or no HSE motifs were constructed, named pGL3-nSWD-M1, pGL3-nSWD-M2, and pGL3-nSWD-M12, respectively (**Fig. 5E**). The reporter assay results showed that the ectopic expression of LvHSF1 could also improve the promoter activity of pGL3-nSWD-M1 and pGL3-nSWD-M2, whereas the activity of pGL3-nSWD-M12 was not upregulated (**Figs. 5F** and **5f**), suggesting that putative HSE binding motifs play significant roles in LvHSF1-mediated nSWD induction.

To further confirm this, EMSA experiments were performed to investigate the direct interaction between LvHSF1 and HSE motifs of nSWD. Biotin-labeled probes or mutant probes of the HSE motifs were synthesized (**Fig. 5G**). LvHSF1 protein was purified to verify its ability to bind to the HSE motifs (**Supplementary Fig. 3A** and **3B**). The results showed that band shifts of protein-DNA complexes were detected when LvHSF1 protein was incubated with biotin-labeled probe1 but not mutant biotin-labeled probe1 (**Fig. 5H**). Additionally, the band shifts could be competitively reduced when LvHSF1 protein was incubated with wide-type unbiotinylated probe1 at 20- and 100-fold molar excess (**Fig. 5H**). A similar result was observed in the interaction between LvHSF1 protein and the biotin-labeled probe2 (**Fig. 5H**). No band shift was observed in the control set (GST), suggesting that the interaction between LvHSF1 protein and each of the HSE binding motifs is specific. These results strongly demonstrate that LvHSF1 could regulate the expression of nSWD *in vitro* and *in vivo*.

2.5 nSWD Inhibits WSSV Replication and Interacts with WSSV Envelope Proteins

To confirm whether nSWD is involved in WSSV infection, qPCR was performed to determine the expression profile of nSWD. When shrimp were infected with WSSV, the expression of nSWD was upregulated at 4 h (5.66-fold) and 12 h (5.60-fold), remained high at 36 h (5.18-fold) and 48 h (7.90-fold), and slightly downregulated at 72 h (4.74-fold; **Fig. 6A**). To reveal the role of nSWD in WSSV infection at high temperature (32 °C), RNA interference (RNAi) was performed to knock down nSWD expression. The mRNA level of nSWD was effectively suppressed by specific dsNWD in gills, and the value was downregulated to 0.30-fold of the dsGFP (green fluorescent protein) injection group (as a control) (**Fig. 6B**). The viral load of the dsNWD group was considerably higher than the control group (**Fig. 6C**). Additionally, the survival rate of shrimp in the nSWD-silenced group was significantly lower than in the GFP-knockdown group ($p < 0.01$; **Fig. 6D**).

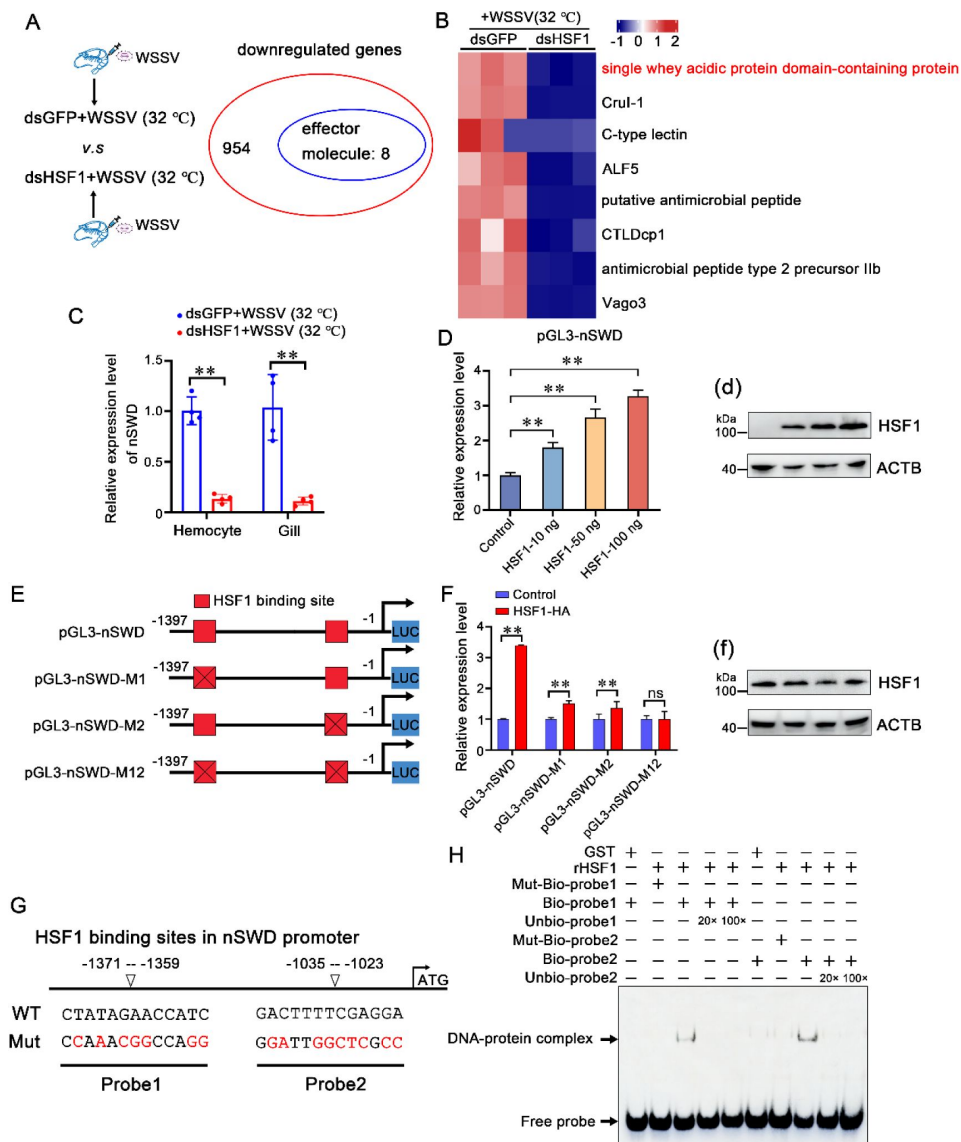


Figure 5.

nSWD is a potential antiviral effector regulated by HSF1.

(A) Venn diagram showing the downregulated genes by RNA-Seq. RNA-seq was performed 24 hours after WSSV infection (NCBI SRA database accession number PRJNA1110613). (B) Heatmap showing the differential expression of downregulated genes encoding potential effector molecules. The color bar indicates the gradient of normalized expression levels. (C) mRNA transcription levels of nSWD in hemocytes and gills. mRNA transcription levels of nSWD in the hemocytes and gills of LvHSF1-silenced shrimp under WSSV challenge. (D) Dual-luciferase reporter assays. Dual-luciferase reporter assays were performed to analyze the effects of overexpression of LvHSF1 on the promoter activities of nSWD in *Drosophila* S2 cells in a dose-dependent manner. Protein expression of LvHSF1 was detected with anti-HA Ab, with β -actin used as a protein loading control (panel d). (E) Schematic diagram of the nSWD promoter regions. Schematic diagram of the nSWD promoter regions in the luciferase reporter gene constructs. The HSF1 binding motif sites are shown in red rectangles. (F) Dual-luciferase reporter assays with mutated HSF1 binding motifs. Dual-luciferase reporter assays were performed to analyze the effects of overexpression of LvHSF1 on the promoter activities of nSWD with mutated HSF1 binding motifs. Protein expression of LvHSF1 was detected with anti-HAAb, with β -actin used as a protein loading control (panel f). (G) Analysis of the nSWD promoter. The HSF1 binding site was analyzed using the online JASPAR database. (H) EMSA assay. LvHSF1 protein interaction with HSF1 binding sites from the nSWD promoter was analyzed *in vitro* by EMSA assay. Competition assays were performed in the presence of excess unlabeled probes. Statistical significance was calculated using the Student's *t*-test (***p* < 0.01, **p* < 0.05). All experiments were conducted with three biological replicates, consistently yielding similar results.

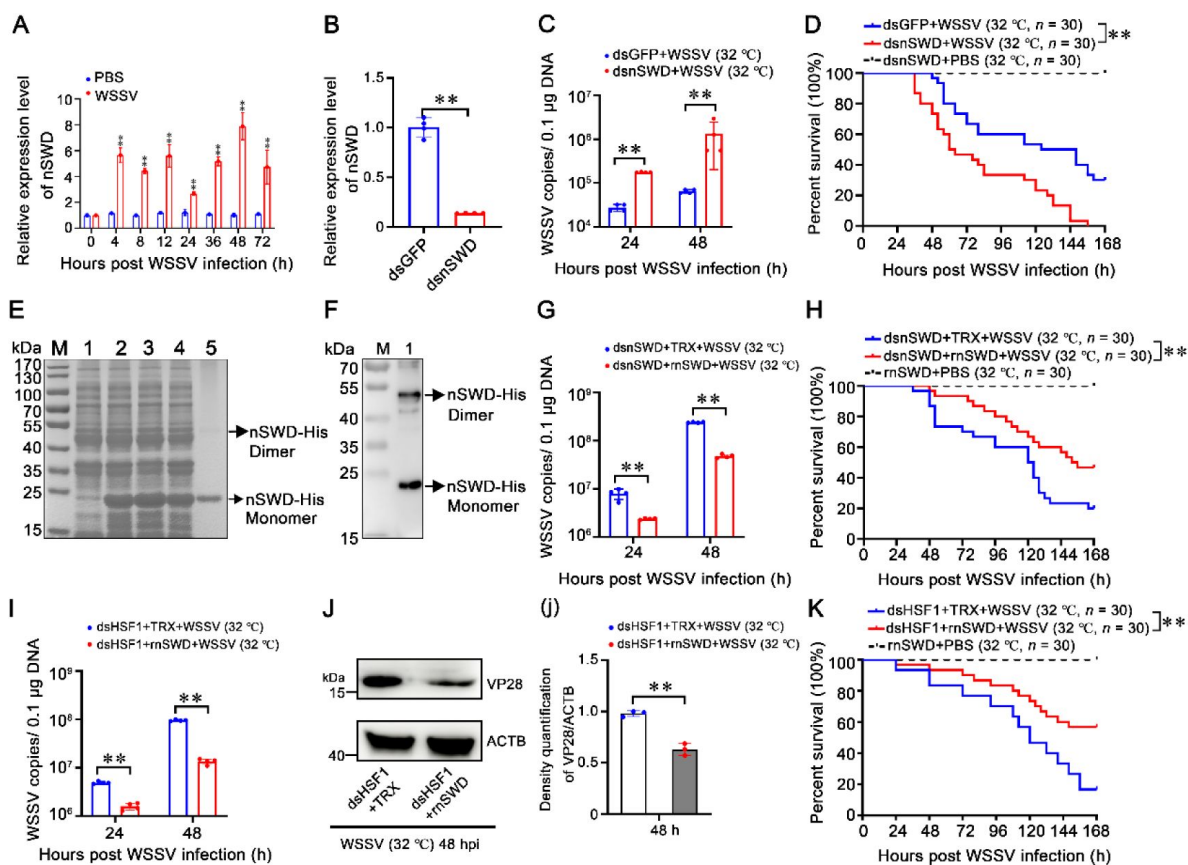


Figure 6.

nSWD possessed potent antiviral activities against WSSV.

(A) Expression of nSWD in gills from WSSV-challenged shrimp. (B) Silencing efficiency of nSWD in gills. Silencing efficiency of nSWD in gills 48 hours after WSSV infection. (C) Quantity of WSSV copies in gills at high temperature (32 °C). The quantity of WSSV copies in gills at high temperature (32 °C) was detected by absolute quantitative PCR at 24 and 48 hours after WSSV infection. DsGFP was used as a control. (D) Survival rates of WSSV-infected shrimp post-nSWD knockdown. Survival rates of WSSV-infected shrimp post-nSWD knockdown at high temperature (32 °C) were monitored, with recordings made every 4 hours. Statistical analysis was performed using the Kaplan-Meier plot (log-rank χ^2 test). (E) SDS-PAGE analysis of recombinant nSWD protein expressed in *E. coli*. Line 1: Uninduced *E. coli* transformed with nSWD; Line 2: Induced *E. coli* transformed with nSWD; Line 3: Supernatant of ultrasonic lysed *E. coli* expressing nSWD; Line 4: Precipitate of lysed *E. coli* expressing nSWD. Line 5: Purified recombinant nSWD protein (black arrow). (F) Western blotting of purified rnSWD protein. Purified rnSWD protein was checked by Western blotting with anti-6 $\times$ -His Ab. (G) Quantity of WSSV copies in shrimp gills following nSWD application post-nSWD knockdown. The quantity of WSSV copies in shrimp gills following 10 μ g nSWD application post-nSWD knockdown at high temperature (32 °C) was measured *in vivo* at 24 and 48 hours using absolute quantitative PCR. (H) Survival rates post-rnSWD application. Shrimp were injected with 10 μ g rnSWD or control protein, mixed with WSSV inoculum post-nSWD knockdown at high temperature (32 °C). Survival rates were recorded every 4 hours. (I) Quantity of WSSV copies in shrimp gills following nSWD application post-LvHsf1 knockdown. The quantity of WSSV copies in shrimp gills following 10 μ g nSWD application post-LvHsf1 knockdown at high temperature (32 °C) was measured *in vivo* at 24 and 48 hours using absolute quantitative PCR. (J) Expression of WSSV VP28 protein. Expression of WSSV VP28 protein was detected by Western blotting in gills at high temperature (32 °C) after WSSV infection. Quantification and statistical analysis of three independent repeats were performed using ImageJ (panel j). (K) Survival rates post-rnSWD application. Shrimp were injected with 10 μ g rnSWD or control protein, mixed with WSSV inoculum post-LvHsf1 knockdown at high temperature (32 °C). Survival rates were recorded every 4 hours. Statistical significance was calculated using the Student's *t*-test (***p* < 0.01, **p* < 0.05). All experiments were conducted with three biological replicates, consistently yielding similar results.

To further demonstrate the actual role of nSWD *in vivo*, RNAi experiments coupled with rnSWD proteins injection were performed. Recombinant proteins of rnSWD and rTrx-His-tag were expressed, purified by SDS-PAGE gels, and confirmed by Coomassie staining (**Fig. 6E** [↗](#)), and the results were further confirmed by Western blot analysis (**Fig. 6F** [↗](#)). After knockdown of endogenous nSWD, shrimp samples were treated with WSSV mixed with rnSWD or rTrx-His-tag by intramuscular injection at high temperature (32 °C). Consequently, the viral load of those co-injected with rnSWD was significantly reduced compared to the control group ($p < 0.01$; **Fig. 6G** [↗](#)). Additionally, the survival rates of shrimp co-injected with rnSWD were significantly higher than the control group ($p < 0.01$; **Fig. 6H** [↗](#)). Therefore, nSWD plays a crucial role in the immune defense against WSSV infection at high temperature (32 °C) *in vivo*.

Next, to verify whether the anti-WSSV function of nSWD was mediated by LvHSF1 at high temperature (32 °C), *in vivo* RNAi-LvHSF1 experiments coupled with rnSWD injection were performed. After knockdown of LvHSF1, shrimp samples were treated with WSSV mixed with rnSWD or rTrx-His-tag by intramuscular injection at high temperature (32 °C). The results showed that the viral load of those co-injected with rnSWD was significantly reduced compared to the control group ($p < 0.01$; **Fig. 6I** [↗](#)). Additionally, VP28 protein levels of those co-injected with rnSWD were significantly decreased (**Fig. 6J** [↗](#), 6j). The survival rates of shrimp co-injected with rnSWD were significantly higher than the control group ($p < 0.01$; **Fig. 6K** [↗](#)). These results suggest that the anti-WSSV function of nSWD at high temperature (32 °C) is mediated by LvHSF1.

To reveal the possible anti-WSSV mechanism of nSWD, pull-down assays were performed to detect whether rnSWD protein could interact with WSSV envelope proteins. The main envelope proteins of WSSV, including VP19, VP24, VP26, and VP28 with GST-tags, were from a previous study. In the GST-tagged pull-down assays, rnSWD could interact with VP24 and VP26 based on SDS-PAGE gels with Coomassie blue staining (**Fig. 7A** [↗](#)), and the result was further confirmed by Western blot analysis with the 6×His antibody (**Fig. 7B** [↗](#)). Based on the His-tagged pull-down assays, VP24 and VP26 could precipitate rnSWD (**Fig. 7C** [↗](#)), and this result was further confirmed by Western blot analysis with GST antibody (**Fig. 7D** [↗](#)). Additionally, confocal analysis confirmed that nSWD colocalized with VP24 (**Fig. 7E** [↗](#) and **7e** [↗](#)) and VP26 (**Fig. 7F** [↗](#) and **7f** [↗](#)) in *Drosophila* S2 cells. Collectively, these results suggest that nSWD could inhibit WSSV replication and bind to VP24 and VP26 of WSSV.

2.6 Conservation of the Febrile Temperature-Inducible HSF1-AMPs Axis Conferring Virus Resistance in Arthropods

To demonstrate whether HSF1 regulation of antimicrobial peptides is a conserved defense mechanism induced by elevated temperature in arthropods, experiments were performed in an invertebrate model system (*Drosophila* S2 cells). Literature reports that the temperature range for optimal growth of *Drosophila* S2 cells is 25–28 °C, and S2 cells tolerated a normal growth temperature of 30 °C. Therefore, S2 cells were cultured at normal temperature (27 °C) or high temperature (30 °C) with or without DCV infection. The results showed that S2 cells were adherent to the wall and grew well at 27 °C or 30 °C without DCV infection, and the cytopathic signs of S2 cells were milder at 30 °C than the control group at 27 °C when infected with DCV (**Supplementary Fig. 4A** [↗](#)). Additionally, the mRNA and protein expression of DCV was significantly lower at 30 °C than the control group at 27 °C (**Supplementary Fig. 4B** [↗](#) and **4C** [↗](#)). These results suggest that elevated temperature could restrict the replication of DCV in *Drosophila* S2 cells.

To determine whether DmHSF1 is related to high temperature (30 °C) and DCV infection in S2 cells, its expression level was also determined. The results showed that the mRNA expression of DmHSF1 was significantly upregulated 4.53-fold and 6.48-fold at high temperature (30 °C) or infected with DCV, respectively (**Fig. 8A** [↗](#)). Antimicrobial peptides have been proven to participate in the innate immunity of *Drosophila* in response to viral infection. The expression of

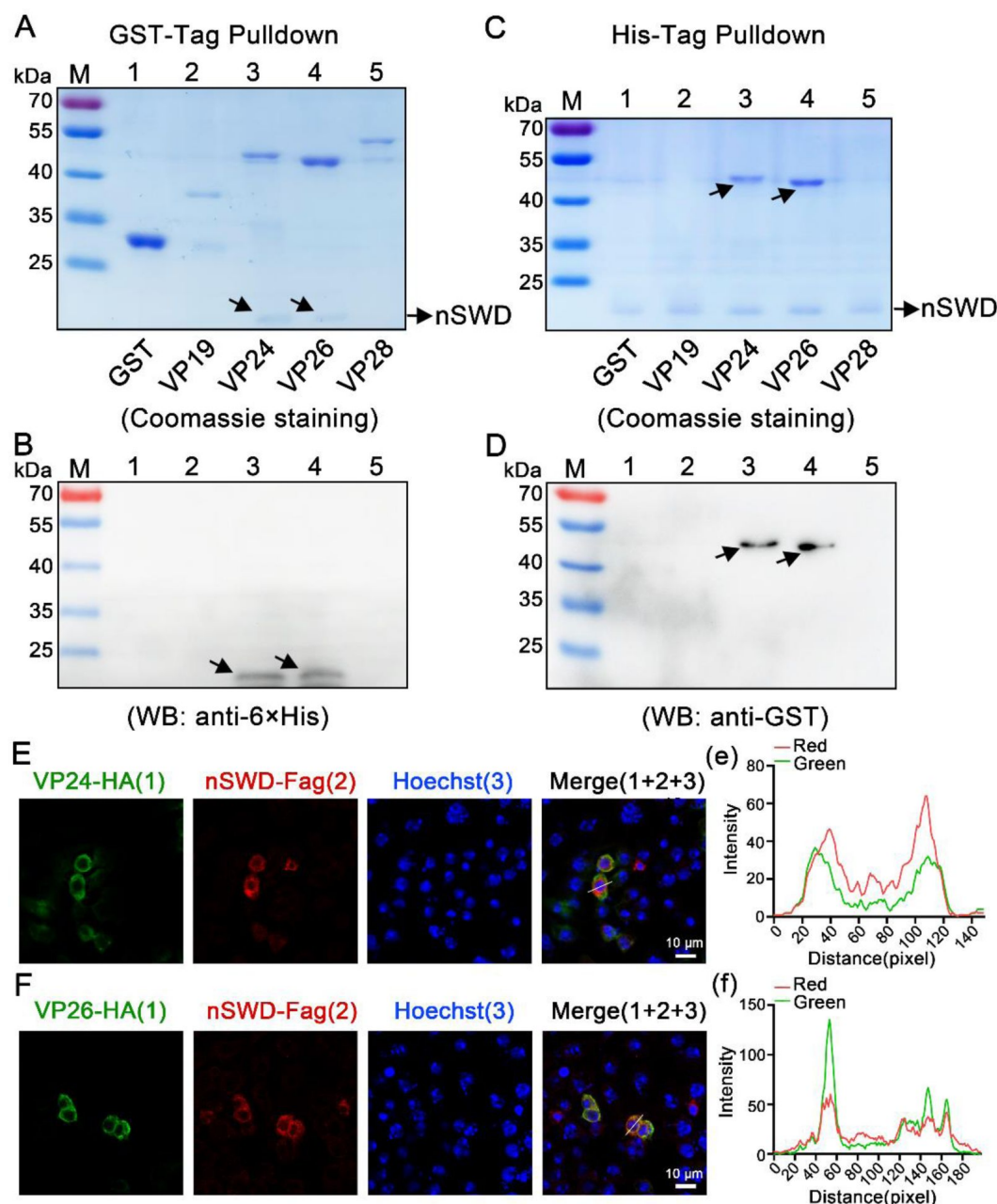


Figure 7.

nSWD interacts with envelope proteins of WSSV.

(A, B) GST pull-down assay for the detection of the interaction between rnSWD with VP19, VP24, VP26, and VP28. The results were shown via staining with Coomassie blue (A) or Western blotting using 6x-His Ab (B). The GST-tag protein was used as a control. (C, D) His pull-down assay for the detection of the interaction of rnSWD with VP19, VP24, VP26, and VP28 via Coomassie blue staining (C) or Western blotting using the GST-tag Ab (D). The GST tag protein was used as a control. (E, F) The colocalization of nSWD with VP24 and VP26 in S2 cells, 24 hours post-plasmid transfection. VP24 and VP26 was detected with rabbit anti-HA antibodies and anti-rabbit Alexa Fluor 488, while nSWD was identified using anti-Flag antibodies and anti-mouse Alexa Fluor 594. DAPI staining highlighted the nuclei. The scale bar represents 10 μ m. (e-f) Quantitative analysis of fluorescence colocalization. Colocalization intensity was quantitatively analyzed, with complete colocalization indicated by overlapping peaks and maxima shifted by less than 20 nm. All experiments were representative of three biological replicates and yielded similar results.

DmAMPs (Attacin A, Cecropins A, Defensin, Metchnikowin, and Drosomycin) was explored in response to high temperature (30 °C) and DCV infection. The results showed that Atta, CecA, and Def were significantly upregulated 5.77-fold, 4.55-fold, and 5.40-fold at high temperature (30 °C), respectively (**Fig. 8B**). When stimulated by DCV, Atta, CecA, Def, Met, and Drs were remarkably increased 46.84-fold, 123.52-fold, 39.72-fold, 41.14-fold, and 241.40-fold, respectively (**Fig. 8C**). These results suggest that DmAMPs play a crucial role in anti-DCV defense at high temperature (30 °C).

Next, DmHSF1's role in *Drosophila* during DCV infection at high temperature (30 °C) was investigated. Using the RNAi strategy in S2 cells, it was observed that the transcription levels of DmHSF1 can be downregulated by dsRNA-DmHSF1 with or without DCV infection (**Fig. 8D**). After DmHSF1 knockdown, the mRNA and protein expression of DCV was significantly higher at 30 °C than the control group (**Fig. 8E** and **8e**). Additionally, Atta, CecA, and Def were significantly downregulated 0.65-fold, 0.43-fold, and 0.56-fold in DmHSF1 silenced, DCV infected S2 cells at high temperature (30 °C), respectively (**Fig. 8F**). Furthermore, S2 cells were transfected with pAc5.1a-DmHSF1-HA or pAc5.1a-HA plasmid (as control; **Supplementary Fig. 5A**). The results showed that the mRNA and protein expression of DCV was significantly lower at 30 °C than the control group (**Fig. 8G** and **8g**). Accordingly, Atta, CecA, and Def were significantly upregulated 4.38-fold, 1.89-fold, and 3.13-fold at high temperature (30 °C), respectively (**Fig. 8H**). These results show that high temperature (30 °C) and DCV could induce the expression of DmHSF1 and DmAMPs, with DmHSF1 playing crucial roles in defending against DCV at elevated temperatures.

To further verify whether DmHSF1 exerts its anti-DCV function by regulating DmAMPs at high temperature (30 °C), the regulatory roles of DmHSF1 on DmAMPs were investigated through ectopic expression of DmHSF1 and knockdown of DmAMPs during infection (Supplementary data). Firstly, the promoter region of DmAtta, DmCecA, and DmDef was cloned, each containing three conserved HSE motifs (**Fig. 8I**). Dual-luciferase reporter assays showed that the promoter of DmAtta, DmCecA, and DmDef could be upregulated 12.36-fold, 7.76-fold, and 2.71-fold by DmHSF1, respectively. The mutant HSE motifs promoter of DmAtta, DmCecA, and DmDef could not be regulated by DmHSF1 (**Fig. 8J**). Then, to further demonstrate that DmHSF1 exerts its anti-DCV function by regulating DmAMPs, DmAtta was chosen as a representative. After knockdown of DmAtta (**Supplementary Fig. 5B**), S2 cells were transfected with DmHSF1 and then infected with DCV. The results showed that the mRNA and protein expression of DCV was significantly lower at 30 °C than the control group (**Fig. 8K** and **8k**). These results suggest that DmHSF1 exerts its anti-DCV function by regulating DmAMPs at high temperature (30 °C).

3. Discussion

Temperature is a key factor regulating various biological activities, and for aquatic ectotherms, it significantly affects growth rates and is also a crucial determinant of viral disease outbreaks (Marcogliese, 2008). Accumulating evidence has indicated that increased temperature enhances immune competence, resistance to infection, and levels of immune mediators under febrile conditions (Evans et al., 2015; Hasday and Singh, 2000). High temperatures (32-33 °C) have been shown to inhibit the replication of WSSV and reduce mortality in WSSV-infected shrimp (Sun et al., 2014). Studies have reported that WSSV-infected shrimp reared at high temperatures (above 31 °C) exhibit increased total hemocyte count (THC), phenoloxidase (PO) activity, and apoptotic index (Granja et al., 2003; Wu et al., 2015). However, the mechanism contributing to enhanced pathogen resistance in shrimp at high temperatures remains unclear. In this study, our findings revealed the molecular mechanism through which the HSF-AMPs axis mediates host resistance to viruses induced by febrile temperature. Specifically, AMPs are a newly documented type of effector protein targeted by HSF that mediate the host antiviral response beyond the classical heat shock response.

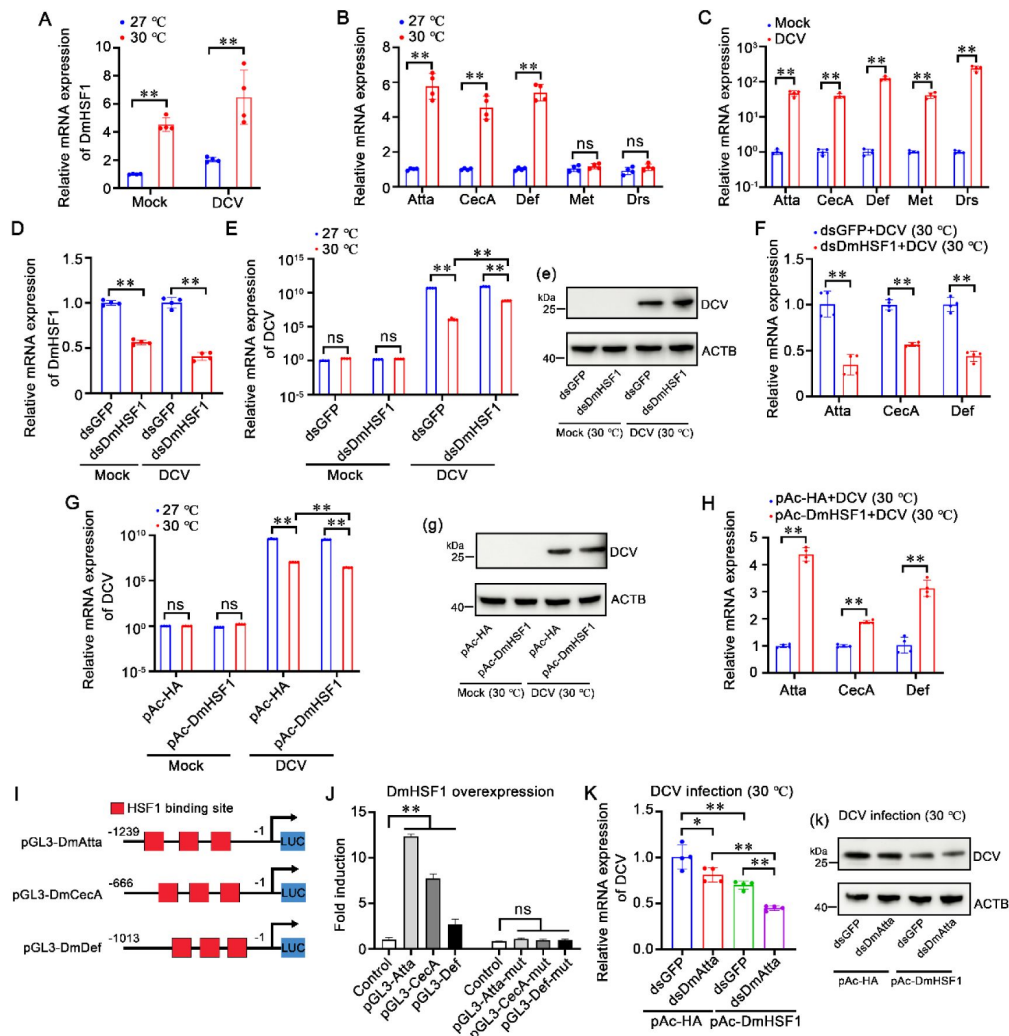


Figure 8.

High temperature-induced DmHSF1-AMPs axis restricts DCV replication in *Drosophila*.

(A) Transcriptional expression of DmHSF1. Transcriptional expression of DmHSF1 in S2 cells with or without DCV infection at low (27 °C) and high (30 °C) temperatures. **(B)** Transcriptional expression of DmAMPs. Transcriptional expression of DmAMPs in S2 cells at low (27 °C) and high (30 °C) temperatures. **(C)** Transcriptional expression of DmAMPs with or without DCV infection. Transcriptional expression of DmAMPs in S2 cells with or without DCV infection. **(D)** Silencing efficiency of DmHSF1. Silencing efficiency of DmHSF1 in S2 cells 24 hours after dsRNA application with or without DCV infection. **(E)** mRNA expression of DCV. mRNA expression of DCV at low (27 °C) and high (30 °C) temperatures after DmHSF1 knockdown, with dsGFP used as a control. **(e)** Protein expression of DCV at low (27 °C) and high (30 °C) temperatures after DmHSF1 knockdown. **(F)** mRNA expression of DmAMPs. mRNA expression of DmAMPs at low (27 °C) and high (30 °C) temperatures after DmHSF1 knockdown and DCV infection. **(G)** mRNA expression of DCV after DmHSF1 overexpression. mRNA expression of DCV at low (27 °C) and high (30 °C) temperatures after DmHSF1 overexpression. **(g)** Protein expression of DCV at low (27 °C) and high (30 °C) temperatures after DmHSF1 overexpression. **(H)** mRNA expression of DmAMPs after DmHSF1 overexpression. mRNA expression of DmAMPs at low (27 °C) and high (30 °C) temperatures after DmHSF1 overexpression and DCV infection. **(I)** Schematic Diagram of DmAMPs Promoter Regions. Schematic diagram of the DmAMPs promoter regions in the luciferase reporter gene constructs. The HSF1 binding motif sites are shown in red rectangles. **(J)** Dual-luciferase reporter assays. Dual-luciferase reporter assays were performed to analyze the effects of DmHSF1 overexpression on the promoter activities of DmAMPs with or without mutated HSF1 binding motifs. **(K)** mRNA expression of DCV following DmHSF1 overexpression post-DmAtta knockdown. mRNA expression of DCV at high (30 °C) temperatures following DmHSF1 overexpression post-DmAtta knockdown. **(k)** Protein expression of DCV at high (30 °C) temperatures following DmHSF1 overexpression post-DmAtta knockdown. Statistical significance was calculated using the Student's *t*-test (** $p < 0.01$, * $p < 0.05$). All experiments were conducted with three biological replicates, consistently yielding similar results.

Regulating body temperature, particularly increasing it (fever), is one of the primary defense mechanisms in homeotherms against infections (Hasday et al., 2014). Ectotherms as diverse as reptiles, fish, insects, and crustaceans also raise their core temperature during infection through behavioral regulation, seeking warmer environments—a phenomenon known as behavioral fever (Hasday et al., 2014). This feature, preserved in endothermic mammals and birds, strongly suggests that febrile temperatures confer a survival advantage (Bicego et al., 2007). In 1975, Kluger and colleagues published the first direct experimental study demonstrating the benefits of elevated temperature for infected animals (Kluger et al., 1975). In this study, *dipsosaurus dorsalis*, being poikilothermic animals, were moved from an environment of 36 °C to 40 °C after bacterial infection, resulting in an increase in survival rates from 25% to 65% (Kluger et al., 1975). Similar results have since been observed in other poikilotherms and some homeotherms. For instance, carp infected with Cyprinid herpesvirus 3 (CyHV-3) move from lower temperatures of 24–28 °C to 32 °C, where their survival rate reaches 100%, and viral load is significantly reduced (Rakus et al., 2017). Behavioral fever responses to microbial infections have also been reported in aquatic invertebrates such as freshwater crayfish (*Cambarus bartoni*), American lobster (*Homarus americanus*), pink shrimp (*Penaeus duorarum*), horseshoe crab (*Limulus polyphemus*), and *Penaeus vannamei* (Casterlin and Reynolds, 1978; 1979; M.E. Casterlin, 1977; Rakhshaninejad et al., 2023). Despite these observations, the antimicrobial immune mechanisms induced by febrile temperatures remain unclear. The prevailing view that febrile temperatures benefit the host primarily suggests that febrile response enhances innate and adaptive immune mechanisms in homeotherms (Hasday et al., 2014). Our findings in the present study further support the enhancement of innate immune mechanisms by febrile temperature in ectotherms such as shrimp and *Drosophila*. Additionally, elevated temperatures can cause some pathogenic microorganisms to lose their optimal growth conditions. However, some microorganisms have evolved strategies to exploit fever to enhance their invasion and proliferation (Freitas Lione et al., 2010; Loh et al., 2013). Paradoxically, this further underscores the benefits of fever for the host, as effective immune strategies exert evolutionary pressure on pathogens, driving them to evolve counterstrategies to hijack or block the febrile response.

HSFs are a type of evolutionarily conserved transcription factors initially known for regulating the heat shock response (Ankar and Sistonen, 2011). However, accumulating evidence indicates multiple additional functions beyond the activation of HSPs, including roles in metabolism, immunity, and disease (Barna et al., 2018). HSF1 acts through a regulated upstream promoter element called the heat shock element (HSE). HSEs are highly conserved, including the inverted repeats of the pentameric sequence nGAAn (Amin et al., 1988). The type of HSEs found in the proximal promoter regions of HSP genes comprises at least three contiguous inverted repeats consisting of nTTCnnGAAnnTTCn (Xiao and Lis, 1988). The discovery of novel HSF1 target genes not involved in the heat shock response suggests that HSEs may be present in multiple genes beyond HSPs (Barna et al., 2018). For instance, HSF1 can bind to the promoters of CUP1, BTN2, SIS1, SGT2, and SSA3 genes, upregulating their transcriptional activity to regulate oxidative stress (Morano et al., 2012). HSF1 also regulates the hypothalamic-specific expression of the heat-inducible ion channel gene TRPV1, suggesting a protective effect during fever (Fan-xin et al., 2012). In the present study, we showed that a newly identified antimicrobial peptide, nSWD, is specifically regulated by LvHSF1 via two HSE motifs with the typical nGAAn sequence. Additionally, the promoters of DmAMPs, such as Atta, Ceca, and Def, also contain several HSE motifs and are regulated by DmHSF1. Previous studies showed that the upstream sequence of CrustinPm5 contains a putative promoter with an HSE motif, and the expression of CrustinPm5 is upregulated upon heat treatment. However, there is currently no evidence that HSF1 directly binds to the HSE motif of AMPs in invertebrates. Notably, our work is the first to show that HSF1 directly binds to the HSE motifs of AMPs in shrimp and *Drosophila*, suggesting this may be a conserved regulatory mechanism in arthropods.

Although febrile temperature is generally believed to enhance host immunity, the role of heat shock factors (HSFs) in viral infections is complex and varies across different species and types of viral infections (Reyes et al., 2022 [DOI](#)). In vertebrates, HSF1 has been reported to participate in the transcription of HIV genes and their reactivation from latency (Pan et al., 2016 [DOI](#)). Additionally, after orthopoxvirus infection, HSF1 is phosphorylated, translocated into the nucleus, and increases the transcription of HSF1 target genes, benefiting the virus by extending the viral genome (Filone et al., 2014 [DOI](#)). Conversely, stress-independent activation of HSF1 can reduce the quantity and infectivity of HIV copies in a lymphoblastic cell line (Nekongo et al., 2020 [DOI](#)). Another study reported that HSF1 could enable apoptosis-stimulating protein to reduce autophagy in hepatocytes and inhibit HBV replication (Wang et al., 2021 [DOI](#)). In invertebrates, DmHSF1-deficient adult flies are hypersensitive to DCV infection, although the underlying mechanism has not yet been uncovered (Merkling et al., 2015 [DOI](#)). HSF1 is also required for tolerance against several pathogenic agents in *Caenorhabditis elegans* (Singh and Aballay, 2006 [DOI](#)). Similarly, the present study showed that HSF1 plays a crucial role in antiviral immunity against WSSV infection, with knockdown of HSF1 *in vivo* leading to higher virus copies and increased susceptibility to WSSV infection at high (32 °C) temperature. Notably, a recent study showed that ammonia stress-induced MjHSF1 upregulates the expression of negative regulators that inhibit the production and function of interferon analogs, enhancing WSSV infection in shrimp *Marsupenaeus japonicus* (Wang et al., 2024 [DOI](#)). This may be because ammonia stress suppresses host immunity and increases sensitivity to pathogenic infection in aquaculture (Weihrauch et al., 2018 [DOI](#)). In fact, previous studies have shown that the optimal water temperature for *M. japonicus* growth ranges from 25 to 32 °C, and a high temperature of 31 °C can reduce mortality of WSSV-infected *M. japonicus* without increasing host immune response activity (You et al., 2010 [DOI](#)). In contrast, the tolerance temperature for *L. vannamei* growth ranges from 7.5 to 42 °C, and our findings showed that elevated temperature can effectively enhance the host's response to WSSV infection (Millard et al., 2021 [DOI](#)). Evidence also supports the involvement of heat shock proteins in WSSV tolerance at elevated temperatures, with heat shock protein 70 (HSP70) playing a crucial role in inhibiting WSSV replication at high temperatures (32 °C) (Lin et al., 2011 [DOI](#); Sun et al., 2014 [DOI](#)). These findings suggest that the mechanism of elevated temperature protection against WSSV infection differs between *M. japonicus* and *L. vannamei*. Therefore, our findings, together with those of other studies, suggest that the benefit of HSF1 can be attributed to either the host or the pathogen, depending on the nature and context of the host-virus-environment interaction.

Antimicrobial peptides (AMPs) are pivotal components of innate immunity in invertebrates, serving as the first line of defense against invading pathogens, including bacteria, fungi, and viruses (Brogden, 2005 [DOI](#)). Typically, the transcription of AMPs is regulated by the classical NF- κ B and JAK/STAT pathways in invertebrates (Carpenter and O'Neill, 2024 [DOI](#)). However, our results revealed that AMPs can also be regulated by an alternative pathway mediated by HSF1 in shrimp and *Drosophila*. In crustaceans, various AMP families, including penaeidins, anti-lipopolysaccharide factors, crustins, and stylicins, have been identified (Matos and Rosa, 2022 [DOI](#)). Among these, the newly identified nSWD belongs to type III crustins, characterized by a single WAP domain (SWD) and a short N-terminal region. The most probable anti-WSSV mechanism of nSWD is its direct interaction with WSSV envelope proteins VP24 and VP26, potentially inhibiting viral entry into target cells. VP24 has been identified as a WSSV receptor-binding protein, interacting with the cellular receptor pIgR to mediate WSSV entry into host cells (Niu et al., 2019 [DOI](#)). VP26 has been reported to bind to SNAP29, inhibiting SNARE complex assembly and autophagic degradation (Liu et al., 2024 [DOI](#)). Additionally, WSSV VP24, VP26, and VP28 form a complex termed an “infectosome”, which is crucial for WSSV infectivity (Li et al., 2015 [DOI](#)). Therefore, the binding of nSWD to WSSV envelope proteins VP24 and VP26 could disrupt WSSV integrity and attenuate viral entry into shrimp cells.

In conclusion, our study demonstrates a conserved antiviral febrile response, where the elevated temperature-induced HSF1-AMP axis plays a crucial role in enhancing host resistance to viruses in shrimp and *Drosophila* (Fig. 9 [DOI](#)). Specifically, febrile temperature-induced HSF1 induces the

expression of AMPs, which directly bind to WSSV envelope proteins and inhibit WSSV proliferation. Additionally, high temperature restricts DCV replication in the invertebrate model system *Drosophila*, also mediated by the HSF1-AMPs cascade. These findings highlight previously unforeseen roles of HSF1 beyond the traditional heat shock response, revealing its involvement in thermal regulation of immunity and the innate immune system's response to thermal stress. Moreover, this study provides new avenues for managing viral infections in arthropods, suggesting that environmental control can effectively enhance disease resistance.

4. Materials and methods

Animal and pathogens

Healthy specimens of *L. vannamei*, averaging 5 g each, were utilized for our experiments. These specific pathogen-free (SPF) shrimp were sourced from Hisenor Company's shrimp farm in Maoming, Guangdong Province, China. The shrimp were maintained in a recirculating water tank system filled with air-pumped seawater at a salinity of 25‰ and a constant temperature of 25 °C. They were fed thrice daily to satiation with a commercial shrimp diet provided by HAID Group, Guangzhou, China. The inoculum for the WSSV, Chinese strain (AF332093), was prepared following previously described methodologies (Li et al., 2018 [DOI](#)).

WSSV was extracted from the muscle tissue of WSSV-infected shrimp and subsequently stored at –80 °C. Prior to injection, the muscle tissue was homogenized to prepare the WSSV inoculum, containing approximately 1×10^5 copies in 50 µl of Phosphate-Buffered Saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). For the pathogenic challenge experiments, each shrimp was administered an intraperitoneal injection of 50 µl of the WSSV solution into the second abdominal segment. Injections were performed using a 1-ml syringe (Becton Dickinson, Shanghai, China).

Drosophila C Virus (DCV) were kindly provided by Professor Jiahuai Han's Lab. The DCV was amplified in the S2 cells, after 3 days of infection, culture medium was collected and centrifuged at 4 °C for 20 min (2,000 × g), then the supernatant was collected and aliquots were frozen at –80 °C.

Cells and cells assays

Drosophila S2 cells (ATCC CRL 1963) were cultured at 28 °C in Schneider's Insect Medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY).

To knockdown *DmHSF1* *in vitro*, 8×10^6 S2 were transfected with 15 µg dsRNA in a 6-well plate by using FuGENE® HD Transfection Reagent (Promega Shanghai, China), and transfected cells were incubated at 27 °C for 3 days before the transfection and DCV infection assays according to a previously published method (Yang et al., 2018 [DOI](#)).

For temperature assays, S2 cells were seeded into 6-well plate and then incubated at 27 °C or 30 °C for 24 h, the transcription of DCV and *DmHSF1* were detected by qPCR. Besides, the same numbers of dsRNA-treated cells were infected with DCV at a multiplicity of infection (MOI) of 10 in a 6-well plate at 27 °C or 30 °C for 24 h and replaced with fresh medium, and the total RNA of S2 cells were extracted for qPCR analysis.

Transcriptomic analysis

Two groups of *L. vannamei* shrimp, Group W and Group TW, each comprising 30 individuals, were initially cultured at 25 °C. For Group W, the shrimp were injected with WSSV and continuously maintained at 25 °C. In contrast, Group TW shrimp were also injected with WSSV, but the culture temperature was raised to 32 °C within 3 hours at 24 hours post-injection (hpi). At 12 hours post-

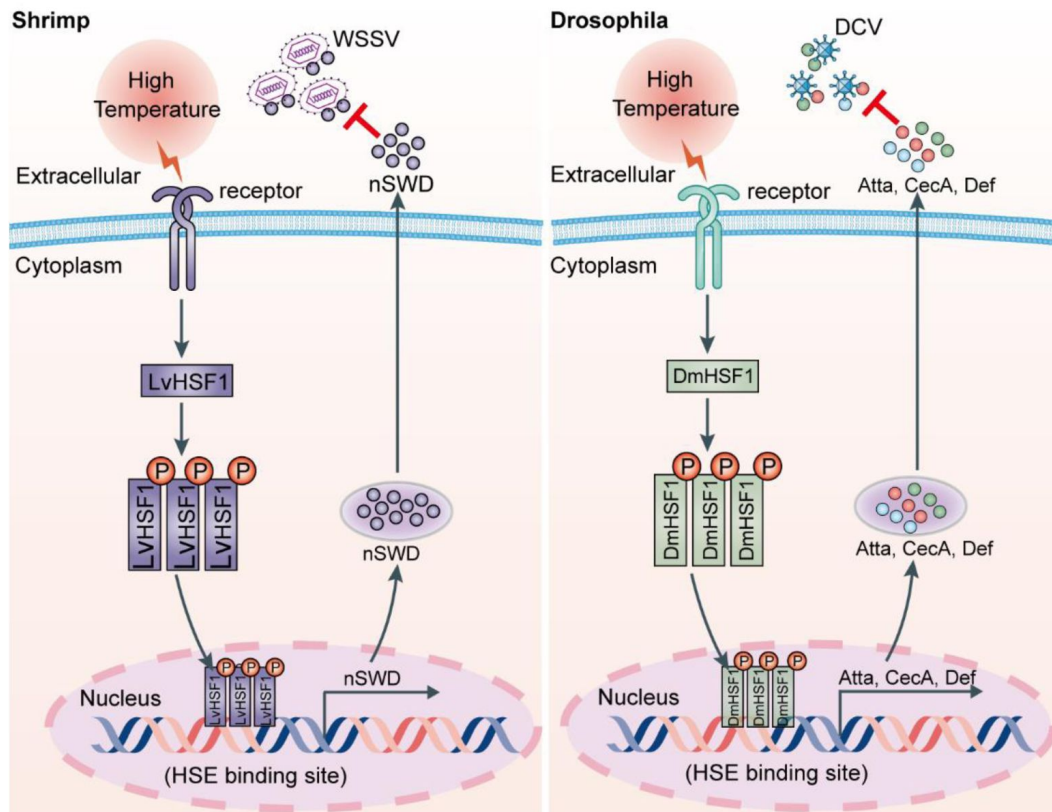


Figure 9.

Model for the HSF1-AMPs-mediated high temperature-induced resistance to viruses in arthropods.

Elevated temperature induces a robust expression of LvHSF1, which in turn specifically induces the expression of the antimicrobial peptide (nSWD) in shrimp. The nSWD directly binds to WSSV envelope proteins and inhibits WSSV replication (left panel). Additionally, elevated temperature induces the expression of DmHSF1, which upregulates the expression of Atta, CecA, and Def in *Drosophila*, subsequently restricting the replication of DCV (right panel). These findings highlight the roles of HSF1 beyond the classical heat shock response, mediating the thermal regulation of immunity and facilitating the innate immune system's response against viruses.

temperature raising (hprr), gill samples from 8 shrimp in both Group W and Group TW were collected. These samples were immediately preserved in liquid nitrogen for subsequent analysis. Transcriptomic sequencing was performed by LianChuan Biotech (Hangzhou, China). The FPKM value was used to represent gene expression levels in each sample. The differential expression between the two groups, each with three biological replicates, was determined using DESeq2 software, accepting criteria of $\log_2$ (Fold Change) ≥ 1 and an adjusted p-value ≤ 0.05 . Gene annotations were provided for the *L. vannamei* shrimp genome (Zhang et al., 2019 [DOI](#)). OmicShare tools (www.omicshare.com/tools [DOI](#)) was used to predict potential transcription factors and generate a heatmap.

To identify the genes whose expression was regulated by *LvHSF1*, healthy shrimp were administered an intramuscular injection of 10 μ g dsRNA targeting the *LvHSF1* gene or *GFP* as a control. After 48 hours, shrimp were injected with 1×10^5 copies of WSSV. At 24 hours post WSSV challenge, gill samples from 8 shrimp in both *dsHSF1* and *dsGFP* were collected. These samples were immediately preserved in liquid nitrogen for subsequent analysis. The sequencing and heatmap were performed similarly to the above procedure.

RNA extraction, cDNA synthesis, and DNA and protein extraction

Total RNA was extracted from different tissues of shrimp using the Eastep Super Total RNA Extraction Kit (Promega, Shanghai, China). The genomic DNA of shrimp tissues was extracted using a genomic DNA extraction kit (Vazyme, Nanjing, China). First-strand cDNA synthesis was performed using a cDNA synthesis kit (Accurate Biology, Hunan, China), according to the manufacturer's instructions. Protein samples from gills and hemocytes were homogenized separately in IP lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol; Thermo Scientific, Shanghai, China) with a protease and phosphatase inhibitor cocktail (Merck, Kenilworth, NJ, USA), and then centrifuged at $12,000 \times g$ for 10 minutes at 4 °C to collect the supernatant for further analysis.

Tissue expression and immune challenge analysis by quantitative real-time PCR (qPCR)

QPCR assays were performed to assess the mRNA levels in tissues expression distribution, immune stimulation or the *in vivo* RNAi experiments. Expression levels of *HSF1* (GenBank accession No. AH113794), *nSWD* (GenBank accession No. PP786678) and *VP28* (GenBank accession No. NP_477943.1) were detected using LightCycler480 System (Roche, Basel, Germany) in a final reaction volume of 10 μ l, which was comprised of 1 μ l of 1: 10 cDNA diluted with ddH₂O, 5 μ l of SYBR Green Pro Taq HS Mix (Accurate Biology, Hunan, China) and 250 nM of specific primers (Supplementary table 1).

For tissue distribution, shrimp were cultured at low temperature (25 °C) and high temperature (32 °C). Shrimp tissues of epithelium, hemocyte, stomach, nerves, eyestalk, gill, antenna, hepatopancreases, muscle, heart, intestine and pleopod were sampled and pooled from 15 shrimp. For immune stimulation, the treated group were injected with 5 μ g polyinosinic-polycytidylic acid (Poly (I: C)) or 50 μ l WSSV ($\sim 1 \times 10^5$ copies), and the control group was injected with PBS solution. Hemocyte, gill and intestine of challenged shrimps were collected at 0, 4, 8, 12, 24, 36, 48, and 72 h after injection. The cycling program were performed as described previously (Xiao et al., 2021 [DOI](#)). The expression of each gene was calculated using the Livak ($2^{-\Delta\Delta CT}$) method after normalization to *EF-1 α* (GenBank accession No. GU136229) and *β -Actin* (GenBank accession No. AF186250). Primer sequences are listed in Supplementary table 1.

The transcription of *DCV*, *DmHSF1* (GenBank accession No. NM_057227.5) and *DmAMPs* were also detect by quantitative real-time PCR. The expression of each gene was calculated using the Livak ($2^{-\Delta\Delta CT}$) method after normalization to *DmRpL32* (GenBank accession No. NP_733340.1). Primer sequences are listed in Supplementary table 1.

Detection of viral loads by absolute quantitative PCR

To measure viral titers in shrimp, absolute quantitative PCR (ab-qPCR) was employed. This process utilized a set of primers, wsv069 (WSSV32678-F/WSSV32753-R), targeting a single-copy gene of the WSSV, along with a Taq-Man fluorogenic probe (WSSV32706), as detailed in previous studies. Gill tissue samples were collected from WSSV-infected shrimp. DNA extraction from these samples was conducted using previously described methods (Fu et al., 2022 [DOI](#)). The concentration of WSSV genome copies was quantified by ab-qPCR, using the specific primers WSSV32678-F/WSSV32753-R and the TaqMan fluorogenic probe, as listed in Supplementary table 1. To ensure accuracy, each shrimp sample underwent three replicates of ab-qPCR. The number of WSSV genome copies was calculated and normalized against 0.1 µg of shrimp tissue DNA.

Recombinant protein expression and purification

The coding sequences of nSWD (without the signal peptide, 1-26 amino acids) was amplified by PCR using corresponding primers (Supplementary table 1) and subcloned into pET-32a (+) plasmid (Merck Millipore, Darmstadt, Germany). After confirmed by sequencing, the recombinant plasmid was transferred into *E. coli* Rosetta (DE3) cells (TransGen Biotech, Beijing, China). Then, positive clones harboring the desired fragment were selected for inducing expression. After 4 hours of induction with 0.1 mM Isopropyl β-D-Thiogalactoside (IPTG) at 30 °C, cells were pelleted by centrifugation and sonicated for 30 minutes on ice water. The supernatant from the sonicated proteins was purified by using Ni-NTA agarose (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. The rTrx-His-tag, GST and HSF1-GST proteins were induced and purified in the same way. The purified proteins were checked by Coomassie staining or Western blot analysis. The concentration of the purified proteins was determined using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China).

SDS-PAGE and western blotting

Western blotting was performed to evaluate the protein levels of LvHSF1, VP28, DCV, the purified nSWD, rTrx-His-tag and LvHSF1-GST. And then the proteins were separated on 12.5% SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). After blocking with 5% nonfat milk diluted in Tris Buffered Saline with Tween 20 (TBST) buffer (150 mM NaCl, 0.1% Tween-20, 50 mM Tris-HCl, pH 8.0) for 1 hour, and the membrane was incubated with 1: 1000 polyclonal rabbit anti-LvHSF1 (produced in Gene Create, Wuhan, China), 1: 1000 polyclonal rabbit anti-VP28 (produced in Abmart, Shanghai, China), 1: 1000 polyclonal rabbit anti-DCV capsid polypeptide (Abcam, Cambridge, UK), 1:1000 mouse anti-6 × His (Sigma, St Louis, MO, USA) or 1:1000 mouse anti-GST (TransGen Biotech, Beijing, China) for 2 hours at 25 °C. The PVDF membranes were washed for three times with TBST and then incubated with 1:5000 goat anti-rabbit IgG (H+ L) HRP or 1:5000 goat anti-mouse IgG (H+ L) HRP secondary antibody (Promega, Shanghai, China) for 1 hour. Membranes were developed using an enhanced chemiluminescent blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA), and the chemiluminescent signal was detected using the 5200 Chemiluminescence Imaging System (Thermo Fisher Scientific, Waltham, MA, USA).

Knockdown of specific genes by RNA interference (RNAi)

The T7 RiboMAX Express RNAi System kit (Promega, Shanghai, China) was employed for the synthesis of double-stranded RNA (dsRNA) targeting *HSF1*, *nSWD* and *GFP*. The specific primers used for dsRNA synthesis are listed in Supplementary table 1. The integrity and quality of the synthesized dsRNA were verified using 1.5% agarose gel electrophoresis and quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Shanghai, China). For the experimental treatments, shrimp were injected with 2 µg/g of body weight of the respective dsRNAs, dissolved in 50 µl of PBS. Control groups received injections of GFP dsRNA and PBS. Hemocytes and gills were collected from the shrimp 48 hours post-dsRNA injection. Total RNA was extracted from the

collected tissue samples. The efficiency of RNAi was evaluated by qPCR using the corresponding primers for each gene. This assessment aimed to confirm the knockdown of the targeted genes in the experimental groups compared to the controls as described previously (Li et al., 2024 [DOI](#)).

Survival assays

Healthy shrimp were divided into two groups ($n = 30$ each) and received an intramuscular injection of 10 μg dsRNA solution (HSF1 dsRNA or GFP dsRNA diluted in PBS). After 48 hours post dsRNA injection, the shrimp in each group were injected again with 1×10^5 copies of WSSV particles diluted in 50 μl PBS. The first group was maintained at low temperature (25 $^{\circ}\text{C}$), the second at a high temperature (32 $^{\circ}\text{C}$). The survival rates of each group were recorded at 4-hour intervals.

To knockdown the expression of nSWD, healthy shrimp were administered an intramuscular injection of 10 μg dsRNA targeting the *nSWD* gene or PBS as a control. After 48 hours, shrimp were injected with 1×10^5 copies of WSSV, with a parallel group receiving a mock challenge with PBS ($n = 30$). To evaluate the effect of recombinant rnSWD on WSSV replication *in vivo*, rescue experiments were conducted. Shrimp ($n = 30$) were co-injected with 10 μg rnSWD or rTRX along with the WSSV inoculum. The survival rates of each group were recorded at 4-hour intervals. Differences in survival between groups were analyzed using the Mantel-Cox (log-rank χ^2 test) method, employing GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Dual-luciferase reporter assays

The partial promoter sequence with 2000 bp upstream of nSWD was cloned using the specific primers (Supplementary table 1) and then linked into pGL3-Basic (Promega, Shanghai, China) to generate pGL3-nSWD (reporter plasmid). The vector pGL3-nSWD-M1, pGL3-nSWD-M2 and pGL3-nSWD-M12 with mutant of the HSF1 binding motif were also generated, respectively. *Drosophila* S2 cell was used instead in order to detect the effects of HSF1 on the promoters of nSWD. For dual-luciferase reporter assays, S2 cells were plated into a 96-well plate, and 12 hours later, the cells of each well were transfected with 0.05 μg of firefly luciferase reporter gene plasmids, 0.001 μg pRL-TK renilla luciferase plasmid (Promega Shanghai, China), or 0.05 μg proteins expression plasmids or empty pAc5.1A plasmids (as controls) using the Fugene HD Transfection Reagent (Promega Shanghai, China) according to the user manual. Forty-eight hours post-transfection, the dual-luciferase reporter assays were performed in order to calculate the relative ratios of firefly and renilla luciferase activities using the Dual-Glo Luciferase Assay System kit (Promega Shanghai, China), according to the manufacturer's instructions. All experiments were repeated six times.

To explore the regulatory activity of DmHSF1 on the DmAMPs (Atta, CecA and Def), we constructed the vector pGL3-Atta, pGL3-CecA and pGL3-Def. And the vector pGL3-Atta, pGL3-CecA and pGL3-Def with mutant of the HSF1 binding motif were also generated, respectively. The dual-luciferase reporter assays were conducted in the same way.

EMSA assay

EMSA was performed using a Light Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA, USA) according to a previously published method (Wang et al., 2020 [DOI](#)). Briefly, the biotin-labeled or unbiotin-labeled probes were designed using the *HSF1* binding motif sequence (5'-CTATAGAACCATC-3' and 5'-GACTTTTCGAGGA-3'). The mutant probe was designed via mutating the HSF1 binding motif sequence. All of the probes were synthesized by Ruibo Technologies (Guangzhou, China), and sequences are listed in Supplementary table 1. In brief, the recombinant proteins (10 μg) of HSF1 were incubated with 20 fmol of the probes for the binding reactions. The reactions were separated on a 5% native PAGE gel, transferred to positively charged nylon

membranes (Roche, Germany), and cross-linked by UV light. Then the biotin-labeled DNA on the membrane was detected by chemiluminescence and developed on x-ray film, followed by enhanced chemiluminescence (ECL) visualization (Tanon, Shanghai, China).

Pull-down assays

Pull-down assays were performed to explore whether the recombinant nSWD could interact with the main envelope proteins of WSSV (VP19, VP24, VP26 and VP28). The recombinant GST-tagged VP19, VP24, VP26 and VP28 were obtained from our previous studies (Xiao et al., 2020 [\[1\]](#)). For GST pull-down assays, 5 µg nSWD was incubated with 5 µg of GST-tagged WSSV protein solution at 4 °C for 3 h by agitation. Subsequently, 20 µl of the GST resin were added and the agitation continued for another 2 h. The resin was washed four times with PBS. Finally, the resins were re-suspended in 50 µl of the SDS-PAGE sample buffer, boiled and analyzed by Western blot analysis using 6 × His antibody. For His pull-down assays, 5 µg nSWD was incubated with 5 µg of GST-tagged WSSV protein solution at 4 °C for 3 h by agitation. Subsequently, 20 µl of the Ni-NTA binding resin were added and the agitation continued for another 2 h. The resin was washed four times with PBS. Finally, the resins were re-suspended in 50 µl of the SDS-PAGE sample buffer, boiled and analyzed by Western blot analysis using GST-tag antibody.

Immunocytochemical staining

Immunocytochemical staining was used to observe the co-localization of nSWD and WSSV VP proteins. *Drosophila* S2 cells were seeded onto poly-L-lysine-treated glass cover slips in a 24-well plate with approximate 40% confluent. The S2 cells of each well were transfected with 0.5 µg pAc5.1-nSWD-Flag, pAc5.1-VP24-HA or pAc5.1-VP26-HA using the MIK-3000 Transfection Reagent (MIKX, Shenzhen, China). Thirty-six hours post plasmid transfection, cell culture medium was removed, and cells washed with PBS three times. Cells were then fixed with 4% paraformaldehyde at 25 °C for 15 min following with methanol permeabilization at - 20 °C for 10 min. After washing sides for three times, cells were blocked with 3% bovine serum albumin (dissolved in PBS) for 60 min at 25 °C and then incubated with rabbit anti-HA antibody (CST, 1:300 diluted in 2% BSA) and mouse anti-Flag antibody (CST, 1:300 diluted in 2% BSA). The slides were then washed with PBS three times and then incubated with anti-rabbit IgG (H+L) Alexa Fluor 488 (CST, 1:1000 diluted in 2% BSA) and anti-mouse IgG (H+L) Alexa Fluor 594 (CST, 1:1000 diluted in 2% BSA) for 60 min at 25 °C in the dark. The cell nuclei were stained with Hoechst (Beyotime, Shanghai, China; cat. no. C1002) for 5 min. Finally, fluorescence was visualized on a confocal laser scanning microscope (Leica TCS-SP8, Wetzlar, Germany).

Statistical analysis

All data were presented as means ± SD. Student's *t* test was used to calculate the comparisons between groups of numerical data. For survival rates, data were subjected to statistical analysis using GraphPad Prism software (Graphpad, San Diego, CA, USA) to generate the Kaplan–Meier plot (log-rank χ^2 test).

Data availability

All reagents and experimental data are available within transparent methods or from corresponding author upon reasonable request.

Acknowledgements

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Additional information

Declaration of interest statement

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

Chaozheng Li, Jianguo He and Bang Xiao conceived and designed the experiments. Bang Xiao, Shihan Chen, Yue Wang, Xuzheng Liao and Chaozheng Li performed the experiments and analyzed data. Bang Xiao, Shihan Chen, Yue Wang, Xuzheng Liao and Chaozheng Li wrote the draft manuscript. Chaozheng Li and Jianguo He acquired finding. Chaozheng Li was responsible for forming the hypothesis, project development, data coordination, and writing, finalizing, and submitting the manuscript. All authors discussed the results and approved the final version.

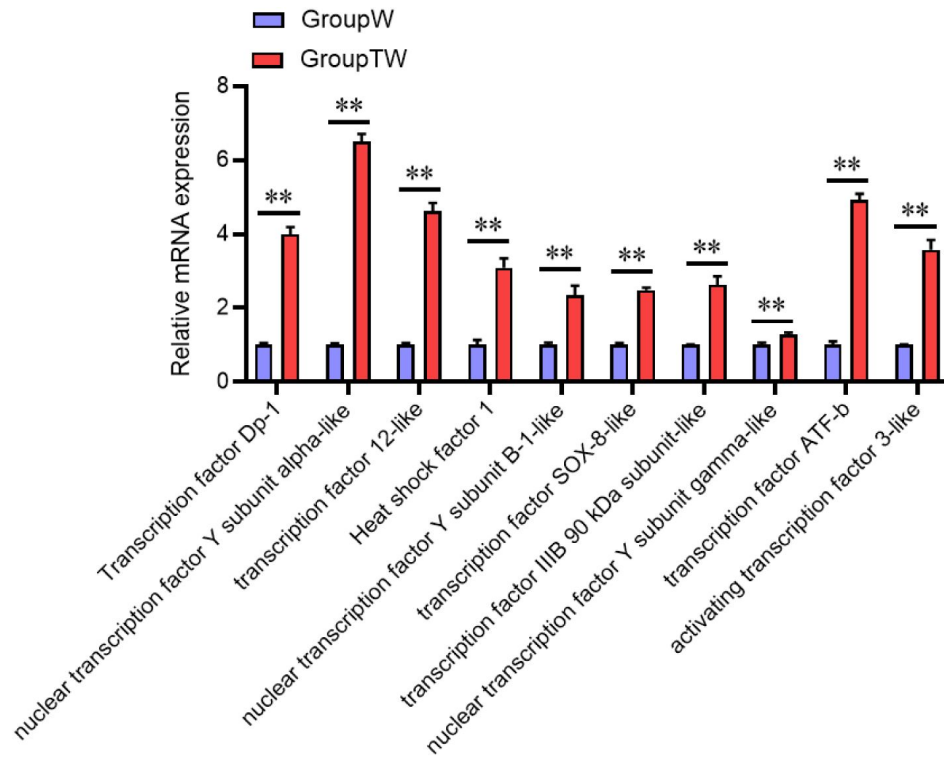
Ethics statement

All animal experiments were approved by Institutional Animal Care and Use Committee of Sun Yat-Sen University (Approval No. SYSU-IACUC-2023-B0005).

Supplementary Figures

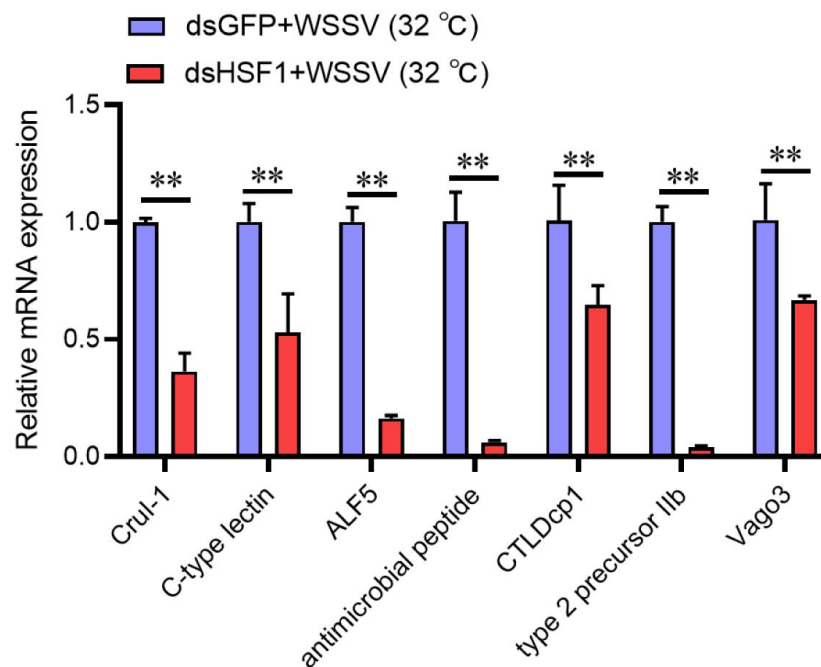
Supplementary Figure 1.

The expression of transcription factors as determined by qPCR.



Supplementary Figure 2.

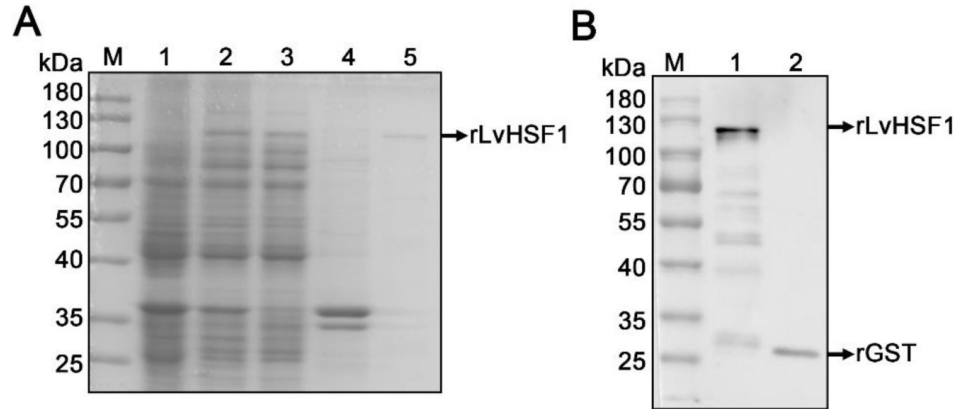
The expression of effector molecules as determined by qPCR.



Supplementary Figure 3.

SDS-PAGE and western blotting analyses of the recombinant LvHsf1 protein expressed in *E. coli*.

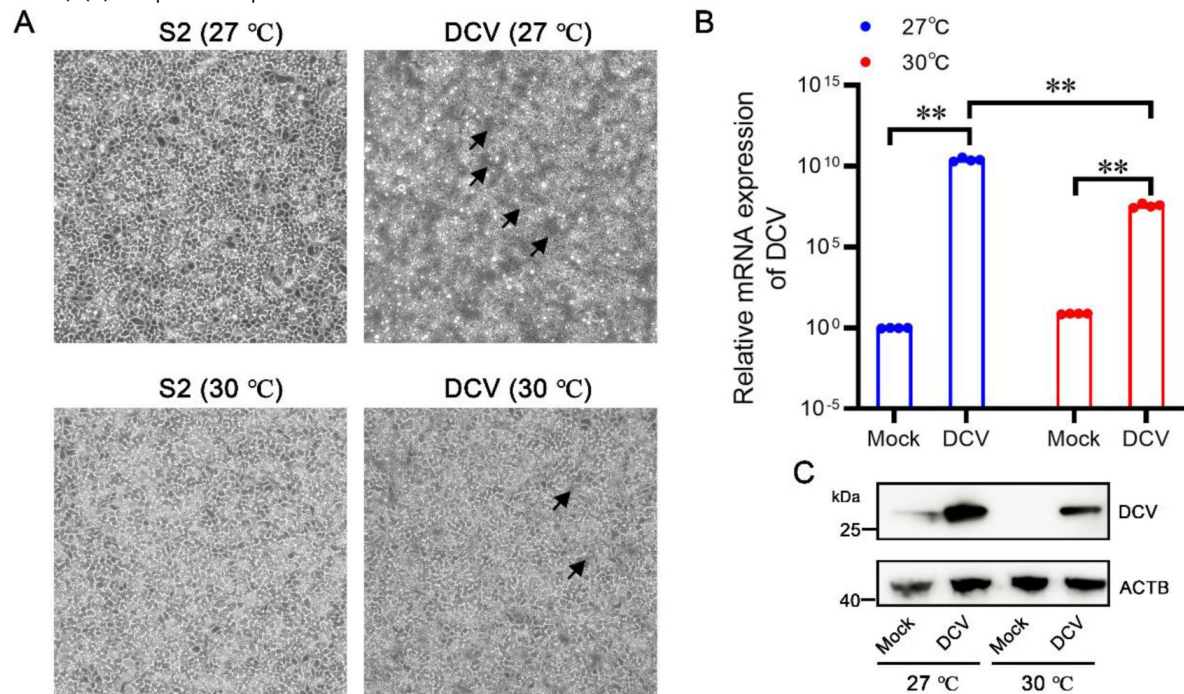
(A) SDS-PAGE analysis of the recombinant LvHsf1 protein. Line 1, uninduced *E. coli* transformed with LvHsf1; line 2, induced *E. coli* transformed with LvHsf1; line 3, supernatant of ultrasonic lysed *E. coli* expressing LvHsf1; line 4, precipitate of lysed *E. coli* expressing LvHsf1; line 5, purified recombinant LvHsf1 protein (black arrow). (B) Western blotting analysis of the recombinant LvHsf1 protein. Line 1, rLvHsf1-GST; Line 2, GST (black arrow). Purified proteins were checked by Western blotting with anti-GST.

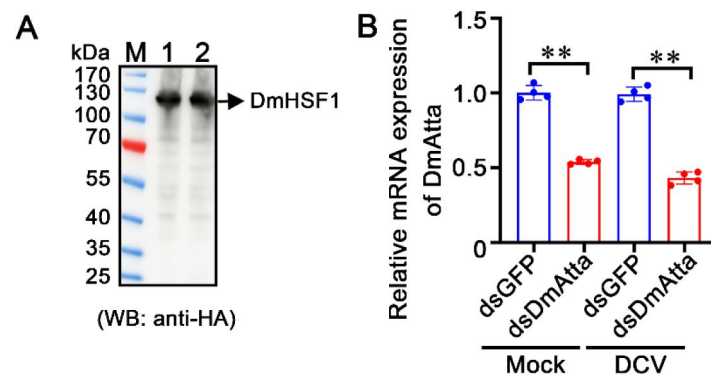


Supplementary Figure 4.

High temperature restricts DCV replication in *Drosophila* S2 cells.

(A) *Drosophila* S2 cells were seeded in 6-well plate and incubated at 27 °C or 30 °C for 24 h, then the cells were infected with DCV at a multiplicity of infection (MOI) of 10 at 27 °C or 30 °C for another 24 h. The cytopathic signs of S2 cells were observed in inverted fluorescence microscope. (B) The transcriptional expression of DCV in S2 cells with or without DCV infection (black arrow). (C) The protein expression of DCV in S2 cells with or without DCV infection.





Supplementary Figure 5.

Transfection in S2 cells.

(A) The protein expression level of DmHSF1 was detected by western blotting at 48 h post-transfection in S2 cells. (B) The silencing efficiency of DmAtta in S2 cells 24 h after dsRNA application with or without DCV infection.

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Editors

Reviewing Editor

Arjumand Ghazi

University of Pittsburgh School of Medicine, Pittsburgh, United States of America

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Satyajit Rath

Indian Institute of Science Education and Research (IISER), Pune, India

Reviewer #1 (Public review):

Summary:

In the manuscript "Heat Shock Factor Regulation of Antimicrobial Peptides Expression Suggests a Conserved Defense Mechanism Induced by Febrile Temperature in Arthropods," Xiao and colleagues examine the role of the shrimp *Litopenaeus vannamei* HSF1 ortholog (LvHSF1) in the response to viral infection. The authors provide compelling support for their conclusions that the activation of LvHSF1 limits viral load at high temperatures. Specifically, the authors convincingly show that (i) LvHSF1 mRNA and protein are induced in response to viral infection at high temperatures, (ii) increased LvHSF1 levels can directly induce the expression of the nSWD (and directly or indirectly other antibacterial peptides, AMPs), (ii) nSWD's antimicrobial activities can limit viral load, and, (iv) LvHSF1 protects survival at high temperatures following virus infection. These data thus provide a model by which an increase in HSF1 levels limits viral load through the transcription of antimicrobial peptides and provides a rationale for the febrile response as a conserved response to viral infection.

Strengths:

The large body of careful time series experiments, tissue profiling, and validation of RNA-seq data is convincing. Several experimental methodologies are used to support the authors' conclusions that nSWD is an LvHSF1 target and increased LvHSF1 alone can explain increased levels of nSWD. Similar carefully conducted experiments also conclusively implicate nSWD protein in limiting WSSV viral loads.

Weaknesses:

Despite this compelling data regarding the protective role of HSF1 in the febrile response, what remains unexplained and complicates the authors' model is the observation that losing LvHSF1 at 'normal' temperatures of 25C is not detrimental to survival, even though viral loads increase and nSWD is likely still subject to LvHSF1 regulation. These observations

suggest that WSSV infection may have other detrimental effects on the cell not reflected by viral load and that LvHSF1 may play additional roles in protecting the organism from these effects of WSSV infection, such as perhaps, perturbations to protein homeostasis. This is worth discussing, especially in light of the rather complicated roles of hormesis in protection from infection, the role of HSF1 in hormesis responses, and the findings from other groups that the authors discuss.

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Reviewer #2 (Public review):

Temperature is a critical factor affecting the progression of viral diseases in vertebrates and invertebrates. In the current study, the authors investigate mechanisms by which high temperatures promote anti-viral resistance in shrimp. They show that high temperatures induce HSF1 expression, which in turn upregulates AMPs. The AMPs target viral envelope proteins and inhibit viral infection/replication. The authors confirm this process in *Drosophila* and suggest that there may be a conserved mechanism of high-temperature mediated anti-viral response in arthropods. These findings will enhance our understanding of how high temperature improves resistance to viral infection in animals.

The conclusions of this paper are mostly well supported by data, but some aspects of data analysis need to be clarified and extended. Further investigation on how WSSV infection is affected by AMP would have strengthened the study.

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Reviewer #3 (Public review):

In the manuscript titled "Heat Shock Factor Regulation of Antimicrobial Peptides Expression Suggests a Conserved Defense Mechanism Induced by Febrile Temperature in Arthropods", the authors investigate the role of heat shock factor 1 (HSF1) in regulating antimicrobial peptides (AMPs) in response to viral infections, particularly focusing on febrile temperatures. Using shrimp (*Litopenaeus vannamei*) and *Drosophila* S2 cells as models, this study shows that HSF1 induces the expression of AMPs, which in turn inhibit viral replication, offering insights into how febrile temperatures enhance immune responses. The study demonstrates that HSF1 binds to heat shock elements (HSE) in AMPs, suggesting a conserved antiviral defense mechanism in arthropods. The findings are informative for understanding innate immunity against viral infections, particularly in aquaculture. However the logical flow of the paper can be improved.

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