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Efficacy of some insecticides on field populations of *Culex pipiens* (Linnaeus) from Egypt

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Abstract The efficacy of the most used insecticides belonging to different groups (organophosphate, carbamate, synthetic pyrethroid and insect growth regulator) was tested against four different field populations of *Culex pipiens*. Results obtained showed that the laboratory colony showed higher susceptibility to the tested insecticides than the mosquito populations collected from Sharkia and Assiut Governorates. Field populations of *Cx. pipiens* from Sharkia were chosen to study the development of resistance (resistant strain) in *Cx. pipiens* to chlorpyrifos toxicity. After 15 generations of selection pressure using chlorpyrifos against the 3rd instar larvae of *Cx. pipiens*, resistance increased by 24.56-fold in the resistant strain as compared with the control. Fractionation of total soluble proteins using SDS–PAGE revealed some differences in the laboratory colony, field populations and resistant strain. Results may indicate that alkaline phosphatase and non-specific esterases were probably responsible for the detoxification of chlorpyrifos in field populations.

Introduction

Mosquitoes are among the most serious insect pests of medical importance. Among these, *Culex pipiens* (Linnaeus) complex, the common and widely distributed mosquitoes across Egypt, has been incriminated as the main vector of bancroftian filariasis (Southgate, 1979) and the Rift Valley fever (RVF) (Meegan et al., 1980).

To control such vectors, insecticides have been extensively produced and widely used. Accordingly, such a large scale use of insecticides against several insect pests has resulted in the development of insect strains resistant to many insecticides.

Trials have been developed to study the nature of resistance and cross-resistance to organophosphorus and synthetic pyrethroid insecticides in resistant strains of *Cx. pipiens* (Ben Cheikh et al., 1998). Detoxification of insecticide by metabolism is the common mechanism that has evolved to protect insects (Price, 1991). Rapid biochemical assays coupled with biological assays were considered as potential tools for the estimation of both the intensity and the frequency of resistance in the field (Brown and Brogdon, 1987). Biochemical monitoring tools such as esterase activity assays can complement bioassays and provide information about the dynamics and evolution of resistance among field populations.

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The present study was undertaken to study the efficacy of four insecticides from the different groups (phosphate, carbamate, pyrethroid and insect growth regulator) which were regularly used in cotton fields against four different populations of *Cx. pipiens*, the development of resistance in *Cx. pipiens* to the insecticide chlorpyrifos as well as the effect of these chemical insecticides on the biochemical contents of *Cx. pipiens* was studied.

Materials and methods

Toxicological studies

Test insects

Four populations of the mosquito, *Cx. pipiens* were used during this study. A laboratory colony (control) was used as a baseline in insecticide and biochemical assays; it was kindly provided by the Research Institute of Medical Entomology, Ministry of Health and Population, Dokki, Giza. This colony was started from egg rafts obtained from colony cultured in the laboratory for several years free from insecticides.

Four populations of *Cx. pipiens* larvae were collected from their drainages in Sharkia, Assiut, Menofia and Gharbia Governorates during the late season of 2005 and 2006. These field populations were exposed regularly to insecticidal applications to cotton fields according to the routine schedule program set annually by the Central Administration for Pest Control, Ministry of Agriculture. This routine schedule was as follows: every year at the beginning of July a mix of cascade (insect growth regulator) and chlorpyrifos was applied for 15 days; kendo (pyrethroids) for 21 days; chlorpyrifos (organophosphate) for 15 days followed finally by thiodicarb (carbamate). The larvae were reared to the adult stage in insectary.

Sharkia field population, which exhibited the highest resistant level to the insecticidal treatments, was selected to study the development of resistance in the 3rd instar larvae of *Cx. pipiens* to an organophosphorus insecticide (Chlorpyrifos) for 15 generations. The susceptibility of Sharkia population at parent as well as the successive generations of *Cx. pipiens* was determined on the basis of LC_{50} values every three generations. The discriminating dose was the double of LC_{99} for the susceptible laboratory colony of the adult stage of *Cx. pipiens* (Gunning et al., 1984).

Insecticides

Commercial formulations of insecticides used are chlorpyrifos (Dursban 48% EC), thiodicarb (Larvin 80% DF), lambda-cyhalothrin (Kendo) and flufenoxuron (Cascade 5% EC). These chemicals were kindly provided by plant protection institute.

Bioassays

For each insecticide five serial concentrations were used against both the 3rd instar larvae as well as the adult stage. Four replicates, 25 insects each were used for each concentration.

Insecticides used were flufenoxuron, chlorpyrifos, lambda-cyhalothrin and thiodicarb. Water and ethanol, insecticide solutions were made and stored at 4 °C for less than 2 months. The technique for measuring the susceptibility level of larvae was adapted according to the WHO technique (WHO, 1975). The late 3rd instar or early 4th instar larvae were used to avoid pupation during exposure period. Test beakers of 500 ml capacity, each containing 249 ml tap water were prepared. In each, 1 ml of ethanol insecticide solution at the desired concentration was infiltrated under the water surface with a pipette. A batch of 20–25 larvae was introduced in each beaker after 30 min of preparing the insecticide mixture. Four replicates per concentration, and five concentrations giving between 20% and 100% mortality were used for each bioassay. The test was run at the same temperature as that at which the larvae were reared. Larvae were left for 24 h and mortality was then recorded. Moribund larvae were considered dead. Larvae pupating during exposure period were excluded from calculation. Tests in which pupation exceeded 10% were repeated. The control tests were following the same procedure using the solvent only (water) and mortality never exceeded 4%.

Adult assays were adapted according to the WHO technique (WHO, 1996) for the evaluation and testing of insecticides. The insecticide's suspension or solution concentration in water or acetone was adjusted and applied to the surface area of test tubes. The sprayed test tubes were stored in open shelves in a room kept at 25 °C, 50–55% relative humidity and constant darkness. Batches of 20 males and non-blood fed females were introduced into test tubes and allowed to alight and rest on the vertical treated surface for 30 min at the same temperature and relative humidity. After the exposure period the mosquitoes were removed and transferred for observation and mortality count after 24 h.

Discriminating dose technique

A discriminating dose technique was used for rapid monitoring of insecticide resistance in field-collected *Cx. pipiens* adults. *Cx. pipiens* adults collected from Sharkia, Assiut, Menofia and Gharbia Governorates, had received four applications of different insecticides, and were subjected to the diagnostic concentration assay. Thirty randomly selected adults from each population were tested with four replicates. Adults were placed in each vial (replicate) pretreated with the discriminating concentration of each insecticide tested. Adult mortality was recorded after 24 h. Percentage of mortality was calculated for each field populations and compared with that of the susceptible laboratory colony.

To calculate the resistance percentages, the following formula was used according to McCutchen et al. (1989)

$$\text{Resistance percentages} = 100 - (\text{MF}/\text{MS} \times 100)$$

where MF, % mortality at discriminating concentration in field-collected adults; MS, % mortality (constant) at discriminating concentration in susceptible adults.

Mortality counts

Mortality counts were made after 24 h. The dosage mortality data were subjected to log dose and probit analysis (LDP) according to Finney (1952). Mortality percentages were corrected according to Abbott's formula (Abbott, 1925). Levels of resistance of the field populations of the insects under investigation were calculated as follows:

$$\text{Resistance ratio(RR)} = \frac{LC_{50} \text{ or } LC_{90} \text{ of the field populations}}{LC_{50} \text{ or } LC_{90} \text{ of the laboratory colony}}$$

The following criteria proposed by Mazzarri and Georghiou (1995) were adopted to classify the resistance level of populations: low (RR < 5), moderate (5 < RR < 10) or high (RR > 10).

Biochemical assay

Insect homogenization

The 3rd instar larvae and the adult stage of *Cx. pipiens* were collected, weighed and counted. Each batch was mechanically homogenized in 10 volumes (w/v) of o.1 M phosphate buffer, pH 7 for 2 min using a Teflon homogenizer surrounded with a jacket of crushed ice. The homogenates were then centrifuged at 4000 rpm for 30 min at 4 °C using cooling centrifuge. The resultant supernatant was used to determine the activities of non-specific esterases and alkaline phosphatase as well as the amount of protein content.

Determination of alkaline phosphatases activity

Alkaline phosphatase activity was determined according to Powell and Smith (1954). The reaction mixture consisted of 1 ml carbonate buffer (pH 10.14), 1 ml of 0.01 M disodium phenyl phosphate (substrate) and 0.1 ml tissue supernatant. It was incubated for 30 min in a water bath at 37 °C. Removed from the bath and 0.8 ml of 0.5 N NaOH, 1.2 ml of 0.5 N $NaHCO_3$, 1 ml of 4-amino-antipyrine solution and then 1 ml of potassium ferricyanide were added. The produced color was measured calorimetrically at 510 nm. The enzymatic activity is expressed as mmole phenol/individual/min.

Determination of non-specific esterases activity

Esterase activity was assayed according to Van Asperen (1962) using α-naphthyl acetate as a substrate. The reaction mixture consisted of 5 ml substrate solution 3×10^{-4} M α-naphthyl acetate, 1% acetone, 0.04 M phosphate buffer (pH 7) and 20 µl tissue supernatant. The mixture was incubated for 15 min at 27 °C. The reaction was terminated by adding 1 ml of diazoblue color reagent (prepared by mixing two parts of diazoblue B and five parts of 5% sodium lauryl sulfate). The color was allowed to develop for 15 min at room temperature. The absorbance value at 600 nm was determined. The enzyme activity was expressed as mmole naphthol released/individual/min.

Determination of total protein content

Total protein content was determined according to the method of Bradford (1976).

Denaturing polyacrylamide gel electrophoresis (SDS–PAGE)

Samples containing equal amount 30 µg) of protein, as determined by Bradford's method from larval and adults tissue extracts of field populations, resistant strain as compared to the laboratory susceptible colony were denatured in sample buffer for 5 min at 100 °C and analyzed on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) along with molecular weight marker proteins. Electrophoresis was carried out at a constant current (25 mA) at room temperature and the gel was stained overnight with 0.025% Coomassie Brilliant Blue R-250 (Lammeli, 1970). After the determination of protein concentration in larval and adult stages samples containing equal amount of protein (30 µg) were selected to separate proteins based on size through the use of a stacking gel and resolving gel of SDS–PAGE. The samples that will be observed are loaded into the wells after being combined with a buffer containing 30% glycerol, SDS. A voltage is then applied (approximately 3–4 v/cm^2). Since a higher charge and the small size of a molecule increase the mobility of that molecule in the stacking gel (pH = 6.8), the resultant migration pattern of the molecules would be molecules with high charge and small size would be in the first row and in the last row would be the small charged large molecules. The rows in between would have the molecules organized by their mobility. The chloride ions in the stacking gel have the greatest mobility and highest charge. The proteins have a lower mobility than the chloride ions, but are faster than the glycine anions from the buffer. The resultant stack would be the chloride ions, proteins, and then the glycine anions. After the icons stack in the stacking gel, they enter the resolving gel (pH = 8.8), which has smaller pore sizes. The first event that occurs is that the higher pH places a greater negative charge on the small glycine anions. This results in the glycine anions migrating faster than the chloride ions. The second event is that the decrease in pore size creates a large frictional component on the mobility of each protein. Since the SDS in the gel creates a charge to mass ration that is equal, the proteins now migrate based on size. The bands created by the proteins of different sizes can be compared to a standard that is run along with the sample. A standard curve can be created from the standard's bands and the samples can then be compared to the standard.

Calculation of the relative mobility (Rm) value

The total length of the separating gel, the distance traveled by the tracking dye in the separating gel and the various distances migrated by the different protein fractions were measured. The relative mobility value of each band was calculated as follows:

$$\text{Relative mobility(Rm)} = \frac{\text{Distance travelled by the protein fraction}}{\text{Distance traveled by the tracking dye}}$$

Statistical analysis

For biochemical data, the analysis of variance was used in completely randomized design (CRD) to test the significant difference as outlined by SAS program (SAS Institute, 1988). In addition, the confidence intervals were done to obtain the significant difference among means using lower and upper values at 0.05 levels. The data were subjected to analysis of variance (ANOVA) with at least three repeats.

Results

Toxicological studies

*Monitoring resistance in **Cx. pipiens** field populations collected from Sharkia and Assiut Governorates*

Results revealed a significant increase of resistance ratio in the 3rd instar larvae of Sharkia population at LC_{50} and LC_{90} level than in the population of Assiut Governorate ($P \leqslant 0.05$). The highest level of resistance to the toxicity of Flufenoxuron at

Table 1 Comparison of four insecticides resistance in the 3rd-instar *Culex pipiens* larvae collected from Sharkia and Assiut Governorates in comparison with laboratory colony.

Insecticides	Population	Slope value	N^a	LC_{50} (ppm) (95% CI[b])	LC_{90} (ppm) (95% CI[b])	Resistance ratio (RR)[c] LC_{50}	Resistance ratio (RR)[c] LC_{90}
Flufenoxuron	Laboratory colony	0.504	125	0.054 ± 0.007^{F} (0.044–0.063)	18.320 ± 0.66^{E} (15.83–20.80)	–	–
	Sharkia	0.539	125	0.148 ± 0.018^{B} (0.12–0.17)	35.112 ± 1.056^{C} (30.14–40.08)	2.74	1.92
	Assiut	0.508	125	0.096 ± 0.008^{D} (0.091–0.10)	31.623 ± 0.812^{D} (0.091–0.10)	1.78	1.73
Chlorpyrifos	Laboratory colony	0.488	125	0.073 ± 0.0065^{E} (0.065–0.080)	30.451 ± 0.726^{D} (25.48–35.41)	–	–
	Sharkia	0.530	125	0.173 ± 0.0115^{A} (0.15–0.20)	44.961 ± 0.98^{A} (42.47–47.44)	2.37	1.48
	Assiut	0.489	125	0.109 ± 0.0045^{C} (0.09–0.16)	42.244 ± 0.622^{B} (37.26–47.19)	1.49	1.39
Lambda-cyhalothrin	Laboratory colony	0.544	125	0.041 ± 0.005^{G} (0.03–0.04)	7.560 ± 0.79^{H} (6.48–8.49)	–	–
	Sharkia	0.555	125	0.072 ± 0.011^{E} (0.06–0.076)	14.527 ± 0.61^{F} (12.04–17.01)	1.76	1.92
	Assiut	0.533	125	0.049 ± 0.0095^{F} (0.046–0.05)	12.304 ± 0.65^{G} (10.5–15.1)	1.20	1.63
Thiodicarb	Laboratory colony	0.634	125	0.027 ± 0.006^{H} (0.02–0.03)	2.780 ± 0.39^{I} 2.5–3.02)	–	–
	Sharkia	0.658	125	0.038 ± 0.018^{G} (0.033–0.042)	4.430 ± 0.62^{I} (2.03–0.65)	1.41	1.59
	Assiut	0.645	125	0.029 ± 0.0045^{H} (0.026–0.031)	2.806 ± 0.48 (2.1–4.1)^{I}	1.07	1.01

[a] Total number of insects used.
[b] Confidence interval.
[c] Resistance ratio(RR) $= \frac{LC_{50} \text{ or } LC_{90} \text{ for the field population}}{\text{Corresponding } LC_{50} \text{ or } LC_{90} \text{ of laboratory colony}}$.
[A] Means with different letters in column of the of LC_{50} and LC_{90} of each insecticide used against Sharkia and Assiut field populations in comparison with laboratory colony are significantly different ($P \leqslant 0.05$, Waller–Duncan).

Table 2 Comparison of four insecticides resistance in the adult stage of *Culex pipiens* collected from Sharkia and Assiut Governorates in comparison with laboratory colony.

Insecticides	Population	Slope Value	N^a	LC_{50} (ppm) ± SE (95% CI[b])	LC_{90} (ppm) ± SE (95% CI[b])	Resistance ratio (RR)[c] LC_{50}	Resistance ratio (RR)[c] LC_{90}
Flufenoxuron	Laboratory colony	0.532	125	0.244 ± 0.017^{D} (0.23–0.25)	62.120 ± 1.06^{E} (57.15–67.08)	–	–
	Sharkia	0.515	125	0.374 ± 0.012^{A} (0.36–0.38)	105.985 ± 0.99^{A} (103.56–108.40)	1.53	1.71
	Assiut	0.518	125	0.342 ± 0.021^{C} (0.337–0.346)	101.794 ± 0.89^{B} (99.30–104.27)	1.40	1.64
Chlorpyrifos	Laboratory colony	0.533	125	0.210 ± 0.055^{F} (0.20–0.214)	53.620 ± 0.81^{F} (46.16–61.07)	–	–
	Sharkia	0.554	125	0.366 ± 0.013^{B} (0.35–0.38)	74.756 ± 0.88^{C} (64.81–84.69)	1.74	1.39
	Assiut	0.519	125	0.226 ± 0.008^{E} (0.216–0.235)	66.783 ± 0.89^{D} (64.29–69.26)	1.08	1.25
Lambda-Cyhalothrin	Laboratory colony	0.560	125	0.104 ± 0.002^{H} (0.94–0.114)	20.170 ± 0.59^{I} (18.43–22.52)	–	–
	Sharkia	0.576	125	0.207 ± 0.054^{F} (0.19–00.21)	34.530 ± 0.77^{G} (30.91–39.1)	1.99	1.71
	Assiut	0.568	125	0.144 ± 0.012^{G} (0. 123–0.156)	25.821 ± 0.91^{H} (23.33–28.30)	1.39	1.43
Thiodicarb	Laboratory colony	0.599	125	0.037 ± 0.009^{K} (0.029–0.044)	5.084 ± 0.53^{L} (4.09–7.23)	–	–
	Sharkia	0.592	125	0.091 ± 0.0205^{I} (0.088–0.093)	13.560 ± 0.78^{J} (11.42–15.60)	2.46	2.67
	Assiut	0.579	125	0.056 ± 0.008^{J} (0.047–0.072)	9.309 ± 0.66^{K} (9.06–9.55)	1.51	1.83

[a] Total number of insects used.
[b] Confidence interval.
[c] Resistance ratio(RR) $= \frac{LC_{50} \text{ or } LC_{90} \text{ for the field population}}{\text{Corresponding } LC_{50} \text{ or } LC_{90} \text{ of laboratory colony}}$.
[A] Means with different letters in column of the LC_{50} and LC_{90} of each insecticide used against Sharkia and Assiut field populations in comparison with laboratory colony are significantly different ($P \leqslant 0.05$, Waller–Duncan.

Table 3 Development of resistance in the 3rd instar *Culex pipiens* larvae collected from the parent Sharkia field populations to the toxic action of chlorpyrifos.

Generations	Slope ± SE	LC_{50} ± SE	Resistance ratio (RR) relative to	
			Lab-strain ± SE[b]	Parent-strain ± SE[c]
Lab-strain	0.488 ± 0.05^{A}	0.073 ± 0.01^{F}	1.00 ± 0^{F}	–
Parent-strain[a]	0.530 ± 0.04^{A}	0.173 ± 0.06^{FE}	2.37 ± 0.19^{EF}	1.00 ± 0.2^{E}
G_3	0.516 ± 0.02^{A}	0.262 ± 0.04^{DE}	3.59 ± 0.15^{E}	1.51 ± 0.11^{E}
G_6	0.502 ± 0.06^{A}	0.411 ± 0.01^{D}	5.63 ± 0.27^{D}	2.38 ± 0.19^{D}
G_9	0.501 ± 0.02^{A}	0.742 ± 0.08^{C}	10.16 ± 0.08^{C}	4.29 ± 0.15^{C}
G_{12}	0.496 ± 0.002^{A}	1.450 ± 0.09^{B}	19.86 ± 0.93^{B}	8.38 ± 0.19^{B}
G_{15}	0.477 ± 0.04^{A}	1.793 ± 0.003^{A}	24.56 ± 0.78^{A}	10.36 ± 0.18^{A}

[a] Sharkia field population collected at the season of 2005 which was used as parent strain for the development of resistant strain in this study.
[b] Resistance ratio (RR) = LC_{50} of the G_n/LC_{50} of lab-strain.
[c] Resistance ratio (RR) = LC_{50} of the G_n/LC_{50} of parent-strain
[A] Means with different letters in column of LC_{50} of chlorpyrifos and the RR relative to Lab-strain and Parent-strain for each generation; G_6, G_9, G_{12} and G_{15} are significantly different ($P \leqslant 0.05$, Waller–Duncan).

Table 4 Response of *Culex pipiens* adults collected from different Governorates as well as chlorpyrifos resistant strain to discriminating concentration of chlorpyrifos using surface film technique.

Field populations	Mortality % (±SE)	Resistance %* (±SE)	Relative resistance** (±SE)
Assiut	87.5 (±0.75)	11.62 (±0.81)	5.47 (±0.24)
Gharbia	85.4 (±0.7)	13.74 (±0.87)	4.62 (±0.11)
Menofia	82.6 (±0.8)	16.57 (±0.79)	3.83 (±0.42)
Sharkia	63.3 (±0.65)	30.06 (±0.53)	2.11 (±0.06)
Chlorpyrifos-resistant strain	36.1 (±0.55)	63.54 (±1.32)	1.00 (±0.2)
Mean	79.70	17.99	4.01
(95% CI***)	(62.01–97.38)	(4.80–31.19)	(1.73–6.29)

* Resistance $\% = 100 - \left[\frac{\%\text{ mortality at discriminating concentration in field populations as well as chlorpyrifos resistant strain}}{\%\text{ mortality at discriminating concentration in the laboratory colony}} \times 100\right]$.
** Relative resistance $= \frac{\text{Highest resistance percentage}}{\text{Resistance percentage of each population}}$.
*** Confidence interval.

LC_{50} was detected in the 3rd instar larvae collected from Sharkia and Assiut Governorates (Table 1).

Data of monitoring resistance in the adult mosquito of Sharkia and Assiut field populations to tested insecticides as compared with the laboratory colony are presented in Table 2. It is obvious that all tested field populations exhibited, relatively low level of resistance ranging between 1.39- and 2.67-fold for Sharkia population and ranged between 1.25- and 1.83-fold for population of Assiut Governorate. The highest level of resistance ratio in the adult stage of field population collected from Sharkia and Assiut Governorates was noticed with Thiodicarb.

Development of resistance in Cx. pipiens 3rd instar larvae to the toxic action of chlorpyrifos

The resistance ratio of the response of the 3rd instar larvae of *Cx. pipiens* to the insecticidal pressure of chlorpyrifos is presented in Table 3.

The slope values illustrated that approximate homogeneity predominated between individuals of generations starting from the 3rd generation. In a descending order of susceptibility, the results evidently proved that the last three of the 15th generations, were the least susceptible to chlorpyrifos, while those of the 3rd generation were the most susceptible.

Monitoring insecticidal resistance in Cx. pipiens adults to chlorpyrifos by discriminating-concentration technique

The discriminating concentration was carried out for rapid monitoring of the insecticidal resistance in the field populations as well as chlorpyrifos resistant strain. The obtained results concerning the response of *Cx. pipiens* adults, which were collected from different governorates as well as chlorpyrifos resistant strain to discriminate concentration of chlorpyrifos are presented in Table 4. The obtained results indicated that the discriminating concentration of chlorpyrifos, i.e. LC_{99} value caused 87.5 ± 0.75%, 85.4 ± 0.7%, 82.5 ± 0.8%, 63.3 ± 0.65% and 36.1 ± 0.55% mortality for Assiut, Gharbia, Menofia, Sharkia field populations and chlorpyrifos resistant strain, respectively. At the discriminating concentration of chlorpyrifos, a significant difference in adult mortalities was achieved between the field populations and chlorpyrifos resistant strain ($P \leqslant 0.05$). However, it is worthy to mention that the maximum mortality level achieved for Assuit field populations was 87.5% and the lowest mortality level was 36.1% for the chlorpyrifos resistant strain. Furthermore, chlorpyrifos selected strain showed the highest resistance value followed by Sharkia field populations. The corresponding resistance ratio was 63.54 ± 1.32% and 30.06 ± 0.53%, respectively. Assiut field populations' adults exhibited the

lowest resistance level to the toxic action of chlorpyrifos in which it was 11.62 ± 0.81. On the other hand, Gharbia and Menofia field populations had moderate resistance ratio (Table 4). It could be concluded that, when using the discriminating concentration of chlorpyrifos (LC_{99}) there was a highly significant difference in the resistance and the resistance ratio of chlorpyrifos resistant strain and the field populations ($P \leqslant 0.05$).

Biochemical aspects in mosquito, Cx. pipiens

Hydrolysis activity

The activities of the determined hydrolyzing enzymes, i.e. alkaline phosphatase and non-specific esterases were determined in the 3rd instar larvae and the adults of the field populations and chlorpyrifos resistant strain of *Cx. pipiens* in comparison with laboratory colony that was not subjected to any insecticide. Determination of the enzymes activity may shed light on the inductive effect of insecticides on the mosquito *Cx. pipiens* (Table 5).

Alkaline phosphatase activity

Adult mosquitoes showed a highly significant difference ($P \leqslant 0.05$) of alkaline phosphatase activity as compared to the 3rd instar larvae in the field populations, chlorpyrifos resistant strain and the laboratory colony of *Cx. pipiens* (Table 5).

Chlorpyrifos resistant strain reached the highest activity ratio values. The enzymatic activity ratio in Sharkia field population was higher than in the other field populations, being 4.68 and 2.73 in the 3rd instar larvae and the adult stage, respectively, whereas Assiut field population showed the lowest activity ratio levels being 1.43 and 1.86 in the 3rd instar larvae and adult mosquitoes, respectively.

Non-specific esterases

The results indicated that, the level of the enzyme activity significantly increased in both the field populations and chlorpyrifos resistant strain as compared with the laboratory colony ($P \leqslant 0.05$). It was noticed that non-specific esterases activity was significantly higher in the adult mosquitoes than the 3rd instar larvae ($P \leqslant 0.05$) (Table 5).

The average of the enzymes activity ratio in the 3rd instar larvae and adult stage was 2.42 and 3.88×10^{-7} mmole α-naphthol released/individual/min, respectively. Furthermore, chlorpyrifos resistant strain attained the highest enzymes activity in both 3rd instar larvae and the adult stage, being 72.3 ± 1.73 and 161.4 ± 5.77×10^{-7} mg α-naphthol released/individual/min, respectively, followed by Sharkia field population where the enzymes activity in the 3rd instar larvae and the adult stage were 60.6 ± 1.73 and 130.6 ± 2.88×10^{-7} mmole α-naphthol released/individual/min, respectively. While, Assiut field population showed the least level of the enzymes activity being 34.1 ± 1.15 and 59.4 ± 0.57×10^{-7} mmole α-naphthol released/individual/min, in both the 3rd instar larvae and adult stage, respectively.

Total protein contents

The amount of the total protein in the 3rd instar larvae was less than in the adult stage in all field populations under study. Chlorpyrifos resistant strain showed the highest amount of total protein content in both the 3rd instar larvae and the adult stage (7.10 ± 0.05 and 8.27 ± 0.05×10^{-3} mg/individual,

Table 5 Alkaline phosphatase, non-specific esterase activity and total protein content in the 3rd instar larvae and adult stage of different field populations as well as chlorpyrifos resistant strain of *Culex pipiens* in comparison with laboratory colony.

Populations	Alkaline phosphatase*		Non-specific esterase**		Total protein***		Activity ratio***				
	Activity × 10^{-7} phenol released/min		Activity × 10^{-7}/α-naphthol released/min		mg × 10^{-3}/individual		Alkaline phosphatase		Non-specific esterase		Total protein ratio
	3rd instar	Adult	3rd instar	Adult	3rd instar	Adult	3rd instar	Adult	3rd instar	Adult	3rd instar / Adult
Assiut	12.94 ± 0.58^{D}	29.04 ± 0.58^{D}	34.1 ± 1.15^{D}	59.4 ± 0.58^{D}	4.61 ± 0.58^{B}	5.87 ± 0.06^{E}	1.43	1.86	1.54	2.12	1.20 / 1.40
Gharbia	19.35 ± 0.58^{C}	35.88 ± 1.15^{C}	49 ± 1.15^{C}	92.9 ± 2.31^{C}	4.71 ± 0.06^{B}	6.36 ± 0.06^{D}	2.13	2.30	2.21	3. 32	1.23 / 1.52
Menofia	21.27 ± 0.88^{C}	36.70 ± 0.58^{C}	52.6 ± 1.15^{C}	100 ± 2.309^{C}	5.28 ± 0.058^{B}	6.67 ± 0.09^{C}	2.35	2.35	2.37	3. 57	1.38 / 1.58
Sharkia	24.34 ± 0.58^{B}	42.57 ± 1.15^{B}	60.6 ± 1.73^{B}	130.6 ± 2.89^{B}	6.57 ± 0.058^{A}	7.28 ± 0.058^{B}	2.68	2.73	2.73	4.66	1.71 / 1.74
Chlorpyrifos resistant strain	27.64 ± 1.73^{A}	49.30 ± 1.15^{A}	72. 3 ± 1.73^{A}	161.4 ± 5.773^{A}	7.10 ± 0.058^{A}	8.27 ± 0.058^{A}	3.05	3.16	3.26	5.76	1.85 / 2.21
Laboratory colony	9.07 ± 0.33^{E}	15.61 ± 0.58^{E}	22.2 ± 1.15^{E}	28 ± 1.15^{E}	3.84 ± 0.058^{C}	4.19 ± 0.00^{F}	–	–	–	–	1.00 / 1.00

Values represent the mean ± SE of three replicates. Other experimental details are given in the Material and methods.

* Alkaline phosphatase activity was expressed as mmole × 10^{-7} phenol released/individual/min.

** Non-specific esterase was expressed as mmole × 10^{-7} α-naphthol released/individual/min.

*** Total protein was expressed as mg × 10^{-3}/individual. Activity ratio = $\frac{\text{Enzymatic activity or total protein in field populations or chlorpyrifos resistant strain}}{\text{Enzymatic activity or total protein in the laboratory colony}}$.

^{A} Means with different letters in column of alkaline phosphatase, non-specific esterases activity and total protein for each population in comparison with laboratory colony are significantly different ($P \leqslant 0.05$, Waller–Duncan).

Table 6 Relative mobility and molecular weight of protein bands detected in the whole homogenates of different field populations and resistant strain of *Culex pipiens* adult in comparison with laboratory colony.

Band No.	Rm	Molecular weight (kDa)	Lab. colony	Sharkia	Assiut	Menofia	Gharbia	Resistant strain
1	0.05	193.345	+	+	+	+	+	+
2	0.08	177.56	–	–	+	+	+	+
3	0.12	148.76	–	–	+	–	+	–
4	0.19	133.59	–	–	–	–	+	+
5	0.15	102.09	+	+	+	+	+	+
6	0.25	98.72	–	–	+	–	–	–
7	0.18	92.50	–	–	–	+	–	–
8	0.19	88.12	+	+	+	+	+	+
9	0.21	83.26	+	+	+	+	+	+
10	0.25	74.71	+	+	+	+	+	+
11	0.30	70.94	–	+	+	+	–	–
12	0.35	62.94	+	+	+	+	+	+
13	0.40	60.57	–	–	–	+	–	+
14	0.43	58.25	+	+	+	+	+	+
15	0.48	54.62	+	+	+	+	+	–
16	0.53	52.01	+	+	+	+	+	–
17	0.60	48.77	+	+	+	+	+	+
18	0.63	45.60	+	+	+	+	+	+
19	0.67	41.77	+	–	–	–	–	–
20	0.70	38.95	+	+	+	+	+	+
21	0.74	38.21	+	–	–	–	–	–
22	0.78	33.38	–	+	–	–	–	+
23	0.81	32.15	+	+	–	+	+	+
24	0.85	30.58	+	+	+	+	+	+
25	0.89	29.20	+	+	–	–	+	–
26	0.90	26.97	+	+	+	+	+	+
27	0.91	25.79	+	–	–	–	+	–
28	0.93	23.46	+	+	+	+	+	+
29	0.95	21.25	–	+	+	–	–	–
30	0.96	20.14	+	–	+	+	–	–
Total number of bands			20	20	21	21	21	18

(+): Present; (–): Absent; Rm: relative mobility; Lab. colony: laboratory colony.

respectively) as compared with the other field populations and the laboratory colony (Table 5).

On the other hand, Sharkia field population exhibited the highest amount of total protein content in the 3rd instar larvae and the adult stage (6.57 ± 0.057 and 7.28 ± 0.05×10^{-3} mg/individual, respectively) as compared with other field population while, Assiut field population had the lowest amount of total protein contents, (4.61 ± 0.57 and 5.87 ± 0.05×10^{-3} mg/individual in the 3rd instar larvae and the adult stage, respectively) as compared with other field populations (Table 5).

It is clear that, the total protein contents in either field populations or chlorpyrifos resistant strain increased significantly than those of the laboratory colony ($P \leqslant 0.05$).

The average of total protein activity ratio in the 3rd instar larvae and the adult mosquitoes of chlorpyrifos resistant strain was 1.40 and 1.58, respectively. This average was slightly higher in the adult than in the 3rd instar larvae.

Resistance level in Cx. pipiens in relation to some biochemical aspects

Chlorpyrifos resistant strain showed the highest resistance percentage (63.54%). This strain exhibited the highest level of enzyme activity as well as total protein contents (Table 4).

The adults enzyme activity level was 49.30 ± 1.15 mmole substrate hydrolyzed/individual/min for alkaline phosphatase and 161.4 ± 5.77 mmole naphthol released/individual/min for non-specific esterases, whereas, the total protein content was 8.27 ± 0.05 mg/adult. On the other hand, Assiut field population exhibited the lowest level of resistance (11.62%) to chlorpyrifos toxicity and also showed the lowest level of the enzymes activity and the total protein content. The corresponding level of the enzymes activity was 29.04 ± 0.57×10^{-7} mmole substrate hydrolyzed/individual for alkaline phosphatase and 59.4 ± 0.57×10^{-7} mmole α-naphthol released/individual/min for non-specific esterases, while the total protein content was 5.87 ± 0.057×10^{-3} mg/adult.

On the basis of the resistance percentage, adult mosquitoes could be descendingly arranged in the following order: chlorpyrifos resistant strain, Sharkia, Menofia, Gharbia and Assiut field populations.

Effect of the tested insecticides on protein profiles (SDS–PAGE)

The total soluble proteins of *Cx. pipiens* adults and larvae using 10% SDS–PAGE gel were size fractionated into 30 bands. There were thirteen common bands and one common band in adults and larvae under investigation, respectively (Tables 6, 7 and Figs. 1 and 2).

Although no significant difference was observed between the protein profiles of the investigated strains in general, there

Table 7 Relative mobility and molecular weight of protein bands detected in the whole homogenates of different field populations and resistant strain of *Culex pipiens* larvae in comparison with laboratory colony.

Band No.	Rm	Molecular weight (kDa)	Lab. colony	Sharkia	Assiut	Menofia	Gharbia	Resistant strain
1	0.065	174.4	−	−	−	−	+	+
2	0.068	169.4	−	+	+	−	−	−
3	0.084	146.4	−	−	+	−	+	+
4	0.09	138.15	−	+	−	+	−	−
5	0.11	122.96	+	−	−	−	−	−
6	0.12	103.2	−	−	−	−	−	+
7	0.13	100.28	−	−	+	+	+	−
8	0.14	95.73	−	+	−	−	−	−
9	0.21	83.85	+	+	+	+	+	+
10	0.31	70.12	+	+	−	−	−	+
11	0.32	69.46	−	−	+	+	+	−
12	0.33	67.36	−	+	−	−	−	+
13	0.35	63.83	+	−	−	−	−	−
14	0.5	36.47	−	−	−	−	+	+
15	0.55	35.33	+	+	+	+	−	+
16	0.61	33.67	−	−	−	−	+	−
17	0.67	32.11	−	−	−	+	−	−
18	0.7	31.42	−	+	−	+	−	−
19	0.74	30.8	−	−	+	+	+	−
20	0.75	30.4	−	+	−	−	−	+
21	0.76	30.22	+	−	−	−	+	−
22	0.78	29.63	−	−	+	+	+	−
23	0.79	29.52	+	+	−	−	−	+
24	0.8	29.51	+	−	−	−	−	−
25	0.81	29.25	−	−	−	+	−	−
26	0.82	29.13	−	−	−	−	+	−
27	0.83	28.94	−	+	−	−	−	−
28	0.84	28.68	+	+	+	−	−	+
29	0.86	28.38	+	−	−	−	−	−
30	0.87	28.13	−	−	−	−	−	+
Total number of bands			10	12	9	10	11	12

(+): Present; (−): Absent; Rm: relative mobility; Lab. colony: laboratory colony

were differences in some subunits (molecular weight 37.00–66.20 kDa). A protein of 62.94 kDa indicated by an arrow in Fig. 1 decreased considerably in the field populations and resistant strain exposed to the tested insecticides as compared to that in the laboratory colony. Further, a protein band of molecular weight 38.95 kDa (indicated by an asterisk) increased in quantity in the field populations and resistant strains (Fig. 1, lanes 2–6) as compared to the respective laboratory colony (Fig. 1, lane 1). Also, extracts from larvae of laboratory colony, field populations and resistant strain were analyzed by SDS–PAGE. The results revealed that, a protein band of molecular weight 67.36 kDa (indicated by arrow) appeared only in Sharkia and the resistant strain (Fig. 2, lanes 2 and 6) while, a protein band of molecular weight = 63.83 kDa (indicated by an asterisk) disappeared in field populations and resistant strain. These results have been repeatedly observed in a few independent analyses. More specific analyses are required to identify these proteins.

Discussion

The objective of the studied resistance-monitoring program is to detect resistance before any control failure occurs. The monitoring program should measure the resistance frequencies as well as monitoring changes in the frequency of resistance with time (Schouest and Miller, 1988). Laboratory bioassays that closely simulate the field situation help to establish a correlation between the laboratory bioassay and the field in order to measure the resistance ratio by establishing consistent log dose and probit mortality (LDP) relationship (Ball, 1981; Roush and Miller, 1986).

Based on the LC_{50} and LC_{90} values of the tested insecticides, a significant difference in the resistance ratio was observed in the adult stage (Table 2) but not in the 3rd larval instar (Table 1) of *Cx. pipiens*. It is interesting to note that the difference in the resistance ratios may be related to the detoxification of the tested compounds by the enzymes activity. In this respect, alkaline phosphatase and non-specific esterases activity exhibited higher levels in the adult stage than the 3rd instar larvae of *Cx. pipiens*.

The toxicity and modifications of resistance with generations

Concerning the development of resistance in *Cx. pipiens* to the pressure of chlorpyrifos; it was found that the LC_{50} values determined against *Cx. pipiens* varied depending on the number of generation, at which such LC_{50} value was determined (Table 3). Thus, the resistance ratio values of chlorpyrifos increased by the subsequence of generations, which further provided evidence that the rate of insensitivity progressed by

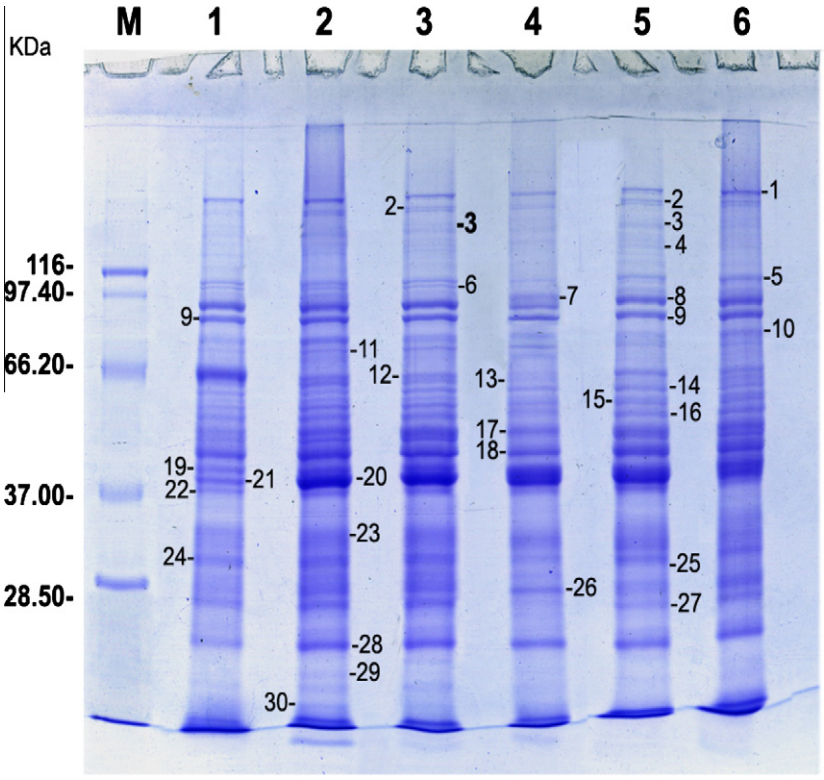

Figure 1 SDS–PAGE analysis of total soluble protein from the whole homogenate of *Culex pipiens* adults. Protein band numbers were indicated on the gel. Thirty micrograms of total soluble proteins (in each lane) was used for electrophoresis. Lane 1: Represents laboratory susceptible strain, lanes 2–5: represent the field strains collected from Sharkia, Assiut, Menofia and Gharbia governorates, respectively, lane 6: represents chlorpyrifos resistant strain. M: Represents the molecular weights of marker protein bands indicated on the left side of the gel. Arrow and asterisk indicate the polypeptides showing variation in their pattern.

succession of generations, irrespective of insect tested. The selected pressure of chlorpyrifos against the 3rd instar larvae of *Cx. pipiens* resulted in 24.56 ± 0.78-fold of resistance after 15 generations. This finding was supported by studies carried out by previous authors (Rathor and Togir, 1981; Mostafa, 1990; Abdel-Badeeh, 2001). Similarly, the resistance of *Cx. pipiens* larvae by fenitrothion pressure for 24 generations resulted in 25.67-fold increase in resistance to this compound despite the number of generations (Hamed et al., 1991). Also, a sample of the Southern house mosquito, *Culex pipiens quinquefasciatus* Say, from Cuba was subjected to lambda-cyhalothrin selection (Bisset et al., 1997), the authors found that high resistance was developed after six generations of selection.

Chlorpyrifos resistant strains of *Cx. pipiens* by LC_{20} concentration seemed moderately resistant (RR < 10) to the compound for the first two generations. Results on susceptibility of parent larvae toward chlorpyrifos revealed a possibility that these larvae had been subjected to some organic phosphate compounds.

Results of the present investigation were confirmed and supported by previous findings which indicated that *Cx. pipiens quinquefasciatus* Say in California developed resistance to chlorpyrifos by 52.2 × the normal LC_{50} (Georghiou et al., 1975). Wirth et al. (1987) working on a mixed population of *Culex tarsalis* (Coq.), *Culex quinque fasciatus* Say, *Culex peus* Speiser and *Culex crythrothorax* Dyer breeding in a sewage-treatment lagoon with organophosphate larvicides, *Cx. tarsalis* displayed high levels of larval resistance to chlorpyrifos (64-fold), methyl parathion (57-fold), temphos (2-fold) and malathion (36-fold)

On the contrary, selection of *Cx. quinquefasciatus* with malathion exhibited 2036- and 2726-fold of resistance as compared with the laboratory strain in the 25th generation at LC_{50} and LC_{90} levels; respectively (Gopalan et al., 1996). Moreover, the resistance to chlorpyrifos in populations of *Cx. pipiens* collected from Tunisia was highly variable, reaching the highest-level >10,000-folds recorded worldwide (Ben Cheikh et al., 1998).

Results associated to insecticidal resistance in *Cx. pipiens* adults by using discriminating concentration of chlorpyrifos revealed that, insecticidal pressure may induce resistance. In this study, the discriminating concentration was implemented for rapid monitoring of the insecticidal resistance in the field as well as chlorpyrifos resistant strains. Chlorpyrifos resistant strain selected by concentration LC_{20} for 15 generations for *Cx. pipiens* exhibited a high level of resistance followed by Sharkia field populations. Higher resistant level was detected in regions where excessive application of chlorpyrifos is common (Armes et al., 1996). This explains the importance of the seasonal differences in chlorpyrifos resistance at several locations under investigations (Kanga et al., 2003).

The possible role of biochemical mechanisms

The relationship between resistance, the level of esterases activity in insects, and insecticidal metabolism, is not always clear.

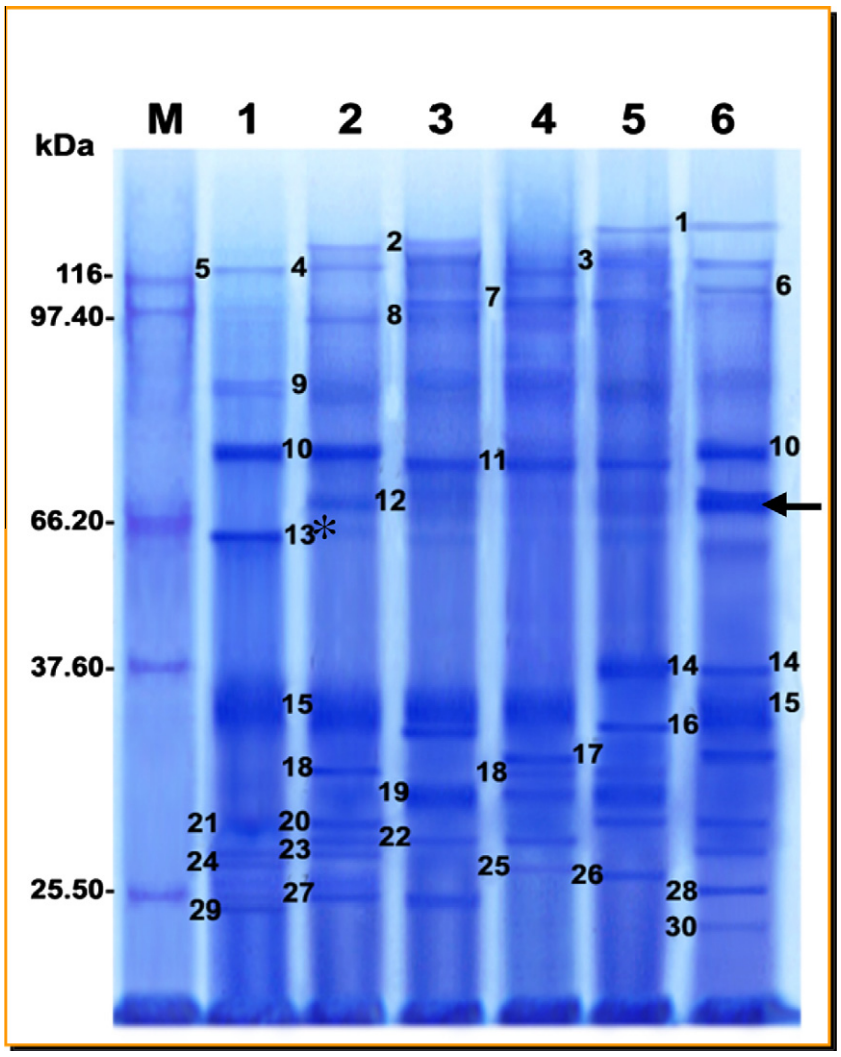

Figure 2 SDS–PAGE analysis of total soluble protein from the whole homogenate of *Culex pipiens* larvae. Protein band numbers were indicated on the gel. Thirty micrograms of total soluble proteins (in each lane) was used for electrophoresis. Lane 1: Represents laboratory susceptible strain, lanes 2–5: represent the field strains collected from Sharkia, Assiut, Menofia and Gharbia governorates, respectively, lane 6: represents resistant strain. M: Represents the molecular weights of marker protein bands indicated on the left side of the gel. Arrow and asterisk indicate the polypeptides showing variation in their pattern.

The organophosphates, carbamates and pyrethroids containing ester groups could be detoxified via hydrolysis of the ester bond. Chlorpyrifos is one of these insecticides that can be hydrolyzed by esterase activity. Activity toward B-naphthyl acetate (2-NA) was increased in resistant populations of *Helicoverpa armigera* compared with susceptible populations (Bues et al., 2005) and a resistant strain of *Aedes aegypti*, generated by deltamethrin showed significant elevation in the activity of alpha- and beta-esterase and glutathione-*S*-transferase (Jagadeshwaran and Vijayan, 2009) Furthermore, esterases might be playing a role in chlorpyrifos resistance in Turkish *Tetranychus urticae* Koch strain selected with chlorpyrifos (Recep and Sibel Yorulmaz, 2010) and the activity of esterase against specific and non-specific substrates is increased in pyrethroid-resistant populations of head lice, *Pediculus humanus capitis* from Argentina (Silvia et al., 2010). In a previous study by our laboratory, a resistant strain of *Pectinophora gossypiella* generated by chlorpyifos showed significant elevation in the activity of alpha-esterases (Abu-El Seoud et al., 2005).

Accordingly, in this study, it was found that there is a positive correlation between the level of the insecticidal resistance and alkaline phosphatase (Table 5) as well as non-specific esterases activity and total protein content in *Cx. pipiens*. Although relatively little information is available about alkaline phosphatase (ALP) as an insecticide resistance mechanism, some lines of evidence suggest an association for ALP with insecticide resistance. For example, a fenvalerate-resistant population of *H. armigera* and four diazinon-resistant populations of *Chilo suppressalis* showed a higher ALP activity than susceptible populations (Srinivas et al., 2004; Zibaee et al., 2009), and parathion and methomyl application increased ALP activity in *H. armigera* (Gao et al., 1996).

The possible role of gene amplification

Moreover, ALPs in *Nilaparvata lugens* and their potential relation with the development and insecticide resistance in this insect pest were evaluated (Wang et al., 2011). These results

strengthen the hypothesis that, the mechanism associated with insecticides resistance had been found in many insects having an increase of esterases activity, probably as a result of gene amplification (Cruz et al., 1997). Also, the high levels of esterases activity involved in organophosphorus insecticides are caused by an enzyme overproduction (Raymond et al., 1989; Piorie et al., 1992). This overproduction is the result of gene amplification and/or gene regulation (Rooker et al., 1996).

Amplification of such esterases genes resulting in an increased enzyme production was responsible for resistance to organophosphorus insecticides in the aphids, *Myzus persicae* and mosquitoes of *Cx. pipiens* complex (Oppenorth, 1984). In both species, different degrees of resistance occur due to different levels of esterase activity hydrolyzing the OP analogs of the insecticides. In this study, the treatment with the tested insecticide produced some differences in SDS protein patterns in the field populations than in the susceptible ones. The results revealed that four specific proteins disappeared in the whole homogenates of the treated larvae of *Cx. pipiens*. The molecular weights of these proteins were 122.96, 63.83, 29.50 and 28.38 kDa, while the proteins with molecular weights 41.77 and 38.21 kDa disappeared in the adult stage. Moreover, many specific proteins to chlorpyrifos appeared in the whole homogenates of the treated larvae and adults of *Cx. pipiens* in one or more field populations, which were collected from four Governorates. Some proteins were characteristic to certain *Cx. pipiens* sample. The protein fraction of 45.6 kDa was detected in both treated larvae and adult homogenates of *Cx. pipiens*. These results agree with the findings of previous study in which a protein of 45 kDa in *Drosophila* head and body tissue and in locust neuronal membranes was detected as a result of Avermectin treatment (Rohrer et al., 1995).

The results of the toxicity lines of the tested insecticides against the field populations of *Cx. pipiens* showed low slope values (> 1.0), indicating that a high degree of genetic heterogeneity exists among these populations. These results are supported by a previous work which indicated that flat lines seem to be a normal response of beet armyworm, *Spodoptera exigua* (Hübner), population to methomyl because it is detoxified by several enzymes in the insect at any given time (Meinke, 1977). The most common mechanism of resistance in the insect species to the tested insecticides is related to several types of metabolic enzymes (Khidr, 1982; Siegfried and Zera, 1994; Wilson and Ashok, 1998). Organophosphates, carbamates and pyrethroids can be detoxified by appropriate hydrolytic enzymes such as esterases. Another type of organophosphates is based on an increase in detoxification capacity resulting from increased phosphatase activity. The resistant larvae of *H. armigera* showed higher activities of esterases, phosphatases and methyl paraoxon hydrolase compared with susceptible larvae (Srinivas et al., 2004). Also, alkaline phosphatases can make insects more resistant to insecticides (Yan et al., 2009). Moreover, the results indicated that non-specific esterases as well as alkaline phosphatase activities might play an important role in the detoxification of the insecticides used against *Cx. pipiens* stages.

Conclusion

It could be concluded that, the changes in the quantity of particular protein fractions and the appearance of specific proteins to chlorpyrifos can be connected with the enhanced phosphatases, non-specific esterases activity and total protein content involved the insect resistance to the OP compound which is caused by an enzyme overproduction as a result of gene amplification. Thus the alkaline phosphatases and non-specific esterases activities might be used as biomarkers in chlorpyrifos resistance in *Cx. pipiens*. Also, the information presented in this study illustrates that the organophosphate chlorpyrifos resistant population in Sharkia Governorate extended throughout the majority of the cotton growing region, making resistance a regional problem. These data indicated that chlorpyrifos was rapidly losing its effectiveness and that alternative control method is needed.

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