Original Article

Insecticide Resistance and Mechanisms of *Culex pipiens* Populations in the Mediterranean and Aegean Regions of Turkey During 2017–2018

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Abstract

Background: *Culex pipiens* has a significant public health importance since it is an important vector of West Nile virus and Rift Valley fever virus. We, therefore, aimed to determine the insecticide resistance level in *Cx. pipiens* populations in the Aegean and Mediterranean regions of Turkey.

Methods: Bioassays have been carried out against Dichlorodiphenyltrichloroethane (DDT) (4%), Malathion (5%), Fenitrothion (1%), Propoxur (0.1%), Bendiocarb (0.1%), Permethrin (0.75%) and Deltamethrin (0.05%). Biochemical analyses have been performed to detect non-specific esterase, mixed function oxidase, glutathione-s-transferase and acetylcholinesterase levels. A knockdown resistance (*kdr)* (L1014F) and Acetylcholinesterase (Ace-1) (G119S) mutations have been detected by using allele-specific primers and a polymerase chain reaction (PCR) amplification of specific alleles (PASA) diagnostic test was performed for detection of F290V mutation.

Results: Bioassay results showed that all *Cx. pipiens* populations were resistant to DDT, Malathion, Fenitrothion, Bendiocarb, Propoxur and some of the populations have started to gain Permethrin and Deltamethrin resistance. Biochemical analyses results revealed that altered glutathione-s-transferases, P450 monooxygenases, esterase levels might be responsible for DDT, organophosphate, carbamate and pyrethroid resistance in *Cx. pipiens* populations. Results showed mild to high frequency of L1014F, low frequency of F290V but no Ace-1 G119S mutation within the populations. Additionally, acetylcholinesterase insensitivity was not significantly high within the most of these populations.

Conclusion: Overall results may help to fulfil the lacking information in the literature regarding insecticide resistance status and underlying mechanism of *Culex pipiens* populations of the Mediterranean and Aegean region of Turkey by using all bioassays, molecular tests and biochemical assays.

Keywords: Kdr; Acetylcholinesterase; Monooxygenase; Glutathione S-Transferase

Introduction

Mosquitoes can transmit many different pathogenic organisms such as viruses, bacteria, protozoa and nematodes and impact over half of the worlds' population through the transmission of harmful diseases to both human and animal. They are also responsible for transmitting malaria, lymphatic filariasis and arboviruses such as yellow fever virus, dengue virus and Zika virus (1). Millions of people are threatened and killed by mosquito-borne diseases every year (2).

Mosquito control strategies have played a major role in reducing the global burden of mosquito-borne diseases since the introduction of dichlorodiphenyltrichloroethane (DDT) in the 1940s (3). Malaria

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control has been achieved during recent years as a result of insecticide-based strategies including indoor residual spraying (IRS), insecticide-treated nets (ITNs) and long-lasting insecticide-treated nets (LLITNs) (4). Current control of mosquitoes is still heavily dependent upon the use of chemical insecticides and four classes of synthetic insecticides called organochlorines (OC), organophosphates (OP), carbamates (CB), pyrethroids (PY) are recommended by World Health Organisation (WHO) for IRS (3). However, pyrethroids are recommended for use in ITNs and LLITNs (3). Despite their environmental pollution concerns and toxicity to non-target organisms and humans, insecticides are still heavily used in current mosquito control strategies. However, this is not sustainable because extensive use of insecticides has also resulted in the development of resistance in mosquito populations around the world.

Culex pipiens is amongst the most important mosquito species and responsible of maintaining several viruses such as West Nile virus (WNV), Rift Valley fever virus (RVFV) and Japanese encephalitis virus (JEV) which can be pathogen of human and livestock animals (5). According to the European Centre for Disease Prevention and Control (ECDC) report, 1605 WNV infection and 166 deaths due to WNV infection was reported from 11 EU/EU member states in 2018 (6). There have been no reported WNV cases until 2009 in Turkey. However, both epidemic and WNV-related central nervous system diseases have been reported in the western part of Anatolia since 2009 (7). A total of 47 and 5 WNV were reported from different regions of Turkey in 2010 and 2011, respectively (8). Recently, one human case of WNV has been reported in 2019 (6).

While many integrative techniques can be used for *Cx. pipiens* control, the fastest and cheapest way to control vector densities in endemic regions, control epidemics and reduce annoying mosquito populations is to apply insecticides. However, as it was stated before, rapidly developing insecticide resistance in mosquito populations is the biggest obstacle in vector management and control. Therefore, it's so crucial to understand the status and mechanisms of insecticide resistance for overcoming or delaying resistance to existing compounds and preventing the development of resistance to new pesticides (9).

To date, a few studies have been reported regarding the insecticide resistance status of Turkish *Cx. pipiens* populations. Akıner et al. (10) reported high DDT resistance in *Cx. pipiens* populations in Antalya, Ankara, Çankırı, Mersin, Hatay, Birecik and Viranşehir. They also reported high Fenitrothion resistance in Birecik, Mersin, Çankırı and Antalya; high Temephos resistance in Birecik, Viranşehir, Mersin, Ankara and Antalya. Similarly, high DDT, Malathion, Permethrin and Deltamethrin resistance have been reported in *Cx. pipiens* populations of Mediterranean region (Mersin, Adana and Antalya). High mixed function oxidase (MFO) and non-specific esterase (NSE) activities have been attributed to high DDT, Malathion and pyrethroid resistance in these populations (11). Taşkın et al. (12) reported high Malathion, Bendiocarb and Dieldrin resistance of *Cx. pipiens* populations collected from the Aegean region. Kdr mutation (L1014F and L1014C) frequencies were also high in these populations. However, Ace-1 (G119S and F290V) and Rdl (A302S) mutation frequencies were too low to explain Malathion and Dieldrin resistance. Finally, Guz et al. (13) reported high frequencies of Diflubenzuron resistance in Muğla for the first time. The Chitin synthase 1 gene mutations (I1043L and I1043M) were ranging from 15.7% to 52.7% and the kdr L1014F mutation was ranging from 40% to 50%.

Turkey is a country with high agricultural activities. Because of the fact that wide ranges of insecticides are still used for different kinds of pest control in Turkey (14). Although many insecticides have been used for many years, studies to regularly monitor resistance development in mosquito populations are not sufficient. Additionally, this study aims to evaluate both the molecular

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and biochemical mechanisms underlying the current insecticide resistance at the same time in *Cx. pipiens* populations of the Mediterranean and Aegean region during 2017 and 2018.

Materials and Methods

Mosquito strains

Larva and adult *Cx. pipiens* samples were collected from a total of 10 locations in both the Mediterranean and the Aegean regions of Turkey between April 2017 and September 2018 (Fig. 1, Table 1). Larval samples were collected using larval dippers and transferred to the transport container until it was brought to the laboratory.

Gravid females were collected from stables and houses by using mouth aspirators and brought to the laboratory in a cardboard cup sealed with a thin cloth. Following the egg laying of gravid females, F_1 generations were obtained. Larval samples were reared to adults under the standard laboratory conditions at 26–28 °C, 12:12 h photoperiod and 70–80% relative humidity in an insectarium. Morphological identifications were performed using an identification key (15). An unfed 3–5 days old F_1 generation females were preferred to use in bioassays, biochemical assays and molecular study. Samples were stored in a -80 °C freezer until biochemical assays have been carried out. We have sensitive *Culex quinquefasciatus*

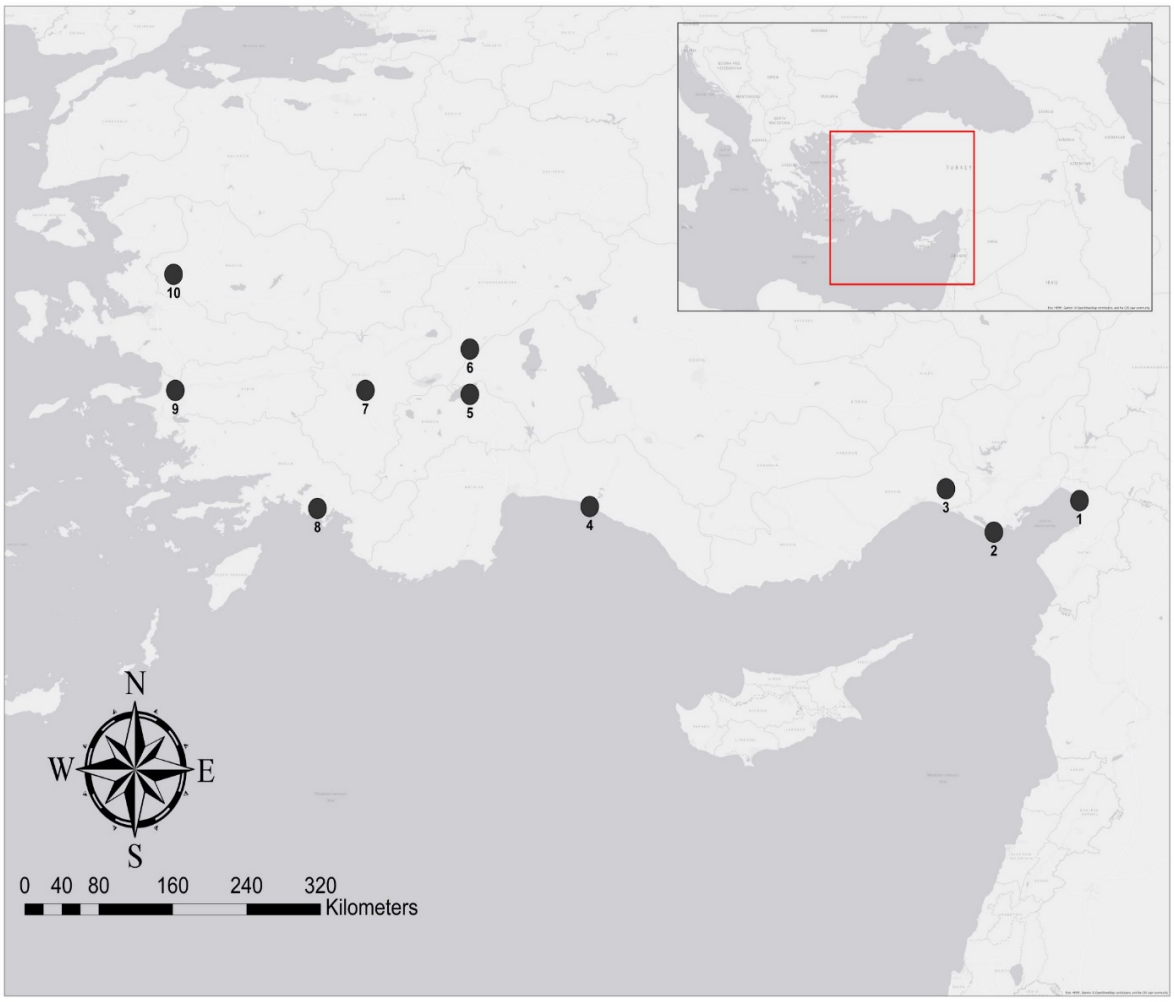

Fig. 1. Sampling localities of *Culex pipiens* populations in Turkey (1. Hatay-Dörtyol, 2. Adana-Karataş, 3. Mersin-Tarsus, 4. Antalya-Manavgat, 5. Burdur-Gölhisar, 6. Afyon-Dinar, 7. Denizli-Honaz, 8. Muğla-Dalaman, 9. Aydın-Söke, 10. Manisa-Hacıhaliller)

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Table 1. Sampling localities of *Culex pipiens* populations in Turkey, 2017-2018

Region	Province	Locality	Abbreviation	Locality No	Field property
Mediterranean Region	Hatay	Dörtyol	HD	1	Tourism and Agriculture
	Adana	Karataş	AK	2	Agriculture
	Mersin	Tarsus	MT	3	Agriculture
	Antalya	Manavgat	AM	4	Tourism and Agriculture
	Burdur	Gölhisar	BG	5	Agriculture
Aegean Region	Afyon	Dinar	AD	6	Agriculture
	Denizli	Honaz	DH	7	Tourism and Agriculture
	Muğla	Dalaman	MD	8	Tourism and Agriculture
	Aydın	Söke	AS	9	Tourism and Agriculture
	Manisa	Hacıhaliller	MH	10	Agriculture

laboratory strain which had not been exposed to insecticides for more than 15 years and raised in Aydın Adnan Menderes university vector biology laboratory. These sensitive *Cx. quinquefasciatus* lab strain was used as a reference strain for comparison in biochemical analyses.

Bioassays

Bioassays have been carried out through WHO's insecticide susceptibility bioassay tubes (16). Susceptibility tests was performed against DDT (4%), Malathion (5%), Fenitrothion (1%), Propoxur (0.1%), Bendiocarb (0.1%), Permethrin (0.75%) and Deltamethrin (0.05%) using WHO papers supplied by the WHOPES collaborating Centre at University Sains Malaysia. Bioassays were performed in the insectarium, under the same physical conditions in which mosquitoes were raised. A total of 660 adult females were used with controls of each of seven types of insecticides, 60 individuals from each population. For each test, we used 20 adult mosquitoes in assay tubes in triplicate. Mosquitoes were transferred into the holding tubes after one-hour exposure to each insecticide-impregnated paper and fed on 10% sugar solution for 24 h. Control group were exposed to insecticide free, impregnated only with the excipient without any active ingredient control papers for one hour. Mean values of three replicates were used to calculate mortality rates. Resistance levels were calculated as susceptible if mortality rates are ≥ 98%, possible resistance if mortality rates are between 90–97% and resistant if mortality rates are lower than 90% (17).

Biochemical assay

A total of 330 (30 samples from each population in addition to the control group) individuals were used for biochemical analyses. An unfed 3–5 days old *Cx. pipiens* F1females were used to perform enzyme assays.

Homogenization was first carried out using liquid nitrogen and then followed by 250 µL 50 mM sodium phosphate buffer onto the ice to keep the samples from heat denaturation. Homogenates were centrifuged at 10.000 g for 10min. at 4 °C. Spectrophotometric analyses were carried out in 96 well microtiter plates using Biotek Elx808 microplate reader (Biotek Instruments, USA). Protein content was measured using a Bradford assay which includes Bradford dye reagent prepared with Coomassie brilliant blue, and the absorbance was measured at 595 nm (18). Total protein content was measured using a standard curve of bovine serum albumin (BSA).

All biochemical analyses including NSE, MFO, glutathione-s-transferase (GST) and Acetylcholinesterase (AchE) assay were carried out by following the test procedure provided by WHO (16). All tests were conducted as two replicates. Alpha-naphthyl acetate, beta-naphthyl acetate and p-nitrophenyl acetate (pNPA) were used as substrates of esterase enzyme for the

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calculation of non-specific esterase activity. Standard curves of alpha- napthol, beta-naphtol and 4-nitrophenol were created and enzyme activity was calculated as enzyme units (EU, µmol/min) using these standard curves of alpha, beta and p-naphthol acetate. Specific enzyme activities were calculated based on enzyme units and stated as EU/mg protein for each esterase. MFO level was calculated using heme-peroxidase assay based on heme-protein amount (16, 19). Heme protein content was calculated using a standard curve of cytochrome C protein to calculate MFO levels. Similarly, WHO (16) were followed for the calculation of GST levels. The extinction coefficient (ε): 4.39 mM^{-1} was used to calculate specific GST enzyme activities as described by WHO (16). Finally, AchE and insensitive AchE levels were measured by following the instructions described by WHO (16). For this assay, the inhibition rate was calculated based on well optic density (OD) and the remaining AchE rates were calculated by dividing the OD of the well with Propoxur by that without Propoxur for the same mosquito, i.e. rate or end point with Propoxur * 100= % remaining activity in Propoxur inhibited replicate rate or end point without Propoxur.

Molecular assays

A total of 95 and 100 DNA was extracted from the Mediterranean and Aegean region, respectively by using Invitrogen PureLink genomic DNA isolation kit. Totally 195 individuals were tested for the presence of kdr (L1014F), Ace-1 G119S and Ace-1 F290V mutations from the study area.

A voltage gated sodium channel (Vgsc 1) generegionwasamplifiedusingprimersCgd1: GTGGAACTTCACCGAACTT C, Cgd2: GCAAGGCTAAGAAAAGGTTAAG, Cgd3:CCACCGTAGTGATAGGAAATTTA and Cgd4: CCACCGTAGTGATAGGAA-ATTTT (20). A polymerase chain reaction (PCR) were performed in a final volume of 25 µL containing 1 µL 2.5 mM dNTP, 2.5 µL 10 reaction buffer, 0.3 µL 20 mM each of the primers, 0.3µL (5 U/mL) Taq DNA polymerase, 1µL template DNA and 19.3 µL dH_2O. The amplification program consisted of an initial denaturation at 94 °C for 15min, 40 cycles of denaturation at 94 °C for 1min, annealing 50 °C for 1 min, extension 72 °C for 2min and followed by 72 °C for 10 min. Subsequently, amplified fragments were loaded on 1% agarose gel and visualized under UV light.

A 194 base paired Ace-1 gene amplicon was amplified using primer pairs ACE1-F1: 5′-CCGGGGGCCACCATGTGGAA-3′ and ACE1-R2: 5′GTTCTCCTCCGAGGCCAG-CGTCCG-3′ (21). The PCR was performed in a final volume of 25 µL containing about 50 ng DNA, 1.5 U Taq DNA polymerase, 20 µM each of primers, 2.5 mM dNTP, and 10X reaction buffer. PCR reaction conditions were denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 sn, extension at 72 °C for 1 min, and the final extension step of 72 °C for 5 min. Since the G119S mutation creates restriction sites, we used AluI enzyme which generates a 120 bp and 74 bp fragment to create fragments and visualize the fragment lengths on agarose gel as a result of the restriction fragment length polymorphism analysis (RFLP). The restriction fragment length polymorphism analysis content consisted of 2 µL 10X buffer, 1 µL Alu-1 enzyme (2 U), 2µL BSA (0,1 mg/ml), 15 µL PCR product and 5 µL dH_2O. This mixture was left in incubation for 4 hours at 37 °C and then the reaction was stopped at 65 °C for 20 minutes. PCR products then were visualized under UV light on 2% agarose gel. A PCR amplification of specific alleles (PASA) diagnostic test was performed for detection of F290V mutation as previously described by Taşkın et al. (12). A 543 bp control band, a 148 bp phenylalanine band and a 435 bp valine band were obtained after a PCR reaction. Amplified fragments were then visualized on 1.5% agarose gel electrophoresis under UV Light.

Statistical analyses

Median enzymatic activities were calculated for *Cx. pipiens* mosquito populations and compared with sensitive *Cx.*

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quinquefasciatus laboratory strain by way of Kruskal-Wallis test using Statistica version 12.

Results

Bioassay

All of the Mediterranean populations were resistant to DDT, Malathion, Fenitrothion, Bendiocarb and Propoxur. Mortality rates ranged between 71.6–85%; 71.6–81.6%; 70–81.6%; 71.6–83%; 78.3–83%; 91.6–98.3% and 91.6–100% for DDT, Malathion, Fenitrothion, Bendiocarb, Propoxur, Permethrin and Deltamethrin, respectively. The only Permethrin and Deltamethrin susceptible population was Gölhisar. All of the populations were possible resistant to Deltamethrin and Permethrin except the Gölhisar population. Mortality rates are given in Fig. 2.

All of the Aegean region populations were resistant against DDT, Malathion, Fenitrothion, Bendiocarb and Propoxur. Mortality rates ranged between 7.6–81.6%; 75–81.6%; 76.6–81.6%; 80–88.3%; 80–83.3%; 96.6–98.3% and 95–98.3% for DDT, Malathion, Fenitrothion, Bendio-

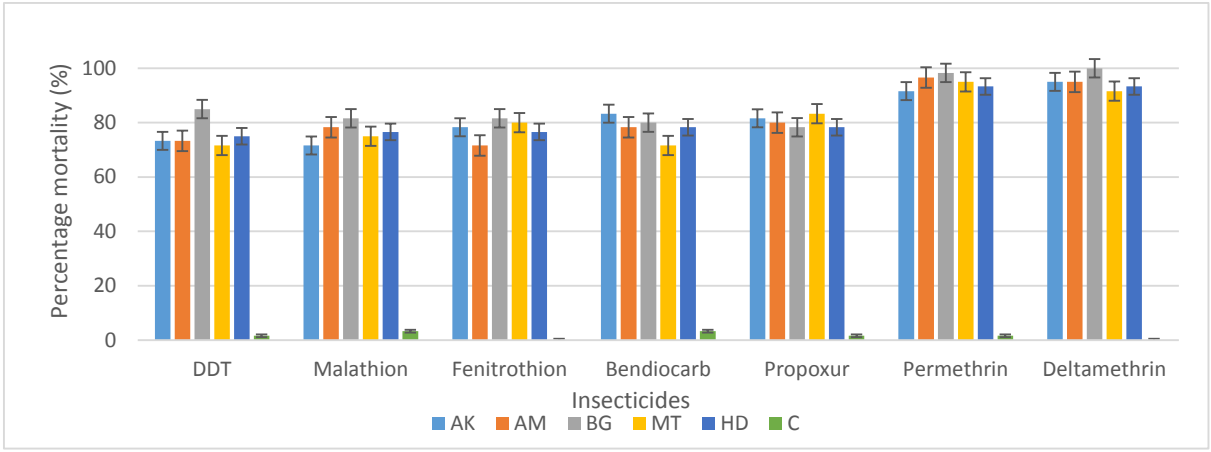

Fig. 2. World Health Organization insecticide susceptibility test results of *Culex pipiens* populations of the Mediterranean region, Turkey (Population name abbreviations represented as: Adana-Karataş (AK), Antalya-Manavgat (AM), Burdur-Gölhisar (BG), Mersin-Tarsus (MT), Hatay-Dörtyol (HD). Calculations are based on the mean results of three replicates. Error bars represent standard deviations.)

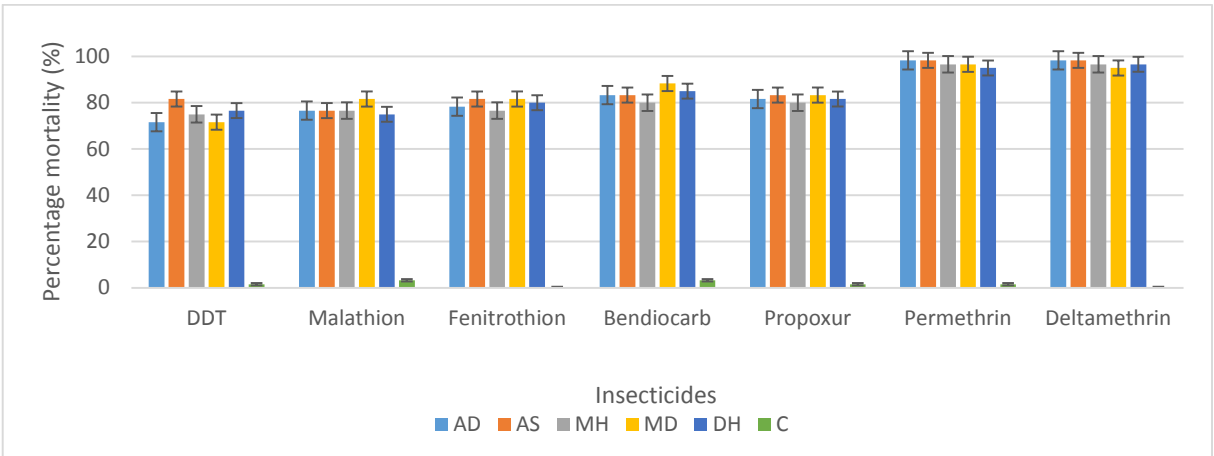

Fig. 3. World Health Organization insecticide susceptibility test results of *Culex pipiens* populations of the Aegean region, Turkey (Population name abbreviations represented as: Afyon-Dinar (AD), Aydın-Söke (AS), Manisa-Hacıhaliller (MH), Muğla-Dalaman (MD), Denizli-Honaz (DH). Calculations are based on the mean results of three replicates. Error bars represent standard deviations.)

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carb, Propoxur, Permethrin and Deltamethrin, respectively. All the Hacihaliller, Honaz and Dalaman populations were possible resistant to Permethrin and Deltamethrin, whereas the Dinar and the Söke populations were still susceptible to Permethrin and Deltamethrin. Mortality rates are given in Fig. 3.

Biochemical analysis results

The median specific alpha, beta and pNPA esterase activities were calculated as 0.242 EU/mg protein, 0.328 EU/ mg protein and 0.265 EU/mg protein in susceptible reference strain, respectively. Median specific enzyme activities for alpha esterase, beta esterase and pNPA ranged between 0.325 EU/mg protein and 1.041 EU/mg protein; 0.508 EU/ mg protein and 0.915 EU/mg; 0.754 EU/ mg protein and 1.526 EU/mg protein in the Mediterranean region populations of *Cx. pipiens*, respectively. Median specific alpha esterase, beta esterase and pNPA activities were significantly increased in all of the Mediterranean populations in relation to reference strain ($p < 0.05$) except the Manavgat population in which median alpha and beta esterase activities were not altered significantly and the Gölhisar population in which alpha esterase and PNPA activities were not significantly increased comparing to reference strain ($p > 0.05$).

Median specific enzyme activities for alpha esterase, beta esterase and pNPA ranged between 0.599 EU/mg protein and

1.590 EU/mg protein; 0.630 EU/mg protein and 1.384 EU/mg; 0.942 EU/mg protein and 1.649 EU/mg protein in the Aegean region populations of *Cx. pipiens*, respectively. All of the populations had significantly higher median alpha, beta and pNPA activities in relation to the reference strain ($p < 0.05$). Median alpha and beta esterase and pNPA activities of the populations are given in Fig. 4, Fig. 5 and Fig. 6, respectively.

The median specific MFO activity was calculated as 2.605 µg cytochrome-c/mg protein in susceptible reference strain. Median MFO activities ranged from 5.310 and 11.373 µg cytochrome-c/mg protein and 9.643 and 21.059 µg cytochrome-c/ mg protein in the Mediterranean and Aegean populations, respectively. All tested populations displayed increased MFO levels ($p < 0.05$) except the Gölhisar population which did not have significantly increased MFO levels in accordance to reference strain ($p > 0.05$) (Fig. 7).

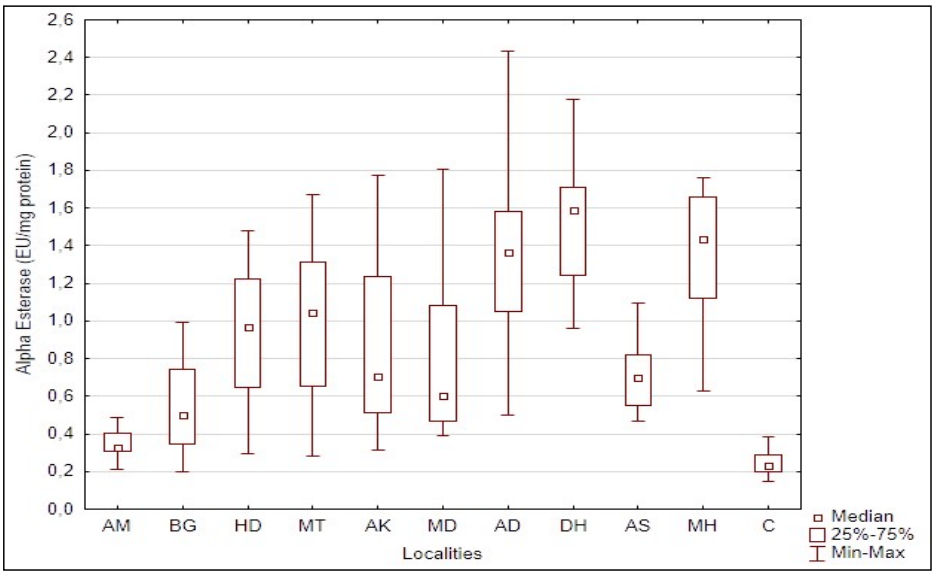

Fig. 4. Median alpha esterase activities of *Culex pipiens* populations (Population name abbreviations represented as: Adana-Karataş (AK), Antalya-Manavgat (AM), Burdur-Gölhisar (BG), Mersin-Tarsus (MT), Hatay-Dörtyol (HD), Afyon-Dinar (AD), Aydın-Söke (AS), Manisa-Hacıhaliller (MH), Muğla-Dalaman (MD), Denizli-Honaz (DH). C: Control)

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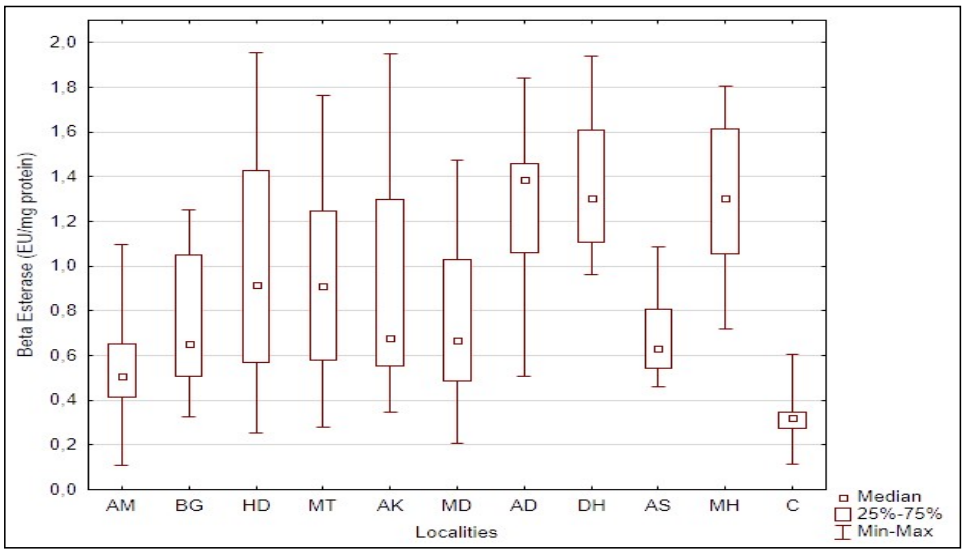

Fig. 5. Median beta esterase activities of *Culex pipiens* populations (Population name abbreviations represented as: Adana-Karataş (AK), Antalya-Manavgat (AM), Burdur-Gölhisar (BG), Mersin-Tarsus (MT), Hatay-Dörtyol (HD), Afyon-Dinar (AD), Aydın-Söke (AS), Manisa-Hacıhaliller (MH), Muğla-Dalaman (MD), Denizli-Honaz (DH).C: Control)

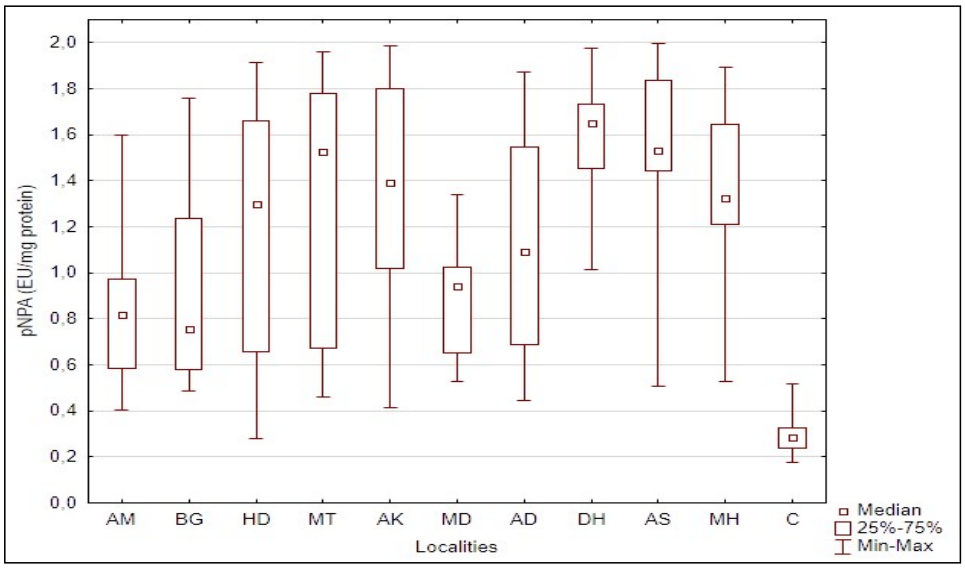

Fig. 6. Median p-nitrophenyl acetate activities of *Culex pipiens* populations (Population name abbreviations represented as: Adana-Karataş (AK), Antalya-Manavgat (AM), Burdur-Gölhisar (BG), Mersin-Tarsus (MT), Hatay-Dörtyol (HD), Afyon-Dinar (AD), Aydın-Söke (AS), Manisa-Hacıhaliller (MH), Muğla-Dalaman (MD), Denizli-Honaz (DH). C: Control)

The median specific GST activity was calculated as 0.138 EU/mg protein in susceptible reference strain. Median specific GST activities changed between 0.217 EU/mg protein and 0.743 EU/mg protein in the Mediterranean and between 0.261 EU/mg protein and 0.795 EU/mg protein in the Aegean region *Cx. pipiens* populations. All of the Mediterranean and the Aegean populations had significantly higher median specific GST activities ($p < 0.05$) except the Tarsus, Manavgat and Gölhisar populations in the Mediterranean and the Honaz population in the Aegean region ($p > 0.05$) (Fig. 8).

The median specific remaining AChE rate was calculated as 7.2% in susceptible

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reference strain. Median remaining AChE rates ranged between 36.9% and 17.4% in the Mediterranean region while they were ranged between 30.5% and 21.3% in the Aegean region. The Tarsus and Karataş populations had insensitive AChE rates which are higher than 30% ($p < 0.05$) while the others did not have significantly altered AChE levels in the Mediterranean region ($p > 0.05$). Median remaining AChE rates changed between 21.3% and 30.5% in the Aegean region. The only populations were the Dalaman and Söke which had insensitive AChE rates expressed as higher than 30% critical level ($p < 0.05$). All of the remaining Aegean populations had sensitive AChE rates ($p > 0.05$). (Fig. 9).

Median levels of detoxifying enzyme activities and p values against control were given in Table 2.

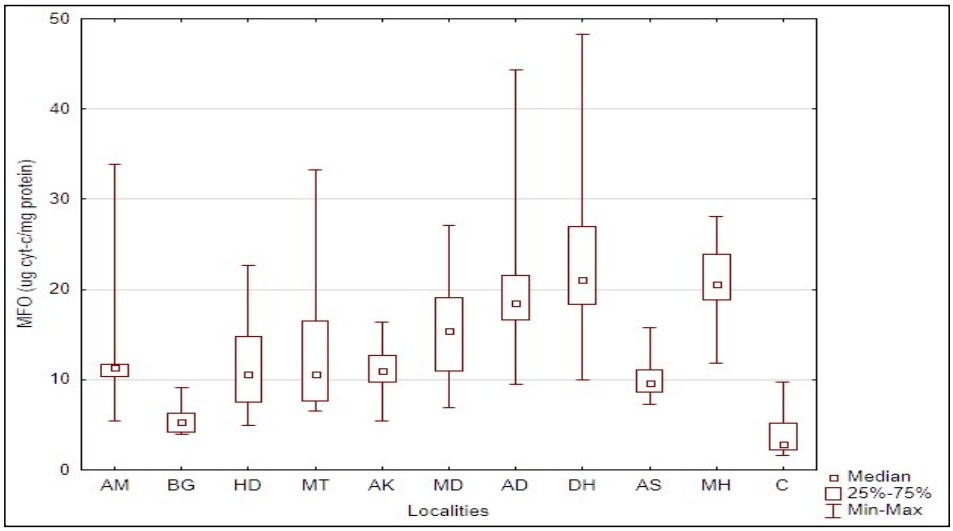

Fig. 7. Median mixed function oxidase activities of *Culex pipiens* populations (Population name abbreviations represented as: Adana-Karataş (AK), Antalya-Manavgat (AM), Burdur-Gölhisar (BG), Mersin-Tarsus (MT), Hatay-Dörtyol (HD), Afyon-Dinar (AD), Aydın-Söke (AS), Manisa-Hacıhaliller (MH), Muğla-Dalaman (MD), Denizli-Honaz (DH). C: Control)

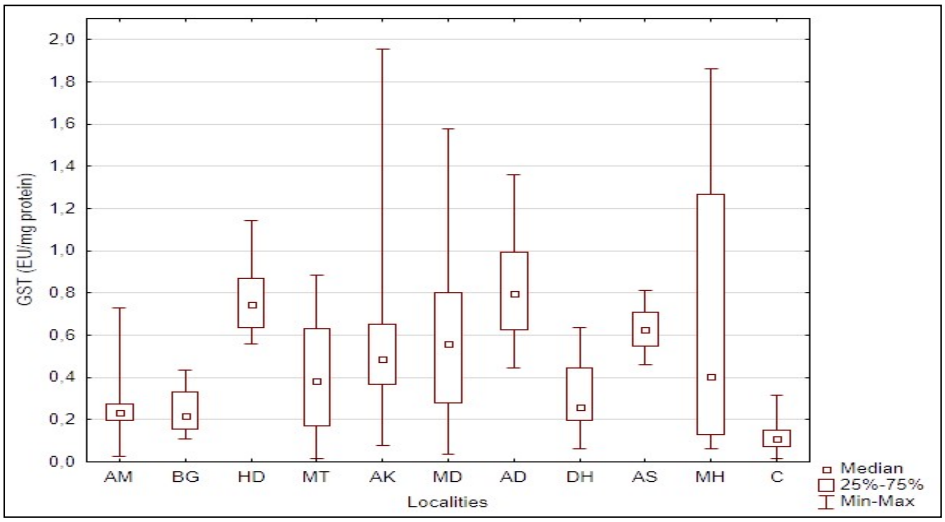

Fig. 8. Median glutathione-s-transferase activities of *Culex pipiens* populations (Population name abbreviations represented as: Adana-Karataş (AK), Antalya-Manavgat (AM), Burdur-Gölhisar (BG), Mersin-Tarsus (MT), Hatay-Dörtyol (HD), Afyon-Dinar (AD), Aydın-Söke (AS), Manisa-Hacıhaliller (MH), Muğla-Dalaman (MD), Denizli-Honaz (DH). C: Control)

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Molecular test results

Both 1014L (TTA) and 1014F (TTT) alleles were detected in the Mediterranean and Aegean populations of *Cx. pipiens*. The highest L1014F allele frequency was calculated in the Gölhisar population as 0.8, however, the population did not show a statistically significant deviation from HW balance ($p > 0.05$). The L1014F allele frequencies were 0.56, 0.55, 0.62, 0.45 in the Dörtyol, Karataş, Manavgat and Tarsus populations, respectively. All of the populations were in HW balance ($p > 0.05$) except the Manavgat and Tarsus populations which had lower heterozygosity rate ($p < 0.05$).

The highest L1014F allele frequency was calculated as 1.00 in the Honaz population in which L1014F allele was fixed. The L1014F allele frequency was 0.90 in the

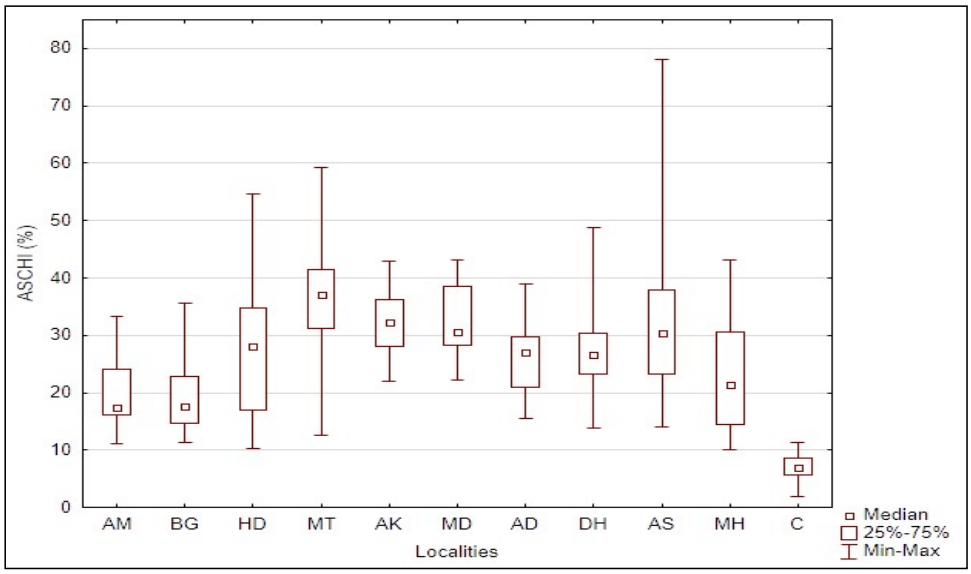

Fig. 9. Median remaining acetylcholinesterase inhibition rates of *Culex pipiens* populations (Population name abbreviations represented as: Adana-Karataş (AK), Antalya-Manavgat (AM), Burdur-Gölhisar (BG), Mersin-Tarsus (MT), Hatay-Dörtyol (HD), Afyon-Dinar (AD), Aydın-Söke (AS), Manisa-Hacıhaliller (MH), Muğla-Dalaman (MD), Denizli-Honaz (DH). C: Control)

Table 2. Median level of detoxifying enzyme activities and p values (2 tailed) in *Culex pipiens* populations collected from the Mediterranean and Aegean region, Turkey.

Region	Province	Locality	Median level of detoxifying enzyme activities and *p* values (2-tailed) against control											
			Alpha esterase EU/mg protein		Beta esterase EU/mg protein		pNPA EU/mg protein		Oxidase µg cytochrome-c/mg protein		GST EU/mg protein		ASCHI (%)	
			Median	P	Median	P	Median	P	Median	P	Median	P	Median	P
Mediterranean	Hatay	Dörtyol	0.966	0.000	0.915	0.000	1.299	0.000	10.652	0.000	0.743	0.000	28.125	0.000
	Adana	Karataş	0.706	0.000	0.675	0.000	1.393	0.000	11.000	0.000	0.486	0.000	32.211	0.000
	Mersin	Tarsus	1.041	0.000	0.908	0.000	1.526	0.000	10.617	0.000	0.384	0.051	36.986	0.000
	Antalya	Manavgat	0.325	1.000	0.508	0.930	0.815	0.051	11.373	0.000	0.232	1.000	17.421	0.031
	Burdur	Gölhisar	0.497	0.510	0.651	0.024	0.754	0.061	5.310	1.000	0.217	1.000	17.586	0.086
Aegean	Afyon	Dinar	1.366	0.000	1.384	0.000	1.091	0.000	18.520	0.000	0.795	0.000	27.048	0.000
	Denizli	Honaz	1.590	0.000	1.303	0.000	1.649	0.000	21.059	0.000	0.261	0.177	26.635	0.000
	Muğla	Dalaman	0.599	0.000	0.667	0.000	0.942	0.001	15.399	0.000	0.559	0.000	30.582	0.000
	Aydın	Söke	0.697	0.000	0.630	0.000	1.530	0.000	9.643	0.005	0.626	0.000	30.444	0.000
	Manisa	Hacıhaliller	1.434	0.000	1.303	0.000	1.325	0.000	20.585	0.000	0.401	0.000	21.388	0.000
Control			0,242	-	0.328	-	0.265	-	2.605	-	0.138	-	7.2	

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Table 3. Genotypes, allele frequencies and exact test results of voltage gated sodium channel 1 gene region of *Culex pipiens* populations in the Mediterranean and Aegean region, Turkey

Region	Locality	No	Genotypes			Allele frequency		p	SE	WC
			SS	SR	RR	S	R			
Mediterranean	Adana-Karataş	20	3	12	5	0.45	0.55	0.6538	0.0018	-0.1875
	Antalya-Manavgat	20	7	1	12	0.375	0.625	0.000	0.000	0.8984
	Burdur-Gölhisar	20	2	4	14	0.2	0.8	0.1269	0.0018	0.3968
	Hatay-Dörtyol	15	4	5	6	0.43	0.56	0.2935	0.0025	0.3519
	Mersin-Tarsus	20	9	4	7	0.55	0.45	0.0079*	0.0005	0.6122
Aegean	Aydın-Söke	20	0	4	16	0.1000	0.9000	1.0000	0.0000	-0.0857
	Muğla-Dalaman	20	0	6	14	0.1500	0.8500	1.0000	0.0000	-0.1515
	Manisa-Hacıhaliller	20	0	4	16	0.1000	0.9000	1.0000	0.0000	-0.0857
	Denizli-Honaz	20	0	0	20	0.0000	1.0000	-	-	-
	Afyon-Dinar	20	0	4	16	0.1000	0.9000	1.0000	0.0000	-0.0857

(SS: homozygous susceptible; RR: homozygous resistant; SR: heterozygous resistant; SE: Standard error; WC: Weir and Cockerham value; Asterisks represent statistical significance ($p < 0.05$))

Table 4. Genotypes, allele frequencies and exact test results of F290V mutations of *Culex pipiens* populations in the Mediterranean and Aegean region, Turkey

Region	Locality	No	Genotypes			Allele frequency		p	SE	WC
			SS	SR	RR	S	R			
Mediterranean	Adana-Karataş	20	20	0	0	1.000	0.000	-	-	-
	Antalya-Manavgat	20	20	0	0	1.000	0.000	-	-	-
	Burdur-Gölhisar	20	18	2	0	0.95	0,050	1.000	0.000	-0.0270
	Hatay-Dörtyol	15	15	0	0	1.000	0.000	-	-	-
	Mersin-Tarsus	20	18	2	0	0.95	0.05	1.000	0.000	-0.0270
Aegean	Aydın-Söke	20	17	3	0	0.925	0.075	1.000	0.000	-0.0556
	Muğla-Dalaman	20	20	0	0	1.000	0.000	-	-	-
	Manisa-Hacıhaliller	20	20	0	0	1.000	0.000	-	-	-
	Denizli-Honaz	20	19	1	0	0.975	0.025	-	-	-
	Afyon-Dinar	20	20	0	0	1.000	0.000	-	-	-

(SS: homozygous susceptible; RR: homozygous resistant; SR: heterozygous resistant; SE: Standard error; WC: Weir and Cockerham value; Asteriks represent statistical significance ($p< 0.05$))

Söke, Hacıhaliller and Dinar and 0.85 in the Dalaman populations. All of the Aegean populations were in HW balance ($p > 0.05$). The kdr genotype, allele frequencies and Fisher's exact test results of *Cx. pipiens* populations along the Mediterranean and Aegean region are given in Table 3.

No Ace-1 mutation was found in either in the Mediterranean or in the Aegean *Cx. pipiens* populations based on RFLP analyses by using Alu-I restriction endonuclease. Sequence analysis results confirmed RFLP results and did not detect any G119S allele in the study area.

F290V mutation frequency was changed between 0–0.075% within the *Cx. pipiens* populations collected from both the Mediterranean and Aegean region.

Genotypes and allele frequencies of F290V mutations are given in Table 4.

Discussion

The Mediterranean and Aegean regions are located in the west and south part of Turkey and both have favourable climate conditions for mosquito survival. The availability of vast and fertile soils within this geographic area leads high agricultural activities therein. In addition to that, intensive human activity,

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tourism and industrialization induce wide spread of mosquito species and accordingly mosquito-borne diseases. Since chemical insecticide-based control management is the primary method for mosquito control efforts in Turkey, understanding the insecticide resistance levels of the mosquito populations and underlying mechanisms are crucial for the deployment of appropriate insecticides. In this study, insecticide resistance levels of *Cx. pipiens* populations from the Aegean and Mediterranean region against various insecticides were investigated.

Results indicated that all of the populations were resistant to DDT even though DDT use was banned in the 1980s in Turkey. DDT resistance has been recorded in many mosquito populations both in Turkey and the world since it was used extensively in the fight against malaria until the 1960s (22-25). Several researchers have previously reported that GSTs that are involved in xenobiotic detoxification, some MFOs that are capable of metabolizing DDT (for example cyp6z1) and a kdr mutation causing receptor structure change in voltage-gated ion channels are all responsible for DDT resistance (26-27). All of the Mediterranean and Aegean *Cx. pipiens* populations showed significantly high GST activity profile except the Antalya and Gölhisar populations, indicating that DDT resistance is still maintained through increased GST activities in most of the populations. However, the Manavgat and Gölhisar populations support the hypothesis that more than one mechanism might play a role rather than the fact that GST alone is responsible for DDT resistance. Increased MFO levels and L1014F allele frequency in the Manavgat population indicates that DDT resistance might be maintained through high MFO levels and kdr mutation even if the GST level is not increased. Similarly, increased mortality rate against DDT, Permethrin and Deltamethrin after treatment with piperonyl butoxide (PBO) in *Cx. pipiens* populations of Mersin demonstrates that MFO is responsible for both DDT and pyrethroid resistance of the Mersin populations (11). Interestingly, high DDT resistance seems to be sustained via L1014F mutation alone in the Gölhisar population in which MFO and GST levels are not significantly increased.

Another interesting conclusion about the Gölhisar population is that both kdr mutation and decreased MFO levels, which are thought to be responsible for pyrethroid resistance in addition to DDT resistance, which is still sensitive to Permethrin and Deltamethrin. In addition to the Gölhisar population, when we consider the Dinar and Söke populations that are sensitive to pyrethroids and have high kdr frequencies because of the DDT resistance, the question of what is the usability of pyrethroids for control purposes comes to mind.

Allele specific primers indicated that mechanism causing kdr resistance of *Cx. pipiens* populations in both the Mediterranean and Aegean regions is the increase of L1014F mutation frequencies. However, Taşkın et al. (12) reported that the kdr resistance responsible for DDT and pyrethroid resistance is maintained by both L1014F and L1014C mutations in *Cx. pipiens* populations collected from the Aegean region. Studies on kdr alleles responsible for DDT and pyrethroid resistance have been conducted in *Cx. pipiens* populations obtained from many countries of the world. For example, both L1014F and L101C kdr mutations were detected in *Cx. pipiens* populations in Greece (28). Additionally, high L1014F allele frequency was reported in *Cx. pipiens* populations obtained from Morocco and Mohammadiye cities of Morocco (29). The Kdr mutation is maintained by L1014S allele in *Cx. pipiens quinquefasciatus* populations obtained from some parts of China (30) by L1014F allele in *Cx. pipiens* populations of New Jersey (31) and by L1014C in *Cx. pipiens* populations of China (32).

Following the occurrence of DDT resistance, in the 1970s, CB and OP insecticides such as Malathion, Fenitrothion, and Bendiocarb and Propoxur began to be applied instead of DDT in the mosquito control studies (33). Bioassay results showed that all *Cx. pipiens* populations from the Mediterranean and Aegean Region are

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resistant to both OP and CB insecticides. This situation can be explained by the intensive use of OP and CB insecticides, especially Malathion, in the control of agricultural pests and as a result, creates a high selection pressure in agricultural areas (34). Similar to Turkish *Cx. pipiens* populations, several researchers from neighbouring countries such as Iran, Russia and Greece reported high insecticide resistance against different insecticides. For instance, Kioulos et al. (2014) reported Temephos and Deltamethrin resistance in some parts of Greece (35). Fenitrothion, DDT, Dieldrin, Propoxur, Bendiocarb, Malathion, Deltamethrin and Permethrin resistance has been reported in *Cx. pipiens* populations collected from the centre of Moscow (36). Resistance t oDDT, Malathion, Bendiocarb, Propoxur, Fenitrothion, Deltamethrin, Permethrin, Lamda-cyhalothrin, Etofenprox and Cyfluthrin has been reported from Iranian *Cx. pipiens* populations (37).

The significant increase in esterase enzyme activity in all *Cx. pipiens* populations of the Mediterranean region except the Manavgat population, indicates that OP and CB resistance is maintained with general esterases in populations of this species. Regarding OP ve CB resistance, molecular and biochemical data results were consistent with each other. ACHE insensitivity was generally lower along populations except the Adana Karataş, Mersin Tarsus, Muğla Dalaman and Aydın Söke populations which had slightly high remaining AChE activity. Consistently, PCR-RFLP did not detect any G119S allele in any of the populations indicating that Ace-1 G119S mutation is not responsible for the OP and CB resistance in *Cx. pipiens* populations. Additionally, OP and CB resistance could not be explained by Ace-1 F290V mutations since the frequency of F290V mutation was also too low. Several researchers reported that individuals carrying *Ace-1* mutation (G119S and F290V) have a fitness cost including longer period time, reduced owerwintering survival, smaller adult size and increased risk of predation (38-41). This might explain why *Culex pipiens* populations in the study area had low Ace-1 (G119S and F290V) mutations. It has been reported that fitness cost might be diminished by duplication of the Ace-1 gene in some *Culex pipiens* populations (42-43). However, we were lack of duplication data set in that study. Similarly, G119S mutation frequency was found to be 0.11 and 0.08 and F290V mutation frequency was found to be 0.05 and 0.06 in *Cx. pipiens* populations obtained from the Aegean and Marmara Regions of our country in 2012 and 2013, respectively (12). The Ace-1 (G119S and F290V) mutations leading to insensitivity to organophosphates and carbamates were detected at low frequencies in *Cx. pipiens* populations in Greece (28). In addition to that, in *Cx. pipiens* populations obtained from urban and rural populations of Morocco, G119S mutation was found to be at a higher frequency in urban areas compared to rural areas due to Temephos (OP) used to fight *Cx. pipiens* larvae. However, the absence of G119S mutation in some individuals who did not die after being treated with OP shows that the only mechanism underlying OP resistance is not the G119S mutation (44). Similarly, Tmimi et al. (2018) found that the G119S mutation frequency was very low in *Cx. pipiens* populations of Morocco (29).

This study demonstrates that multiple insecticide resistance exists in *Cx. pipiens* populations from the Mediterranean and Aegean regions of Turkey. Medium to high kdr (L1014F) mutation frequency and extremely low F290V mutation frequency detected in these populations. However, no G119S mutation was detected within these populations as well as low AChE activity levels have been detected. Effective implementation of insecticide resistance management strategies is needed in order to delay the fixation of resistance alleles currently occurrs within these populations. The data obtained from this study will be valuable for vector control interventions in Turkey.

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Conclusion

Understanding the resistance mechanisms and monitoring resistance patterns of the populations regularly are crucial for insecticide resistance management. We used both WHO's bioassay tests, biochemical assays and molecular markers at the same time to evaluate the insecticide resistance status and underlying mechanisms for the first time for *Cx. pipiens* populations collected from the Mediterranean and Aegean region of Turkey. The Mediterranean and Aegean regions are important agricultural regions as well as high tourism activities. As a result of high insecticide use in these regions, we hypothesised the occurrence of high resistance status against different classes of insecticides which have been used until today. We showed the complicated role of detoxification enzymes. However, one of an important limitation of our study was the absence of synergist assays to get more reliable data set regarding the biochemical mechanisms. We also detected mild to high frequency of kdr L1014F allele frequency as a result of DDT and/or pyrethroid use. However, we did not detect any other kdr alleles probably because of the restricted ability of allele-specific primers to detect different kinds of alleles. Interestingly, we did not detect Ace-1 G119S allele in any populations. Furthermore, we detected too low Ace-1 F290V mutations and insensitive acetylcholinesterase levels in some of the populations. Further studies with higher sample sizes are needed to establish insecticide resistance profiles in order to evaluate more accurately and avoid resistance problems before it is spread to the whole mosquito populations.

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Ethical approval

This article does not contain any studies with animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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