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In the battle of survival: transcriptome analysis of hypopharyngeal gland of the *Apis mellifera* under temperature-stress

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Abstract

Background Temperature is one of the essential abiotic factors required for honey bee survival and pollination. Apart from its role as a major contributor to colony collapse disorder (CCD), it also affects honey bee physiology and behavior. Temperature-stress induces differential expression of genes related to protein synthesis and metabolic regulation, correlating with impaired gland function. This phenomenon has been confirmed in mandibular glands (MGs), but not in Hypopharyngeal glands (HGs), potentially affecting larval nutrition. RNA-seq analysis was performed using HGs tissue at low (23 °C), regular (26 °C), and high (29 °C) ambient temperatures. This study aims to decode molecular signatures and the pathways of the HGs tissue in response to temperature-stress and the rapid genetic changes that impact not only royal jelly (*RJ*) production potential but also other biological functions related to HGs and beyond.

Results From the analyzed RNA-seq data, 1,465 significantly differentially expressed genes (DEGs) were identified across all the temperature groups. Eight genes (*APD-1*, *LOC100577569*, *LOC100577883*, *LOC113218757*, *LOC408769*, *LOC409318*, *LOC412162*, *OBP18*) were commonly expressed in all groups, while 415 (28.3%) of the total genes were exclusively expressed under temperature-stress. The DEGs were categorized into 14 functional groups and significantly enriched in response to external stimuli, response to abiotic stimuli, and protein processing in the endoplasmic reticulum (ER). Pathway analysis of exclusively temperature-stressed DEGs revealed that these genes promote ECM-receptor interaction and fatty acid metabolism while reducing protein processing in the ER, which is related to royal jelly (*RJ*) production and overall nutrition. Although heat-shock protein 90 and gustatory receptor 10 serve as markers for stress and hypopharyngeal glands (HGs) development respectively, their expression varies under temperature-stress conditions.

Conclusions We conclude that with the recent effects of climate change and its contributing factors, honey bee pollination, and reproduction activity is on the verge of halting or experiencing a detrimental decline. Considering the impact of temperature-stress on the expression of the nutritional marker gene (*GR10*), silencing *GR10* in HGs tissue

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could provide valuable insights into its significance in nutritional performance, survival, and beyond. Finally, a broader temperature range in future experiments could help derive more definitive conclusion.

Keywords *Apis mellifera*, DEGs, Temperature-stressed genes, Honey bee, Metabolism, Protein processing, ECM-receptor, *GR10*, *HSP90*

Background

Honey bees are cosmopolitan insects, that contribute to the pollination services [1, 2]. Their population has been declining due to colony collapse disorder (CCD), which is also a massive contributing factor to the abrupt disappearance of honey bee colonies [3, 4]. CCD has caused severe negative impacts due to the decline of pollination [5]. This can affect the internal and external activities of honey bee colonies, including behavioral performance, disease susceptibility, production, and honey bee development [6–8]. The development of honey bees however is influenced by many abiotic factors such as environmental temperature [9]. CCD mechanism remains unknown, although other possible factors such as pesticides, infectious pathogens, genetic factors, and temperature can be considered [10, 11].

Climate change appears to be one of the major factors in temperature changes and a major concern for agriculture and beekeeping [12]. It is one of the possible contributors to the decline in pollinators, including honey bees [13]. Higher air temperatures elevate colonial net gain rates and increase the efficiency of honey storage rates by lowering metabolic rates [14]. Ambient air temperature also affects the honey bee flight activity [15]. However, there is growing interest regarding climate impact on honey bee mortality and social behaviors such as nursing and foraging [16, 17]. For example, climate change comes with instant temperature variation (Higher and Lower) resulting in "temperature-stress". Temperature-stress induces different expressions of genes related to protein synthesis and metabolic regulation in insects, and protects the body from thermal damage [18]. Changes in gene expression correlate with impaired gland function, potentially affecting larval nutrition [19]. This was confirmed in mandibular glands (MGs), but not in hypopharyngeal glands (HGs) [19]. HGs and MGs are the two exocrine glands for royal jelly (*RJ*) secretion, which is the main source of food for larvae [20]. Therefore, understanding the impact of climate change on honey bees necessitates a closer look at the organs directly involved in colony survival, such as the HGs, which are critical for brood care.

HGs in honey bees are paired glands that develop as bees develop [21]. They are exclusively located in the head region of worker bees and play a significant role in various social behaviors through the production of different secretions [22, 23]. Each gland consists of small oval bodies called acini that are connected to terminal or axial secretory ducts. The HGs usually become fully developed in young workers (6–13 days) with large functional secreting acini [24, 25]. Once the honey bee starts foraging behavior, the glands degenerate [26]. Honey bee development affects gland activity from producing the primary *RJ* proteins in young nurse bees to manufacturing carbohydrate-metabolizing enzymes like α-glucosidase in forager bees [27]. *RJ* is not just a protein-based food fed to larvae but also helps to produce healthy queens [28]. However, these glands are vulnerable to various stresses such as *Varroa* infestation and heat, which may result in the reduction and degeneration of the gland, and subsequently their biological and genetic functions [29].

Temperature stress is one of the major factors affecting honey bee mortality, however, survived through morphological changes [12]. Summer foragers exposed to extreme heat stress show less HGs activity than nurses who are not exposed to the same stress [30]. The reduced HGs activity resulted in a low protein synthesis rate or decomposition [31]. This results in slowing down or repairing the damaged proteins by increasing the content of protective substances [32, 33]. Protective substances like heat shock proteins (*HSP*s) play a vital role in the stress responses of many organisms [34]. They act as a molecular chaperone to protect the protein from damage during protein synthesis, and folding [35]. *HSPs* of various sizes and functions exist including *HSP60*, *HSP70*, and *HSP90* [36]. On the other hand, superoxide dismutase (*SOD1*) and Vitellogenin (*VG*) have also been suggested as candidates for a stress marker whereby they provide a protective role against oxidative stress and control by a juvenile hormone, which is also related to stress responses [37, 38]. Further, as the size of HG is known to be related to nursing behavior [39], gustatory receptor 10 (*GR10*) is equally known to be related to nursing behavior [40]. Therefore *GR10* expression under temperature-stress might explain the HG performance related to the nursing behavior. Overall, heat stress triggers the expression of various genes that are involved in some biological processes including metabolism, and translation [41]. Surprisingly, little research has been performed on the relationship between climate change and the genetic functions of honey bees concerning HGs performance [42]. Especially for its nutritional function related to nursing behavior, and biological adaptation, under various unfavorable conditions such as temperature. Although Feng et al. described the protein profile of

HG within the whole range of development under natural conditions, suggest verifying it by the transcriptome approach for a comprehensive understanding of HG development [43].

Transcriptome analysis provides a comprehensive view of gene expression changes in response to environmental stressors, making it an ideal tool for identifying molecular pathways affected by temperature stress in HGs. Therefore, we observed the HG development, transcriptomes, and honey bee survival rate under various temperature-stress and compared the transcriptome of *Apis mellifera* (*A. mellifera*) HGs at Low-temperature, High-temperature, and Regular-temperature using RNA sequencing technology and detected the sequence pattern of differentially expressed genes (DEGs). Our data aim to compare the DEGs from one another among the three conditions to better understand the regulatory mechanisms in honey bees' physiological and biological activities especially related to the HG nutritional and survival activities. Our result identified important temperature-stressed genes and canonical pathways that affect HGs' biological and molecular activities. The changes in the gene expression in the varying temperatures can be related to thirteen functions other than its traditional functional role in royal jelly production.

Methods

Rearing condition of *Apis mellifera*

The *A. mellifera* used in this study were bred at the Incheon National University Apiary in June 2019, a honey bee breeding facility equipped with an enclosed space designed to maintain a stable temperature. The ambient temperature of space was regulated at an average monthly temperature ± 3 °C to investigate the physiological response of bees to climate change factors. To monitor the environmental conditions inside the bee colony, temperature/humidity sensors (BO-H100, Aosong Electronics, China, -40 ~ 80℃ (± 0.5℃), 0 ~ 99.9% (± 3%)), and a carbon dioxide sensor (BO-C100, Winsen Electronics Technology, China, 0 ~ 10,000ppm (± 50ppm + 5%)), were installed. The larval development period was investigated by inducing the queen bee treated in the ambient-temperature range monthly average temperature ± 3 °C) to lay eggs, and then observing the opening of the larval chamber. After emergence, 30 newly emerged bees were collected and transferred to wood frame cages (W10cm × L12cm × H15cm) made for longevity testing. Honey bees were reared and the development period from eggs to adults was investigated in an incubator under the temperature of 33 ± 1 °C and humidity of 55 (± 5) %, surrounded by three different ambient temperature conditions 23 °C, 26 °C, or 29 °C until eclosion and adulthood. The honey bees were maintained by being fed 50% sugar solution and pollen rice cake as food during the experimental period. All the inside and outside hive temperatures were properly maintained, with a proper relative humidity both in the center and surrounding of the hive as mentioned in (Supplementary Table S1). To maximize the impact of temperature-stress, honey bees were further, exposed to a wider ambient temperature range of 25 °C and 35 °C. This was proceeded for validating the expression of some selected stress marker genes using the qPCR method. A control sample that was not exposed to any temperature stress factor was used.

Hypopharyngeal gland measurements

Hypopharyngeal glands (HGs) from developed workers (Nurse bees) were dissected. The development stage was properly monitored both at pupa and adult stages (Supplementary Fig. S2). The acini size was majored using the area of the 9 acini (mm^2) using the Leica Applications Suite v.3.8.0 software (Supplementary Fig. S3). All experiments were repeated three times in June, July, and September 2019.

Statistical analysis

The statistical analysis was conducted for developmental stages, acini size, and survival rate. Kaplan-Meier analysis and post-hoc log-rank test were used to derive and compare overall survival and temperature variation curves between the groups (Supplementary Fig. S1). Multivariate analysis for survival was performed using the Cox-Mantel Model. A p-value of < 0.05 was considered to indicate statistical significance. Graphs were created using GraphPad Prism software v7.03.

RNA extraction and cDNA synthesis

RNA was extracted from each temperature condition Nurse bee (Three for each temperature condition) by dissecting hypopharyngeal glands (HGs) at -75 ℃ to -80 ℃ using dry Liquid Nitrogen to prevent denaturation by RNeasy® Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. cDNA was synthesized from 1 µg of RNA. First, DNase treatment of RNA samples before RT-PCR was performed by RQ1 RNase-Free DNase (Promega, WI, USA). Then reverse transcription was performed with oligo dT with Superscript III enzyme (Invitrogen, CA, USA) (Supplementary Table S2).

RNA sequencing and quality control

The total RNA of each temperature sample (Three replicates per group) was prepared at 2 µg per sample before experiments. RNA Quality control was examined with 2100 Expert Bioanalyzer (Agilent Technologies, CA, USA) using mirVana™ miRNA Isolation Kit (Ambion, TX, USA), and RNA 6000 Nano Kit (Agilent Technologies, CA, USA) according to the manufacturer's protocol. Once all samples passed, library preparation was

performed by Truseq Stranded mRNA Library Prep kit (Illumina, CA, USA). Sequencing was performed on the Novaseq 6000 (Illumina, CA, USA) in 100 bp PE configuration according to the manufacturer's instructions. Quality control of raw reads was performed through FastQC v0.12.0 [44]. Adapter sequences were trimmed out and low-quality reads were discarded using Trim Galore v0.6.7 [45]. Trimmed reads were then mapped to the reference genome (NCBI RefSeq: GCF_003254395.2 of Amel_HAv3.1) for gene mapping related to *Apis mellifera* using the default parameters [46].

Differentially expressed genes (DEGs) analysis

Gene expression level Fragments Per Kilobase of transcript per Million mapped reads (FPKM) value was measured with CLC Genomics Workbench 11.0 (QIAGEN, Germany) using RNA-seq analysis tools. Fold change value (> 1 fold) and p-value < 0.05 were analyzed by calculating the Normalized FPKM value using the Differential Expression for RNA-Seq tool using DESeq2 v1.34.0 package in R Studio. The differentially expressed analysis was performed by comparing the three temperature groups. EnhancedVolcano R-package was used to generate a volcano plot for significant DEGs, and Venny 2.1 was used to identify the commonly shared genes between the three groups.

Functional analysis

GO (Gene Ontology) terms including BP (Biological process), CC (Cellular component), and MF (Molecular function) as well as KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway ID were identified using DAVID (Database for Annotation Visualization and Integrated Discovery) 2021 tool [47]. Genes were also classified into 14 categories by function through a literature review. The log2FC value of RNA-seq data ranked the genes, and the ggplot2 R-package was used to present the GO terms. Pheatmap R-package was used for heatmap construction.

Construction of protein-protein interaction network

The PPI (protein-protein interaction) network was constructed using the Search Tool for the Retrieval of Interacting Genes/Protein (STRING) v11.5 database [48]. The network was visualized using the STRING plugin from Cytoscape v3.9.1 for network analysis [49]. Cytoscape plugins including CytoHubba v0.1 for the identification of highly connected genes (Hub-genes) based on their degree of connectivity were used.

Quantitative RT-PCR

Following the above-mentioned protocol for HG-tissue RNA extraction. The HG samples from 25 °C to 35 °C were processed for RT-PCR validation. Quantitative RT-PCR was performed using the StepOnePlus machine (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocol with SYBR green qRT-PCR measurement for each gene repeated with three independent biological samples, and quantitative analysis was conducted by StepOne plus Software V.2.0 (Applied Biosystems). The transcript level of the gene was calculated by the standard curve method. The primer sequences for the *HSP70*, *HSP90*, *SOD1*, and *GR10* were mentioned in (Supplementary Table S3).

Results

Survival rate and morphological development

Under the variable temperature condition described (Supplementary Table S1), there was a low survival rate of the HGs tissue when exposed to higher and lower ambient temperatures. On the other hand, a high survival rate was observed at regular ambient temperature with a statistically significant result **(Supplementary Fig. S1)**. The acini size checked in adults shows faster development when subjected to a high temperature as compared to the other two conditions **(Supplementary Fig. S2)**. This indicated the effect of high temperature-stress on acini development. This result remained the same both in pupa and adult honey bee's development, indicating less development at high-temperature stress (Supplementary Fig. S3). Overall, both low and high temperature affects the honey bee's development as well as survival rate.

Identification of differentially expressed genes

A DEGs (Differentially Expressed Genes) analysis was conducted on 10,612 genes from all three groups (comparing Low-temperature with High-temperature, Regular-temperature with High-temperature, and Regular-temperature with Low-temperature, respectively) among 12,332 genes registered in Amel_HAv3.1. Volcano plots for the DEGs showed different genetic profiles between each group (Fig. 1A-C). In addition, the Venn diagram represented the combination of DEGs (> 1 fold change and p-value < 0.05) used for the downstream analysis. Therefore, 8 genes were identified as commonly differentially expressed genes (cDEGs) shared among the three groups (Fig. 1D). DEGs from the three groups indicated more down-regulated genes in regular versus low and regular versus high-temperature groups, while more up-regulated genes were observed between low versus high-temperature groups (Fig. 1D). Further, DEG results from the three groups were classified into fourteen categories based on their functions identified through a literature review. 40 Up-regulated genes and 39 Down-regulated genes were identified when comparing the low-temperature and high-temperature groups (Fig. 2A-B). Their collective functions were evaluated and presented based on the Average Log_2 fold change. These included neurons, major royal jelly protein (*MRJP*), and

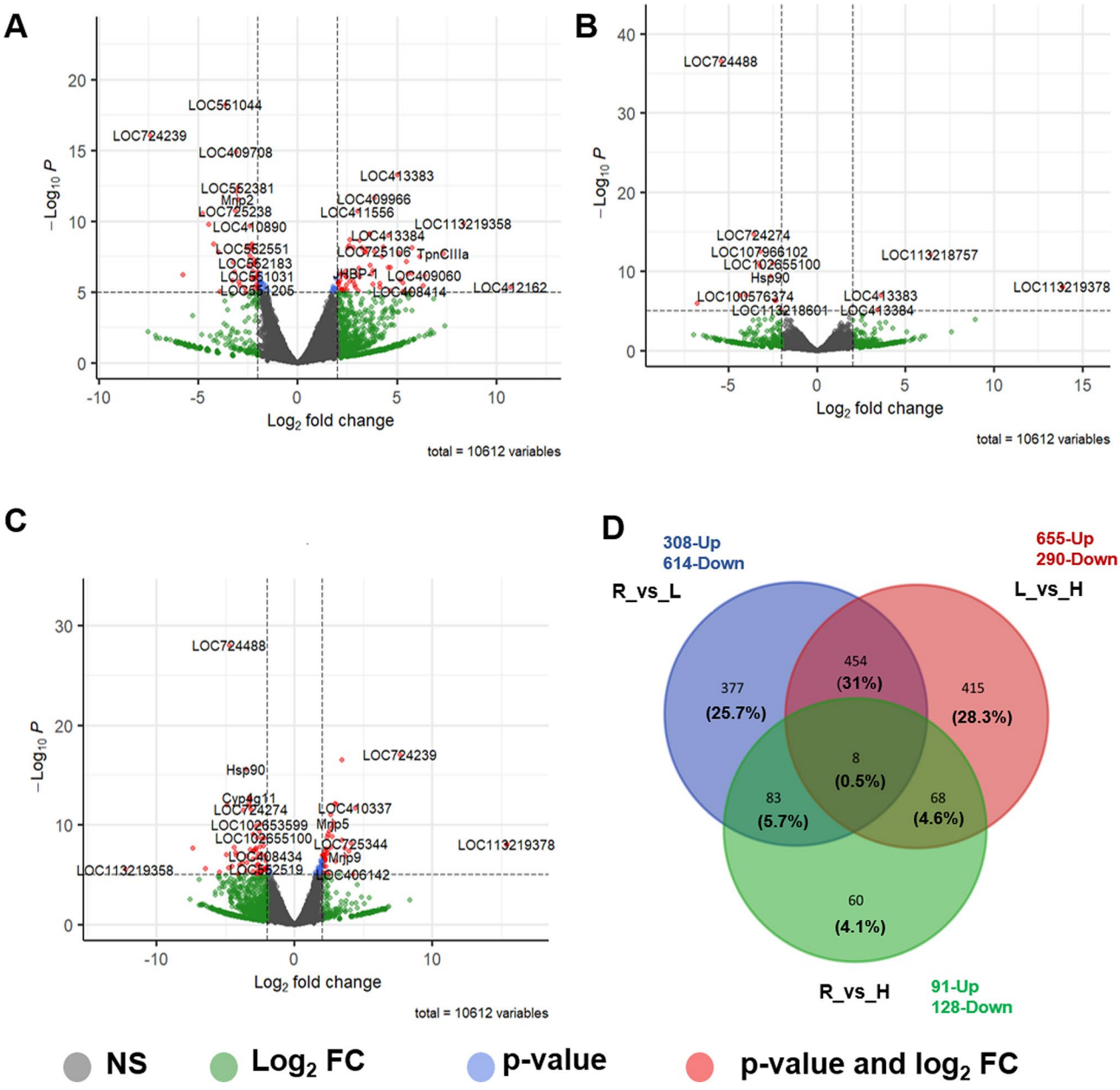

Fig. 1 **A** Enhanced volcano plot of differentially expressed genes identified between the Low-temperature group and High- temperature group. **B** Enhanced volcano plot of differentially expressed genes identified between the Regular temperature group and High-temperature group. **C** Enhanced volcano plot of differentially expressed genes identified between the Regular-temperature group and Low-temperature group. Green color dots indicate differentially expressed genes (DEGs) based on Log2 fold change >1. Blue color dots indicate significant genes based on the p- vahie >0.05. Pink color dots indicate genes with -value and log2 fold change (FC). Genes with positive log2 FC are up-regulated, while genes with negative log2 FC are down–regulated. **D** The Venn diagram indicates the shared genes between the groups. Genes are included based on their fold change >1 and p-value of 0.05. The total number of up- regulated and down-regulated genes from each group was indicated

gustatory receptor (*GR*) related genes as down-regulated. Honey, zinc finger protein, and immune-related genes are up-regulated (Fig. 2C). Also, 7 up-regulated genes and 10 down-regulated genes were identified when comparing the regular-temperature and high-temperature groups (Fig. 3A-B). Their collective functions were evaluated and presented based on the Average Log_2 fold change. These included stress, heat shock protein (HSP), and Gr-related genes as down-regulated, while neuron protein (NP), honey, immune, and zinc finger protein-related genes as up-regulated (Fig. 3C). Further, 38 up-regulated genes and 32 down-regulated genes were identified when comparing the regular-temperature and low-temperature groups (Fig. 4A-B). Their collective functions were evaluated and presented based on Average Log_2 fold change. These included honey, *HSP*, hormone, and

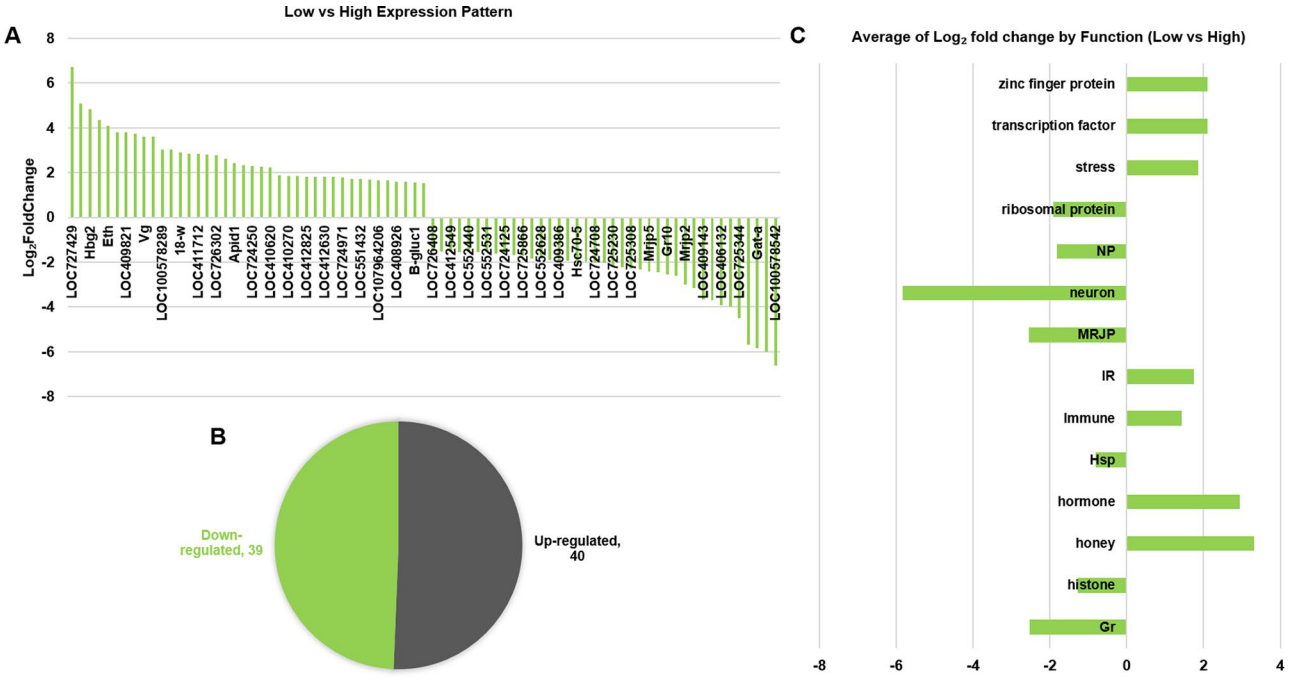

Fig. 2 Identified genes based on 14 essential functions among Low vs High-temperature DEG groups. **A** Shows a list of up-regulated genes and down-regulated genes. **B** Shows the number of the up-regulated and down-regulated genes. **C** Shows their fold change variation among the functional genes

immune-related genes as up-regulated, while neuron, *MRJP*, histone, and ribosomal protein-related genes as down-regulated (Fig. 4C). Overall, no common functional gene was found between the three groups although certain genes were shared between one another (Supplementary Fig. S4A-B).

Functional enrichment analysis for common DEGs

The DAVID was used to annotate the cDEGs found in at least two groups based on their (BP), (CC), and (MF). Top ten (10) highly enriched gene ontology (GO) terms were represented including mitochondrial translation (GO: 0032543), and G-protein coupled receptor signaling pathway (GO: 0007186) for BP. CC-GO terms include sarcomere (GO: 0030017), myofibril (GO: 0030016), and contractile fiber part (GO: 0044449) among others. The top ten (10) enriched MF include translation elongation factor activity (GO: 0003746), lipid transporter activity (GO: 0005319), and calcium ion transmembrane transporter activity (GO: 0015085) among others (Fig. 5A). KEGG was analyzed, and six (6) enriched pathways were found including glycan degradation (ame00511), ascorbate and aldarate metabolism (ame00053), longevity regulating pathway (ame004213), and protein processing in endoplasmic reticulum (ame04141) among others (Fig. 5B).

Commonly differentially expressed genes

The 8 commonly shared genes expressed in all three groups were identified. These include *APD-1*, *LOC100577569*, *LOC100577883*, *LOC113218757*, *LOC408769*, *LOC409318*, *LOC412162*, and *OBP18* were presented together with their respective expression values among the three groups (Table 1. Their respective functions were evaluated (Fig. 6). *LOC113218757* remained up-regulated irrespective of the temperature change. All eight genes were exclusively up-regulated under temperature-stress conditions of Low versus High-temperature. *APD-1* (Apidermin 1), *LOC100577883* (Cytochrome P450 4aa-like), *LOC408769* (Excitatory amino acid transporter), *LOC409318* (Kv channel-interacting protein 2like), and *OBP18* (Odorant binding protein 18) remained exclusively up-regulated only under Low versus High-temperature stress.

Protein-protein interaction analysis to identify hub genes

Using the PPI network analysis, highly connected genes (Genes commonly found in at least two groups) from the large set of DEGs were identified. Hub genes were pinned by using *A. mellifera's* specific species name in constructing the gene interaction network. From the PPI network, 450 nodes with 489 edges were identified. The topological assessment analysis from the network revealed 10 hub genes (*LOC408328*, *LOC410200*, *HSC70-4*, *LOC552531*, *HSC70-5*, *LOC552572*, *LOC410620*, *RPL39*, *LOC411351*, and *HSP90*) using degree matrics (Table 2; Fig. 7).

Functional analysis for temperature-stressed genes

The cDEGs observed above contained common responsive genes in response to temperature-stress (Low/ High/

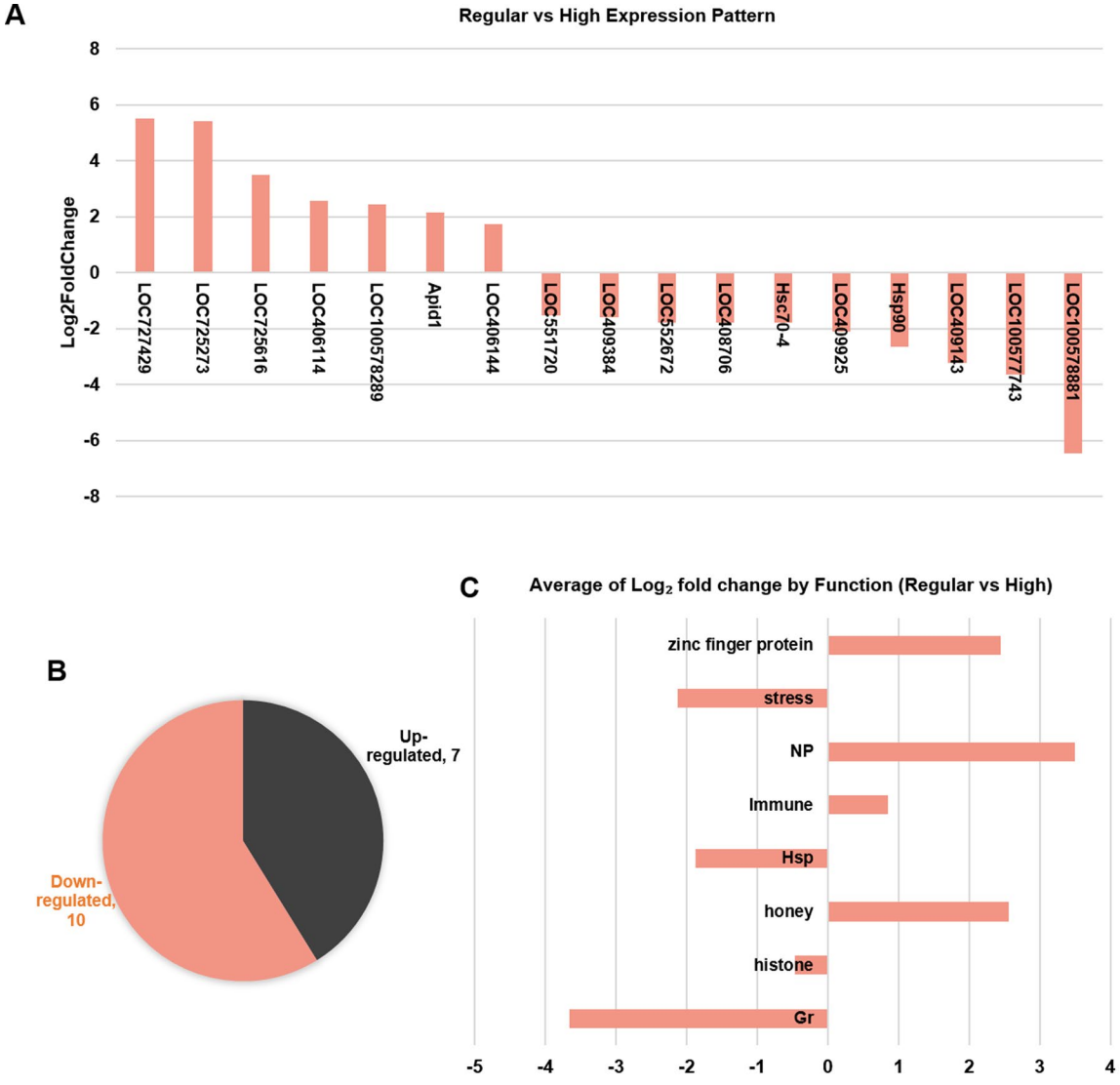

Fig. 3 Identified genes based on 14 essential functions among Low vs High-temperature DEG groups. **A** Shows a list of up-regulated genes and down-regulated genes. **B** Shows the number of the up-regulated and down-regulated genes. **C** Shows their fold change variation among the functional genes

Regular), however, more curiosity is developed regarding the specific genetic response of honey bees under low versus high-temperature stress conditions. Having said that, temperature-stressed genes identified only from the Low Vs High-temperature group and not shared between the other two groups were evaluated. The Venn diagram from (Fig. 1D) identified 415 exclusively unique genes. Among them, 286 were up-regulated, while 129 were reported down-regulated. GO-terms of these genes were evaluated based on their expression and show the up-regulated genes are enriched in cell adhesion, biological adhesion, and neurogenesis among the enriched BP. The extracellular region and focal adhesion were among the enriched CC. For MF, metalloendopeptidase activity, and calcium ion binding among others are among the highly enriched terms. Down-regulated genes were found to be highly enriched in protein folding, peptide metabolism, and protein processing among the enriched BP. The endoplasmic reticulum and signal peptidase complex were the identified enriched CC. The protein disulfide isomerase activity and unfolded protein binding were among the enriched MF. Further, KEGG pathway analysis revealed that down-regulated genes were enriched in the protein processing in the endoplasmic reticulum. While the up-regulated genes were enriched in ECM-receptor interaction, fatty acid metabolism, and Glycine, serine, threonine metabolism pathways respectively (Fig. 8A-D).

Validation of some stressed marker genes response

Stress markers including *HSP70* and *HSP90* as well as *GR10*, a marker for nursing behavior and HG development were analyzed under wider temperature-stress

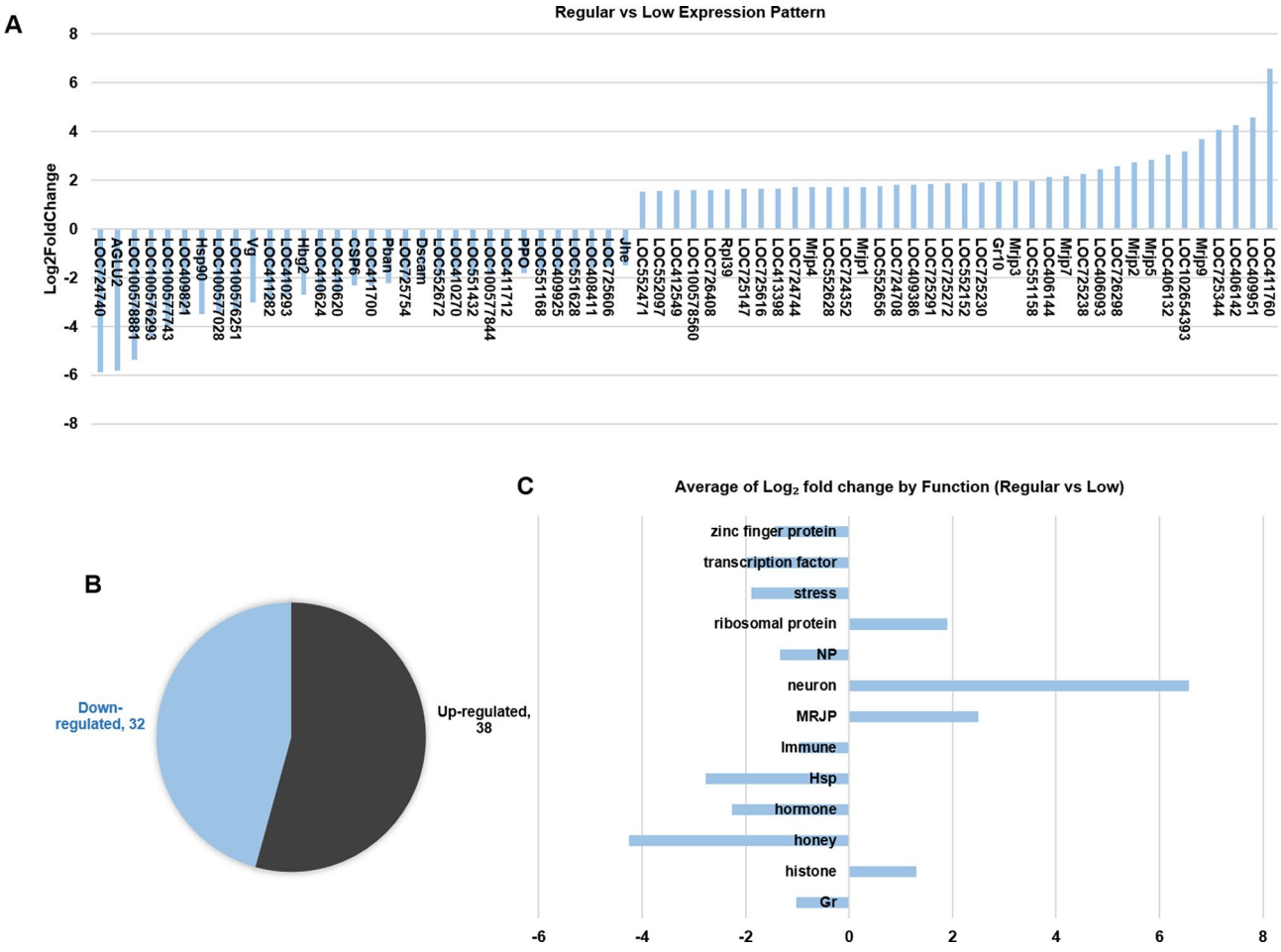

Fig. 4 Identified genes based on 14 essential functions among Low vs High-temperature DEG groups. **A** Shows a list of up-regulated genes and down-regulated genes. **B** Shows the number of the up-regulated and down-regulated genes. **C** Shows their fold change variation among the functional genes

conditions. In addition, *SOD1* was not found in our DEG data, however, it is relevance in terms of other stress factors like chemical-stress attracts our attention for comparing with the temperature-stress marker genes. The temperature difference in the DEG data was 6 °C between the low and high High-temperature, while a wider temperature gap of 10 °C was used for qPCR between the low and high temperatures. Their relative expression quantified using qPCR indicated their responses to temperature change. *HSP70* showed a low response when subjected to a higher temperature. Therefore no significance was observed concerning the temperature-stress condition. *HSP90* and *SOD1* showed response under temperature-stress conditions, although the relative response between the lower and higher temperatures were not that significant as compared with *GR10*. In *GR10*, high significant response was observed when subjected to a temperature-stress condition as shown in (Fig. 9). Their respective expression level from the DEGs analysis were equally compared (Supplementary Fig. S4).

Discussion

Discovery of functional genes

Although a wider temperature range might have a more significant impact on cellular activities and genetic modification, a smaller temperature-stress derived from a 6 °C interval significantly affects the biological function of HG by altering the expression of its nutrition and stress marker genes, as well as its overall development. The impact of abiotic factors, particularly temperature, on honey bees has been previously reported [15, 50]. At the individual level, temperature fluctuations cause honey bees to expose their brood to overheating inducing certain genes such as *HSPs* to resist any passive effect [51]. An increased level of *HSPs* was observed among worker bees exposed to a variable temperatures ranging from 4 to 50 °C, highlighting the influence of temperature on genetic changes in bees. This finding aligns with our results, which demonstrate the effect of temperature on the *HSPs* expression [52]. Accurate determining gene expression levels and gene functions is essential for

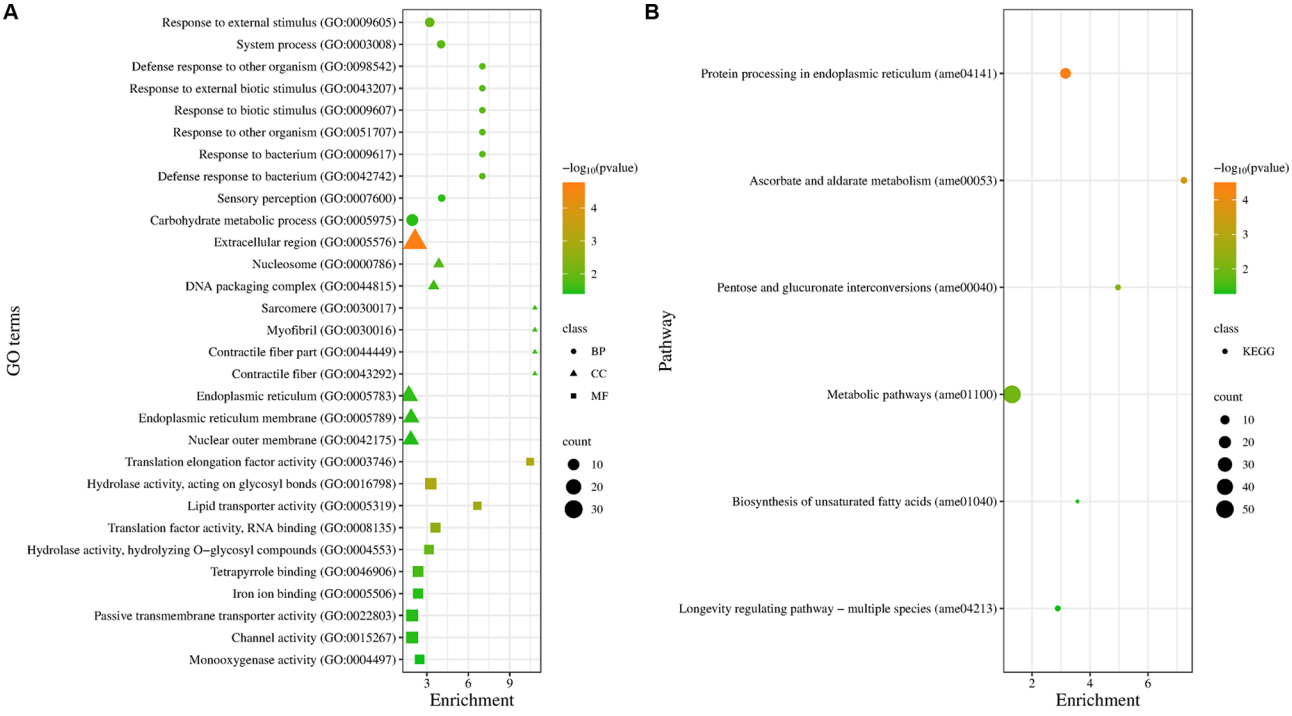

Fig. 5 **A** Bubble chart indicating the top ten (10) Gene Ontology (GO) enriched terms for the commonly differentially expressed genes (cDEGs) from at least two groups. Ulis includes BP; Biological process. CC; Cellular component. MF; Molecular function. Significant GO terms ar e based on the number of genes involved. **B** Bar chart for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the cDEGs found in at least two groups. Their significance was determined based on enrichment values and respective p-values

Table 1 List of eight (8) cDEGs regularly expressed in all three temperature groups with their respective Log_2 fold change values in each respective group. Positive values indicate up-regulated genes, while negative values indicate down-regulated genes. Their descriptions were investigated and mentioned under the description column

Genes	L_vs _H (Fold change)	R_vs_H (Fold change)	R_vs_L (Fold change)	Description
APD-1	2.5	-1.78	-4.29	Apidermin 1
LOC100577569	3.72	1.72	-1.99	Uncharacterized
LOC100577883	2.08	-3.2	-5.29	Cytochrome P450 4aa1-like
LOC113218757	4.65	6.4	1.75	Uncharacterized
LOC408769	2.41	-1.83	-4.24	Excitatory amino acid transporter
LOC409318	2.11	-5.43	-7.55	Kv channel-interacting protein 2-like
LOC412162	10.7	8.94	-1.75	Armadillo repeat-containing protein 4
OBP18	1.63	-1.85	-3.48	Odorant binding protein 18

identifying genes putatively involved in the physiological response to temperature-stress in nurse bees.

Previous research focused mainly on morphological, physiological, biochemical, age-dependent, and proteomics network analysis to track protein changes during the HGs development under natural conditions [43]. This provides a basis to verify the proteomic data by transcriptome profile of the HGs for more understanding of the HGs development. However, subjecting the *A. mellifera* HGs to various temperature-stress leads to significant genetic changes related to biological and nutritional activities. Under temperature-stress (Low Vs High-temperature group), more genes were up-regulated, while down-regulated genes were more prevalent in other temperature groups. Certain genes are only induced by stress factors and are more responsive to proteostatic stresses [53]. Similar to our findings, while considering the effect of thermal stress in inducing tissue damage, various signaling cascades are expected to be activated especially related to cell survival, apoptosis, and protein correction [54]. This can contribute to more gene proliferation under the temperature-stress. Moreover, previous research has shown that, cold stress but not heat stress, reduces stored sperm viability in queen honey bees, affecting survival and development [55]. The overall function of the DEGs indicates variation in expression pattern. For example, *MRJP* expression was reduced, highlighting the effect of temperature-stress on *RJ* production. On the other hand, increased expression of *MRJP* functional genes was noticed in the regular versus low-temperature group. Ribosomal proteins are related to *RJ* production since most protein synthesis takes

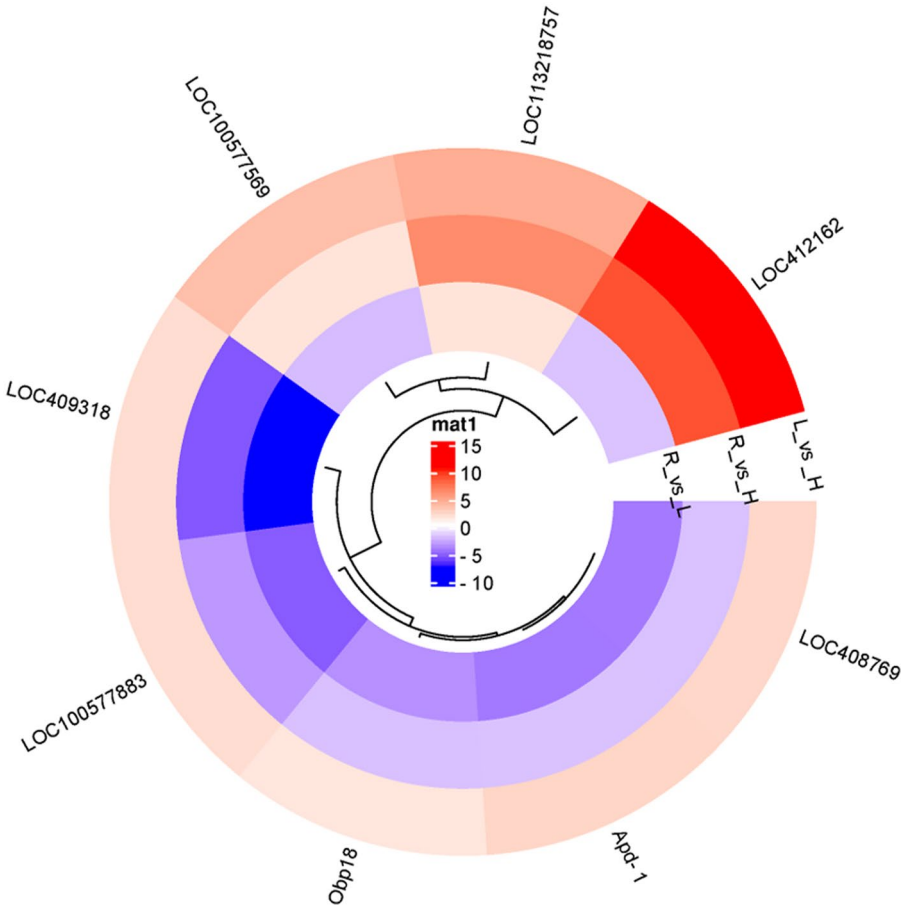

Fig. 6 Circular lieatmap showing the expression pattern of 8 cDEGs found nr all three groups. The red color indicates up-regulated genes, while the blue color indicates down-regulated genes. The list of commonly differentiated genes found among the three groups with then respective functions

Table 2 List of ten (10) hub genes identified from the protein-protein interaction (PPI) network derived using the complete set of differentially expressed genes (DEGs) in all three groups. The top 10 hub genes were generated based on their degree of connectivity matrix employed using the Cytoscape plugin (CytoHubba). Their respective expression pattern (Up-regulated or Down-regulation) derived using Log_2 fold change values in each respective group were shown. Their descriptions were investigated and mentioned under the description column and functions under the function column

Genes	L_vs _H Regulation	R_vs_H Regulation	R_vs_L Regulation	Description	Function
LOC408328	Down	Down	Up	Elongation factor Tu, mitochondrial-like	Uncharacterized
LOC410200	Down	Down	Up	mRNA turnover protein 4 homolog	Uncharacterized
HSC70-4	Down	Down	Down	Heat shock protein cognate 4	Uncharacterized
LOC552531	Down	Down	Down	10 kDa heat shock protein, mitochondrial	Uncharacterized
HSC70-5	Down	Down	Up	heat shock protein cognate 5	Uncharacterized
LOC552572	Down	Down	Up	Eukaryotic translation initiation factor 5 A	Uncharacterized
LOC410620	Up	Down	Down	Heat shock protein Hsp70Ab-like	HSP
RPL39	Down	Up	Up	Ribosomal protein L39	Ribosomal protein
LOC411351	Down	Up	Up	Elongation factor G, mitochondrial	Uncharacterized
HSP90	Down	Down	Down	Heat shock protein 90	HSP

place in the ribosome. From our definitive finding, their expression pattern was exactly similar to the *MRJP* in all three conditions. This supports a similar finding stating thermal stress results in alterations in genes involved in ribosome biogenesis [56]. Ribosome biogenesis requires a series of cellular mechanisms, including protein folding before the final stage of production. This important cellular cascade involved the *HSPs* primarily serving as molecular chaperones, aiding in the folding of proteins, averting protein aggregation, and guiding misfolded proteins toward specific degradation pathways [57]. In the honey bee, the enhanced expression of *HSPs* was

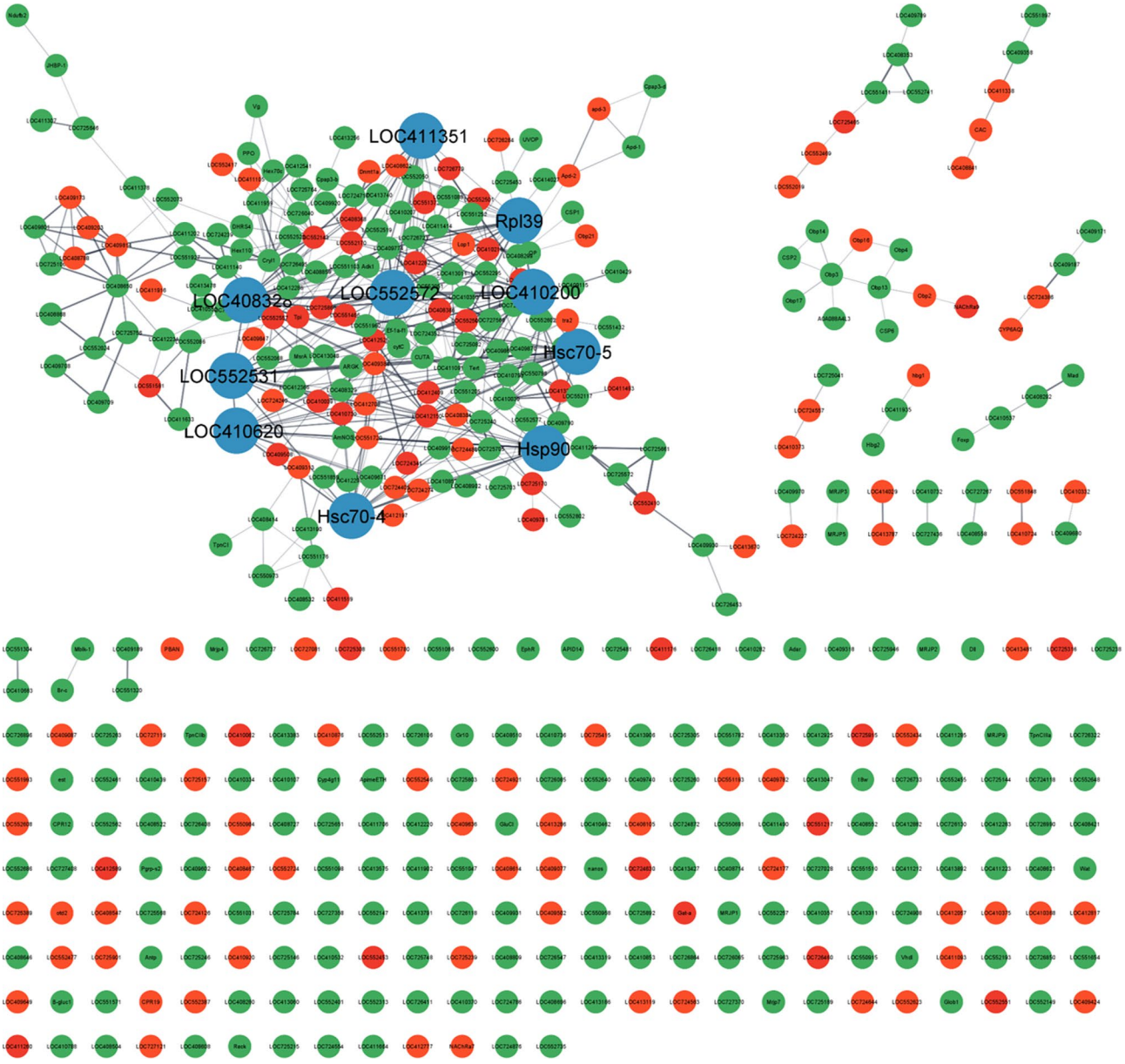

Fig. 7 **A**Protein-protein interaction (PPI) network (Cytoscape view). Green color nodes represent up-regulated genes from all groups. Red color nodes represent down-regulated genes from all groups. Blue color nodes represent the ten (10) hub genes. The expression level of the hub genes has been explained in Table 2 together with their functions

reported under temperature-stress conditions, reported as a defense mechanism against stress conditions [51, 58]. *HSPs* have been widely identified in worker larvae, embryos, the head and brain of workers, the hemolymph, and the venom gland respectively [59, 60]. However, their presence in HGs suggests their role in cell maintenance and protein synthesis related to *RJ* secretory activity, for honey bee survival and nursing activities. Interestingly, most of the identified hub genes belong to the *HSP* family including the *HSP70*, and *HSP90*. Overall, honey bee *HSPs* are responsive to various proteostatic stresses and potentially promising biomarkers of honey bee stress [53].

From our findings, temperature-stress affects the Gustatory receptor (*GR*) gene family expression negatively. *GR10* a family member of *GR* was found primarily involved in nursing behavior in honey bees, explaining how the *GR* protein family in social insects mediates the synthesis of *RJ* and contributes to behavior in the hive [61]. As HG development is correlated with honey bee age and social role, *A. mellifera GR10* (*AmGR10)* knockdown accelerated the transition of honey bees from nursing to foraging establishes a correlation between

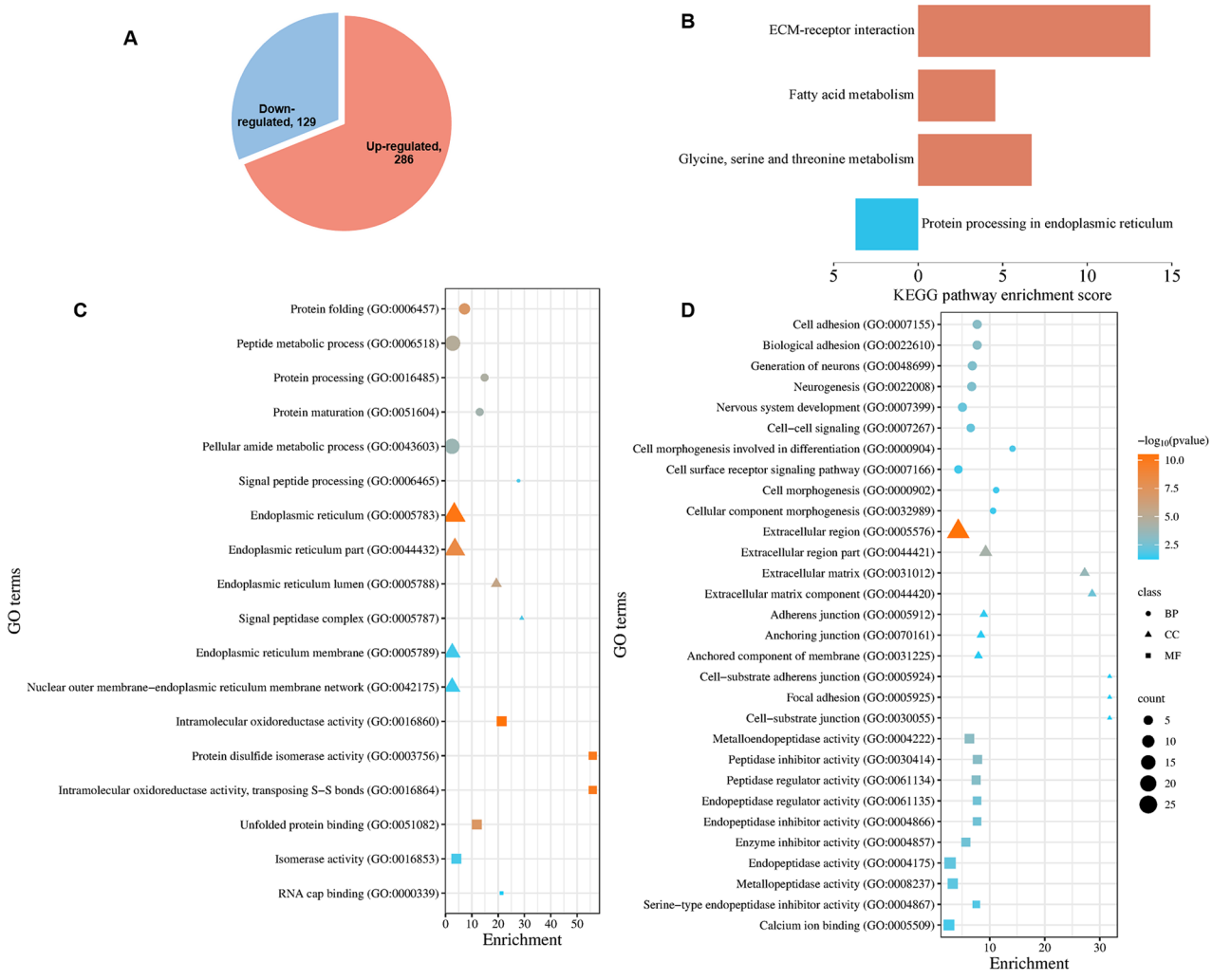

Fig. 8 **A** Pie-chart showing the number of up-regulated and down-regulated genes found exclusively under the Low versus High temperature-stress group. **B** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicates the significant pathways. The blue-color represents enriched pathways from the down-regulated genes, while the brown-color represents enriched pathways from the up-regulated genes. Gene Ontology (GO) terms include their BP: Biological process. CC; Cellular component. MF; Molecular function for the down-regulated genes **C** and up-regulated genes **D**. Their significance was determined based on enrichment values and respective p-values

AmGR10 and HG development [61]. *GR*s expressed in insect taste neurons, signal the presence of carbohydrates, sugar alcohols, and bitter compounds [62]. Their functional role decreases in all temperature conditions. *GR10* in particular shows a significant low expression in Low versus High-temperature conditions. The qPCR data verified its responsiveness with temperature-stress as highly significant. Together, the down-regulation of *GR10* under temperature-stress conditions might affect HG's nursing behavior and honey bee nutrition in general. Other genes such as *SOD1* which is highly responsive to pesticide-stress were absent under temperature-stress [63]. This might indicate gene peculiarity with stress factors and or tissue role.

A high immune activity was observed in HG under temperature-stress conditions. This correlates with a significantly high expression of Vitellogenin (*VG*). *VG* serves as a marker for bee health [64]. It plays a role in the transfer of immune elicitors from the gut to HG in *A. mellifera* [65]. This emphasizes the *VG* protein immunoreactivity in the midgut and HG under temperature-stress. On the other hand, low immune functional genes were observed in the Regular versus Low-temperature group, which equally correlates with low expression levels of *VG*. It is worth knowing that, the fat body is the primary site of *VG* synthesis and storage [66]. It regulates the metabolic and immunological state of the organism and plays a central role in immune response [67]. With that, *VG* can be a central player mediating between fat body and HG for a cross-talk related to nutritional activity and honey bee health in a gut-brain-axis manner. A statement which was confirmed by Virginia et al., states

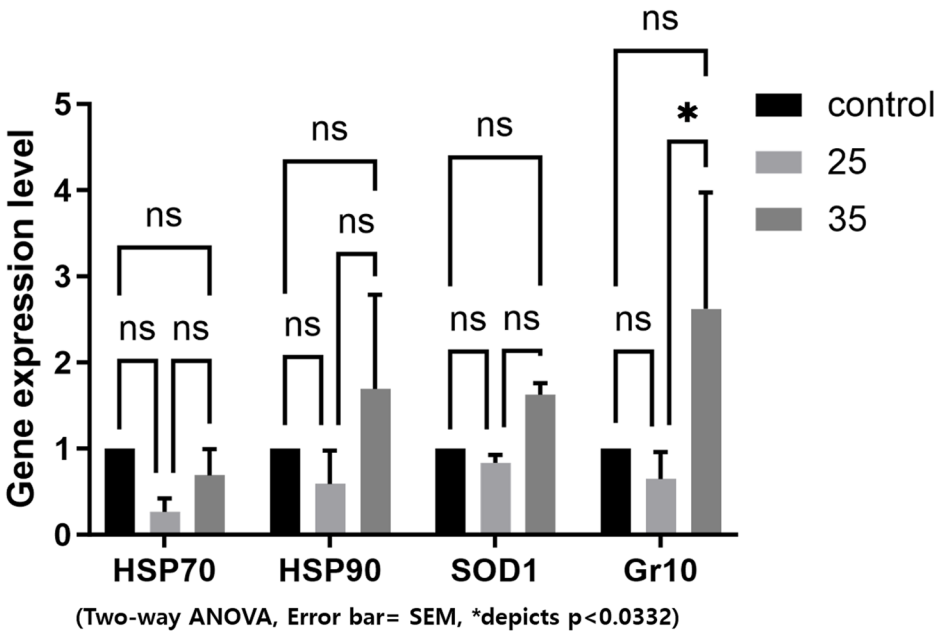

Fig. 9 **A** Effect of high, temperature-stress on hypopharyngeal glands (HGs) tissue for it is marker genes. Eleat shock protein 70 (HSP70), Heat shock protein 90 (HSP90), and superoxide dismutase (SOD1) are stress markers. While gustatory receptor 10 (GrlO) is an HGs marker for nursing behavior and development. RNA samples from HGs subjected to temperature of 25°C and 35°C with control were pooled with 3 samples from each group respectively. Their expressions were calculated (Two-way ANOVA, Error bat= SEM, * depicts p>0.0332)

that *VG* receptor performs the transport of *VG* to the HGs contributing to the understanding of it is role in the social context of honey bees [68].

Commonly differentially expressed genes (cDEGs)

In the past, proteomics and transcriptomic analysis in Chinese white wax insects (*Ericerus pela*) from various climatic regions found 2386 DEGs, involved in catalytic activity, and response to stimuli [61]. Similar to our finding, response to external stimuli was the most enriched BP among cDEGs from at least two groups. The effect of temperature in HG among 1465 significantly identified DEGs leading to a higher number of up-regulated genes from the temperature-stress group only. The affected genes among cDEGs were enriched in translation elongation factor activity and Lipid transporter activity as an essential MF related to protein synthesis. This is similar to other findings indicating 12 identified proteins enriched in protein and lipid metabolism related to HG activity [43]. The endoplasmic reticulum emerged as the predominantly enriched pathway impacted, serving as a site for the processing of proteins. This is followed by metabolic pathways. It is not surprising the detected genes are involved in various metabolic pathway processes as the main energy source of the honey bee is glucose, fructose, sucrose, and maltose [69]. In addition, the observed upregulated genes involved in metabolic processes and protein folding suggest activation of stress-response pathways, potentially mediated by transcription factors such as *HSF1* (Heat shock factor 1). Failure of HG to maintain the nutrient sources can affect the nurse's bee biological activity, survival, and even longevity. Data from our results shows a decline in survival rate under high, and low-temperature conditions. This is reflected in our enriched pathway result showing the temperature effect on longevity regulating pathways (ame04213). This is similar to Bach et al. findings, indicating the impact of thermal stress in inducing tissue damage and a broad shift in regenerative signaling pathways, suggesting a shared program of damage response [54].

The present study identified eight cDEGs that are consistently expressed across all groups (Table 1) and perform various functions, all of which are up-regulated in temperature-stress conditions. Cytochrome P450 acts in the detoxification process of phytochemicals in honey, pollen, and mycotoxins in the hive environment [70]. Involvement of various Cytochrome enzymes in the detoxification activity under natural conditions may be used as a stress indicator upon pesticide exposure [71]. With our current result, we also propose the role of Cytochrome P450 as a temperature-stress indicator. Apidermin 1 exhibits antibacterial and antifungal activities, by inducing structural damage through binding to bacterial and fungal cell walls [72]. In a previous study, *APD-1* was identified as a highly expressed gene in the heads of newly emerged bees, correlated to its current expression in the HGs [73]. The excitatory amino acid transporter (*EAAT*) expression was found in different body compartments

of the honey bee, with the highest expression pattern in the brain [74]. Their role in honey bees resembles that in humans for glutamate transportation [75]. Still, much work needs to be done before the structure of *EAAT* is clearly understood in terms of function [74]. Odorant sensing is one of the hallmarks of honey bee feeding and HGs' biological functions. *OBP18* was found in high concentration in the mandibular glands of drones [76]. *OBPs* mediate both the perception and release of chemical stimuli in insects [76]. The genome of honey bees contains 21 genes encoding *OBPs* [76]. From our transcriptome analysis, 9 *OBPs* were discovered among which 8 were up-regulated under temperature-stress conditions while *OBP2* was found significantly down-regulated. As a family member of *OBPs*, *OBP18* was involved in the identification of odor molecules and plays an important role in olfaction [77]. Showing high expression levels in the head of resistance Chinese honey bees when being challenged by *Varroa destructor* resistant to parasitism, might indicate its involvement in the progress of mite resistance [78]. This functional role might be affected when honey bees are subjected not only to biological challenges but also to temperature-stress challenges. This information can be extended to humans as well, facing deficiency with their odorant sensation to consider the *OBP18* homolog gene as a target gene for odorant deficiency-related diseases.

Identification of exclusively temperature-stress genes

High proliferation of genes observed from the set of DEGs exclusively expressed under temperature-stress negatively affects protein processing in the endoplasmic reticulum, and might highlight reduced turn-out of protein production which is related to *RJ* production by the HG. Heat shock-induced changes in protein metabolism in the endoplasmic reticulum (ER) by suppression of secretory protein synthesis [79]. This process requires protein folding for proper transportation to the Golgi complex for the final destination [80]. From the result obtained in this analysis, the reported down-regulated genes affect these processes negatively considering the enriched BP including protein folding and processing, and CC related to ER and its membrane. Contrary to that, up-regulated genes from this category are likely to affect BPs related to neurogenesis, and nervous system development. Li et al. stated that *Nosema ceranae* infection in the HG up-regulated the expression levels of neuropeptide genes [81].

The KEGG pathway annotation of the 286 up-regulated DEGs revealed significant enrichment in three pathways, including the extracellular matrix-receptor (ECM-receptor) interaction pathway which was the most significantly enriched pathway, followed by fatty acid metabolism and glycine, serine, and threonine metabolism pathways. *LOC725946*, *LOC408393*, *LOC412663*, *LOC726736*, and *LOC408552* are the ECM-receptor interaction pathway-enriched genes from our data. The Interaction between the ECM and receptors on the cellular surface regulates cell behavior and plays a significant role in cell communication, proliferation, adhesion, and migration [82]. In the honey bee, *LOC725946* functions as an integrin_alpha9 domain belonging to the integrin alpha chain family, while *LOC408393* (agrin-like) is highly expressed in queen and worker bees [83]. Once the integrin in the interior signal transduction chemical pathways is triggered, the mechanical status and chemical composition of the ECM will be changed and can lead to an activated transcription response including regulation of the cell shape, cell cycle, and motility [84]. Therefore, the identified DEGs in this study encoding for integrins in the "ECM-receptor interaction" pathway might suggest that integrins are important receptors in response to HG temperature-stress response. A similar result was reported under *Ascosphaera apis* infection in *A. mellifera* [85]. Overall, to the best of our knowledge, this study is the first to evaluate the transcriptomes of HGs under temperature-stress. The key nodes identified are likely to regulate the translation process for the regular development of HG and *RJ* production. However, at individual gene expression levels, exploring interactions between the up-regulated *HSP90* and *HSP70* marker genes, which respond to temperature-stress, and the *GR10* marker genes, which are involved in secretion, may provide insights into how these changes could impair HG functionality. In addition, *GR10* responsiveness correlates with HG developmental changes and may represent a distinct phenomenon sepearate from general HGs development. With the involvement of *GR10* in sensing and nutrition and with reference to the significant involvement of the fatty acid metabolism pathway, we proposed it as a potential signaling pathway related to thermal adaptation, together with the protein processing pathway in the endoplasmic reticulum, which requires the involvement of *HSP*s. Further studies on physiological assessment to validate the functional implications of the DEGs would be worth exploring. Although various proteomics analyses on the HG were performed [43, 86]. Performing proteomics and metabolomics characterization will equally be good for a better understanding of the effect of temperature-stress on honey bee nutritional values and survival, which serve as the limitation of our finding. Despite the variation in several genes' expression observed due to temperature change, a wider ambient temperature should be implemented for more validation.

Conclusion

Our results showed that temperature variation affects the biological and genetic functions of HGs in *A. mellifera* by altering the gene expression patterns and developmental retardation. Overall, the observed changes in DEGs indicate that there are major genes that can monitor the HGs development under temperature-stress conditions, and modulate cell survival. Furthermore, we noticed that temperature-stress affects protein synthesis negatively, while positively increasing cell proliferation, adhesion, and migration, as well as metabolism. Our results might not only be restricted to HG cells but can be extended to various types of tissues likely to be exposed to underlying stress conditions. The finding of Ji et al. was groundbreaking and produced a comprehensive view of how HGs differentially and developmentally expressed genes and secret proteins based on age and developmental stages [86]. Our work extended the functions of the HGs genes based on survival under temperature-stress conditions. The impact of temperature on honey bee HGs physiology correlates with the expression of its nutritional marker gene (*GR10*) and stress marker genes (*HSP70*, *HSP90*), mirroring the effects of pesticides. Consequently, silencing *GR10* in HGs tissue could help in exploring its significance in nutritional performance, survival, and beyond. Finally, a broader temperature range in future experiments could help derive more definitive conclusions.

Abbreviations

CCD	Colony Collapse Disorder
HG	Hypopharyngeal Glands
RJ	Royal Jelly
ER	Endoplasmic Reticulum
DEG	Differentially Expressed Genes
HSP	Heat-Shock Protein
GR10	Gustatory Receptor 10
AmGR10	Apis mellifera Gustatory Receptor 10
VG	Vitellogenin
SOD1	Superoxide Dismutase
GO	Gene Ontology
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
DAVID	Database for Annotation Visualization and Integrated Discovery
PPI	Protein-Protein Interaction
STRING	Search Tool for the Retrieval of Interacting Genes
MRJP	Major Royal Jelly Protein
NP	Neuron Protein
BP	Biological Process
CC	Cellular Component
MF	Molecular Function
KEGG	Kyoto Encyclopedia of Genes and Genomes
OBP18	Odorant Binding Protein 18
°C	Degree Centigrade
RNA-seq	Ribonucleic acid-sequencing
cDEG	Commonly Differentially Expressed Genes
PCA	Principal Component Analysis
FC	Fold Change
EAAT	Excitatory Amino Acid Transporter

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11322-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Author contributions

H.W.K. designed research; A.Y.M., J.H.L., and S.L. performed research; S.L. sampling; A.Y.M., Y.Y.and S.L. analyzed data; A.Y.M., Wrote the original draft of the paper. H.W.K. J.H.L S.L., and Y.Y Writing-review and editing. All authors reviewed the manuscript. The authors read and approved the final manuscript.

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Data availability

The RNA-seq data presented in this publication have been deposited in NCBI's Sequence Read Archive (https://www.ncbi.nlm.gov/sra) under the project accession number PRJNA1150281. Data generated or analyzed during this study are included in this published article. Data are provided within the manuscript or supplementary information files. Further details can be obtained from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

All insects materials were collected following national and international standards and local laws and regulations. No specific permission is required to collect all samples described in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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