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RESPONSES OF ANTIOXIDANT ENZYMES AND HEAT SHOCK PROTEINS IN *DROSOPHILA* TO TREATMENT WITH A PESTICIDE MIXTURE

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Abstract: The effects of a mixture of seven pesticides were examined on the expression of antioxidant enzymes, Mn superoxide dismutase (Mn-SOD), catalase (CAT), glutathione synthetase (GS), and heat shock proteins (HSP) 26, 60, 70 and 83 in adult fruit flies (*Drosophila melanogaster* Oregon R). The flies were reared under controlled conditions on artificial diets and treated with a mixture of seven pesticides (molinate, thiobencarb, linuron, phorate, primiphos-methyl, fenvalerate and lambda-cyhalothrin) commonly found in water, at concentrations of 0.1, 0.5 and 1 parts per billion (ppb) for 1 and 5 days. Quantitative real-time PCR (qRT-PCR) analysis of Mn-SOD, CAT and GS expression revealed that the analyzed markers responded significantly to pesticide-induced oxidative stress, in particular on the 5th day of treatment. On the 1st day of treatment, the relative expression of HSP26 and HSP60 genes increased only after exposure to the highest concentrations of pesticides, whereas HSP70 and HSP83 expression increased after exposure to 0.5 and 1 ppb. After five days of treatment, the expression of all HSP genes was increased after exposure to all pesticide concentrations. A positive correlation was determined between the relative expression levels of some HSPs (except HSP60), and antioxidant genes. The observed changes in antioxidant enzyme and HSP mRNA levels in *D. melanogaster* suggest that the permissible limits of pesticide concentrations for clean drinking water outlined in the regulations of several countries are potentially cytotoxic. The presented findings lend support for reevaluation of these limits.

Key words: Pesticide; qRT-PCR; antioxidant genes; HSP; *Drosophila*

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INTRODUCTION

Pesticide release into the environment increases with the growth of the human population and the anthropogenic activities required to enhance plant food production (Katagi, 2010). Pesticides are one of the major contaminants of groundwater and drinking water, and are generally introduced into the environment through careless anthropogenic and farming practices. As water is the final destination for pesticides, these contaminants generally occur more often as complex mixtures than individual compounds (Relyea, 2009; Silins and Högberg, 2011). Although organisms using these contaminated waters are exposed to mixtures of these substances, pesticide toxicity studies are generally focused on the effects of each individual pesticide on an organism. Therefore, it is important to determine the effects of pesticide mixtures on living cells. These contaminants can enter into animal or human bodies via breathing, drinking and eating contaminated air, water or food, as well as contact with them (Katagi, 2010).

It is well known that exposure to single pesticides causes reproductive, teratogenic, carcinogenic, oncogenic and mutagenic effects (Didla et al., 2011). Additionally, it has been documented that excess production of reactive oxygen species (ROS) (Bagchi et al., 1995; Mathew et al., 1992), changes in the expression of antioxidant and heat shock protein genes (Doganlar, 2012; Ceyhun et al., 2010) and DNA damage (Lee and Steinert, 2003) can result from pesticide exposure. Therefore, to avoid unlimited pesticide use and its toxic consequences, countries and some

agencies such as the United States Environmental Pollution Agency (EPA) and World Health Organization (WHO), have enforced limitations on the total amounts of pesticide residue in water. For example, the total amounts of pesticide residue permitted by the Turkish Government, as other countries, are 0.5, 1 and 100 ppb in type 1, type 2 and type 3 waters, respectively, as published in the "Regulation of Surface and Waste Water" guidelines of 2005.

Organisms such as *Mus musculus, Danio rerio, Caenorhabditis elegans, Drosophila melanogaster* and others (Paes and Oliveira, 1999; Franco et al., 2009; Mela et al., 2010; Singh et al., 2010) have been used in environmental toxicity research in view of their rapid growth and established genetic and physiological pathways. *D. melanogaster* is a good choice for research because it has about 75% functional homology with human disease-causing genes (Pandey and Nichols, 2011; Demir et al., 2013). The developmental, cellular and molecular mechanisms of *Drosophila* are well understood and, therefore, it has been extensively used in drug, immunity, personalized medicine and toxicological studies for elucidating human diseases (Reiter et al., 2001; Koh et al., 2006; Wolf et al., 2006). Moreover, the European Center for the Validation of Alternative Methods has approved the use of *D. melanogaster* for research on genetic and metabolic disorders in humans (Siddique et al., 2005).

Previous studies reported that exposure to pesticide and other toxic chemical compounds induced oxidative stress via an excess production of reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, hydrogen peroxide and singlet oxygen in animal (*Drosophila*) and cell-line studies (Dreiem et al. 2002; Stone et al., 2003; Posgai et al., 2011). In rodent models, an alternation of ROS induced by solvent toxicity were reported in the liver, kidney and immune system (Myhre and Fonnum, 2001; Baydas et al., 2003; Dreiem et al., 2002). At the cellular level, excessive amounts of ROS induce multiple stress comebacks and injure different cell components, such as proteins, genomic and mitochondrial DNA and several membrane systems (Au et al., 1999; Jimi et al., 2004; Doganlar et al., 2014). ROS can be scavenged by enzymatic (such as superoxide dismutase, catalase, and peroxidase) or non-enzymatic (such as glutathione, ascorbate, and tocopherol) antioxidant systems of an organism.

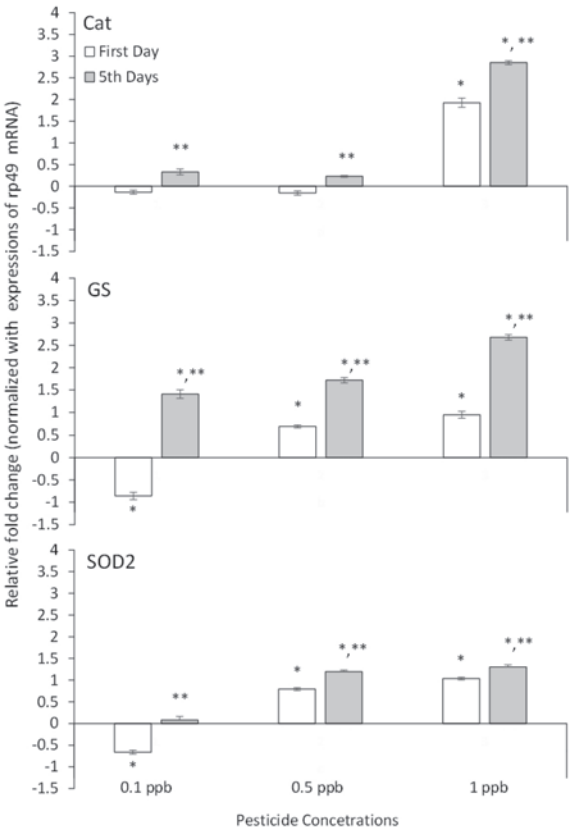

Fig 1. Quantitative real time PCR (qRT-PCR) analysis of catalase (CAT), glutathione synthetase (GS), and Mn-superoxide dismutase (Mn-SOD) expression after fruit fly exposure to pesticides for 1 and 5 days. All data are normalized with Rp 49 expression and given as relative to the control (control = 1, not shown in figure); * indicates significantly different values compared to their respective controls, analyzed by one-way ANOVA and Duncan's test ($p \leq 0.05$); ** indicates significantly different values between treatment times (T-test; $p \leq 0.05$).

HSPs are one of the most abundant cellular proteins found under non-stress conditions and are expressed in response to various biological stresses, including toxic chemicals, heavy metals, osmolarity, anoxia and viral infections (Singh et al., 2009; Desai et al., 2010; Singh et al., 2010). HSP60, HSP70, HSP83 and HSP90 are part of a family of proteins known as

"chaperones," which help other proteins to fold and assume their proper functions (Gehrmann et al., 2008; Desai et al., 2010; Qian et al., 2012), participate in receptor binding and proteolysis (Zarate and Bradley, 2003; Ceyhun et al., 2010), and have important roles in repair mechanisms to incorrectly coded proteins (Eckwert et al., 1997; Gupta et al., 2010; Singh et al., 2009; Desai et al., 2010; Singh et al., 2010).

Some genotoxic stressors not only disturb genetic stability but also directly or indirectly affect gene expression. For this reason, more attention should be given to investigating the genotoxic effects of environmental pollutants (Doganlar and Doganlar, 2014). The aims of the present study were to investigate the responses of *D. melanogaster* to exposure to a mixture of pesticides with regard to changes in the expression of antioxidant protein (Mn-SOD, CAT and GS) and HSP (Hsp26, Hsp60, Hsp70 and HSP83) genes, and to assess the correlations between antioxidant systems and HSPs.

MATERIALS AND METHODS

Drosophila stocks and treatment

The wild type *D. melanogaster* strain (Oregon R) was reared on standard Drosophila medium (DM) (cornmeal, agar, sugar, yeast, water, and propionic acid) at 26±1°C, in constant humidity and light conditions. 150 two-day-old adult male flies were housed in 16-mL test tubes (5 flies/tube and 3 tube/group). Three herbicides (molinate, thiobencarb, linuron), three insecticide-acaricides (phorate, primiphos-methyl, fenvalerate) and one insecticide (lambda-cyhalothrin) were preferred as commonly used for crop protection. The 1000-ppb stock solutions were prepared with ultra-pure water for each pesticide; 100 µl of each stock solution was pipetted into 3 clean 50-ml falcon tubes; 0.7 mL mix-solution was diluted with 4.3, 9.3 and 49.3 ml ultra-pure water to obtain 20, 10 and 2 ppb mixtures of pesticides, respectively. These mixtures were immediately applied to the liquid DM at doses of 0.1, 0.5 and 1 ppb (µg/L) for 1 and 5 days. The mixtures were vortexed vigorously and allowed to stand at room temperature for 2 h until solidification. Ten treatment groups (five treatment groups per application time) were as follows: control (2 ml DM), negative control (100 µl ultra-pure water /1.900 ml DM), 0.1 ppb mixture of pesticide (100 µl-2ppb mix/1.900 ml DM), 0.5 ppb mixture of pesticide (100 µl-10 ppb mix/1.900 ml DM), 1 ppb mixture of pesticide (100 µl-20 ppb mix/1.900 ml DM). The concentrations of pesticide mixtures were selected based on permissible limits of total amounts of pesticide residue for human consumption. The CAS formula, mode of actions and number of Dr. Ehrenstorfer reference materials of pesticides are given in Table 1.

Table 1. The types of pesticide, CAS formula, modes of action and number of Dr. Ehrenstorfer reference materials of pesticides used in this study.

Pesticide	Type of pesticide	CAS formula	Mode of Actions	Reference No.
Molinate	Herbicide	*S-ethyl hexahydro-1H-azepine-1-carbothioate*	Inhibits photosystem II	XA15280000CY
Thiobencarb	Herbicide	*S-[(4-chlorophenyl)methyl] N,N-diethylcarbamothioate*	Inhibits photosystem II	L17470000CY
Linuron	Herbicide	*3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea*	Inhibits photosystem II	XA 14640000AL
Phorate	Insecticide-Acaricide	*O,O-Diethyl S-[(ethylsulfanyl)methyl] phosphorodithioate*	Inhibits Acetylcholinesterase (AChE) and pseudocholinesterase	L16080000CY
Primiphos-methyl	Insecticide-Acaricide	*O-[2-(Diethylamino)-6-methyl-4-pyrimidinyl] O,O-dimethyl phosphorothioate*	Inhibits Acetylcholinesterase (AChE)	L16270000CY
Fenvelerate	Insecticide-Acaricide	*Cyano(3-phenoxyphenyl)methyl 2-(4-chlorophenyl)-3-methylbutanoate*	Sodium channel modulator	L13630000AL
Lambda-cyhalothrin	Insecticide	*(R)-cyano(3-phenoxyphenyl)methyl (1S,3S)-rel-3-[(1Z)-2-chloro-3,3,3-trifluoro-1-propenyl]-2,2-dimethylcyclopropanecarboxylate*	Some repellant properties Sodium channel modulator	L11860000CY

Isolation of total RNA and cDNA synthesis

At the end of each treatment period, three flies were individually collected from the test tubes and total RNA was isolated from each single adult *D. melanogaster* specimen using the PureLink® RNA Mini Kit (Life Technologies, USA) according to the manufacturer's instructions. The extracted RNA concentrations were measured by Qubit® Fluorometer (Life Technologies, USA). The concentration of total RNA was adjusted to 50 ng/µL for synthesis of the first strand of cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). cDNA synthesis was performed using the Applied Biosystems® Veriti® thermal cycler (step 1: 25°C, 10 min; step 2: 37°C, 120 min; step 3: 85°C, 5 min). Finally, 20 µl cDNA were obtained from each replicate. The cDNA was stored -20°C for subsequent analysis.

Quantitative real-time PCR (qRT-PCR) assays and statistical analyses

Expression levels of HSPs (HSP26, HSP60, HSP70 and HSP83) and antioxidant enzymes (Mn-SOD, CAT and GS) genes in response to pesticide mixture treatment were analyzed by qRT-PCR using SYBR® Select Master Mix (Applied Biosystems) on an ABI Step One Plus Real-Time PCR system (1 cycle of 2 min at 50°C and 10 min at 95°C followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min) with the primer pairs given in Table 2. Gene expression was determined as the relative fold change compared the control and normalized with rp49 mRNA expression. The comparative cycle threshold (ΔCt) method (User Bulletin 2, Applied Biosystems, CA) was performed to analyze the expression levels of mRNAs. Additionally, differences in fold-change resulting from changes in gene expression in response to exposure to mixtures of pesticides were compared using analysis of variance (ANOVA) with Duncan's separation of means test using SPSS 18 software at a significance level of $p \leq 0.05$. Differences in the relative expression levels of antioxidant and HSP genes between both treatment times were analyzed by t-test, $p \leq 0.05$. Correlations between the relative expression levels of antioxidants and HSP genes were analyzed by a bivariate correlation test with Pearson correlation coefficient and a two-tailed test of significance using SPSS 18 software at significance levels of $p \leq 0.05$.

RESULTS

In the present study, we determined the effects of a pesticide mix on the expression of antioxidant enzymes and HSP genes in adult flies of *D. melanogaster*, and the results are given in Figs. 1 and 2. Both control and negative control gave the same results and therefore a single control (type) was used in all

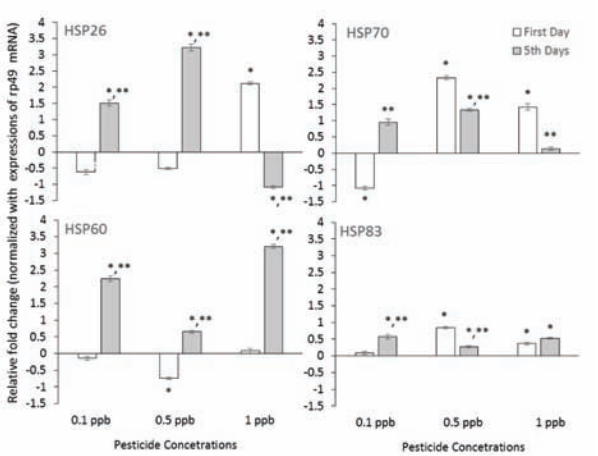

Fig 2. Quantitative real time PCR (qRT-PCR) analysis of HSP26, HSP60, HSP70 and HSP83 expression after fruit fly exposure to pesticides for 1 and 5 days. All data are normalized with RP49 expression and given as relative to the control (control=1, not shown in the figure); * indicates significantly different values compared to the respective controls analyzed by the one-way ANOVA, Duncan's test ($p \leq 0.05$); ** indicates significantly different values between treatment times (T-test; $p \leq 0.05$).

experiments. Significant increases in the expression of CAT genes were observed with the 1 ppb pesticide mix exposure in *D. melanogaster* at both days 1 the treated groups after both treatment periods (except 0.1 ppb on day 1) compared to their respective controls, and the relative expression of GS were sig and 5 (Fig. 1). Compared with the control, the highest increase (2.85-fold) was determined for the 1 ppb pesticide mix treatment on the fifth day. A significant increase in GS mRNA levels was observed in all of

nificantly lower in the 1-day treated group compared to the 5-day group, except at the 0.1 ppb level of exposure. The maximum increase (2.67-fold when compared to the control) in the relative expression of GS was determined at the highest pesticide concentration and exposure time. While a significant decrease in the mRNA level of Mn-SOD was observed in fly tissues on the first day, similar to GS genes, a significant increase in the expression of Mn-SOD was determined for exposures to 0.5 and 1 ppb pesticides at both treatment times. The highest increase in the expression of the Mn-SOD gene (1.3-fold) was determined for the 1 ppb pesticide mix after 5 days of treatment (Fig. 1).

Compared with the control, while the 0.1 and 0.5 ppb pesticide mix exposure caused decreases in the relative expression of HSP26 during the first day of treatment, the same pesticide mix concentrations caused increases in the expression levels of HSP26 on the 5th day of exposure (Fig. 2). However, compared with their respective controls, statistically significant increases and decreases were determined for the highest pesticide mix exposure, after 1 and 5 days, respectively. The maximum expression of HSP26 (compared to the control, 3.2-fold) was determined in the 0.5 ppb mix after 5 days of treatment. Compared with the control, the HSP60 transcription level was decreased significantly (0.7-fold) by 0.5 ppb after one day of exposure. However, significant increases were determined after 5 days for all exposure concentrations (Fig. 2). While the HSP70 expression level increased significantly at 0.5 and 1 ppb on the first day of exposure, its expression was observed to decrease at 0.1 ppb. After 5 days, the HSP70 gene expression significantly increased only after exposure to the 0.1 and 0.5 ppb pesticide mix. The expression of HSP83 was significantly increased after all pesticide concentrations and exposure times (except 0.1 ppb on day 1) and the maximum increase was determined to be for exposure to 0.5 ppb (2.3-fold), after one day of exposure, as compared with the control.

In this study, positive correlations were found among the relative expression levels of HSP26 and CAT and GS genes at both treatment times. On the first day, the relative expression of both HSP70 and HSP83 positively correlated with expression of CAT and GS genes. While Mn-SOD expression showed a significant correlation only with HSP26 on the first day of treatment, positive correlations were determined for HSP70 and HSP83 on the 5th day of treatment. Interestingly, no significant correlations were determined between HSP60 and antioxidant enzymes (Table 3).

DISCUSSION

Environmental stresses, such as toxic metals, volatile organic compounds and pesticides, cause the excess production of ROS (Chen et al., 2008; Verma and Rana, 2008). Toxic ROS accumulation due to an inadequate antioxidant system can lead to genotoxic effects such as DNA polymorphisms and gene induction/repression (Ochsendorf, 1999). Recent studies have shown that under stressful conditions, a differential regulation of genes encoding proteins involved in antioxidant enzymes (SOD, CAT, GS) and heat shock proteins exists (Singh et al., 2010; Tsuda et

Table 2. Genes and primer sequences of antioxidant enzymes and HSPs used in expression studies.

Antioxidant Systems		Heat Shock Proteins	
Genes	**Primer Sequences**	**Genes**	**Primer Sequences**
CAT	F: 5'-TACGAGCAGGCCAAGAAGTT-3'	***Hsp26***	F: 5' GCCCCGCAGCCCCATCTACGAG 3'
	R: 5'-ACCTTGTACGGGCAGTTCAC-3'		R: 5' GAGCACGCCATCCGACGACAGC 3'
Mn-SOD	F: 5'-TCTGAAGAAGGCCATCGAGT-3'	***Hsp60***	F: 5' GTCGCGCCCCGTTAGCAC 3'
	R: 5'-GCAGATAGTAGGCGTGCTCC-3'		R: 5' CATCGCGTCCCACCTTCTTCAT 3'
GS	F: 5'-TGGGACCAGCAAGTAAAACC-3'	***Hsp70***	F: 5' CGAGETCGACGCATTGTTTG 3'
	R: 5'-TCGCGAATGTAGAACTCGTG-3'		R: 5' GAGTGGATCCGCCGACGAGTA 3'
Control-rp49	F: 5'-GCTAAGCTGTCGCACAAATG-3'	***Hsp83***	F: 5' CCGGAGGCTCTTTCACAGTC 3'
	R: 5'-TGTGCACCAGGAACTTCTTG-3'		R: 5' CTTCTCGCGCTCCTTCTCTAC 3'

al., 2010). To scavenge ROS, SOD is the first and most important enzyme of the antioxidant system, catalyzing the dismutation of superoxide anions to hydrogen peroxide (H_2O_2) and water. In the second step, CAT catalyzes the decomposition of H_2O_2 to water and oxygen (Chelikani et al., 2004). Mechanisms involving glutathione neutralize toxic xenobiotic and endobiotic electrophiles so that the secondary products can be metabolized and excreted (Circu and Aw, 2010). Additionally, the overexpression of a GS gene, by conjugation with glutathione in the wild type *Drosophila*, increased resistance to oxidative stress. Decreases in GS expression in the TRX-2 mutation resulted in decreased resistance to oxidative stress (Tsuda et al., 2010), and high levels of antioxidant protein gene expression are biomonitors of cell oxidative stress (Silins and Högberg, 2011). Moreover, the antioxidant capacity of the cells correlated with the antioxidant enzymes, which responded rapidly and reached high enough levels (in the case of GS, CAT, SOD and peroxidase, etc.); however, if these conditions persisted in the cell, oxidative damage to the DNA would occur, despite ROS scavenging by the antioxidant enzymes.

HSPs are called cellular stress proteins expressed in bacteria to humans (Lindquist and Craig, 1988; Singh et al., 2009). Over the last few years, many studies have attempted to characterize the gene expression profiles of HSPs during stress responses, such as in heat stress, oxidative stress, heavy metal and pesticide toxicity (Singh et al., 2009; Gupta et al., 2010; Singh et al., 2010). However, different HSP exhibit different responses to biotic and abiotic stressors depending on the duration and severity of stress. In the current studies, while the relative expression of the HSP26 and HSP60 genes increased only at the highest concentrations of pesticides; HSP70 and HSP83 expression increased after exposure to 0.5 and 1 ppb on the 1st day of treatment. However, the expression of all HSP genes was increased by all pesticide concentrations after five days of treatment (except HSP26 at 1 ppb). This result suggests that only the highest concentration was toxic after one day of exposure, and that exposure to the pesticide mixture caused pronounced cytotoxic effects after five days of treatment. The increasing expression of HSPs shows that the cytoprotective mechanisms involve the degradation of misfolded proteins (Hayes and Dice, 1996). In this study, we showed that exposure to different concentrations of pesticides and treatment times caused changes in the expression of HSP genes in *D. melanogaster*. However, the relative mRNA levels decreased in a time-dependent manner. According to Harboe and Quayle (1991) and Pratt (1993), HSPs reprogram cellular metabolic activity transiently, assuming a protective in stress.

It has been established that pesticides can cause increases in ROS via uncoupling of oxidative phosphorylation, which results in increases in components of the enzymatic and non-enzymatic antioxidant system. In the present study, increased antioxidant enzyme gene expression showed that the pesticide mixture caused oxidative stress in *D. melanogaster* (Fig. 1). Because

Table 3. Interactions between gene expressions of HSP and antioxidant enzymes

		1st Day			5th Day		
		CAT	GS	Mn-SOD	CAT	GS	Mn-SOD
HSP26	PC	.979**	.645*	.643*	.965**	.584*	0.54
	Sig.	0.0001	0.024	0.024	0.0001	0.046	0.07
HSP60	PC	-0.127	-0.177	0.574	0.487	-0.067	-0.322
	Sig.	0.694	0.581	0.051	0.109	0.835	0.308
HSP70	PC	.916**	.926**	0.32	0.312	0.482	.887**
	Sig.	0.0001	0.0001	0.31	0.323	0.112	0.0001
HSP83	PC	.658*	.693*	-0.012	0.067	0.52	.808**
	Sig.	0.02	0.012	0.971	0.835	0.083	0.001

PC – Pearson correlations; Sig – significant degree (2-tailed); * correlation is significant at the 0.05 level; ** correlation is significant at the 0.01 level.

ROS attack vital proteins and cause their oxidation or aggregation (Kalmar and Greensmith, 2009), pesticide-induced oxidative stress can cause an increasing expression of HSPs. Additionally, we hypothesized that the reasons for the HSP synthesis under oxidative stress include the prevention of proteins damaged by ROS and correction and/or degradation of misfolded proteins. We thought that the correlations between the expression of HSPs and antioxidant enzyme genes might support this hypothesis (Table 3).

In conclusion, we determined that the permissible concentration limits of pesticides in the mixture, as given in the regulations of several countries for drinking water, may cause cellular toxicity in *D. melanogaster*. According to our study, the expression of Mn-SOD, CAT and GS genes can serve as biomarkers of oxidative stress in fruit fly exposed to pesticide pollution. Additionally, we suggest that changes in the relative expression levels of the HSP26 and HSP70 genes in *Drosophila* could be used to monitor cellular response to exposure to toxic pesticide mixtures.

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