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Original research article

Thermal Stress-related Genes in Honey Bees (*Apis mellifera* Linnaeus) by RNA-seq Analysis

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Abstract The honey bee, *Apis mellifera*, a vital pollinator for agricultural crops, has experienced a decline in numbers over the past decades due to colony collapse disorder (CCD). The suspected causes of CCD have included climate change, parasites, and pesticide use. This study focused on the impact of climate change, as insect body temperature, which is regulated by environmental conditions, is highly susceptible to thermal stress. We investigated the molecular response processes of *A. mellifera* by comparing gene expression profiles under low (10°C) and high (38°C) temperature stress with those of an untreated control (25°C). RNA-seq analysis identified 349,489 assembled transcripts. Differentially expressed genes (DEGs) with a $\log_2$ fold change ≥ 2 were compared between the temperature conditions. We identified 2,838 DEGs at 10°C and 3,634 DEGs at 38°C. Specifically, 2,077 and 2,007 genes were upregulated, while 1,438 and 3,393 genes were down-regulated at 10°C and 38°C, respectively, compared to 25°C. The honey bees exhibited increased expression of *HSP70* and *HSP60* genes under high-temperature stress, whereas *ApoID*, *HR38*, and *ABRA* genes were highly expressed under low-temperature stress. *ETH*, *TTLL4*, and *OR* genes were highly expressed under both temperature conditions. Our findings demonstrate that honey bees exhibit distinct transcriptomic responses to thermal stress, with *HSPs* mediating heat tolerance and calcium/lipid-associated genes supporting cold tolerance.

Keywords *Apis mellifera*, Climate change, Colony collapse disorder, DEG, Thermal stress, Pollinator

INTRODUCTION

Honey bees (*Apis mellifera* Linnaeus) play a crucial role in pollinating the nectar of various crops and flowers, with approximately one-third of crops relying on honey bee pollination (Gallai *et al*., 2009; Hou *et al*., 2014). Also, honey bees have economical effect estimated about US$200-500 billion per annual (Lautenbach *et al*., 2012). Colony collapse disorder (CCD), which is responsible for destroying colonies of honey bees, has been reported as a global concern. For example, the number of honey bee colonies in the USA declined from 4.6 million in 1970 to 2.7 million in 2014, in Europe from 21.1 million in 2014 and from 21.1 million to 17.7 million during the same period (FAO, 2017; Paris *et al*., 2017). More recently, significant colony losses have been reported in South Korea. According to Lee *et al*. (2022), approximately 43% of bee hives in Jeollanam-do were affected, resulting in the loss of about 105,900 colonies. In Gyeongsangnam-do, 11.1% of hives were lost, totaling about 38,400 colonies, while in Jeju, 15.1% of bees disappeared from 74,200 colonies, affecting 31.3% of beekeeping farms. The suspected causes of CCD include global climate change, pesticide exposure, nutritional deficiencies, and pathogens (vanEngelsdorp *et al*., 2009; Baines *et al*., 2017; Mahanhuda and Tiwari, 2024).

Global climate change gives many negative effects on honey bees, particularly because they are ectotherms

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which rely on environmental heat sources for their body temperature (Hilderbrand and Goslow Jr., 2001). When global warming gives thermal stress to honey bees, heat shock protein (HSP) family do key play role on heat stress tolerance. HSPs play role of heat hardening in high temperature, which in turn provides protection from subsequent and more severe temperature (Matz *et al*., 1995). HSP includes large HSPs, HSP90, HSP70, HSP60, HSP40, and small HSPs. Many HSPs perform chaperone functions by stabilizing new proteins to promote correct folding or by helping to refold proteins that were damaged by cellular stress (De Maio, 1999). HSP family is essential for relieving physiological stress (Ceylan *et al*., 2023). To regulate the expression of HSPs, heat shock factors (HSFs) are considered transcription factors of HSPs within the cellular system (Watanabe and Tanaka, 2018). In *Caenorhabditis elegans*, HSF regulates approximately 942 genes during heat stress for homeostasis beyond the immediate stress response (Brunquell *et al*., 2016). *HSP90* gene participates the protein conformational maturation (Hu *et al*., 2022). *HSP70* gene is conserved molecular chaperon ubiquitously expressed in prokaryote and eukaryote organism (Rosenzweig *et al*., 2019). Seventeen kinds of *HSP70* genes are encoded in human (Brocchieri *et al*., 2008). *HSP60* gene is divided into two groups of chaperonins which are expressed in different locations within cells. *HSP60* is categorized into 2 groups of chaperonins located in different parts of the cell. Group I of *HSP60*s are found in prokaryotes, as well as in the mitochondria and chloroplasts of eukaryotes. Group II of *HSP60*s are present in archaea and the cytosol of eukaryotic cells (Balchin *et al*., 2018). Octopamine coordinates thermo-protection of an insect nervous system (Armstrong and Robertson, 2006).

Despite climate change resulting in colder winter, insects have developed seasonal adaptations to cold stress, including environmentally programmed periods of dormancy called diapause (Denlinger *et al*., 2005; Kostál, 2006), and rapid cold hardening (RCH), which provides cold resistance against rapidly decreased temperatures (Lee Jr. *et al*., 1987). A remarkable feature of RCH is that cells directly respond to low temperature in the absence of stimulation from the brain and hormones. Although the mechanism and down-stream of cold-hardening pathway are unknown (Yi and Lee Jr., 2004; Teets *et al*., 2008), chilling causes a rapid melanogaster, a mutation of membrane protein, dystroglycan, have elevated intracellular calcium levels, which correlates with improved performance and survival at low temperatures. To regulate localization of calcium in rapid cold condition, calcium and calmodulin-dependent signaling event have important roles (Teets *et al*., 2013).

In this study, we investigated the transcriptional responses of honey bees to thermal stress. Transcriptome analysis identified differentially expressed genes (DEGs), and selected candidates were validated using quantitative real-time PCR (RT-qPCR). Our results demonstrate that honey bees exhibit distinct transcriptomic responses to thermal stress, with heat shock proteins mediating heat tolerance and calcium/lipid-associated genes supporting cold tolerance.

MATERIALS AND METHODS

1. Honey bee rearing and thermal stress treatment

The honey bee apiaries were maintained at the experimental apiary of Gyeongkuk National University. Worker bees were collected using an insect net for experiment and placed in insect breeding cages (120 × 80 mm) (SPL, Pocheon, Korea) to stabilize honey bees in a new environment (Ulziibayar *et al*., 2025). Collected bees were transferred to the laboratory and maintained at 25 ± 2°C with 50–60% of relative humidity, and provided with a 40% sugar solution as food for one day. To expose the honey bees to thermal stress conditions, they were kept at 28°C, 10°C, and 38°C for 12 h. Following these treatments, the honey bees were used for RNA extraction.

2. RNA extraction and cDNA library construction

Total RNA from *A. mellifera* was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. To generate cDNA library, TrueSeq stranded total RNA with Ribo-Zero H/M/R_ Gold (Illumina, California, USA) was used. The sequencing was performed on a Novaseq (Illumina, California, USA) platform by Macrogen (Seoul, Korea). The sequences were trimmed using Trimmomaic 0.38. The trimmed

sequences were assembled by Trinity (version trinityseq_r20140717, bowite 1.1.2) via *de novo* sequencing. Total reads were mapped using RSEM version 1.3.1, bowtie 1.1.2 from http://genontology.org/, and BLAST version 2.9+ (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and DIAMOND version 0.9.21 (https://github.com/bbuchfink/diamond/releases?after=v0.9.21) were used for gene annotation. For DEG analysis, size factors were estimated from count data and normalized using the trimmed mean of M-values (TMM) method in edgeR library in R (Bioconductor, Seattle, WA, USA).

3. *In silico* analysis

The mRNA sequencing was performed using the Nova Seq platform. The sequenced reads were processed to remove adapter sequences using Trimmomatic 0.38. Subsequently, *de novo* assembly of the RNA sequences was conducted using Trinity (version trinityseq_r20140717, bowtie 1.1.2). Gene expression levels were quantified by mapping the *de novo* assembled RNA sequences with RSEM (version v1.3.1, bowtie 1.1.2). Gene identification was performed through the DIAMOND, using databases obtained from the Gene Ontology (GO) knowledgebase (http://geneontology.org/) and the BLAST. The mapped data were then analyzed for differentially expressed genes (DEGs) using the edgeR library. The Fragmented per Kilobase of transcript per Million mapped reads (FPKM) values were utilized for DEG analysis.

Table 1. Primer information in this study

Gene	Primer	Sequence (5′ to 3′)	Annealing temp (°C)	Amplicon size (bp)
HSP70	AmHSP70 F AmHSP70 R	GTACTCGTTGGAGGTTCTAC CTTGTTCCCCAGAAAGTACA	50	151
HSP60	AmHSP60 F AmHSP60 R	AGAACAAAGTTGGGGTAGTC TACAGGATTGGCACCTTTAC	51	223
HR38	AmHR3 F AmHR3 R	ATTTCGCAAAAAGTCGATCC ACCTGAGATATCCTGAACGA	51	203
ETH	AmETH F AmETH R	TCCTGAAAATCGCCAAGAAT CCCGCCTTTTTATAGGTCTT	51	168
VDCC	AmVDCC F AmVDCC R	TCGCTTTTGAATTTCAGTCG CGTGATGTGTTAGTTCTTGC	51	191
ABRA	AmABRAP F AmABRAP R	GGCTACTATCCAACTTCGTT GAAGAGCCAGACAAATTGTTA	50	153
TTLL4a	AmTTLL4a F AmTTLL4a R	TCGTGCCTCGATTAGTTTAG TATCCTGACAAACACCTTCG	51	152
TTLL4b	AmTTLL4b F AmTTLL4b R	GAGGAGAGAACGAGAGAAAC AGAAATATCCCGACAAGACG	50	221
ApolD	AmApolD F AmApolD R	GGTAAAACAGAGGGGGATAC ATTGGAAATACCTCTCAGCC	50	266
OR Oa	AmOROa F AmOROa R	TATGTTCATCGTTAGCCTCG CCTGGTCACAGCTAAGTATC	51	205
OR1	AmOR1 F AmOR1 R	GCCAATAGAAATCCCTCGAT AACCGAAAAATCCCCCTATC	51	169
RL32	AmRL32 F AmRL32 R	CGTCACCAGAGTGATCGTTACA CCCCATGAGCAATTTCAGCAC	50	250

4. Quantitative real time polymerase chain reaction (RT-qPCR)

cDNA was synthesized by Max RT premix kit (Intron, Seongnam, Korea) with random primer for RT-qPCR. The RNA was dissolved in diethyl pyrocarbonate (DEPC) treated water, and 500 ng of this RNA was used for cDNA synthesis in a total volume of 20 μL. Reverse transcription and annealing were conducted at 45°C for 60 min. Subsequently, inactivation of reverse transcriptase was performed at 95°C for 5min. RT-qPCR was performed cDNA with iSYBR green premix (Bio-Rad) with the following conditions: initial denaturing at 95°C for 3 min, followed by 40 cycles of 95°C denaturing for 30 s, 50°C annealing for 30 s, 72°C extension for 30 s, and melting step. Each PCR reaction mixture (20 μL) contains 10 μL of iQ SYBR Green Supermix, 1 μL of each 10 pmol forward and reverse primer set (Table 1), 2 μL of cDNA (200 ng), and 6 μL of water. RNA expression levels were normalized following Comparative Ct ($^{\Delta\Delta}$CT) method (Livak and Schmittgen, 2001) against *EF-1* as reference gene.

5. Graphics and statistical analysis

The heatmaps were demonstrated by R with heatmap library. Statistical values in the graphs are presented as mean ± standard deviation. The results were visualized using GraphPad Prism 9 (Dotmatics, Boston, USA). One-way ANOVA was performed to analyze the means with the SAS program (SAS Institute Inc., 1989). Differences among treatment means were assessed using the LSD test, with a significance level set at $P < 0.05$.

RESULTS

1. Abundance of transcriptome

The transcriptome of honey bees was analyzed and generated about 12.0 GB of sequences from each treatment (Fig. 1). 100 Mb pairs of reads were retrieved after trimming (Fig. 1A). After sequences assembly, about 73.68–87.00% sequences were mapped to genes. Total 349,489 assembled transcripts were annotated through DIAMOND version 0.9.21. Principal component analysis (PCA) was performed to compare honey bees exposed to high (38°C), low (10°C), and normal (control, 25°C) temperatures (Fig. 1B).

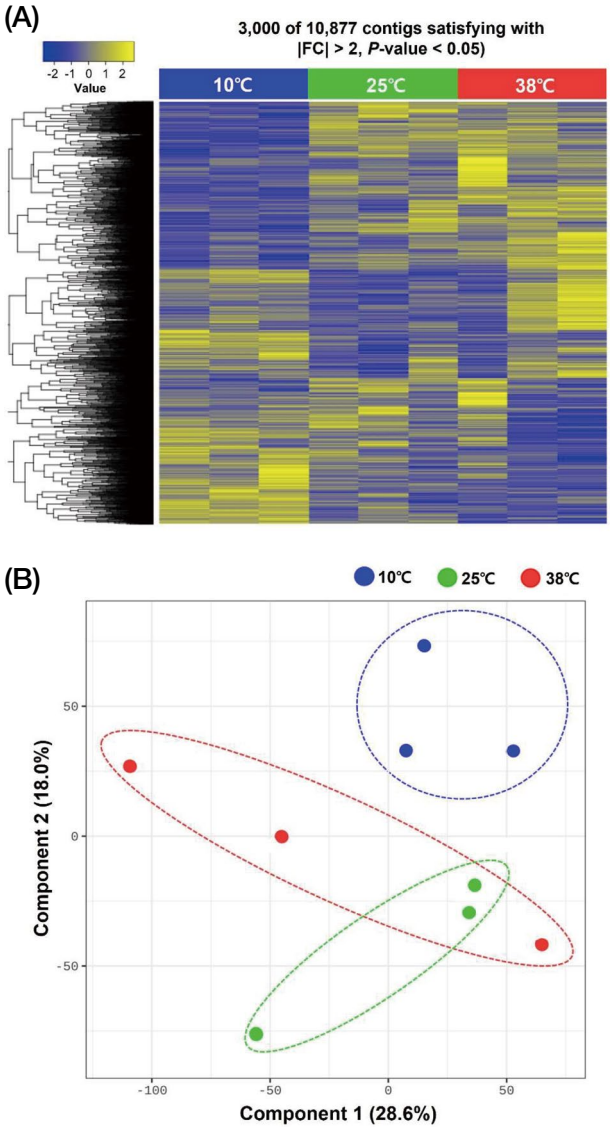

Fig. 1. Heatmap and multidimentional scaling (MDS) analysis of differentially expressed genes in *Apis mellifera* among 3 different temperatures exposed workers. (A) The heatmap was generated using Z-scores transformed from Fragments per Kilobase of transcript per Million mapped reads values. (B) The MDS plot was generated using R programming language.

2. Analysis of differentially expressed genes (DEGs)

Transcriptional abundance of 38°C and 10°C were compared to control (25°C) via RSEM by using FPKM to perform DEG (Fig. 2). DEGs were selected based on a

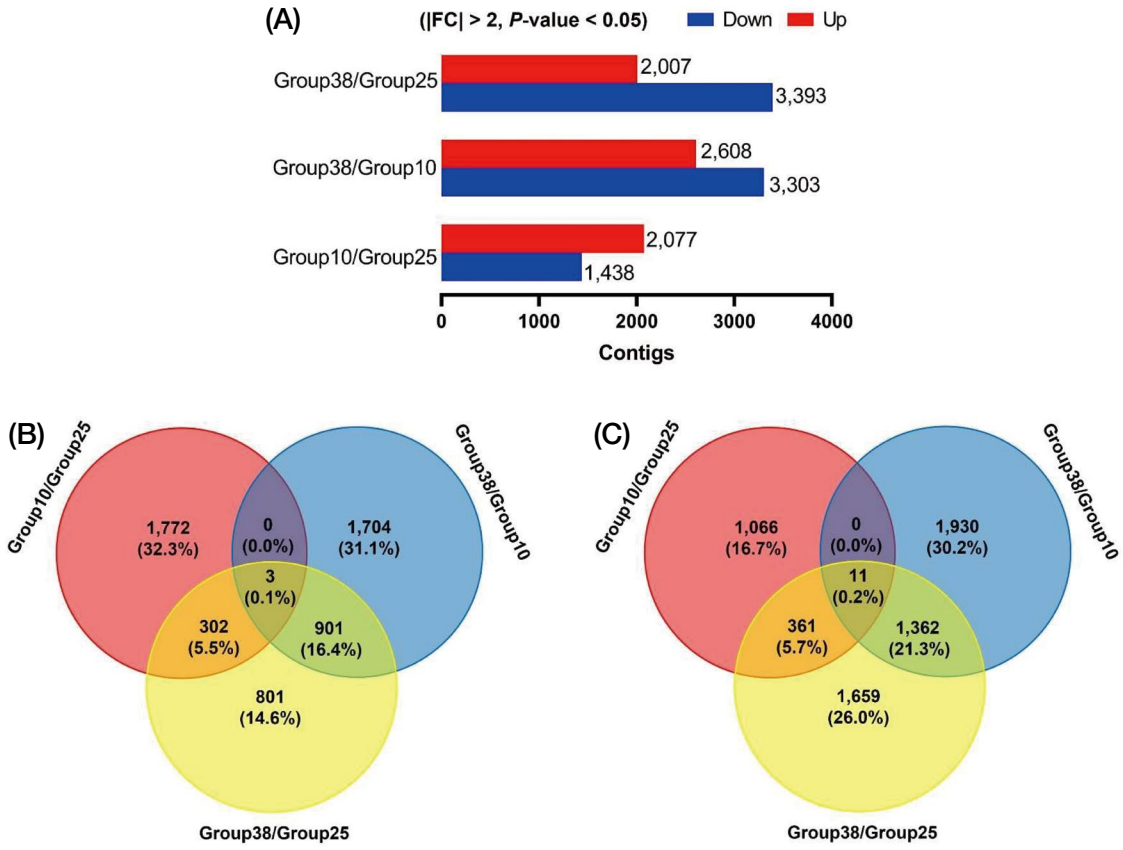

Fig. 2. Analysis of differentially expressed genes (DEGs) in *Apis mellifera* among 3 different temperatures exposed workers. (A) DEGs abundance of *A. mellifera* among different temperature group. (B) Venn diagram analysis. The number of specific and shared DEGs were showed between three different temperature groups. All data were selected from threshold levels of >2-folds change and *P*-value<0.05 in Fragments per Kilobase of transcript per Million mapped reads values.

Table 2. RNA-Seq results of *Apis mellifera* workers, comparing among different temperatures

Sample	Rep	Total reads bases	Trimmed reads	Mapped reads (%)	Genes
10°C	1	12,621,052,920	124,960,920	107,444,518 (87.00)	349,489
	2	13,222,446,512	130,915,312	106,248,704 (82.41)	
	3	13,291,471,730	131,598,730	106,354,222 (82.09)	
25°C	1	11,611,932,428	114,969,628	85,605,990 (75.64)	
	2	11,175,831,194	110,651,794	86,865,012 (79.67)	
	3	12,761,369,998	126,350,198	91,986,196 (73.85)	
38°C	1	12,881,649,080	126,541,080	111,481,534 (88.86)	
	2	12,979,548,784	128,510,384	93,127,868 (72.46)	
	3	13,295,705,852	131,640,652	95,754,564 (73.90)	

threshold of a 2-fold change (FC) value and a *P*-value of less than 0.05. Fig. 2A showed numbers of up- and down-regulated genes. Up- and down-regulated genes were generated 2,077 and 1,438 at 10°C compared to 25°C, respectively (Fig. 2B and 2C). At 38°C compared to 10°C, a total of 2,608 genes were up-regulated and 3,303 were

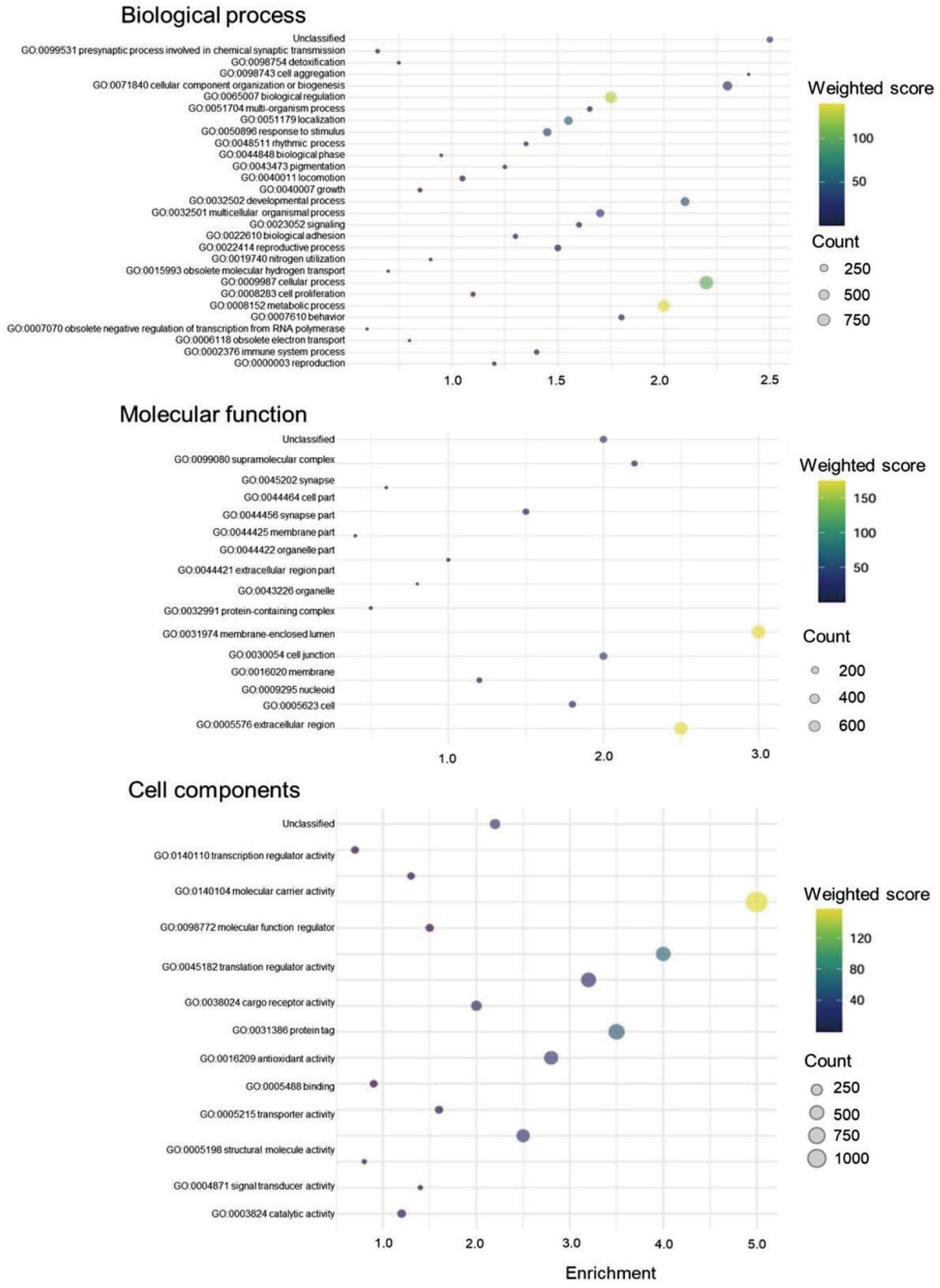

Fig. 3. Gene Ontology (GO) classification of all annotated unigenes. 10,877 unigenes were assigned to three main GO categories of 'Biological process with 29 functional subcategories', 'Molecular function with 16 functional subcategories', and 'Cellular component with 13 functional subcategories. Some unigenes were not classified and were shown as 'Unclassified' in each category.

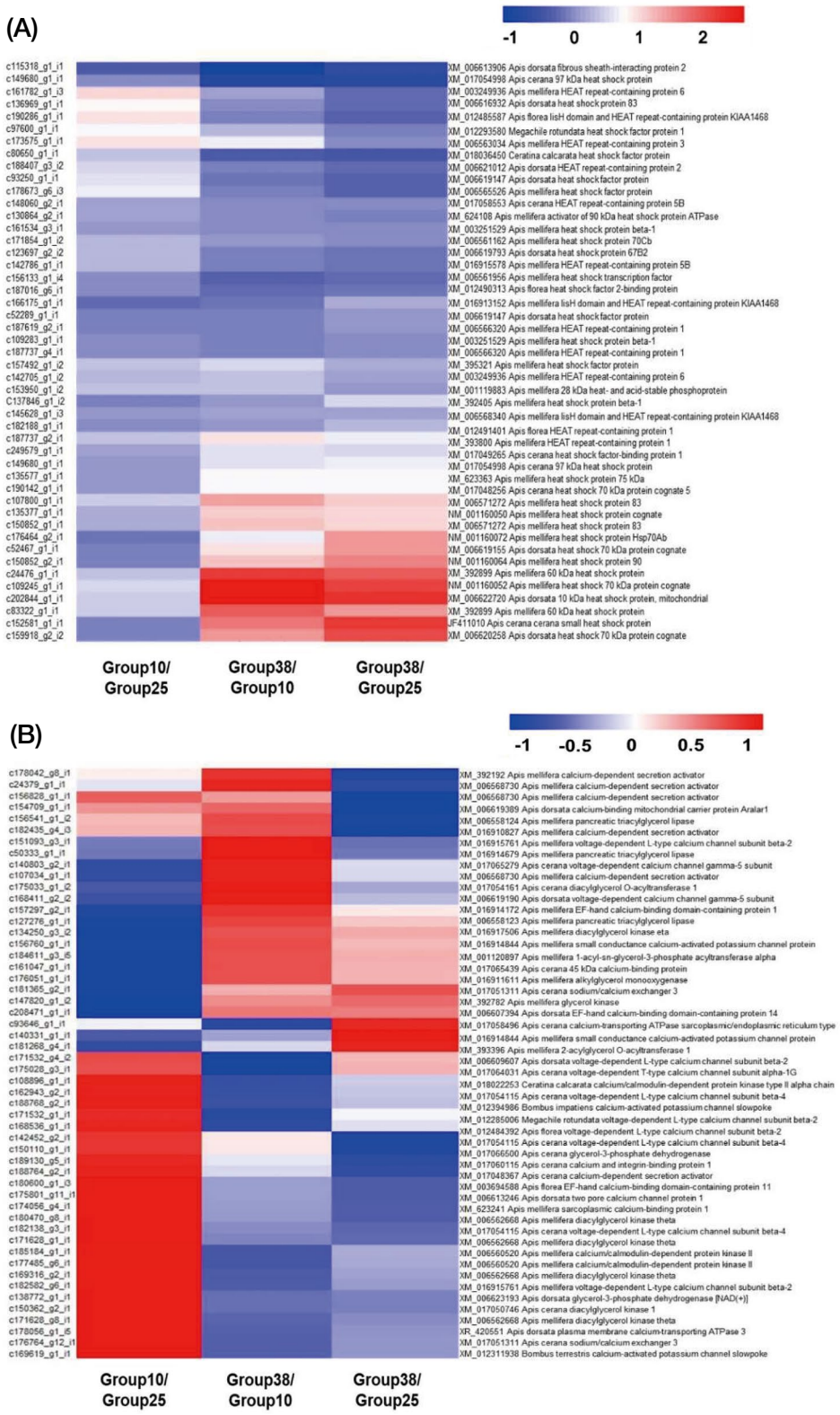

Fig. 4. The heatmap analysis of heat shock protein and calcium related proteins in *Apis mellifera* among 3 different temperatures exposed workers. (A) Heat shock proteins from differentially expressed genes (DEGs) for high temperature tolerance. (B) Calcium related proteins from DEGs for low temperature tolerance.

down-regulated, whereas at 38°C compared to 25°C, 2,077 genes were up-regulated and 3,393 were down-regulated (Fig. 2B and 2C). Total DEGs were performed GO term analysis followed by biological process, cellular components, molecular function. GO term analysis was displayed via bubble plot (Fig. 3). In case of biological process, GO:0008152 metabolic process showed the highest weighted score (139.06). GO:0009987 cellular process showed the highest number of count (900). In case of cellular component, GO:0044464 cell part showed the highest weighted score (156.61) and count (1007). In case of molecular function, GO:0005488 binding activity showed the highest weighted score (174.64) and count (651).

3. Thermal stress related genes

HSP and cold stress responsible genes were selected from DEGs to analyze thermal stress responsible genes (Fig. 4). In high temperature exposed group, *HSP70* (c109245_g1_i1), *HSP90* (c150852_g2_i1), *HSP83* (c107800_g1_i1), *HSP60* (c24476_g1_i1) were highly expressed compared to normal temperature exposed group (Fig. 4A). These *HSP*s showed more than 2 FC value. In low temperature exposed group, calcium channel related proteins and calcium binding proteins were analyzed by heat map with FC values. Some genes were selected what is more than 2 FC value, *Apolipolin D*, *Odorant receptor* (*OR*), *Hormone receptor 38* (*HR38*), *Ecdysis trigger hormone* (*ETH*), *Tubulin tyrosine ligase like 4* (*TTLL*), *Voltage dependent L-type calcium channel* (*VDLCC*), and *Actin binding Rho-activating* (*ABRA*) from DEGs. RT-qPCR was conducted to validate the expression of genes associated with thermal stress (Fig. 5). In the group exposed to 38°C, *HSP60*, *HSP70*, *OROa*, *ETH*, and *TTLL4a* exhibited significantly different expression levels compared to the control as 25°C ($P < 0.05$; Fig. 5A). In the group exposed to 10°C, *TTLL4b*, *ApolD*, *VDLCC*, *ABRA*, and *HR38* also showed significantly altered expression relative to the normal temperature as control (Fig. 5B).

High-temperature exposure markedly upregulated genes related to proteolysis (trypsin-1), neurotransmission (octopamine receptor, GABA transporter), and stress adaptation (ecdysis triggering hormone, chemosensory

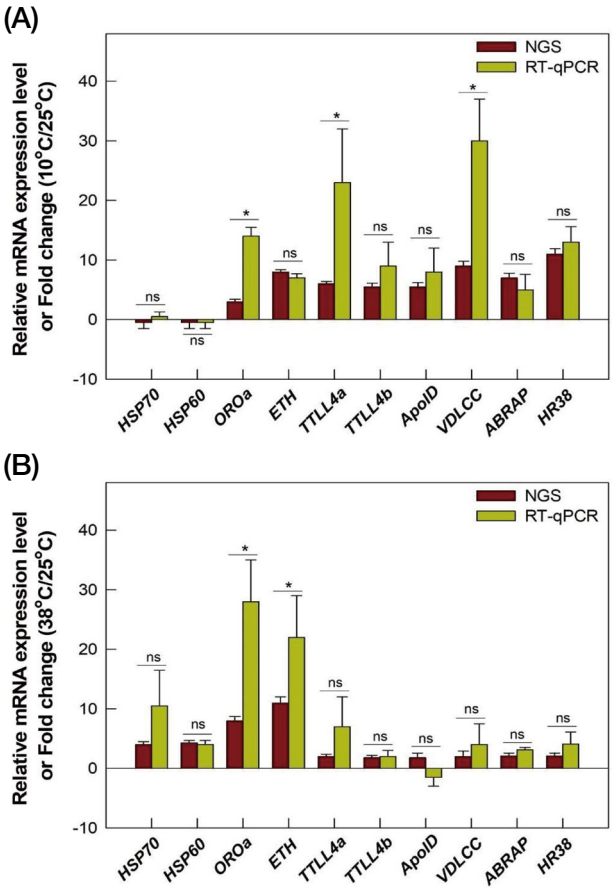

Fig. 5. Validation of differentially expressed genes (DEGs) associated with thermal stress in *Apis mellifera* by RT-qPCR. (A) Gene expression of honeybee when honey bee got heat stress. (B) Gene expression of honeybee when honeybee got cold stress. All the samples were replicated independently three times. *HSP70* and *60*; *Heat* shock protein *70* and *60*, *OROa*; *Octopamine receptor_Oa*, *ETH*; *Ecdysis triggering hormone*, *TTLL4a* and *b*; *tubulin polyglutamylase TTLL4a* and *b*, *VDLCC*; *Voltage dependent L-type calcium channels*, *ABRAP*; *Actin binding rho activating protein*, *HR38*; *hormone receptor 38*. Asterisks above standard deviation bars indicate significant difference between groups at Type I error = 0.05 (LSD test). The letter "ns" stands for a non-significant difference between groups.

proteins), with the highest FC observed for *Aphis dorsata* trypsin-1 in Table 3, while in low-temperature exposure, genes involved in developmental regulation (nuclear hormone receptor HR38, distal-less), muscle function (troponin H/I, muscle LIM protein 1), and cuticle formation (pupal cuticle protein-like, laccase 2) were predominantly upregulated, with the largest change detected for *A. mellifera HR38* in Table 4.

Table 3. Gene list of differentially expressed genes with highly altered Fragments per Kilobase of transcript per Million mapped reads (FPKM) values under high-temperature condition

Contig	NT	group38/ group25.fc	-log *P*-value
c175756_g1_i3	XM_006621261 PREDICTED: *Apis dorsata* trypsin 3A1-like	21.39	4.15
c176088_g1_i1	XM_003400443 PREDICTED: *Bombus terrestris* guanine nucleotide-binding protein subunit gamma-e	16.98	3.63
c173091_g1_i1	XM_016916767 PREDICTED: *Apis mellifera* M-phase inducer phosphatase 1-like	12.77	3.21
c187268_g4_i1	XM_001120450 PREDICTED: *Apis mellifera* DC-STAMP domain-containing protein 1-like	12.59	8.83
c117587_g1_i2	XM_001120633 PREDICTED: *Apis mellifera* lipase maturation factor 2-like	12.36	3.90
c185576_g1_i1	XM_017065179 PREDICTED: *Apis cerana* neuropeptides capa receptor-like (LOC108003086), partial mRNA	12.20	3.83
c159758_g1_i1	NM_001142607 *Apis mellifera* ecdysis triggering hormone (Eth), mRNA	11.56	3.62
c173209_g1_i1	XM_006565223 PREDICTED: *Apis mellifera* leucine-rich repeat-containing protein 15	9.02	2.16
c181508_g1_i4	NM_001011565 *Apis mellifera* octopamine receptor (Oa1), mRNA	8.76	5.20
c188711_g3_i2	XM_012320203 PREDICTED: *Bombus terrestris* tyrosine-protein phosphatase 99A	8.04	4.01
c19821_g1_i1	XM_006565270 PREDICTED: *Apis mellifera* protein msta-like	7.96	5.70
c184464_g2_i1	XM_391929 PREDICTED: *Apis mellifera* sodium- and chloride-dependent GABA transporter 1	7.63	2.09
c169128_g1_i4	XM_392599 PREDICTED: *Apis mellifera* V-type proton ATPase 21 kDa proteolipid subunit-like	7.60	2.12
c181038_g4_i1	KJ404272 *Apis mellifera* octopamine receptor 1 (oa1) gene, partial cds	7.48	3.62
c185668_g5_i1	XM_006563079 PREDICTED: *Apis mellifera* mucin-2-like	7.16	3.53
c169959_g3_i8	XM_016915948 PREDICTED: *Apis mellifera* protein argonaute-3	7.13	4.32
c134250_g3_i2	XM_016917506 PREDICTED: *Apis mellifera* diacylglycerol kinase eta	7.13	2.53
c161202_g1_i1	XM_016917860 PREDICTED: *Apis mellifera* sodium-dependent nutrient amino acid transporter 1-like	7.10	2.78
c176499_g12_i1	XM_001120112 PREDICTED: *Apis mellifera* trypsin-1	7.04	3.40
c180904_g2_i1	XM_006557878 PREDICTED: *Apis mellifera* chemosensory protein 4 (CSP4)	6.80	4.02
c173176_g2_i1	XM_006560375 PREDICTED: *Apis mellifera* carboxypeptidase B-like	6.75	2.32
c225804_g1_i1	XM_006612424 PREDICTED: *Apis dorsata* protein disulfide-isomerase A6-like	6.70	9.73
c5013_g1_i1	XM_017056961 PREDICTED: *Apis cerana* octopamine receptor Oamb	6.64	4.50
c193069_g1_i1	XM_001122093 PREDICTED: *Apis mellifera* putative ATP-dependent RNA helicase DHX33	6.41	2.46
c167921_g1_i2	XM_016912575 PREDICTED: *Apis mellifera* trypsin-7	6.30	3.22
c179505_g6_i3	XM_017052087 PREDICTED: *Apis cerana* phospholipase A1 member A	6.21	2.20
c197807_g1_i1	NR_039451 *Apis mellifera* microRNA 3757 (Mir3757), microRNA	6.18	3.15
c169928_g1_i5	XM_012486113 PREDICTED: *Apis florea* neuromodulin-like	6.09	2.93
c185282_g1_i2	XM_016914086 PREDICTED: *Apis mellifera* dynein heavy chain 10, axonemal	6.08	2.74
c185626_g6_i5	XM_016915846 PREDICTED: *Apis mellifera* UNC93-like protein	6.00	4.69

DISCUSSION

Insects are the most diverse and successful group of animals, playing indispensable roles in terrestrial ecosystems (Foottit and Adler, 2017). Their life histories are strongly influenced by abiotic factors, among which temperature is particularly critical. Temperature governs insect population abundance and distribution (Li *et al*., 2020) and affects population dynamics and geographic ranges by altering developmental rates, life cycles, and the availability of host plants (Bale *et al*., 2002). Under global warming, more frequent extreme thermal events can directly and indirectly impact insect survival and disrupt a wide array of physiological and metabolic

Table 4. Gene list of differentially expressed genes with highly altered Fragments per Kilobase of transcript per Million mapped reads (FPKM) values under low-temperature condition

Contig	NT	group10/ group25.fc	-log *P*-value
c171851_g8_i1	XM_016917762 PREDICTED: *Apis mellifera* probable nuclear hormone receptor HR38	10.87	4.31
c171532_g1_i1	XM_012285006 PREDICTED: *Megachile rotundata* voltage-dependent L-type calcium channel subunit beta-2	10.37	3.67
c187448_g4_i1	XM_006560304 PREDICTED: *Apis mellifera* distal-less (Dll)	8.62	2.69
c185582_g2_i2	BK005282 TPA_inf: *Apis mellifera* troponin H (TpnH) and troponin I (TpnI)	7.90	3.026
c172856_g1_i1	KC853324 *Tetragonisca weyrauchi* isolate Barcode13 cytochrome *c* oxidase subunit I (COI)	7.66	2.37
c159758_g1_i1	NM_001142607 *Apis mellifera* ecdysis triggering hormone (Eth)	6.98	2.41
c171666_g2_i1	XM_017054470 PREDICTED: *Apis cerana* muscle LIM protein 1	6.92	2.53
c170008_g1_i1	XM_006568271 PREDICTED: *Apis mellifera* pupal cuticle protein-like	6.87	2.47
c159502_g1_i3	XM_016912418 PREDICTED: *Apis mellifera* arylsulfatase B-like	6.60	2.25
c99350_g1_i1	XM_006562254 PREDICTED: *Apis mellifera* laccase 2	6.01	4.38
c165372_g2_i1	XM_006558163 PREDICTED: *Apis mellifera* set1/Ash2 histone methyltransferase complex subunit ASH2	6.01	3.58
c185837_g4_i1	XM_016913631 PREDICTED: *Apis mellifera* paired mesoderm homeobox protein 2A	5.86	2.56
c186016_g3_i3	XM_006560372 PREDICTED: *Apis mellifera* tensin-1	5.72	1.63
c144638_g3_i1	XM_016917868 PREDICTED: *Apis mellifera* transcription factor GAGA	5.70	1.96
c144231_g1_i1	XM_003250555 PREDICTED: *Apis mellifera* actin-binding Rho-activating protein-like	5.65	3.18
c169968_g1_i3	XM_012491227 PREDICTED: *Apis florea* 26S proteasome non-ATPase regulatory subunit 2-like	5.62	2.14
c161202_g1_i1	XM_016917860 PREDICTED: *Apis mellifera* sodium-dependent nutrient amino acid transporter 1-like	5.51	2.18
c176764_g5_i1	XM_020862846 PREDICTED: *Bombus terrestris* nuclear receptor coactivator 6	5.41	1.64
c169128_g1_i4	XM_392599 PREDICTED: *Apis mellifera* V-type proton ATPase 21 kDa proteolipid subunit-like	5.37	1.54
c184362_g3_i1	XM_016914787 PREDICTED: *Apis mellifera* protein muscleblind-like	5.36	2.10
c147341_g2_i1	KX421581 *Apis mellifera ligustica* early growth response protein variant 3 (Egr)	5.34	2.47
c170823_g1_i1	NR_039510 *Apis mellifera* microRNA 3724 (Mir3724), microRNA	5.24	1.62
c177304_g3_i3	HQ633047 *Bombus terrestris* clone BAC BT_CBa-88K23 antennapedia-like protein (Antp)	5.21	3.54
c131444_g4_i1	XM_016916806 PREDICTED: *Apis mellifera* histone-lysine N-methyltransferase 2D-like	4.88	2.83
c182571_g5_i1	BK006346 TPA_exp: *Apis mellifera* sex determination locus	4.84	2.29
c147341_g1_i1	KX421581 *Apis mellifera ligustica* early growth response protein variant 3 (Egr)	4.80	2.26
c174586_g1_i2	KM418986 *Chalybion californicum* isolate 6991cha ultra conserved element locus uce-1037 genomic sequence	4.76	1.97
c174208_g5_i1	XM_006560858 PREDICTED: *Apis mellifera* tubulin polyglutamylase TTLL4-like	4.74	2.71
c180118_g5_i1	XM_016916893 PREDICTED: *Apis mellifera* homeotic protein antennapedia (Antp)	4.68	2.95
c184283_g7_i1	BK005282 TPA_inf: *Apis mellifera* troponin H (TpnH) and troponin I (TpnI)	4.64	1.75

functions, including feeding, digestion, detoxification, reproduction, growth, and development (Garrad *et al*., 2016).

Previous studies have demonstrated that thermal stress elicits distinct transcriptional responses depending on whether insects are exposed to high- or low-temperatures (Vatanparast and Park, 2021, 2022; Vatanparast *et al*., 2021). Metabolizable energy is essential for maintaining homeostasis and supporting growth (Richard, 1986). Exposure to temperatures above the optimal range impairs both energy reserves and metabolic process (Belhadj *et al*., 2015). Under heat stress, the synthesis of most proteins decline, including ATPases involved in major metabolic pathways such as glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation (Li *et al*., 2020).

In our study, high-temperature exposure prominently induced genes related to proteolysis, neuronal modulation, and general stress adaptation, whereas low-temperature exposure favored genes involved in developmental regulation, muscle function, and cuticle remodeling. These patterns are consistent with established principles of insect thermal physiology: heat often drives rapid metabolic turnover and neuromodulation, while cold necessitates developmental, structural, and contractile adjustments to maintain function (Li *et al*., 2020).

HSP family plays a key role in protecting cellular proteins under high-temperature conditions. Elevated temperatures can disrupt stabilizing disulfide bonds, leading to protein denaturation and loss of function (Matz *et al*., 1995). Consistent with this, honey bees subjected to heat stress in our study exhibited significantly upregulated expression of *HSP70* and *HSP80*, indicating activation of molecular chaperone systems to mitigate heat-induced protein damage (Fig. 5).

Interestingly, *ETH* (c15958_g1_i1), *OROa* (c181508_g1_i4), and *TTLL4* (c182436_g1_i1) were upregulated under both high- and low-temperature conditions. In *Drosophila*, *ETH* expression increases under high temperatures to promote reproduction (Meiselman *et al*., 2017), and in the western black spider, *20E* is elevated under high-temperature conditions (Moen *et al*., 2022). Our results suggest that elevated *20E* levels may stimulate *ETH* expression under heat stress. However, *ETH* was also highly expressed under cold conditions, indicating a potential role in promoting survival under stressful environments, which warrants further investigation. *TTLL4* accumulation in organelles can induce hyperglutamylation-related neurodegeneration, as observed in mice, suggesting a negative response to harsh conditions (Bodakuntla *et al*., 2021).

Cold tolerance in insects often relies on lipid accumulation, which can lower the freezing point of cells and protect against winter-induced damage (Trenti *et al*., 2022). In honey bees, *apolipoprotein D* expression increases under cold stress, potentially influencing hemolymph viscosity and circulation. Similarly, *Abra* plays an important role in supporting blood flow in human (Troidl *et al*., 2009). *HR38* has been shown to regulate carbohydrate metabolism during mosquito reproduction (Dong *et al*., 2018).

Overall, extreme temperatures induce dynamic transcriptomic changes in honey bees. Under heat stress, HSPs facilitate protein folding and confer heat tolerance, whereas under cold stress, calcium signaling and lipid metabolism contribute to reducing cellular freezing points and enhancing cold tolerance. *TTLL4* appears to mediate negative effects under harsh conditions. This transcriptomic analysis provides fundamental insights into the physiological mechanisms that enable honey bees to withstand extreme temperatures.

CCD has caused widespread damage to honey bee populations worldwide, yet its precise causes remain unclear. Climate change, pesticide overuse, and honey bee pests have been suggested as potential contributing factors. In this study, we investigated the comparative transcriptomic responses of honey bees under different temperature conditions to elucidate the effects of thermal stress, which is closely related to climate change. Collectively, these findings offer a valuable foundation for understanding honey bee thermal adaptation and have important implications for mitigating CCD.

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