

RESEARCH ARTICLE

Studies on Overwintering Behavior and Cold Stress Related Unigenes of *Wohlfahrtia magnifica*

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Abstract

The overwintering behavior and unigenes related to cold stress were studied in this paper. The pupae of *Wohlfahrtia magnifica* were placed at room temperature, 4°C, -5°C, -10°C, -15°C and -24°C respectively, and the recovery experiment after low temperature induction was carried out. The hatching of the pupae was counted, the shell interior pupae at -5°C, -10°C, -15°C and -24°C were photographed and recorded. Transcriptome sequencing was performed on the pupae at room temperature (PA), -5°C (PA1) and -10°C (PA2). The results were analyzed by Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), and the HSP67Bc, HSP23, HSP27, HSC70-4 and HSP70Ba were verified by Q-PCR. The results showed that the expression level of heat shock proteins (HSPs) in PA1 and PA2 were significantly higher than in PA, and HSP23, HSP27, HSP67Bc, HSP70Ba, HSP60, HSP83 and heat shock protein homologous (HSCs) such as HSC70-4, HSC7-5 were highly expressed in the pupae under low temperature stress. 13168 and 11161 entries were annotated in GO and KEGG, respectively. Q-PCR result showed that except HSP67Bc, the analysis results of the other four unigenes were consistent with the data of transcriptome analysis. Therefore, the overwintering behavior of *Wohlfahrtia magnifica* was in the form of pupa. HSPs played an important role in the overwintering process of the *Wohlfahrtia magnifica* pupa.

Keywords: GO, HSPs, Low temperature stress, Transcriptome, *Wohlfahrtia magnifica*

Wohlfahrtia magnifica'nin Kışlama Davranışı ve Soğuk Stresiyle İlgili Unigenleri Üzerine Çalışmalar

Öz

Bu çalışmada, *Wohlfahrtia magnifica*'nin kışlama davranışı ve soğuk stresi ile ilgili unigenleri incelenmiştir. *Wohlfahrtia magnifica*'nin pupaları sırasıyla 4°C, -5°C, -10°C, -15°C ve -24°C'de oda sıcaklığına yerleştirildi ve düşük sıcaklık induksiyonundan sonra geri kazanım deneyi yapıldı. Pupalardan çıkan erişkin sinekler sayıldı, -5°C, -10°C, -15°C ve -24°C'deki kabuk iç pupalar fotoğraflandı ve kaydedildi. Pupalarda, oda sıcaklığında (PA), -5°C (PA1)'de ve -10°C (PA2)'de transkriptom sekanslama yapıldı. Sonuçlar, Gen ontolojisi (GO) ve Kyoto Gen ve Genom Ansiklopedisi (KEGG) ile analiz edildi ve HSP67Bc, HSP23, HSP27, HSC70-4 ve HSP70Ba, Q-PCR ile doğrulandı. Sonuçlar, PA1 ve PA2'deki ısı şoku proteinlerinin (HSP'ler) ekspresyon seviyelerinin PA'ya göre önemli ölçüde yüksek olduğunu ve HSP23, HSP27, HSP67Bc, HSP70Ba, HSP60, HSP83 ve HSC70-4 ve HSC7-5 gibi ısı şoku protein homologlarının (HSC'ler) düşük sıcaklık stresi altındaki pupalarda yüksek oranda eksprese edildiğini gösterdi. GO ve KEGG'de sırasıyla 13168 ve 11161 kayıtlarına açıklama yapıldı. Q-PCR sonucu, HSP67Bc dışındaki diğer dört unigenin analiz sonuçlarının, transkriptom analiz sonuçları ile tutarlı olduğu saptandı. Bu nedenle, *Wohlfahrtia magnifica*'nin kışlama davranışı pupa formunda olmuştur. HSP'ler, *Wohlfahrtia magnifica* pupalarının kışlama sürecinde önemli rol oynamıştır.

Anahtar sözcükler: GO, HSP, Düşük sıcaklık stresi, Transkriptom, *Wohlfahrtia magnifica*

INTRODUCTION

Wohlfahrtia magnifica is the main pathogen causing hemorrhagic trauma and natural openings of human and

animals. In the Mongolian plateau, *Wohlfahrtia magnifica* is the only pathogen causing Bactrian camel vaginal myiasis. The 1st, 2nd and 3rd instar larvae of *Wohlfahrtia magnifica* parasitize in the camel vaginal, causing vaginal

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diseases of Bactrian camel [1]. The annual vaginal myiasis of Bactrian camel incidence rate is 20%~30%, and the mortality rate is about 2% [2], it has caused great economic losses to local camel farming. It has been reported that *Wohlfahrtia magnifica* maggot may infect foot of diabetic patients causing muscle trauma and may also cause periungual myiasis in girls [3,4].

Chen et al. [5] and Reynolds et al. [6] compared brain proteome differences between diapause and non-diapause *musca domestica* Linnaeus pupae, and found high expression of HSPs in diapause, but phosphoenolpyruvate synthase, fatty acid-binding protein and endonuclease expression levels decreased.

Heat shock proteins (HSPs) are highly conserved heat stress proteins that widely exist in prokaryotes and eukaryotic cells. They can assist in protein refolding to maintain protein stability and cell integrity, and acting as essential regulators of diverse constitutive metabolic processes [7-9]. Many animals have HSPs, and insects produce HSPs to maintain cell homeostasis when they are stimulated by cold and heat, including non-ATP-dependent small heat shock proteins and large ATP-dependent heat shock proteins, such as HSP70, HSP90 and HSP60 [10]. Small HSPs are the most least conservative of all HSPs [11]. At present, more and more HSPs have been proved to be responsive to temperature stress, and the expressions of HSP70, HSP90 and HSP60 were increased after low-temperature induction of insects [12-15]. The expression of HSP26 in the brain of *Sarcophaga crassipalpis* was up-regulated after cold treatment [16].

Some freeze-tolerant insects produce antifreeze proteins (AFPs) for self-adaptation to resist cold. AFPs are specific proteins, carbohydrates and polypeptides produced by different organisms that enable cells to survive in subzero conditions [17]. It is the earliest biological antifreeze material found in polar fish [18], which mainly in hemolymph, intestinal juice and intracellular fluid. It can bind to the surface of ice crystals and inhibit the growth of ice crystals [19]. It has the function of thermal hysteresis, inhibiting the recrystallization of ice crystal and strengthening the interaction between cell membrane and membrane proteins [20]. AFPs exist in fish, insects, plants and microorganisms [21], and the antifreeze ability of arthropods is higher than that of fish. AFPs have potential application value in gene transformation and tumour freezing, and it can be used to produce economical and efficient fish, animals and plants in agriculture, and it also can be used to resist extreme low temperatures [22].

Different insects have different cold-resistant strategies. In behavior, some insects overwinter with one insect state pupae, others with multiple insect state; physiologically, they can improve their cold tolerance by reducing water content and storing more cold-resistant substance [23], such as the physiological mechanism of protecting diapause

larvae from dehydration may also increase their cold tolerance [24]. Although the overwinter morphology of insects is different, winter with pupae is the most secure and safest, which may be due to the metabolic inhibition of diapause pupae [25].

The overwintering behavior and unigenes related to cold stress were studied in this paper, further reveal the overwintering behavior and molecular mechanism of *Wohlfahrtia magnifica*.

MATERIAL AND METHODS

Ethical Statement

All experimental procedures were approved by the Animal Protection and Use Committee of Inner Mongolia Agricultural University and strictly followed animal welfare and ethical guidelines.

Studies on the Overwintering Behavior of *Wohlfahrtia magnifica*

The adult flies and each stage larvae of *Wohlfahrtia magnifica* were collected, and some of the 3rd instar larvae were put into culture medium to pupate. Putting the pupae, adult flies and remaining each stage larva into 4°C to observe. When their activity weakens to no activity, they were removed and returned to room temperature, then observed for their survival.

Emergence Rate of the Pupae of *Wohlfahrtia magnifica* at Different Ambient Temperature

Collecting the larvae of *Wohlfahrtia magnifica* and cultured them to mature the 3rd instar larvae, and then put them into culture medium to pupate. After 2~3 days, the pupae were cultured at room temperature, 4°C, -5°C, -10°C, -15°C and -24°C, and 80 pupae were used in each temperature gradient. Five groups of pupae except the room temperature group were slowly let hypothermia in refrigerator to induce low temperature stress. After falling to the target temperature, the pupae were kept for 5 days, then they were returned to room temperature and the hatching of them were counted. The shell interior morphology of the pupae was photographed and recorded by superview imager and anatomical microscope at target temperatures.

Studies on Transcriptome Sequencing of *Wohlfahrtia magnifica* at Room Temperature and Low Temperature Stress at -5°C and -10°C

The 3rd instar larvae of *Wohlfahrtia magnifica* were divided into three groups after pupation. One group was preserved at room temperature, and the others were kept at -5°C and -10°C for 5 days, respectively. Then the three group samples were put into liquid nitrogen quickly for transcriptome sequencing.

According to the reagent instruction, Trizo reagent (Invitrogen, CA, USA) was used to extract total RNA. The quantity and purity were analyzed by Bioanalyzer 2100 that RIN >7 and RNA 1000 Nano LabChip kits (Angelen, California, USA). Using magnetic beads that adhered to poly-T oligonucleotides, poly(A) RNA was purified from total RNA through two rounds of purification. After purification, the mRNA sections were transformed into small sections using bivalent cations at high temperature, and the lytic RNA sections were reverse transcribed according to the procedure of mRNAseq sample preparation kit (Illumina, San Diego, USA) to establish the final cDNA library. The average insert size of the match terminal library was 300bp (± 500 bp). Then the pairing end sequencing was performed on Illumina Hiseq4000 from LC Sciences in the USA according to the vendor recommended protocol.

Unigene Annotation and Functional Classification

First, entrails Cutadapt and perl libretto to delete reads that contain adapter pollution, inferior base pairs and indefinite base pairs, then FastQC to verify sequence quality, including Q20, Q30 and GC content of clean data were used. All downstream analyses were based on high-quality clean data. All the packaged unigenes were compared with SwissProt's non-redundant proteins and GO, KEGG and eggNOG used the data base of DIAMOND and threshold Evalue <0.00001.

Differential Unigene Expression Analysis

Salmon^[26] performed the expression level of unigenes by calculating TMP^[27]. The differential expression unigenes were selected by edgeR^[28], where $\log_2 > 1$ or $\log_2 < -1$, and the difference was statistically significant ($P < 0.05$). Then the differential expression unigenes were analyzed by GO and KEGG enrichment analysis by entrails perl libretto.

Statistical Analysis of HSP23, HSP27, HSP70Ba, HSP67Bc and HSC70-4 Unigenes Expression Under Different Temperature Stress

All the selected HSPs and their homologues were chosen, and four highly expressed and significantly different HSPs (HSP23, HSP27, HSP70Ba, HSP67Bc) and a HSP homologue unigene (HSC70-4) were chosen from the HSPs and their homologues, then they were verified by FQ-PCR.

RESULTS

Studies on the Overwintering Behavior of *Wohlfahrtia magnifica*

Through the experiment, it was found that the 1st instar larvae and the 2nd instar larvae were hardened and stiffen on the day when the temperature dropped to 4°C, and after recovered to room temperature, they could not survive. The 3rd instar larvae hardened and stiffened at 4°C after one and a half day and did not survive after recovery to room temperature. The adult flies showed no signs of life after surviving at 4°C for 4 days, and there was no resuscitation when they were recovered to room temperature. The pupae were placed at 4°C for 7 days and then restored at room temperature, it was observed that flies began to hatch on the 7th day after being taken out from 4°C. The results of the experiment showed that the pupa has the property of cold-tolerance ability.

Emergence Rate of the Pupae of *Wohlfahrtia magnifica* at Different Ambient Temperatures

The emergence rate at room temperature, 4°C, -5°C, -10°C, -15°C and -24°C are 75%, 50%, 48.75%, 47.5%, 3.75% and 0%, respectively (Table 1). The pupae peeled off their shell cuticle and found that interior morphology was intact after low temperature stress at -5°C (Fig. 1) and -10°C (Fig. 2). While at the temperature of -15°C, it was found that most of the pupae blackened and suppured (Fig. 3), a small part of the pupae developed to the third stage but failed to emerge from shell, and at the temperature of -24°C, all pupae wizened and died (Fig. 4). It can be concluded that the emergence rate of *Wohlfahrtia magnifica* is affected by ambient temperatures.

Studies on Transcriptome Sequencing of *Wohlfahrtia magnifica* at Room Temperature and Under Low Temperature Stress at -5°C and -10°C

- Analysis of Overall Unigenes Expression Level

In order to make the data more reliable, three biological replicates were used for transcriptome sequencing in each group (Fig. 5). It was found that the upper quartile, median and lower quartile of unigene expression level in each group were basically at the same level, and it shows

Table 1. Number of emergence and emergence rate of the pupa of *Wohlfahrtia magnifica* after different temperature stresses

Temperature (°C)	Number	Number of Emerge	Emergence Rate (%)
-24	80	0	0
-15	80	3	3.75
-10	80	38	47.50
-5	80	39	48.75
4	80	40	50.00
Room temperature	80	60	75.00

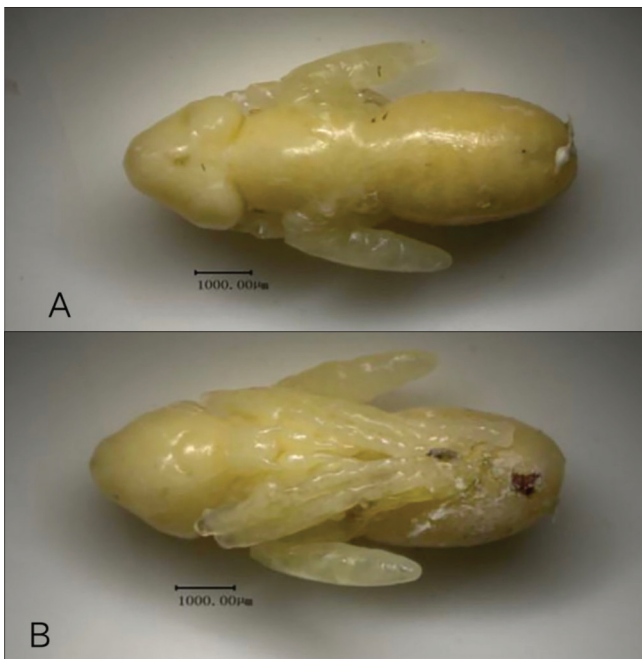


Fig 1. Morphology of the pupa after low temperature stress at -5°C in the shell (A, B)

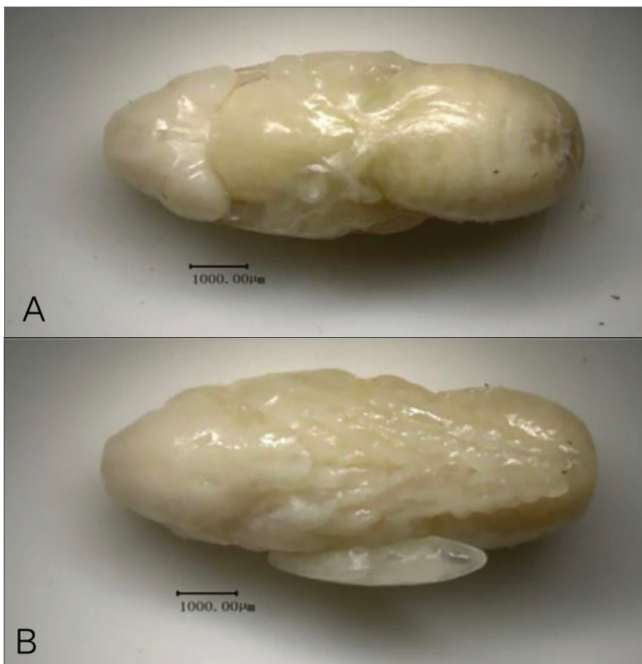


Fig 2. Morphology of the pupa after low temperature stress at -10°C in the shell (A, B)

that the repeatability of the sample is good and the next experimental analysis can be carried on.

Differential Unigene Expression Analysis

- Differential Unigene Expression

Differential unigene expression profile analysis showed that the transcripts were compared with each other (blue

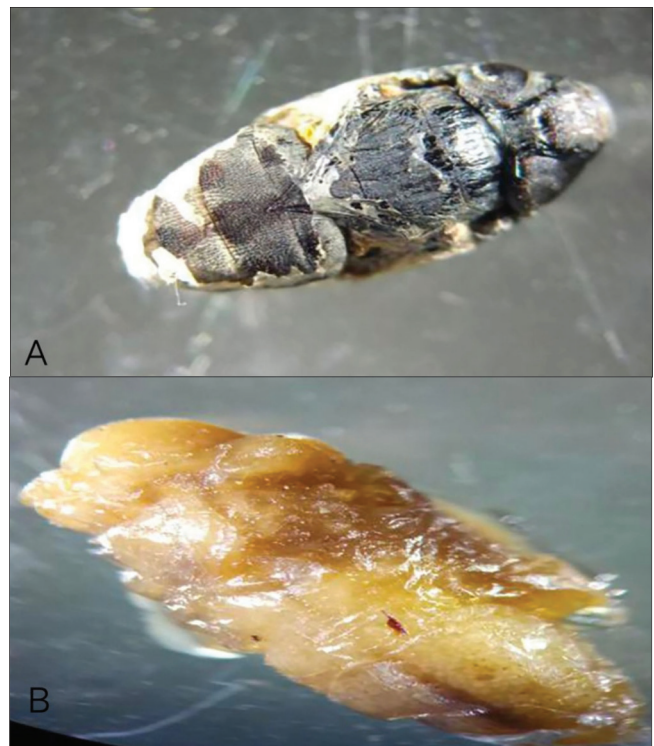


Fig 3. Morphology of the pupa after low temperature stress at -15°C in the shell (A, B)

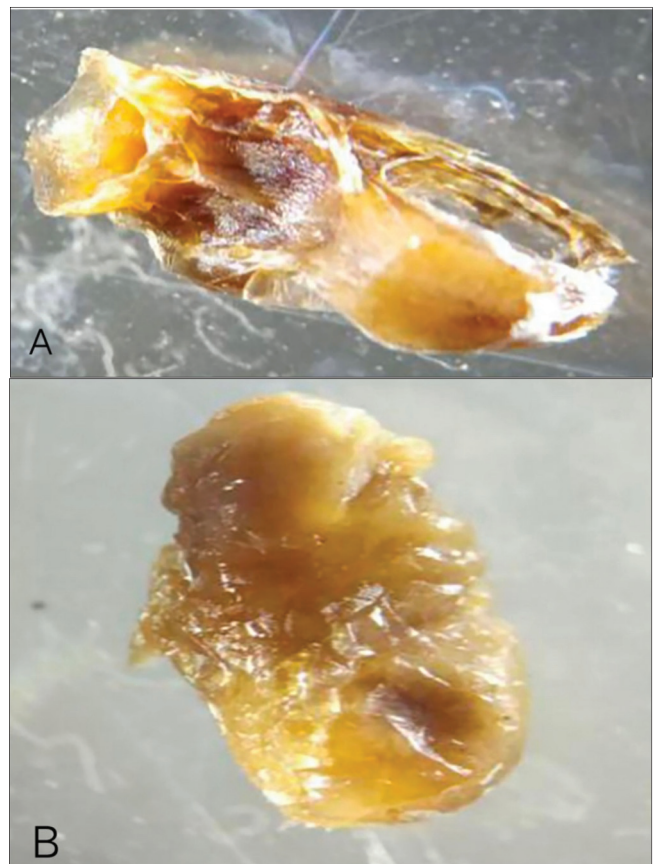


Fig 4. Morphology of pupa after low temperature stress at -24°C in the shell (A, B)

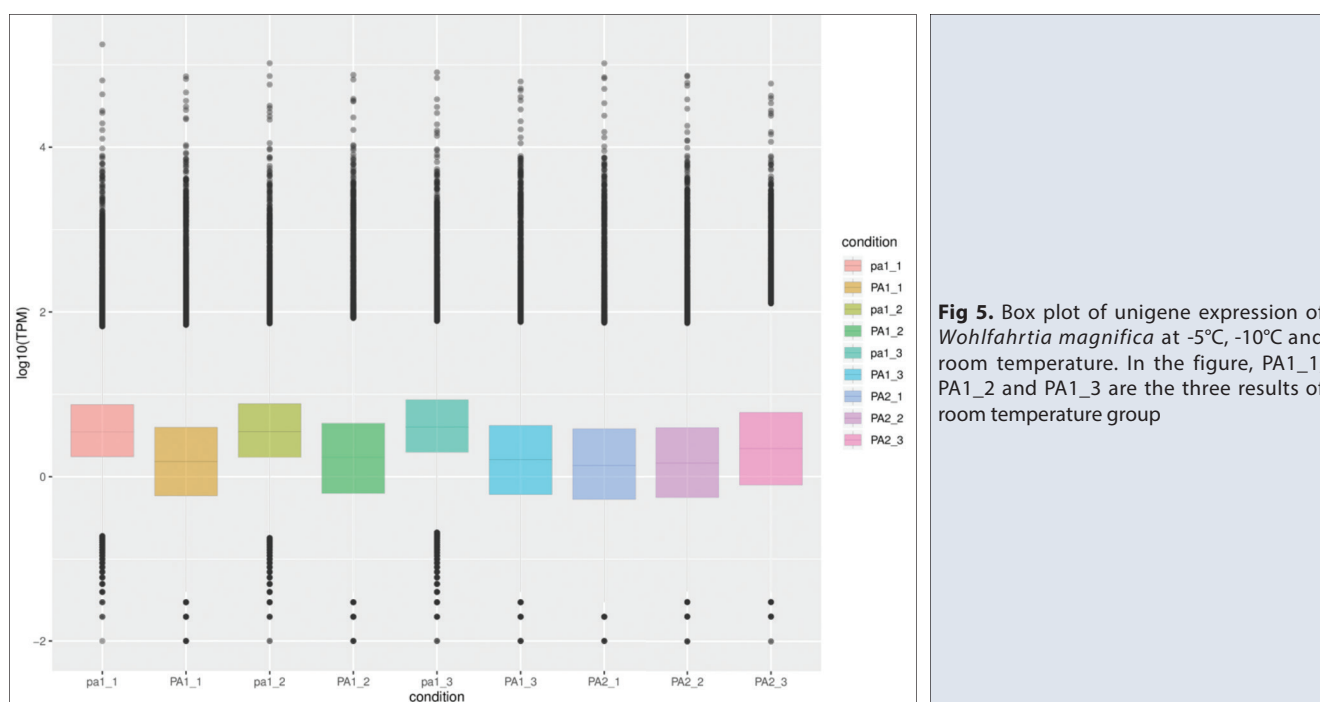


Fig 5. Box plot of unigene expression of *Wohlfahrtia magnifica* at -5°C , -10°C and room temperature. In the figure, PA1_1, PA1_2 and PA1_3 are the three results of room temperature group

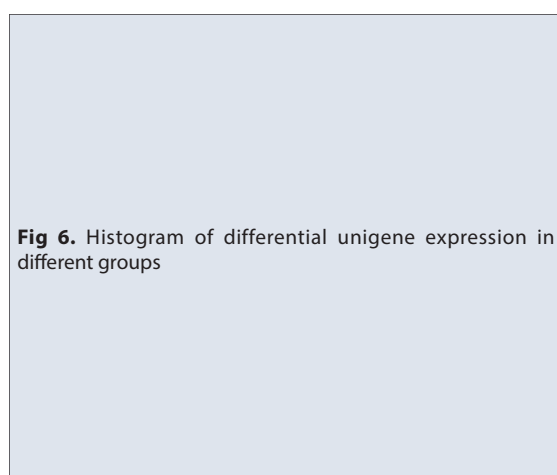
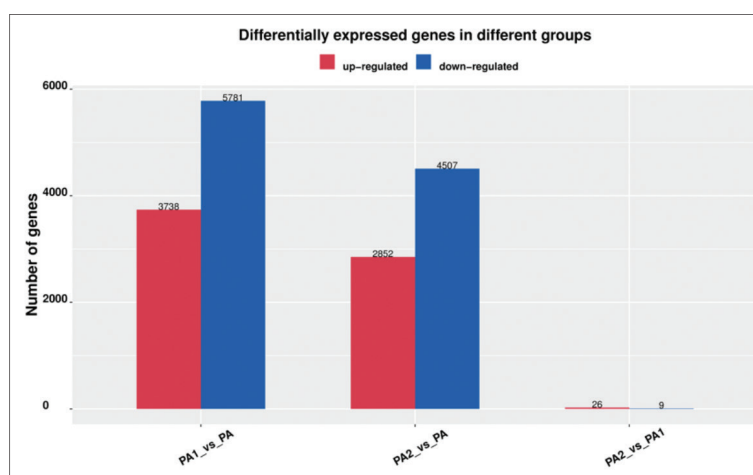


Fig 6. Histogram of differential unigene expression in different groups



column for down-regulation, red column for up-regulation (Fig. 6). Compared with PA (at room temperature), 9519 and 7359 unigenes were found to be significantly different in the PA1 and PA2 (at -5°C , -10°C), including 3738 and 2852 up-regulated unigenes, and 5781 and 4507 down-regulated unigenes, respectively; 35 unigenes difference were found between PA1 and PA2, of which 26 different unigenes were up-regulated and 9 different unigenes were down-regulated.

By processing the differential unigenes among PA, PA1 and PA2 groups and making the Venn diagram (Fig. 7), it was found that PA1, PA2 and PA contained 9159 and 7359 differential unigenes respectively, and PA1 and PA2 contained 35 unigenes. PA1 and PA2 contained 3026 and 872 unigenes separately from PA; PA1 and PA2 separately contained 7 unigenes; Together PA1_vs_PA with PA2_vs_PA contains 6469 unigenes; Together PA1_vs_PA with PA1_

vs_PA2 contains 24 unigenes, together PA2_vs_PA with PA1_vs_PA2 contains 18 unigenes and together PA1_vs_PA with PA2_vs_PA and PA1_vs_PA2 contains 7 unigenes.

- GO Enrichment Analysis of Differential Unigenes

PA1 and PA2 separately were enriched 286 and 223 unigenes in biological process, 678 and 452 unigenes in cytoplasm, 611 and 402 unigenes in nucleus, 419 and 314 unigenes in molecular function, and 386.258 unigenes in protein binding from PA (Fig. 8, Fig. 9).

- The Analysis of HSPs and HSCs Unigene

Through the GO enrichment, it was found that HSPs and HSCs were enriched in biological process, molecular function and cellular component respectively, in which HSPs participated in cold acclimation, cold translation, protein binding transport, ATP binding transport and other 177

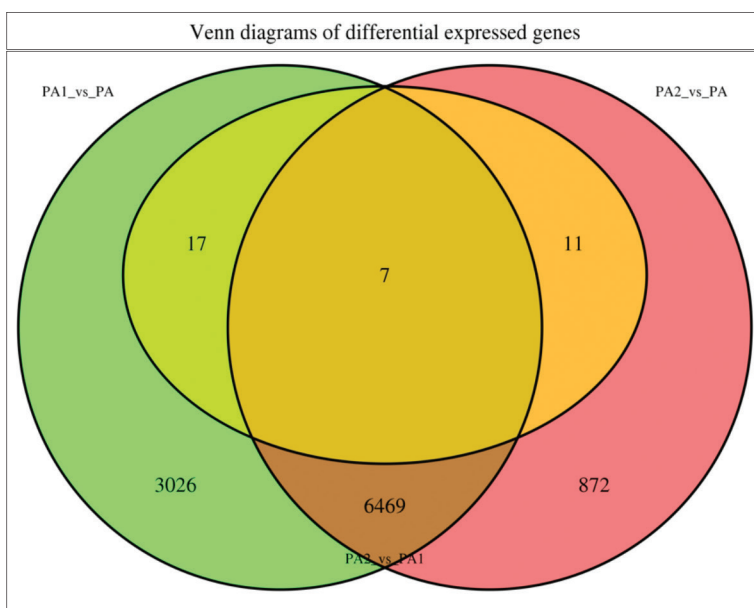
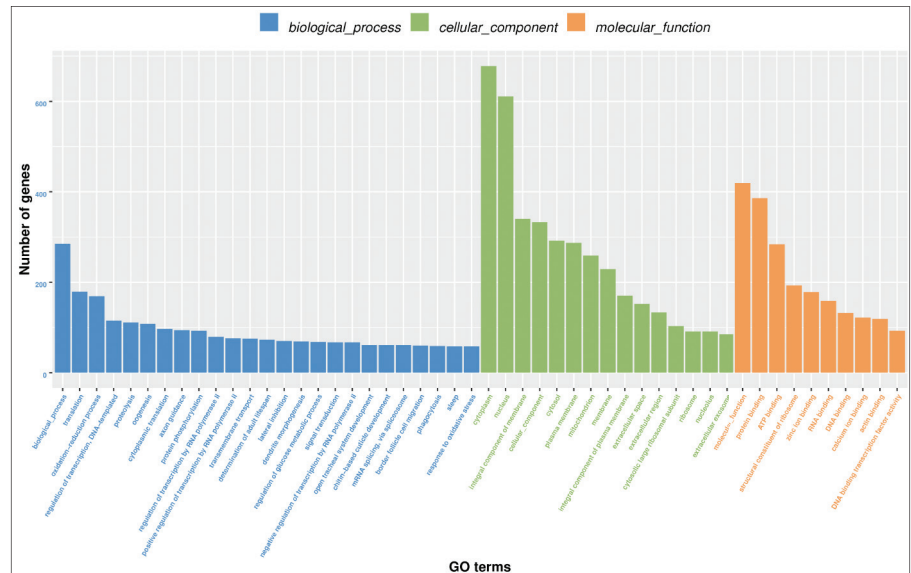


Fig 7. Venn diagram of differential unigene expression

Fig 8. GO enrichment map of PA1_vs_PA differential unigene



small items; while HSCs participated in 47 small items such as chaperones mediated protein folding, cell response to heat, protein renaturation and TAP binding.

The results of sequencing showed that the HSPs were highly expressed in pupae under low temperature stress, in which 4 unigenes of HSP23 and 1 unigene of HSP83 were involved in cold acclimation of pupae of *Wohlfahrtia magnifica*. Differential HSCs were found in both -5°C and -10°C treatment groups: HSC70, HSC70-1, HSC70-2, HSC70-3, HSC70-4, HSC70-5 and HSCB; of which HSC70 and HSC70-1 were down-regulated, the rests were up-regulated. The HSC70-4 and HSC70-5 with $P < 0.05$ and $\log_2FC > 2$ were chosen, and there were 52 and 58 differential HSPs in the -5°C and -10°C treatment groups compared with the room temperature group, respectively. Among them, 2 unigenes were down-regulated, 50 unigenes were up-regulated at -5°C; and 13 unigenes were down-regulated,

and 45 unigenes were up-regulated at -10°C. There were 22 unigenes among the HSPs which chosen for $\log_2FC > 2$ and $P < 0.05$ (Fig. 10, Fig. 11).

The HSP67Bc, HSP23, HSP27, HSC70-4 and HSP70Ba were selected, and the unigenes expression TMP of their transcriptome sequencing data were performed variance analysis. It was found that the unigene expression levels of HSP67Bc ($P=0.002$), HSP23 ($P=0.003$), HSP27 ($P=0.00$), HSC70-4 ($P=0.00$) and HSP70Ba ($P=0.00$) were significantly different between pupae under low temperature stress and pupae at room temperature (Fig. 12).

Statistical Analysis of HSP23, HSP27, HSP70Ba, HSP67Bc and HSC70-4 Unigenes Expression Under Different Temperature Stress

The mRNA treatment results of candidate unigenes under

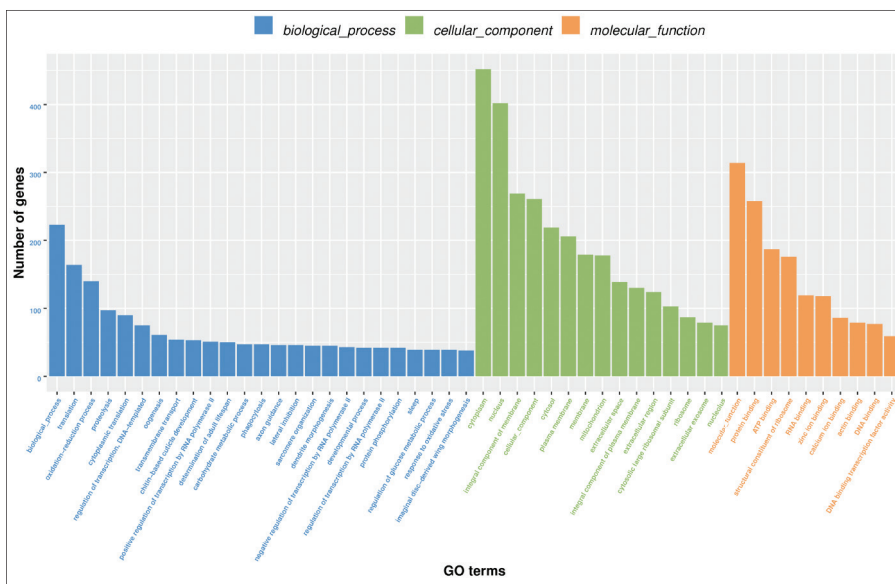


Fig 9. GO enrichment map of PA2_vs_PA differential unigene

Fig 10. Line graph of HSPs expression

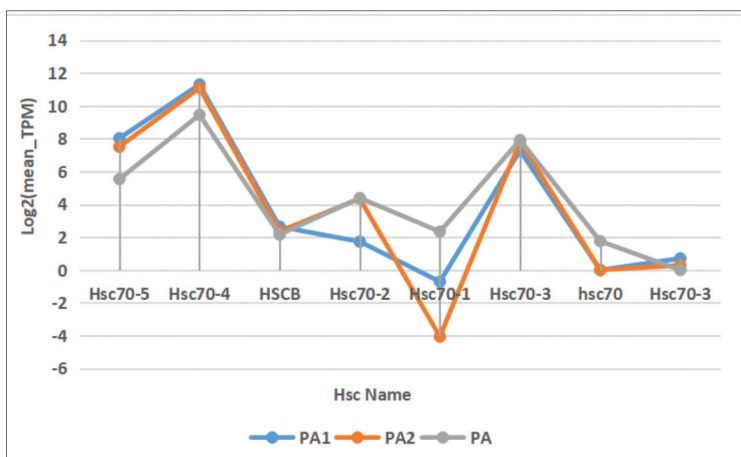
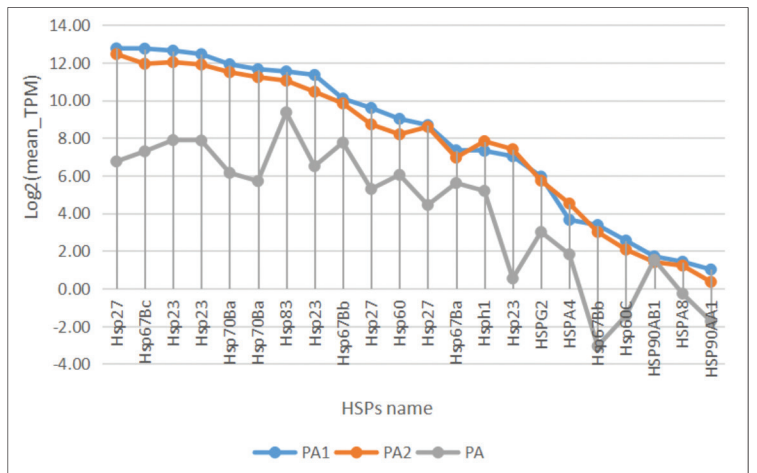


Fig 11. Line graph of HSCs expression. The two HSC70-3 in the figure represent two different sequences respectively

different treatment conditions were shown in Fig. 13. PASWStatistics18 was used for variance analysis of the relative expression levels of the same unigene under different temperature conditions, and Duncan's new multiple range test (MRT) was used for pairing test of mean difference. Through the analysis and detection of

the expression profiles of five HSPs of the screened cold-tolerance related unigenes of pupae at different temperatures, and the results showed the screened five HSPs were expressed to different degrees: The expression of HSP27 was significantly different ($P=0.009<0.01$) and the expression of HSP23 was significantly different ($P=0.037<0.05$)

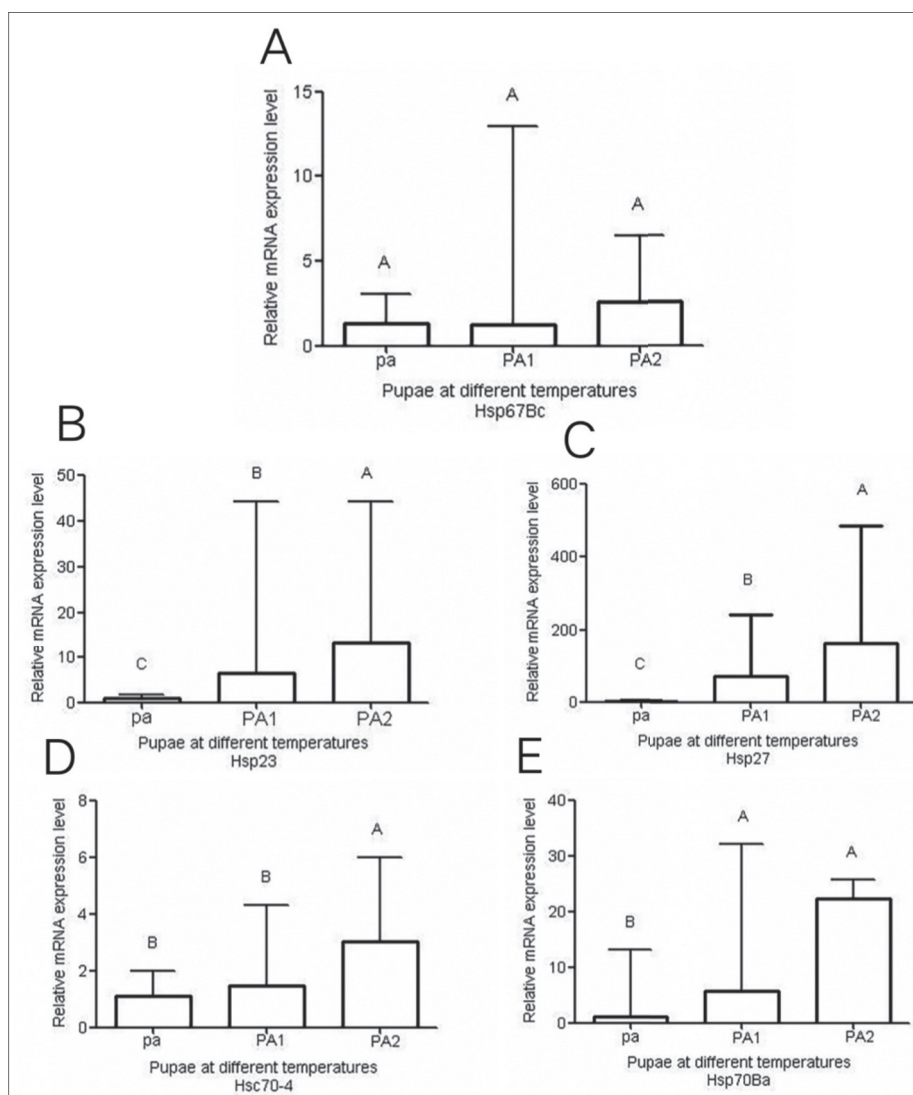


Fig 12. Transcription sequencing expression of different unigenes at different ambient temperature. A- Transcription sequencing expression of HSP67Bc at different ambient temperature, B- Transcription sequencing expression of HSP23 at different ambient temperature, C- Transcription sequencing expression of HSP27 at different ambient temperature, D- Transcription sequencing expression of HSC70-4 at different ambient temperature, E- Transcription sequencing expression of HSP70Ba at different ambient temperature

under low temperature; The expression of HSC70-4 was significantly different at -10°C ($P=0.026<0.05$), while in the -5°C treated group, its expression was higher than that of the room temperature group, but it was not significantly different; Though the expression of the HSP67Bc in the cryogenic treatment pupae was higher than that of the room temperature pupae, but the difference was not significant ($P=0.271>0.05$); The expression of the HSP70Ba in pupae under low temperature was significantly different ($P=0.039<0.05$).

The results showed that the expression of HSP23, HSP27, HSP70Ba, HSP67Bc and HSC70-4 in pupae were different at different temperatures. The results of Q-PCR showed that the analysis results of HSP23, HSP27, HSP70Ba and HSC70-4 were in accordance with the data of transcriptome analysis, but the Q-PCR results of HSP67Bc were significantly different from the results of transcriptome analysis, due to the deviation may be caused by intraspecific repeatability. Among them, the expression of HSP27 in the pupa at -5°C and -10°C were significantly higher than that of the room temperature, the expression of HSP23 and HSP70Ba in the

pupae at -5°C and -10°C were significantly higher than that of the room temperature. It was found that HSP27, HSP23 and HSP70Ba were related to the overwintering cold-tolerance of *Wohlfahrtia magnifica*. The expression of HSC70-4 in the pupae at -10°C were significantly higher than that of the -5°C and room temperature group, while in the -5°C treated group, which was slightly higher than that of the room temperature group, the difference was not significant. It was shown that unigenes in *Wohlfahrtia magnifica* play an important role in the cope with cold.

DISCUSSION

The results showed that the overwintering behavior of *Wohlfahrtia magnifica* was in the form of pupa, in which the HSPs played an important role.

Through transcriptome sequencing and comparative database analysis of low temperature stressed pupae, it was found that the pupae at -5°C and -10°C had 9519 and 7319 different transcripts compare with the pupae at room temperature, respectively. Through comparison

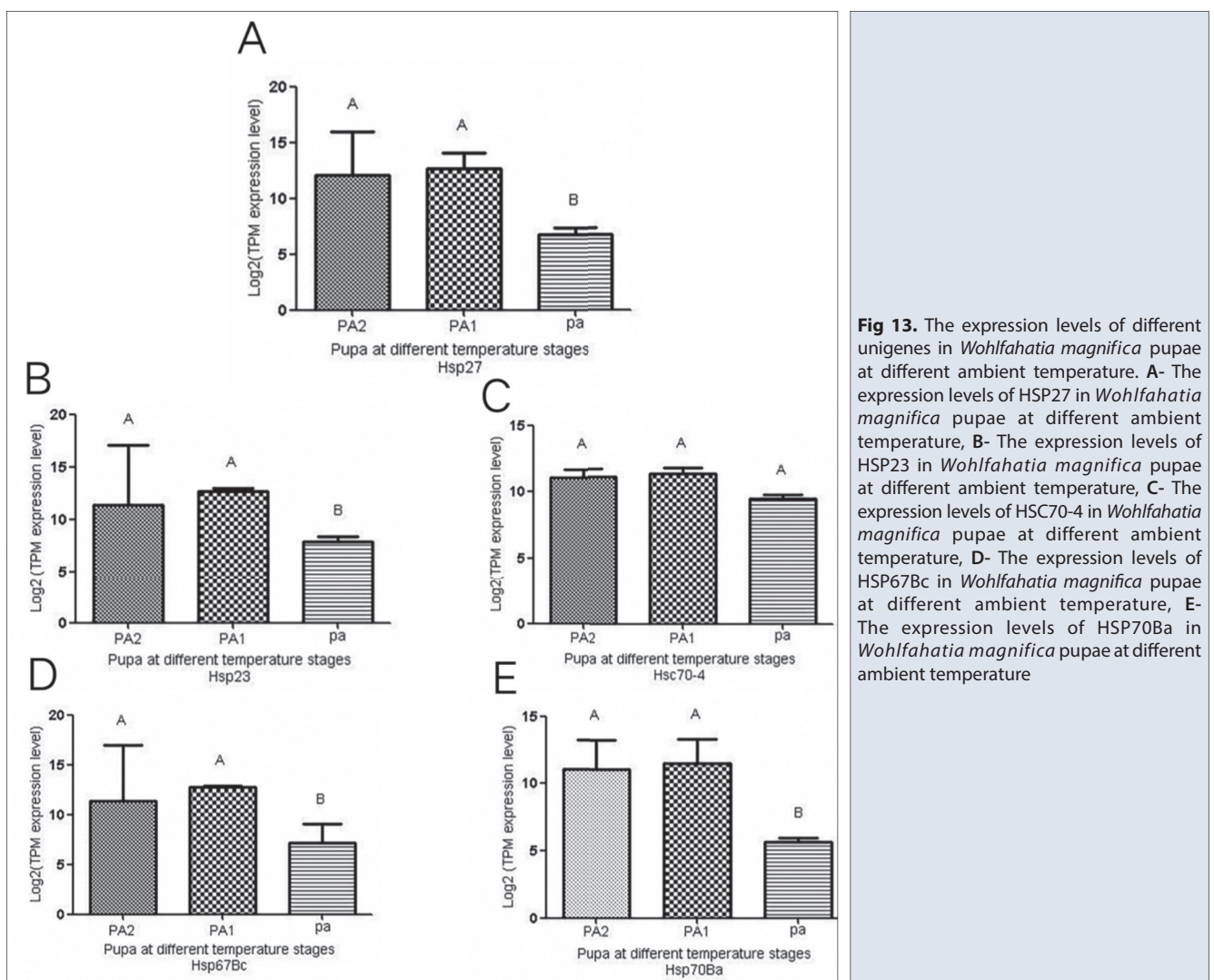


Fig 13. The expression levels of different unigenes in *Wohlfahrtia magnifica* pupae at different ambient temperature. **A-** The expression levels of HSP27 in *Wohlfahrtia magnifica* pupae at different ambient temperature, **B-** The expression levels of HSP23 in *Wohlfahrtia magnifica* pupae at different ambient temperature, **C-** The expression levels of HSC70-4 in *Wohlfahrtia magnifica* pupae at different ambient temperature, **D-** The expression levels of HSP67Bc in *Wohlfahrtia magnifica* pupae at different ambient temperature, **E-** The expression levels of HSP70Ba in *Wohlfahrtia magnifica* pupae at different ambient temperature

with major databases, we found many homologous unigenes were similar to that in other insects (such as *Lucilia cuprina*^[29]), and among them there were many kinds of differential unigenes, indicating that the transcriptome data for the pupae of *Wohlfahrtia magnifica* under low temperature stress is very important to study its cold-tolerance related unigenes.

In this study, RNA-seq and Q-PCR technology were used to explore the molecular mechanism of the pupae survival under low temperature. Through GO enrichment analysis, HSP67Bc, HSP23, HSP27, HSC70-4 and HSP70Ba were found to be enriched in proteins folding and cold stress that related to cold-tolerance in pupae under -5°C and -10°C stress, but no AFPs were found. These results showed that HSPs play an important role in the cold-resistant environment of *Wohlfahrtia magnifica* pupa.

Q-PCR results showed that HSP23, HSP27, HSC70-4 and HSP70Ba were significantly differential expressed. It was reported that the cold-tolerance of *Drosophila melanogaster* was decreased after the HSP23 was knocked^[30],

indicating that this unigene plays an important role in the cold-tolerance in *Drosophila melanogaster*. Results showed the pupae under low temperature stress, the expression of HSP23 was significantly different, indicating that the HSP23 is very important in the overwintering of *Wohlfahrtia magnifica* pupa. Studies on the cold-tolerance of HSP27 in flies have not been reported at present, but when the *Drosophila mediterranean* are heat stressed, this unigene will maintain cell stability^[31], speculating that the HSP27 plays an important role in maintaining cell stability of *Wohlfahrtia magnifica* pupa during the overwinter. HSP67Bc is similar to the earliest found human HSPB8, it enhances the dissolution of denatured protein and amyloid protein^[32]. Although the expression of HSP67bc was not significantly different in this experiment, but its expression level increasing with the decreasing temperature means that HSP67Bc plays a certain role in the overwintering process of *Wohlfahrtia magnifica* pupa. The expression of HSP70 and HSC70 in the *Drosophila melanogaster* were increased after it was cold-shocked at -10°C^[33]. Results showing that the expression of HSC70-4 and HSP70Ba were significantly different in the cold stressed pupae,

inferring that they are very important to the overwintering process of *Wohlfahrtia magnifica* pupa.

When insects are stimulated by cold stress, AFPs can reduce the freezing point of aqueous solution to produce freeze-tolerance and promote the survival of insects in cold conditions [34]. It is reported that true AFPs with high thermal hysteresis are found in freeze-avoiding animals (those that must prevent freezing, as they die if frozen), especially marine fish, insects and other terrestrial arthropods act as preventing freezing below the temperature at which the organism normally experiences [35], such as *Dorcus hopei binodulosus* (*Dhb*) synthesizes at least six hyperactive AFPs (*Dhb* AFP) to enhance its cold-tolerance [36]. Some insects respond to cold stress by using HSPs, because HSPs play an important role in the anti-freezing ability of insects, which improves the survival and adaptability of insects in low temperature environment [37], for example, the expression of HSPs increased in larvae of *Eurosta solidaginis* before and during winter diapause [38]. Insects cold-tolerance are also related to the up-regulation of proline [39], such as no antifreeze protein was found in *Drosophila melanogaster* [40], while high concentration of proline made *Drosophila melanogaster* have cold-tolerance [23]. In addition, pyrroline-5-carboxylate reductase and hyaluronoglucosaminidase precursor in *Culex piniens* were up-regulated during its overwintering diapause [39].

This study showed that HSPs play an important role in the overwintering process of *Wohlfahrtia magnifica*, that laid a foundation for the research of molecular mechanism of *Wohlfahrtia magnifica* overwintering.

AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current study available from the corresponding author and can be provided on your request.

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COMPETING INTERESTS

The authors declared that there is no competing interests.

AUTHORS' CONTRIBUTIONS

H.B.L. performed the experiment research in detail; summarized and analyzed the experiment results. J.Q.X.

consulted a large number of literature and completed the writing of the paper. D.E. planned the experimental research program and implementation process; gave guidance to the writing of the paper. B.X.H. put forward valuable suggestions on the revision and improvement of the paper.

REFERENCES

1. Wangchao Li H, Oyun G, Bao H, Yunzhang L, Yang B, Liu T, Demtu TE: Morphological and scanning electron microscopic (SEM) studies of the pupae of *Wohlfahrtia magnifica*. *J Camel Pract Res*, 27 (1): 17-22, 2020. DOI: 10.5958/2277-8934.2020.00003.X
2. Li H, An X, Zhou J, Ba L, Cha H, Bao H, Yang B, Li Y, Er D: Morphological observation of the *Wohlfahrtia magnifica* in mongolia plateau. *J Camel Pract Res*, 27 (3): 351-357, 2020. DOI: 10.5958/2277-8934.2020.00051.X
3. Villaescusa JM, Angulo I, Pontón A, Nistal JF: Infestation of a diabetic foot by *Wohlfahrtia magnifica*. *J Vasc Surg Cases Innov*, 2 (3): 119-122, 2016. DOI: 10.1016/j.jvsct.2016.04.007
4. Boscarelli A, Levi Sandri GB: Periungual myiasis caused by *Wohlfahrtia magnifica* mimicking an ingrown toenail. *Transl Pediatr*, 5 (2): 95-96, 2016. DOI: 10.21037/tp.2016.03.01
5. Chen W, Geng SL, Song Z, Li YJ, Wang H, Cao JY: Alternative splicing and expression analysis of HSF1 in diapause pupal brains in the cotton bollworm, *Helicoverpa armigera*. *Pest Manag Sci*, 75 (5): 1258-1269, 2019. DOI: 10.1002/ps.5238
6. Reynolds JA, Bautista-Jimenez R, Denlinger DL: Changes in histone acetylation as potential mediators of pupal diapause in the flesh fly, *Sarcophaga bullata*. *Insect Biochem Mol Biol*, 76, 29-37, 2016. DOI: 10.1016/j.ibmb.2016.06.012
7. Santana E, de Los Reyes T, Casas-Tinto S: Small heat shock proteins determine synapse number and neuronal activity during development. *PLoS One*, 15 (5): e0233231, 2020. DOI: 10.1371/journal.pone.0233231
8. Zhou C, Yang XB, Yang H, Long GY, Wang Z, Jin DC: Effects of abiotic stress on the expression of Hsp70 genes in *Sogatella furcifera* (Horvath). *Cell Stress Chaperones*, 25 (1): 119-131, 2020. DOI: 10.1007/s12192-019-01053-4
9. Fregonezi NF, Oliveira LT, Singulani JL, Marcos CM, Dos Santos CT, Taylor ML, Mendes-Giannini MJS, de Oliveira HC, Fusco-Almeida AM: Heat shock protein 60, insights to its importance in *Histoplasma capsulatum*: From biofilm formation to host-interaction. *Front Cell Infect Microbiol*, 10: 591950, 2020. DOI: 10.3389/fcimb.2020.591950
10. Zhao J, Huang Q, Zhang G, Zhu-Salzman K, Cheng W: Characterization of two small heat shock protein genes (Hsp17.4 and Hs20.3) from *Sitodiplosis mosellana*, and their expression regulation during diapause. *Insects*, 12 (2): 119, 2021. DOI: 10.3390/insects12020119
11. Bai J, Liu XN, Lu MX, Du YZ: Characterization of genes encoding small heat shock proteins from *Bemisia tabaci* and expression under thermal stress. *Peer J*, 7: e6992, 2019. DOI: 10.7717/peerj.6992
12. Yi J, Wu H, Liu J, Lai X, Guo J, Li D, Zhang G: Molecular characterization and expression of six heat shock protein genes in relation to development and temperature in *Trichogramma chilonis*. *PLoS One*, 13 (9): e0203904, 2018. DOI: 10.1371/journal.pone.0203904
13. Farahani S, Bandani AR, Alizadeh H, Goldansaz SH, Whyard S: Differential expression of heat shock proteins and antioxidant enzymes in response to temperature, starvation, and parasitism in the Carob moth larvae, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). *PLoS One*, 15 (1): e0228104, 2020. DOI: 10.1371/journal.pone.0228104
14. Teets NM, Dalrymple EG, Hillis MH, Gantz JD, Spacht DE, Lee Jr RE, Denlinger DL: Changes in energy reserves and gene expression elicited by freezing and supercooling in the antarctic midge, *Belgica antarctica*. *Insects*, 11 (1): 18, 2019. DOI: 10.3390/insects11010018
15. Wu YK, Zou C, Fu DM, Zhang WN, Xiao HJ: Molecular characterization of three Hsp90 from *Pieris* and expression patterns in response to cold and thermal stress in summer and winter diapause of *Pieris melete*. *Insect*

Sci, 25 (2): 273-283, 2018. DOI: 10.1111/1744-7917.12414

16. Chen K, Tang T, Song Q, Wang Z, He K, Liu X, Song J, Wang L, Yang Y, Feng C: Transcription analysis of the stress and immune response genes to temperature stress in *Ostrinia furnacalis*. *Front Physiol*, 10:1289, 2019. DOI: 10.3389/fphys.2019.01289

17. Eskandari A, Leow TC, Rahman MBA, Oslan SN: Antifreeze proteins and their practical utilization in industry, medicine, and agriculture. *Biomolecules*, 10 (12): 1649, 2020. DOI: 10.3390/biom10121649

18. Kim HJ, Lee JH, Hur YB, Lee CW, Park SH, Koo BW: Marine antifreeze proteins: Structure, function, and application to cryopreservation as a potential cryoprotectant. *Mar Drugs*, 15 (2): 27, 2017. DOI: 10.3390/md15020027

19. Meister K, Moll CJ, Chakraborty S, Jana B, DeVries AL, Ramløv H, Bakker HJ: Molecular structure of a hyperactive antifreeze protein adsorbed to ice. *J Chem Phys*, 150 (13): 131101, 2019. DOI: 10.1063/1.5090589

20. Cheung RCF, Ng TB, Wong JH: Antifreeze proteins from diverse organisms and their applications: An overview. *Curr Protein Pept Sci*, 18 (3): 262-283, 2017. DOI: 10.2174/1389203717666161013095027

21. Ye Q, Eves R, Campbell RL, Davies PL: Crystal structure of an insect antifreeze protein reveals ordered waters on the ice-binding surface. *Biochem J*, 477 (17): 3271-3286, 2020. DOI: 10.1042/bcj20200539

22. Eslami M, Shirali Hossein Zade R, Takaloo Z, Mahdevar G, Emamjomeh A, Sajedi RH, Zahiri J: afpCOOL: A tool for antifreeze protein prediction. *Heliyon*, 4 (7): e00705, 2018. DOI: 10.1016/j.heliyon.2018.e00705

23. Toxopeus J, Sinclair BJ: Mechanisms underlying insect freeze tolerance. *Biol Rev Camb Philos Soc*, 93 (4): 1891-1914, 2018. DOI: 10.1111/brv.12425

24. Shivananjappa S, Laird RA, Floate KD, Fields PG: Cross-tolerance to desiccation and cold in Khapra Beetle (Coleoptera: Dermestidae). *J Econ Entomol*, 113 (2): 695-699, 2020. DOI: 10.1093/jee/toz316

25. Kivelä SM, Gotthard K, Lehmann P: Developmental plasticity in metabolism but not in energy reserve accumulation in a seasonally polyphenic butterfly. *J Exp Biol*, 222 (Pt 13): jeb202150, 2019. DOI: 10.1242/jeb.202150

26. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C: Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods*, 14 (4): 417-419, 2017. DOI: 10.1038/nmeth.4197

27. Zhao S, Ye Z, Stanton R: Misuse of RPKM or TPM normalization when comparing across samples and sequencing protocols. *RNA*, 26 (8): 903-909, 2020. DOI: 10.1261/rna.074922.120

28. Chen Y, Lun AT, Smyth GK: From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. *F1000Res*, 5:1438, 2016. DOI: 10.12688/f1000research.8987.2

29. Anstead CA, Korhonen PK, Young ND, Hall RS, Jex AR, Murali SC, Hughes DS, Lee SF, Perry T, Stroehlein AJ, Ansell BRE, Breugelmans

B, Hofmann A, Qu J, Dugan S, Lee SL, Chao H, Dinh H, Han Y, Doddapaneni HV, Worley KC, Muzny DM, Ioannidis P, Waterhouse RM, Zdobnov EM, James PJ, Bagnall NH, Kotze AC, Gibbs RA, Richards S, Batterham P, Gasser RB: *Lucilia cuprina* genome unlocks parasitic fly biology to underpin future interventions. *Nat Commun*, 6:7344, 2015. DOI: 10.1038/ncomms8344

30. Gu X, Chen W, Perry T, Batterham P, Hoffmann AA: Genomic knockout of hsp23 both decreases and increases fitness under opposing thermal extremes in *Drosophila melanogaster*. *Insect Biochem Mol Biol*, 139: 103652, 2021. DOI: 10.1016/j.ibmb.2021.103652

31. Economou K, Kotsiliti E, Mintzas AC: Stage and cell-specific expression and intracellular localization of the small heat shock protein Hsp27 during oogenesis and spermatogenesis in the Mediterranean fruit fly, *Ceratitis capitata*. *J Insect Physiol*, 96: 64-72, 2017. DOI: 10.1016/j.jinsphys.2016.10.010

32. Jabłońska J, Dubińska-Magiera M, Jagla T, Jagla K, Daczewska M: *Drosophila* Hsp67Bc hot-spot variants alter muscle structure and function. *Cell Mol Life Sci*, 75 (23): 4341-4356, 2018. DOI: 10.1007/s00018-018-2875-z

33. Rinehart JP, Yocum GD, Denlinger DL: Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochem Mol Biol*, 30 (6): 515-521, 2000. DOI: 10.1016/s0965-1748(00)00021-7

34. Drori R, Davies PL, Braslavsky I: When are antifreeze proteins in solution essential for ice growth inhibition? *Langmuir*, 31 (21): 5805-5811, 2015. DOI: 10.1021/acs.langmuir.5b00345

35. Duman JG: Animal ice-binding (antifreeze) proteins and glycolipids: An overview with emphasis on physiological function. *J Exp Biol*, 218 (Pt 12): 1846-1855, 2015. DOI: 10.1242/jeb.116905

36. Arai T, Yamauchi A, Miura A, Kondo H, Nishimiya Y, Sasaki YC, Tsuda S: Discovery of hyperactive antifreeze protein from phylogenetically distant beetles questions its evolutionary origin. *Int J Mol Sci*, 22 (7): 3637, 2021. DOI: 10.3390/ijms22073637

37. Quan G, Duan J, Fick W, Candau JN: Molecular characterization of eight ATP-dependent heat shock protein transcripts and their expression profiles in response to stresses in the spruce budworm, *Choristoneura fumiferana* (L.). *J Therm Biol*, 88:102493, 2020. DOI: 10.1016/j.jtherbio.2019.102493

38. King AM, MacRae TH: Insect heat shock proteins during stress and diapause. *Annu Rev Entomol*, 60, 59-75, 2015. DOI: 10.1146/annurev-ento-011613-162107

39. Kang DS, Cotten MA, Denlinger DL, Sim C: Comparative transcriptomics reveals key gene expression differences between diapausing and non-diapausing adults of *Culex pipiens*. *PLoS One*, 11 (4): e0154892, 2016. DOI: 10.1371/journal.pone.0154892

40. Neelakanta G, Hudson AM, Sultana H, Cooley L, Fikrig E: Expression of *Ixodes scapularis* antifreeze glycoprotein enhances cold tolerance in *Drosophila melanogaster*. *PLoS One*, 7 (3): e33447, 2012. DOI: 10.1371/journal.pone.0033447