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Molecular Genetic Studies on Honeybees in Response to Some Environmental Stresses

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Abstract: The honeybee, *Apis mellifera*, is a widespread pollinator insect. Colony collapse disorder (CCD) has recently caused a significant decline in honeybee numbers worldwide. The beekeeping industry, agriculture, and world biodiversity are threatened by the honeybee population decline. We examined the mRNA levels of *heat shock protein gene 60* (*hsp60*), *acetylcholinesterase 1*, and *acetylcholinesterase 2*, as biomolecular markers of the insect response to three separate forms of environmental stress, including high temperature, low temperature, and insecticide. The obtained results indicated increased levels of *hsp60* and *acetylcholinesterase 2* after the exposure of worker bees to temperatures of 17, 32, and 40°C for one hour, as well as exposure to a low dose of pesticide for 24 h ingestion of imidacloprid solution (2 ppm); meanwhile, *acetylcholinesterase 1* showed remarkable expression decrease under heat and cold stresses but increased under pesticide treatment. The responses of honeybees to *hsp60*, *acetylcholinesterase 1*, and *acetylcholinesterase 2* are diverse and very specific to various stimuli. According to this research, the distinct expression profiles of *hsp60* and *acetylcholinesterase 2* can serve as useful instruments for tracking honeybees' vulnerability to varied environmental effects. Due to its insufficient responsiveness, *acetylcholinesterase 1* expression is not permitted for use as a biomarker.

1 Introduction

Honeybees are pollinators that offer core services to preserve ecosystems and systems of agriculture by maintaining crop yields that depend on insect pollination. Honeybees create a variety of useful goods, including wax, pollen, royal jelly, honey, venom, and propolis, in addition to the pollination service they provide. In 2015, it was estimated that honeybees' economic contribution to American agriculture ranged from $21.22 to $54.75 billion annually (Bartling et al 2021, Vercelli et al 2021).

According to FAO, during the period of April 1, 2018, to April 1, 2019, the controlled bee population fell by 40.7% colony collapse disorder (CCD), which was first noted in the United States in late 2006, it is the occurrence of rapidly declining honeybee colonies

(CCD) significantly harmed the agricultural sector (Kim et al 2019). Despite numerous potential causes being put out, the mechanisms behind CCD are still unknown (Koo et al 2015).

Climate change is a complex worldwide issue that has a negative impact on the abundance and distribution of a wide variety of ecosystems and creatures, including plants and pollinators. Temperature fluctuations between high and low can directly affect the dynamics of insect populations by affecting the insects' ability to survive, reproduce, and disperse. Pollinating insects, their pollination activity, and effectiveness are all impacted by climate change (Vercelli et al 2021). Bee populations and biodiversity are also significantly reduced. Additionally, colony collapse has been linked to climate change. Understanding how bees are reacting to the new climate change situation is crucial to overcoming this obstacle (Flores et al 2019, Zhao 2021).

The majority of pesticide applications are not directed at honeybees; however, they may be exposed to pesticides while gathering pollen and nectar from flowers and resin from various plants (Gregorc 2012). Imidacloprid, a neurotoxic insecticide that belongs to the class of substances known as neonicotinoids, affects the nicotinic acetylcholine receptors in the central nervous system of insects. The latter exhibit strong insecticide activity and are used against several organisms harmful to agriculture; however, due to their action against non-target organisms, including pollinating insects, notably bees (De Smet 2017). imidacloprid binds to acetylcholine receptors and reduces the amount of acetylcholine produced (Grünewald and Siefert 2019).

Under diverse stress settings, several stress markers, such as cellular, physiological, and behavioral stress responses, measure the stress levels of honeybees. Recently, it was used to assess the level of stress in honeybees subjected to a variety of stimuli, including high temperature and low temperature (climate change), and pesticides (Kim et al 2019).

The objectives of the present study were to measure changes in gene expression of some genes (*hsp60, acetyl cholinesterase1,* and *acetylcholinesterase 2*) under several stresses (heat, cold, and pesticide), and use changes in gene expression as biomarkers to detect and quantify levels of cell stress by using the qRT-PCR method (quantitative real-time reverse transcription polymerase chain reaction).

2 Materials and Methods

2.1 Materials

2.1.1 Honeybee workers

The population of honeybees was taken from breeding colonies at the Faculty of Agriculture, Ain Shams University; it was a typical mix of the Apis mellifera subspecies found in Cairo, Egypt. The beehive was opened in April, and female bees of any age were taken from the frames.

2.1.2 Chemicals and Reagents

All chemicals and reagents utilized in this search will be listed in the procedures of techniques.

2.2 Methods

All tested individuals were chosen from healthy clean colonies which were kept in the garden at the faculty of agriculture, Ain Shams University, Egypt. All experiments were carried out inside cages, where honeybees were selected, kept in transparent plastic containers (15 workers/jar), and supplied with 70% sucrose solution in a 5 ml test tube. The typical test environment for this investigation required that the bees be kept at 33°C with 50% relative humidity out of the light. Every day, the 70% sucrose solution was changed. All stress experiments were carried out using three biological replicates, along with the required controls.

2.2.1 High and low-temperature stress

Worker bees are exposed to a wide variety of temperatures for heat shock or cold shock treatments. To ensure that the insects were homogeneous, workers were taken from one hive. Three high-temperature degrees, i.e., 36, 38, and 40°C as well as three low temperatures; 27, 22, and 17°C were used for one hour incubation. To evaluate the tolerance for tested bees of heat shock or low shock. The worker bees exposed to 17 and 40℃ for one hour were selected and collected to study gene expression because they adapted more efficiently. Meanwhile, the workers exposed to 32°C were used as a negative control for thermal treatments.

2.2.2 Pesticide stress

In order to treat imidacloprid pesticide, honeybees were given 50% sucrose solutions containing 0, 1, 2, and 4 ppm imidacloprid over the course of 1, 4, 8, 24, and 48 hours, while other conditions being equal to the standard. After 24 hours of incubation (32°C), treated bees 2 ppm only imidacloprid were collected.

2.3 Molecular techniques

2.3.1 Primers

Four pairs of primers (forward and reverse) were used in this study. The gene name, sequence of each primer length, and references are shown in **Table 1**. The chosen primers were tested for hairpin formation and self-annealing consulting blast online tool.

2.3.2 RNA isolation

Pure total RNA was isolated from abdomen tissues (n=1) from 9 insects in three technical replicates with three biological and from each treatment and sampling time point, this procedure was followed as described by EasyPure® RNA Kit- Cat. No. ER101- TransGen Biotech Company-China.

2.3.3 cDNA synthesis

Used approximately 1 µg of RNA to create the cDNA by EasyScript® First-Strand cDNA Synthesis SuperMix kit-Cat. No. AE301- TransGen Biotech Company- China.

2.3.4 qPCR assay

Typically, the process followed the kit instructions: The PerfectStartTM Green qPCR SuperMix kit (Cat. No. AQ601- TransGen Biotech Company-China) was utilized as a template for quantitative PCR to ascertain the levels of expression of the genes of interest utilizing 1 µL of cDNA in 40 cycles for each individual gene. Real-time PCR primers for each gene were created using the NCBI database's identified sequence (**Table 1**), and qPCR amplifications were carried out in a reaction volume of 20 µL that contained 1 µL of cDNA as a template, 1 µL of gene-specific primers, 7 µL of distilled water, 10 µL of SYBR Green, and 1 µL of Master Mix. The Stratagene MX3000 P (Agilent Technologies) machine was designed to continually monitor the amplified signals. initial denaturation at 92°C for 2 minutes, followed by 40 cycles of denaturation for 5 seconds, annealing for 15 seconds, and extension for 30 seconds.

2.4 Data analysis

The relative concentrations of *hsp60, acetylcholinesterase 1, acetylcholinesterase 2, and small ribosomal subunit 5 (rps5)* were determined using qPCR threshold cycle (Ct) values. According to Livak and Schmittgen (2001), the formula: $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ treatment\ -\ \Delta Ct\ reference]}$ was used to analyze the data. Gene mRNA levels in the same samples were normalized by rps5 mRNA levels. Gene expression increased when the value >1. Using Micros of Excel 2010 (Micros of Corporation, Redmond, WA, USA), gene expression data were examined. In order to create a map, comparisons across treatments were based on the means of three biological and three technical replicates.

3 Results and Discussion

The effects of high, low temperature, and low doses of insecticide stresses were tested on the response genes of stress. worker insects were exposed to different temperatures or insecticidal neonicotinoid imidacloprid. Different gene expression levels were measured at various stresses by (qRT-PCR) reaction compared to the ribosomal small subunit 5 gene that serves as a reference to the three genes (*heat shock proteins 60, Acetylcholinesterase 1, and Acetylcholinesterase 2*). Jeon et al (2020) confirmed that the rps5 gene is one of the best genes used as a reference for the genes in honeybees. The different numbers indicate significant gene expression differences between treatments in **Table 2**. The results were the following:

3.1 *hsp60* gene expression under several stresses

The following gene expression result was obtained by assessing the thermal shock protein 60 gene expression under three different types of stress: Gene expression was found to be substantially greater than normal (5.3 times) in the case of heat stress (high temperature 40°C), as well as for low-temperature exposure (17°C) after a maximum of one hour, gene expression was 2.7 times higher than usual, and gene regulation was higher than usual (4.7 fold) in response to pesticide exposure (2 ppm) (**Fig 2 and Table 2**).

3.2 Gene expression of *acetyl cholinesterase1* gene under several stresses

When measuring gene expression of *acetylcholinesterase1* under high temperature (40°C) no change was observed in the gene expression of normal, but under low temperature (17°C) was less regulated than normal after one hour (downregulated 0.5-fold), and gene expression under pesticides (2 ppm) was above normal (upregulated 2.1-fold), which means that this gene cannot be used as an indicator of thermal stress (**Fig 3 and Table 2)**.

Table 1. qRT-PCR primers for *Apis mellifera* the gene's name, each primer's sequence, length, melting temperature, and accession number according to Kim et al (2019)

Gene name	Primer Name	Sequence 5′→3′	length (bp)	Accession Number	Tm ©
ribosomal small subunit 5	rps5-F	AATTATTTGGTCGCTGGAATTG	22	XM_006570237.2	56.6
	rps5-R	TAACGTCCAG-CAGAATGTGGTA	22		60.3
heat shock protein 60	hsp60-F	AATGCAGGCGTGGATGCTAG	20	XM_392899.6	60.5
	hsp60-R	CCGTACGCACTACTTTCGTTG	21		61.3
Acetylcholinester-ase 1	ace1-F	GATCGACGGCGCTTTCCTCG	20	KU532288.1	64.6
	ace1-R	GACCCGTCGATGTGGAACAAC	21		63.3
Acetylcholinester-ase 2	ace2-F	ATATCCGTTCAACAGTG-GAACAGT	24	KU532289.1	61.8
	ace2-R	CTCGTTGTTACCGATCAG-TATCT	23		61.1

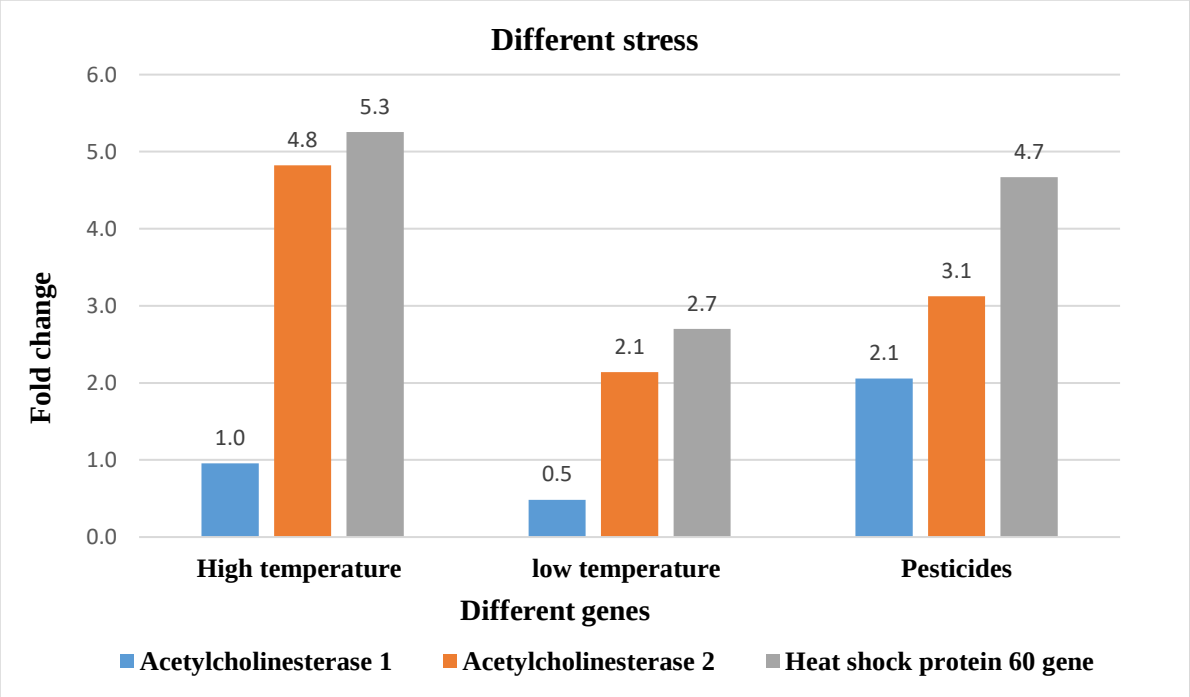

Fig 1. The effect of high temperature, low temperature and pesticides on *acetylcholinesterase1*, *acetylcholinesterase 2*, and *hsp 60* genes. The strong induction of genes encoding heat shock protein 60 and acetylcholinestrase2 are observed. The *hsp60* gene was strongly induced by pesticide, heat, and cold stress. The *acetylcholinestrase2* gene was strongly influenced by heat stress, *acetylcholinestrase2* gene was only moderately upregulated by pesticide, slightly above by cold stress.

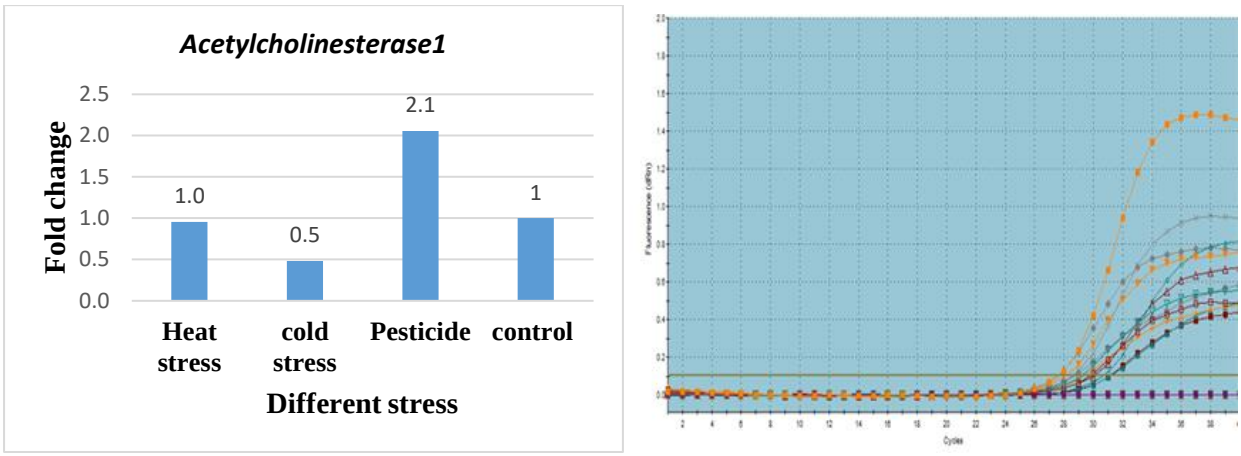

Fig 2. Gene expression of *acetylcholinesterase1* gene under several stresses, *acetyl cholinesterase1* gene was strongly induced by pesticide only, down-regulation of expression to acetylcholinesterase 1 by cold stress and gene expression does not change by heat stress.

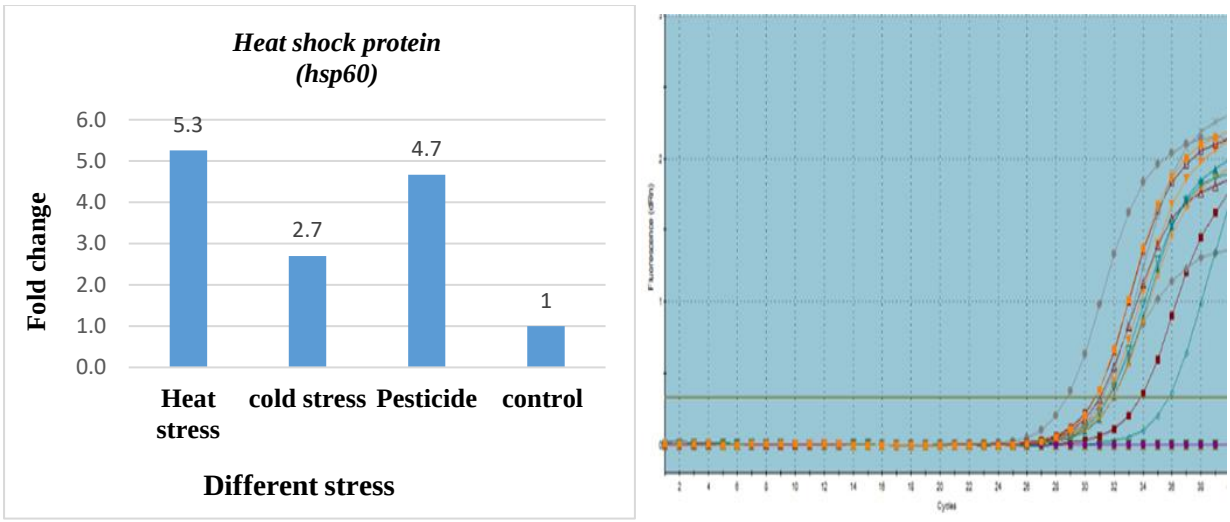

Fig 3. Gene expression of *heat shock protein 60* gene under several stresses. It is expressed above average under several stresses but in various degrees.

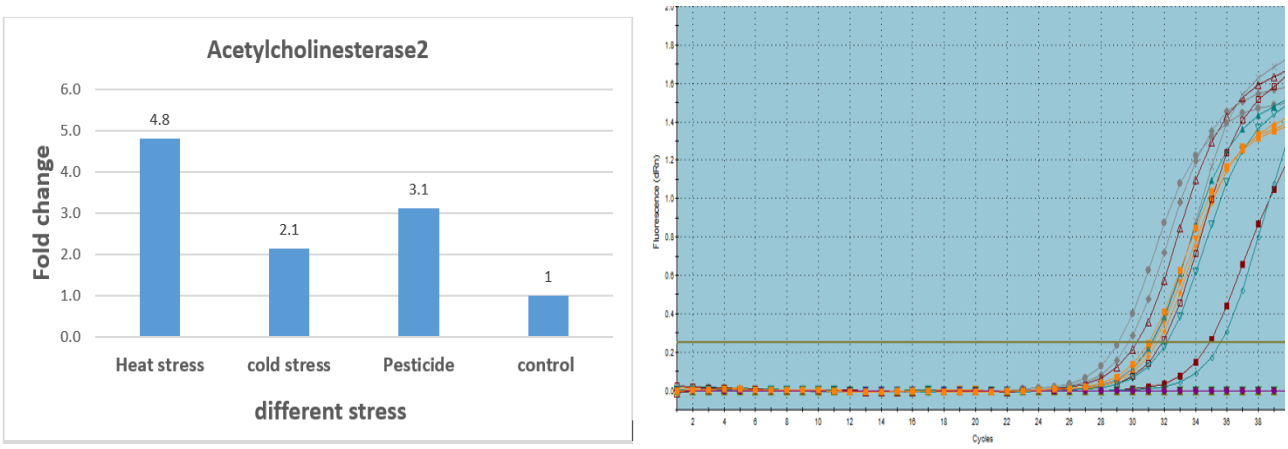

Fig 4. Gene expression of *acetylcholinesterase2* gene under several stresses. It is expressed above normal under several stresses but in various degrees.

Table 2. Different gene expression levels of genes that were measured at various stresses by (qRT-PCR) compared to the ribosomal small subunit 5 gene that serves as a reference to three genes (heat shock proteins 60, acetylcholinesterase one and acetylcholinesterase two). The different numbers reflect significant differences between treatments

Genes / Stress	Heat stress	Low stress	Pesticide
Heat shock proteins 60	5.3 - fold	2.7 - fold	4.7 - fold
Acetylcholinesterase 1	No change (1)	0.5 - fold	2.1 - fold
Acetylcholinesterase 2	4.8 - fold	2.1 - fold	3.1 - fold

3.3 Gene expression of *acetylcholinesterase 2* gene under several stresses

The gene expression of acetylcholinesterase 2 was studied under several different pressures whether high heat, low heat, or exposure to the pesticide. The results showed the following: in the case of high temperature (40°C), the gene expression increased above normal (4.8- fold), but under low temperature (17°C) for one hour, the increment was 2.1- fold, while under the effect of pesticides (2 ppm), it was up-regulated (3.1- fold) **(Fig 4 and Table 2).**

3.4 Effect of diverse stress factors on gene expression of tested genes

Heat or cold-shock-dependent induction of putative genes were examined. Strong induction of *hsp60* and *acetylcholinesterase 2* was observed. *Acetylcholinesterase 1* induction did not appear due to cold stress. However, Heat stress caused this gene to be less active compared to conditions at the control temperature (32°C) (**Fig 1**).

Using samples of bee abdomen, qRT-PCR was used to examine the three genes expression encoding heat shock proteins 60, acetylcholinesterase one, and acetylcholinesterase two in order to assess low pesticide dose effect (imidacloprid) (2ppm) on the stress responses of honeybees. A strong genetic response to the three genes was observed on varying degrees; the genetic regulation of heat shock protein 60 was stronger than the other genes as shown in **Fig 1.**

The impacts of heat stress, cold stress, and pesticides on the honeybee were shown by the measurement of gene expression levels. The results showed that a particular mechanism within honeybees to combat pesticide, heat, and cold stresses was activated.

The results reflected an increase in the heat shock protein 60 gene expression above normal under several different stresses, but to varying degrees which means that this gene can be used as an indicator of stress (biomarker). This result agrees with Koo et al (2015), who confirmed that as indicators for the ecotoxicity of pesticides, the levels of hsp 60 protein and transcripts had been studied. As a result, the analysis of *hsp60* expression profiles can be utilized to show how a particular substance affects cellular stress responses. Mckinstry et al (2017) agreed with our results and their research paper explains that temperatures that honeybees experience during everyday activities lead to strong heat shock response induction and enhanced expression of the majority of HSF-dependent core homologs reported in other systems. In a prior investigation, it was also discovered that heat stress increased *hsp60* transcription. The overworked protein biosynthesis pathway is well supported by the excessive transcription of *hsp60*, which likely denotes a faster rate of protein translation. The over-expression of *hsp60* in response to pesticide stress also suggests that *hsp60* functions as a chaperone to shield or repair stress-damaged proteins in worker bees. High induction by heat shock points to their significant function in preventing stress on cells. Kim et al (2019) agreed with our results, they confirmed the use of *hsp* expression profiles as a generic stress marker.

We attempted to assess the potential of the *acetylcholinesterase1* gene as a general stress marker in honeybees by analyzing the *acetylcholinesterase1* expression profiles in response to various stress stimuli. These findings imply that *acetylcholinesterase I* is not a suitable general stress marker. The expression of *acetylcholinesterase 1* does, however, seem to be linked to the heat shock response (HSR), at least in part because *acetylcholinesterase1* controls the Acetylcholine (Ach), particularly in the neural tissues, it likely provides essential information on physiological changes in honeybees. This is because it affects the signal cascades mediated by membrane acetylcholine receptors

(mAChR). According to Kim et al (2017) both neuronal and non-neuronal tissues, such as the fat body and hemolymph, contain *acetylcholinesterase 1*. Considering this, rather than the neuronal cholinergic system controlling acute stress response, the actions of *acetylcholinestrase1* appear to be related to the non-neuronal cholinergic system mediating chronic stress response via ACh sequestration. Since most of the *acetylcholinesterase 1* expressed in the belly comes from body fat. Kim et al (2019) agree with our results, they confirmed that the expression of *AmAChE1* is not appropriate as a stress marker. Despite its poor responsiveness, it appears that *AmAChE1* expression is connected to other pathways and the heat shock response, at least in part. It is expected that *AmAChE1* alters the ACh titer, particularly in non-neuronal tissues, which in turn modifies the signal cascades mediated by mAChR, and that the *AmAChE1* expression profile in diverse circumstances provides essential information on its physiological roles in honey bees.

According to Kim et al (2019), this gene regulates the cholinergic signal pathways by reducing the overall quantity of ACh through sequestration or degradation. Therefore, acetylcholinestrase1's lowering of ACh concentration in hem-lymph would specifically affect the physiology of various organs. Our results found that the difference in the gene expression of *acetylcholinesterase2* means that the gene could be used as a molecular marker. This result agrees with Kim et al (2017) who suggested that *acetylcholinesterase 2* is responsible for synaptic transmission in honeybees by Western blot analysis. When honeybees are exposed to stress, the neural signal delivered by AChE2 increases, and therefore the gene expression of *acetylcholinesterase 2* is produced to communicate the neural signal, so when the bees are exposed to different types of stress, the gene expression of the enzyme increases over normal. Grünewald and Siefert (2019) suggested that the mechanism of the acetylcholine (ACh) gene is the main excitatory neurotransmitter in the central nervous system (CNS) of insects. generated by the cholinergic system's choline acetyltransferase. The non-neuronal cholinergic system and the neuronal cholinergic system coexist in animals. The functional principles of both systems are largely the same. There are two AChEs: one is membrane-bound (AmAChE2), located in the CNS, soluble (AmAChE1), and was found also, in non-neuronal tissue in the thorax, abdomen, and leg, as well as in the peripheral nervous system. Neonicotinoids are nAChR agonists and are mostly utilized in crop protection. Neonicotinoids appear to alter bee developmental processes independently of neuronal AChRs, as evidenced by their effects on behavior.

4 Conclusion

According to (qPCR) reaction data, the expression patterns of the stress marker genes varied depending on the type of stress. Our findings show that different stressors, such as high temperature, low temperature, and pesticides, cause diverse changes in the expression of *hsp60*, *acetylcholinesterase2*, and *acetylcholinesterase1* in worker honeybees. Our research implies that individual *hsps60* and *acetylcholinesterase 2* expression patterns may be useful tools for assessing the susceptibility of honeybees to various environmental stresses. It also implies that *acetylcholinesterase 1* expression may not be a useful tool for assessing honeybee susceptibility to various environmental stresses.

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