*Russian Journal of Genetics, Vol. 40, No. 11, 2004, pp. 1215–1222. From Genetika, Vol. 40, No. 11, 2004, pp. 1475–1482.*


**GENERAL GENETICS**

# The Genetic Basis of Malathion Resistance in Housefly (*Musca domestica* L.) Strains From Turkey*

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Received October 31, 2003

**Abstract**—Organophosphate insecticide (parathion/diazinon) resistance in housefly (*Musca domestica* L.) is associated with the change in carboxylesterase activity. The product of *MdαE7* gene is probably playing a role in detoxification of xenobiotic esters. In our research, we have isolated, cloned and sequenced the *MdαE7* gene from five different Turkish housefly strains. High doses of malathion (600 μg/fly) were applied in a laboratory environment for one year to Ceyhan1, Ceyhan2, Adana, and Ankara strains while no insecticide treatment was performed in the laboratory to Kirazli strain. $Trp^{251} \longrightarrow$ Ser substitution was found in the product of *MdαE7* gene in all malathion-resistant and Kirazli stocks. In addition, we checked the malathion carboxylesterase (MCE), percent remaining activities in acetylcholinesterase (AChE), glutathion-S-transferase (GST), and general esterase activities in all five strains used in this study. In comparing with universal standard sensitive control WHO, a high level of MCE and GST activities were observed while lower level of general esterase activities was detected in the tested strains. In addition, a higher percent remaining activities in AChE than WHO susceptible strain were observed in all malathion-resistant strains.

## INTRODUCTION

Organophosphate (OP) esters are being used as pesticides and chemical warfare agents. These are very toxic chemicals since one molecule of OP inhibits one molecule of acetylcholinesterase enzyme (AChE) at the active site serine residue which cause muscle paralysis and seizures and may lead to death of the organism [1]. OP insecticides have been used from the beginning of 1960s for *Lucilia cuprina* (sheep blowfly) and *Musca domestica* (housefly). The first OP resistant insects were determined at mid 1960s and have risen to a high frequency [2, 3].

Malathion [S-(1,2-dicarboethoxyethyl) O,O-dimethyldithiophosphate)] is one of the most frequently used OP because of its low acute toxicity compared to other OP insecticides. It is an exceptional OP by containing two carboxylester groups and phosphotriester moiety that define it as an OP. Malathion preparations contain traces of several oxon OPs and it is activated in vivo to malaoxon by mixed function oxidases. Activated oxon forms of OPs including malaoxon are powerful inhibitors of malathion carboxylesterase (MCE) and AChE activities [4].

In aphid (*M. persiace*) and mosqitoes (*Culex quinquefasciatus* and *C. pipiens*) resistance to OPs is achived by tandem gene amplifications of up to 300 copies of a particular carboxylesterase gene (nearly 3% of the total protein of the organism). Even though, the enzyme has negligible OP hydrolytic activity, overexpression enables it to sequester sufficient amount of OPs for the organism to survive, which prevents the inhibition of target site AChE [2, 5, 6].

In *L. cuprina* and *M. domestica* strains resistance to diazinon and malathion is mainly caused by a carboxylesterase gene on the fourth and second chromosomes, respectively [7]. This major OP resistance gene (in *L. cuprina* named as *LcαE7*, in *M. domestica* named as *MdαE7*) encodes the major ali-esterases of *L. cuprina* and *M. domestica* which are also known as esterase isozymes, ALI and E3, respectively [8, 9]. The *MdαE7* and *LcαE7* genes are located in a cluster of closely related esterase genes, called α-esterase cluster [10].

The *LcαE7* and *MdαE7* genes were isolated, cloned and expressed in vitro, and a $Gly^{137} \longrightarrow$ Asp change was found in the active site of the enzyme from diazinon resistant *M. domestica* [8] and *L. cuprina* strains [11, 12]. A different amino acid substitutions $Trp^{251} \longrightarrow$ Leu were found in the *LcαE7* gene in malathion resistant *L. cuprina* strains [3, 9].

Products of both alleles can detoxify a broad and overlapping range of oxon OPs. The resistance in these two types are associated with OP hydrolase activity (defined as the hydrolysis at phosphotriester moiety of the insecticides) and low ali-esterase activity (defined as the hydrolysis of aliphatic esters such as methyl butyrate) [3, 4, 9]. This led to a proposal that each type of OP resistance in these organisms is the result of a mutation in a specific carboxylesterase that enhanced the enzyme's turnover rate of certain OP compounds but at the same time compromised the enzyme's activity toward artificial ester substrates (the "mutant ali-esterase hypothesis") [3, 9, 12, 13].

* This article was submitted by the authors in English.

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**Table 1.** List of the fly strains, their origins and resistance status (including the $LD_{50}$ values of malathion resistant strains)

| Strain | Origin | Resistance status | $LD_{50}$ (μg/μl/fly) |
|---|---|---|---|
| Ankara | Ankara | Malathion | 384.64 |
| Ceyhan1 | Ceyhan | Malathion | 300.38 |
| Ceyhan2 | Ceyhan | Malathion | 316.89 |
| Adana | Adana | Malathion | 356.12 |
| Kirazli | Bursa | Unknown | |

In insecticide resistant housefly strains, the OP resistance is associated with elevated levels of gluthation-S-transferase (GST) activity compared with susceptible flies [14, 15]. GST enzymes can play an important role in detoxification of xenobiotic compounds including insecticides and these enzymes have been studied by [6, 16–19].

AChE, encoded by the *Ace* gene, is the primary target for OP and carbamate insecticides. In housefly, the insensitivity of AChE against OPs has been studied [5, 20–25] and was shown to be the result of point mutations of $Gly^{342}$ and $Tyr^{407}$ [23, 26]. In malathion resistant *L. cuprina* and *M. domestica* strains, high levels of malathion carboxylesterase (MCE) activity have also been measured [4, 9, 27].

The first objective of this study was to examine the changes in *MdαE7* gene from a great level of malathion resistant (Ceyhan1, Ceyhan2, Adana, and Ankara) and Kirazli (no insecticide treated in the laboratory environment) housefly strains. Our first task was to sequence the *MdαE7* gene (from exon2 to exon6) from these strains to test whether these high resistance levels are due to additional amino acid substitutions. Also, to perform southern blot hybridization analysis to find out whether there is a duplication of *MdαE7* gene in the housefly genome. The second objective of this study was to understand the biochemical basis of malathion resistance by assaying MCE, GST, general esterase (hydrolase) and percent remaining AChE activities. Since this study combines the biochemical evidences with the sequence data of *MdαE7* gene of the strains obtained from their natural environments and selected in laboratory for their malathion resistance, it is valuable.

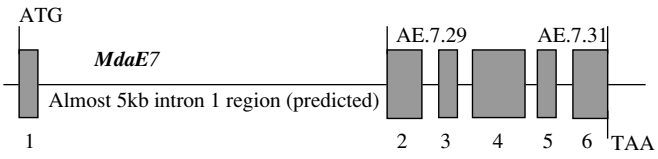


**Fig. 1.** The *MdαE7* gene and the primers used for amplification. The exons are boxed and numbered 1–6. The ATG initiation codon and TAA termination codon are marked.

## MATERIAL AND METHODS

*Housefly stocks.* Houseflies were collected from garbage at 4 different locations (Ankara, Adana and two different regions from Ceyhan; Ceyhan1 and Ceyhan2) in Turkey and kept in separate cages so as to prevent interbreeding in the laboratory environment. Technical malathion (98%) was dissolved in pure acetone and applied to the dorsal face of the thorax of 5–6 days old flies (anesthetized under flow of carbon dioxide) by topical application. One microliter of different doses of 98% technical malathion in acetone was applied to each fly and, at each generation, the resistant flies were mated with each other. At the end of the selection period 600 μg/fly malathion solutions were applied to all stocks and living ones mated with each other and offsprings were frozen and stored at –80°C until used. Kirazli strain was kept in the laboratory without exposure to other strains or insecticide pressure. Laboratory selected malathion resistant housefly strains and their $LD_{50}$ values are given in Table 1.

*Laboratory conditions.* Adana, Ceyhan1, Ceyhan2, and Kirazli strains were kept at 25 ± 3°C, in 50–70% relative humidity and constant illumination. Adult flies were fed with sugar, milk powder and water. Eggs were collected from the cages and cultured in larval medium, which consisted of 26% suncured alfalfa, 40% extracted residue of barley malt, and 33% of wheat bran. One kg of this mixture was added to two liters of water containing 50 g bakers yeast and 50 g powdered milk.

*Genomic DNA extraction and the amplification conditions of MdαE7 gene.* Genomic DNAs were isolated from 5–6 days old flies by using lifton method [28]. The region from the beginning of exon 2 to the end of exon 6 was amplified by using primers AE7.29 and AE7.31 (Fig. 1). PCR was conducted in a Corbett Research FTS-1 thermal cycler. For PCR reactions, procedure mentioned by [8] was followed. The PCR products were visualized and isolated from the 1% agarose gel by using Qiagen Qiaquick PCR purification kit following manufacturer's instructions. 5 μl of the purified amplicons were visualized on a 1% agarose gel and the remainder stored at –20°C prior to either direct sequencing or cloning. Purified PCR products were inserted into T-tailed P-GEM-T plasmid vector following standard techniques [29]. The products of at least two PCR reactions or three individually cloned plasmid DNA preparations from individual flies were sequenced by using appropriate gene primers (from exon 2 to end of exon 6) and primers complementary to the T7 and SP6 promoters in the vector. DNA was sequenced using TaqFS dye terminator chemistry (Applied biosystems) on the Applied biosytems model 373A automated DNA sequencer. The *MdαE7* gene was sequenced from the clones and PCR amplified genomic DNAs by direct sequencing.

*Southern blot hybridization.* Southern blot hybrizations were performed using the standard procedure of [29]. Genomic DNAs were digested with ApaL1 (cuts

nearly in the middle of the *MdαE7*) and *Xba*I (does not cut the gene). The X-ray films were developed 24 h later to decide longer or shorter exposure was required.

*Malathion carboxylesterase activity assay*. Procedure from [4] was followed and ten individual flies were used.

*Acetylcholinesterase activity assay*. AChE activities were measured according to the method of Moores [22]. In this assay ten inidividual housefly heads were used and percent remaining activities were calculated as: Mean inhibited activity × 100/uninhibited activity.

*Glutathion-S-transferase activity assay*. GST activities were measured with some modifications according to [30]. For the measurements of GSTs activities ten individual fly heads and thoraces were homogenized in 300 µl of 225 mM Tris-HCl buffer (pH 7.5). To the 100 µl of homogenized sample, 300 µl of 225 mM Tris-HCl buffer, 100 µl of 9 mM CDNB and 400 µl of 5 mM GSH were added. Enzyme activities were measured as nmol/min/fly at 344 nm by using Shimatzu spectrometer.

*General (non-specific) esterase activity assay*. The measurements were done according to the method of Gomori [31] with some modifications. Ten individual house fly heads and thoraces were homogenised in 200 µl of 0.2 M pH 7.5 phosphate buffer. After adding 200 µl of same phosphate buffer containing 0.1% triton X-100, this mixture was incubated for an hour at 4°C. Activity measurement were carried on by the addition of 400 µl of same phosphate buffer, 300 µl of 0.06% fast garnet GBC salt, 100 µl of 1.8 mM α-naphtylacetate to the 100 µl of the sample solution. Activities were measured as µM/min/fly at 365 nm by using Shimatzu spectrophotometer.

*Protein assay*. The protocol from Bradford [32] was followed; 0.1 ml protein solution in appropriate buffer was added to 5 ml of protein reagent (final concentrations in the reagent were 0.01% (w/v) comassie brilliant blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid) the test tube and the contents mixed by inversion. The absorbance at 595 nm was measured after 2 min and before 1 hr in 1 ml cuvettes against reagent blank prepared from 0.1 ml of the approprite buffer and five ml of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in standart curve used to determine the protein in samples.

*Computer methods*. Staden and Genetics Computer Group (GCG) (GCG; [33]) and DNA Bio Star software programs were used for sequence assembly, alignments, and analysis.

## RESULTS

### *MdαE7 from Malathion Resistant Strains*

The segments of the *MdαE7* gene starting from the beginning of exon 2 to the end of exon 6 (total 1915 bp) were amplified by using primer pairs AE7.29 and AE7.31 (Fig. 1), and directly sequenced from all Turkish housefly strains by using appropriate internal primers.

**Table 2.** At six different positions malathion resistant and diazinon resistant "Rutgers" and WHO (susceptible) *M. domestica* strains have amino acid changes in MdαE7

| Position of amino acid | $Mal^R$ (Ceyhan1, Ankara, Adana, and Ceyhan2) | $Dia^R$ (Rutgers)* | WHO (susceptible)* |
|---|---|---|---|
| 32 | Asn | Thr | Asn |
| 137 | Gly | Asp | Gly |
| 250 | Thr | Ser | Thr |
| 251 | Ser | Trp | Trp |
| 304 | Ile | Met | Met |
| 359 | Ile | Ile | Asn |

* From [8].

**Table 3.** At four different positions malation resistant and Kirazli Turkish house fly strains have amino acid changes in *MdαE7*

| Position of amino acid | Ankara | Adana | Ceyhan1 | Ceyhan2 | Kirazli |
|---|---|---|---|---|---|
| 309 | Met | Thr | Met | Met | Met |
| 358 | Ser | Leu | Ser | Leu | Leu |
| 366 | Val | Ala | Val | Ala | Ala |
| 384 | Ser | Thr | Ser | Thr | Thr |

To sequence the *MdαE7* gene (except intron 1 and exon 1 regions) from the clones, products of PCR reactions were inserted into a PGEM-T easy vector and then sequenced from these clones. In this sequencing method, in addition to internal primers and plasmid vector specific Sp6 and T7 primers were also used.

The *MdαE7* sequences from susceptible strain, WHO, compared with Turkish malathion resistant and Kirazli house fly strains, $Trp^{251} \longrightarrow Ser$ change was identified in the active center. In addition to this $Ser^{250} \longrightarrow Thr$ and $Asp^{137} \longrightarrow Gly$ substitutions was observed in *MdαE7* of Turkish house fly strains in comparison with diazinon resistant Rutgers strain (Table 2). Comparing all the amino acid coding sequences of *MdαE7's*, most of the changes were in exon 2, 3, and 4. Exon 5, 6 were relatively conserved among the strains. Probably, these regions code for functionally important residues irrespective of resistance status. Malathion resistant and Kirazli house fly strains differed at four positions in inferred amino acid sequence of *MdαE7* (Table 3).

Like in *L. cuprina* and *D. melanogaster* *αE7* genes, all the *MdαE7* alleles that we have sequenced, contains a number of motifs that are characteristic for carboxyl/cholinesterases.

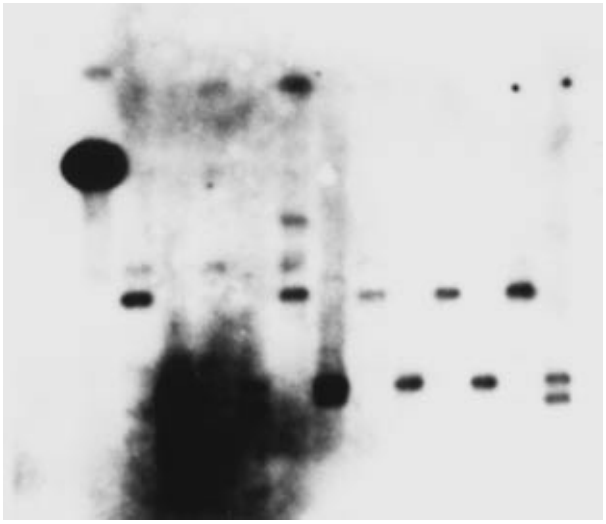


**Fig. 2.** Southern blot gel analysis of Ceyhan1 strains, 6 individuals were used. The first big dot is marker. In lanes *1–3–5–7–9–11 Apa*LI, *2–4–6–8–10–12 Xba*I restriction enzymes were used.

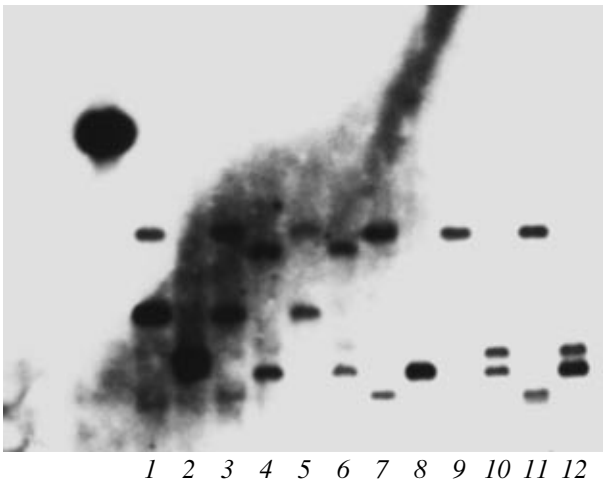


**Fig. 3.** Southern blot gel analysis of Ceyhan2 strains, 6 individuals were used. The first big dot is marker. In lanes *1–3–5–7–9–11 Apa*LI, *2–4–6–8–10–12 Xba*I restriction enzymes were used.

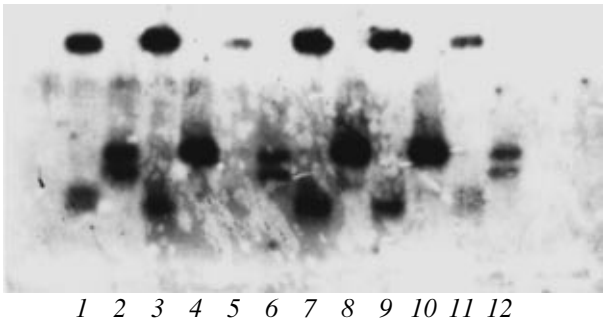


**Fig. 4.** Southern blot gel analysis of Ankara strains, 6 individuals were used. In lanes *1–3–5–7–9–11 Apa*LI, *2–4–6–8–10–12 Xba*I restriction enzymes were used.

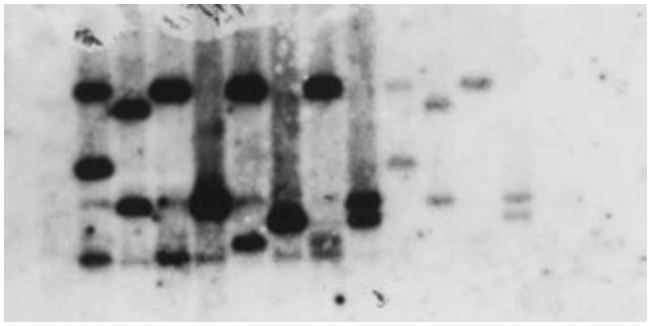


**Fig. 5.** Southern blot gel analysis of Adana strains, 6 individuals were used. In lanes *1–3–5–7–9–11 Apa*LI, *2–4–6–8–10–12 Xba*I restriction enzymes were used.

### *Southern Analysis*

The genomic DNAs from malathion resistant strains were digested with 2 different restriction enzymes *Apa*LI and *Xba*I. *Apa*LI cuts the *MdαE7* gene once (at the middle) in malathion resistant Turkish house fly strains. *Xba*I does not cut the *MdαE7* gene. From the beginning of exon 2 to the end of exon 6 (1915 bp region) PCR amplified genomic DNA was used as a probe. In blots with *Apa*LI digestion 2 or 3 bands, with *Xba*I digestion 1 or 2 bands were observed (Figs. 2–5).

### *Malathion Carboxylesterase (MCE) Assay*

The malathion carboxylesterase activities were assayed by using whole fly homogenates of adults from the malathion resistant (Ankara, Adana, Ceyhan1, and Ceyhan2) and Kirazli strains. The mean MCE activities are given in Fig. 6. Kirazli strain, which was not treated with malathion in the laboratory environment, had the lower malathion carboxylesterase activity (704 pmol malathion/min/mg) than the malathion resistant strains. Among the resistant strains, Ceyhan2 strain showed the highest MCE activity (1419 pmol malathion/min/mg, almost 8 times higher than WHO susceptible strain), while Ceyhan1 had the lowest activity (867 pmol malathion/min/mg, almost five times higher than WHO) among the resistant strains. Ankara and Adana strains showed 944 pmol malathion/min/mg and 1331 pmol malathion/min/mg MCE activities, respectively.

### *Acetylcholinesterase (AChE) Activity*

Mean percent activities were calculated according to Moores *et al.* [22]. The results were given in Fig. 7. Kirazli strain was found (12% remaining activity) near-

ly as sensitive as WHO which has 11% remaining activity. Ceyhan1 strain with 43% remaining activity was detected as the most insensitive strain to maloxon (by considering this assay), being nearly four times more resistant than WHO. Ceyhan2 strain showed the lowest remaining activity (23%) among the malathion resistant strains.

### *Glutathion-S-transferase (GST) Activity Assay*

In this assay, higher GST activities were detected in all malathion resistant strains than WHO susceptible strain (39.96 metabolized CDNB nmol/min/mg). Ankara strain had the highest GST activity (75.0 nmol metabolized CDNB/min/fly) among all the strains tested. Adana (60.66 nmol metabolized CDNB/min/mg), Ceyhan1 (65.33 nmol metabolized CDNB/min/mg) and Ceyhan2 (60.14 nmol metabolized CDNB/min/mg) strains showed closer activities. Kirazli strain had 43.55 nmol metabolized CDNB/min/mg activity (Fig. 8).

### *General (Non-Specific) Esterase Activity Assay*

Lower general esterase activities were observed in all malathion resistant house fly strains than WHO control strain. Among all the tested strains Kirazli flies, which were not exposed to any insecticides in the laboratory environment, showed closest activity (10.53 μM produced α-napthol/min/mg) to WHO which had 12.78 μM produced α-napthol/min/mg activity. Ankara (8.15 μM produced α-napthol/min/fly), Adana (7.19 μM produced α-napthol/min/fly), Ceyhan1 (7.93 μM/min/fly), and Ceyhan2 (7.47 μM produced α-napthol/min/fly) strains resulted in closer activities to each other being lower than Kirazli (Fig. 9).

## DISCUSSION

The *MdαE7* gene (except intron 1 and exon 1 regions) has been sequenced from the high malathion resistant Turkish housefly strains. The inferred amino acid sequence of *MdαE7* protein (from exon 2 to the end of exon 6) from all sequenced five strains contained all characteristic motifs of catalytically active members of carboxyl/cholinesterases; the acid turn, the histidine loop, the catalytic triad, the nucleophilic elbow and the oxyanion hole [34, 35].

One amino acid change ($Trp^{251} \longrightarrow$ Ser) was identified in the active site of *MdαE7* from all Turkish house fly strains in comparison with the susceptible WHO strain. It was previously reported by Newcomb *et al*. [12] and Campbell *et al*. [3, 9] that the Trp $\longrightarrow$ $\longrightarrow$ Leu substitution, at the position of 251, was recovered in *LcαE7* from malathion resistant *L. cuprina* strains which is diagnostic for malathion type OP resistance. The proposed mechanism of $Trp^{251} \longrightarrow$ Ser substitution for malathion hydrolysis in *L. cuprina* is given in Campbell *et al*. [3]. Probably, the replacement of a

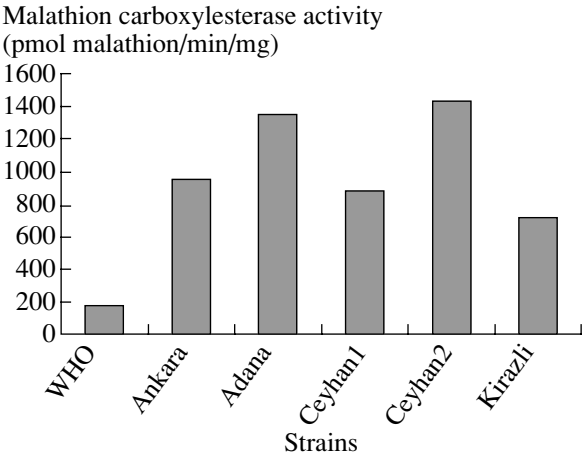


**Fig. 6.** Bar diagram of mean malathion carboxylesterase activities of susceptible and malathion resistant housefly strains.

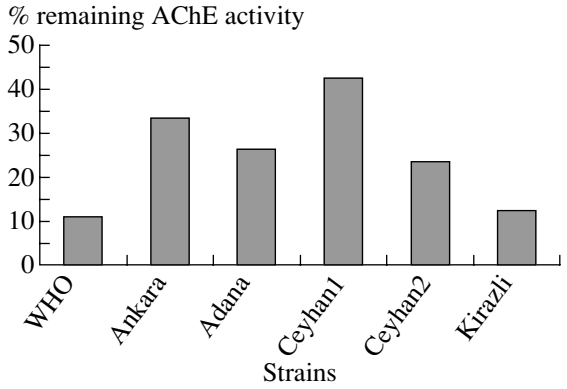


**Fig. 7.** Bar diagram of percent remaining activities of AChE enzyme of susceptible and malathion resistant housefly strains.

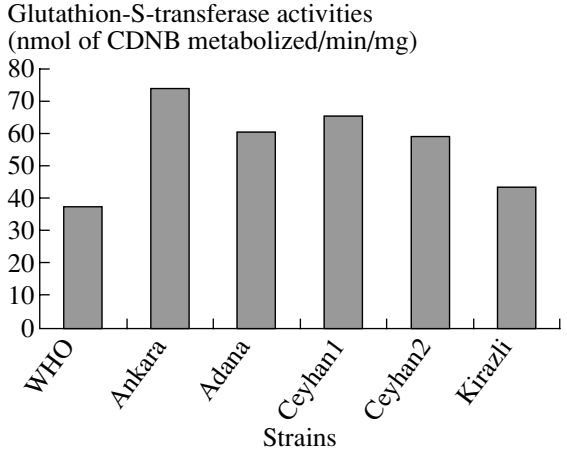


**Fig. 8.** Bar diagram of mean glutathion-S-transferase activities of susceptible and malathion resistant housefly strains.

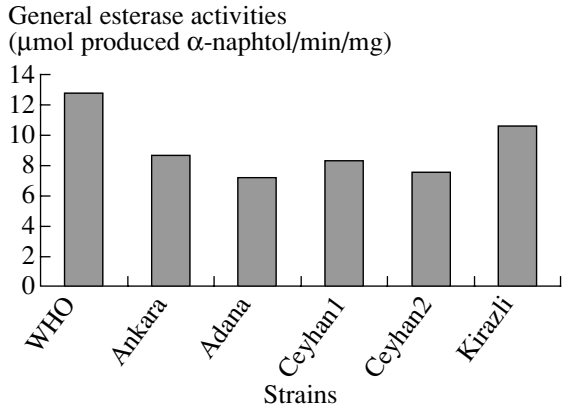


**Fig. 9.** Bar diagram of mean general esterase activities of susceptible and malathion resistant housefly strains.

bulky hydrophobic Trp with a smaller residue like Gly, Ser, or Leu at this site in *Lcα E7* and *Mdα E7* may enhance the malathion hydrolysis. From a malathion resistant strain of wasp (*Anisopteromalus calandrae*) Trp ⟶ Gly (corresponding to position of $Trp^{251}$ in E3/ALI) substitution was identified in a carboxylesterase, which has the same characteristic motifs of α-esterase [15]. In orthologous members of a large multigene family, selection was operated for malathion type resistance to select similar amino acid mutations independently and this implies that the range of genetic options available for selection of resistance by the mechanism of the acquisition of OP hydrolase activity is very limited [2, 27].

High MCE activities were found in malathion resistant Turkish house fly strains like in malathion resistant *L. cuprina* strains. The difference in activity between sussceptible and resistant *M. domestica* strains is similar to that of malathion resistant *L. cuprina* strains [3, 4, 9, 27]. Lower general esterase activities were observed in malathion resistant Turkish house fly strains than WHO susceptible control strain. Campbell *et al.* [4] reported that OP resistant *M. domestica* strains exhibited 25–50% less general esterase activities than susceptible strains. The reason for this lower activity (than the control) might be explained by the mutant ali-esterase hypothesis.

In this study, in all malathion resistant strains the mean percent remaining activities of AChE were higher than that of the WHO susceptible strain. The results are consistent Moores *et al.* [22]. In his study, the percent remaining activities of AChE by using OP insecticides on different *M. domestica* strains were measured and wide range of percent remaining activities were reported (from 1.8 to 81.1%). It is likely that, another malathion resistance mechanism in our house fly strains might be the target site (AChE) insensitivity mechanism.

It was stated by Claudianos [27] that the mutant form of *Lcα E7* is not alone sufficient to protect the organisms adequately against the high dose of OPs applied in the field. In *M. domestica* the first resistant strains (based on mutant ali-esterase of ALI/E3) was reported within two to three years of the first use of OPs. And then several other mechanisms have been identified from other species which altogether can confer very high resistance levels [2]. In *M. domestica* as well as ali-esterase and GST there are now several target site mutations in AChE and enhanced detoxifications due to the cytochrome P450s [21].

The expression of α-esterases may be integrated with other complementary detoxification systems. In *M. domestica* there might be a genetic association between OP resistant ALI and upregulated cytochrome P450s and GST transferases [6, 8, 12, 36]. It was suggested by Plapp [37] that in housefly, and may be in other insects, a single major locus which is located on the chromosome II is responsible for the regulation of the insecticide metabolising enzymes in co-ordination. Supposedly, it interacts with minor genes on other chromosomes to confer metabolic resistance to many types of insecticides by inducing synthesis of appropriate detoxifying enzymes. In addition, it was hypothesised by Feyereisen [36] that a regulatory gene may control the over expression of GST genes of *MdGST-1*, *MdGST-3* and other P450 genes. This regulatory gene is mapped on chromosome II close to the chromosome 2 markers *aristapedia* and *carnation eye*, where the resistance to DDT and diazinon also maps.

Our findings for GST activity assay indicated that in malathion resistant house fly strains 1–2 times higher GST activities than WHO control strain. These results are similar with the findings of Franciosa and Berge [38]. They reported that resistant *M. domestica* strains had 1.3 times more GST activity than susceptible strains.These high GST activities may support the Plapp's hypothesis [37] mentioned above.

In malathion resistant Turkish house fly strains higher $LD_{50}$ values might have implied us that there might be a duplication for *Mdα E7* gene in the fly genome. However, southern blot analysis results indicated that probably, instead of duplication of the *Mdα E7* gene in the genome, there might be restriction enzyme cutting site polymorphism outside of the gene at both alleles in all malathion resistant house fly strains.

In order to better understand the molecular mechanisms of insecticide resistance and evolution of resistance in *M. domestica* and *L. cuprina* further studies should be carried out involving biochemical mechanisms. These will help to develop strategies in fighting with insect pest populations.

## ACKNOWLEDGMENTS

This study was partially supported by a research grant (METU, AFP-99.06.02.21) and an integrated Ph.D. fellowship program from TUBITAK.

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