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RESEARCH ARTICLE

DETOXIFYING AND SERINE ENZYME ACTIVITIES IN DEVELOPING *CULEX PIPIENS* RESISTANCE AGAINST CARBARYL (SEVIN), MALATHION AND *BACILLUS THURENGIENSIS* SEROVAR H-14

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ABSTRACT

In Egypt, chemical insecticides are used repeatedly in mosquito control programs leading to increase insect resistance in the field especially against carbamate, Organophosphates and Pyrethroids, *B.t.i.* strains are restrictly used. In order to find a quick method for detecting elevation of resistance to the old insecticides or newly used biological insecticides, our research correlated between level of detoxifying and serine enzyme activity with elevation of resistance in Culex pipiens larvae against carbamate, DP and biological insecticides. Results proved that α and β-esterases showed decrease during early formation of resistance when using both carbamate and OP insecticides while no great change in *B.t.i.* resistant generations. Glutathione S- transferase showed positive significant difference between susceptible and carbamate and OP and *B.t.i.* resistance populations. Serine protease were good indicators for measuring OP and *B.t.i.* resistance elevation.

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INTRODUCTION

Culex pipiens is considered the major mosquito vector of *Bancroftian filariasis* and Rift Valley virus epidemics in Egypt. Globally, it is responsible for transmitting arboviruses (Hamer *et al.,* 2008 and (Kilpatrick *et al.,* 2010), Filarial parasites (MichalsKi*et al.,* 2010) and Protozoa(Kimura *et al.,* 2010). Larval control of mosquitoes achieved by two ways, either by reduction the source of breeding places, or by using larvicides as chemical and biological insecticides. The combination of both treatments is a preferable method for reducing adult mosquitoes in many areas of the world (Mulla *et al*., 2001). The intensive use of chemical insecticides as (organ phosphorus and carbamates) to achieve control of the pests in agriculture and vectors control has been widely used for the past 20 years led to the development of resistance between insects, (Zhang *et al.,* 2012). Insect populations may survive under the effect of toxic chemical compounds by different physiological mechanisms including reduced target site sensitivity and elevated detoxifying enzyme production (Linna Shi *et al.,* 2015)**.**

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The difficulties of controlling insect vectors for diseases are faced by the resistance of some vector populations, not only to chemical insecticides, but recently to some famous biological insecticides. An important alternative method for control of these insect vectors is through introducing *Bacillus thuringensis* subsp. *israelensis*, which is toxic to different mosquito and black fly species (Margalith and Ben-Dov, 2000). Insecticide, toxicity bioassays, and microplate assays were performed on *Culex pipiens* mosquitoes to determine evolutionary mechanisms of resistance, through determining the level of susceptibility of mosquito *Cx. pipiens* larvae in our habitats to chemical (Malathion and Sevin) and biological (*B.t.* H14) insecticides, with detecting the level of variation in detoxifying enzymes activity as proteolytic esterases and glutathione s-transferases (GST) enzymes as families in resistance formation(David *et al.,* 2013 andSomwang*et al.,* 2011).

MATERIALS AND METHODS

Insect colonies

Culex pipiens larvae were collected from El-Moassasa, Cairo governorate and reared to adults for subsequent production and from one egg reared in laboratory to F15 to ensure the

homogeneity of the colony. Then this colony is used to drive 3 other colonies against (Sevin, Malathion, and B.t. H14). Bioassays were performed using World Health Organization (WHO) methods for larvae with the diagnostic doses of 3 insecticides (2chemical and one biological).

Insecticides used

Sevin: is a carbamate insecticide prepared as different concentrations. It is used in malaria control programs.
Malathion: it is from the class of phosphorothiolothionate compounds and it is used in Egypt against agricultural and health pests
***Bacillus thuringiensis*:** *B.t.i.* is used as a technical powder produced by Pasteur Institute – France.

Bioassay test for *Cx. pipiens* parental generation

Pure colony was considered as the parental generation where the three resistance lines were driven. The susceptibility of the parental generation to two chemical insecticides, Carbaryl (Sevin) and Malathion, and one biological insecticide (*Bacillus thuringiensis*) was determined by bioassaying different concentrations of the tested insecticides against 3rd larval instar of *Culex pipiens.*

Resistance development in *Cx. pipiens* to chemical and biological insecticides

The selection of resistance experiments were undergone according to the method described by (Yangyang Liu *et al.,* 2011) Bioassays were performed on third instar larvae by standard WHO larval susceptibility test (WHO, 2005). The susceptibility of the parental generations, were determined by bio-assay tests (24hrs or 48hrs mortality of third instars for chemical and biological insecticide respectively). Lethal concentrations for 50% and 90% mortality levels, with 95%confidence limit (CL), chi-square, and line parameters of log dose-probit response lines (ld-p lines) were determined using a probit analysis computer program.(Sakine Ugurlu Karaagac, 2012). The parental colony was divided into four lines, three subjected to selection pressure and the other one was cultured without exposure to insecticides.

Total protein determination in *Culex pipiens* larvae (Protein assay)

Total protein content for susceptible and resistant larval samples, was determined from an aliquot using, the Bio-Rad Protein Assay derived from the Commassie Brilliant Blue dye G-250 Bovine serum albumin (BSA) was used as the standard protein, (0.2-1.4 mg/ml distal water).

Esterases activity

The detoxifying enzyme activity of acetylcholine esterase, GST, α and β-esterases and proteolytic enzymes (trypsin and aminopeptidase). Survived larvae from the chemical and biological insecticide were measured for each generation. Esterase activities from whole insect body homogenates were determined using α and β-Naphthyl acetate as substrates, using an adaptation of the method used by**.** The assays were run in microtitre plates with α-esterase substrate α-Naphthyl acetate (NA). Enzyme activity was read at 630 nm as an endpoint on Vmax microtitre plate reader. The amount of Naphthol produced from the esterase reactions were calculated from standard curves of α-Naphthol and the results expressed as mmol. product/min/ mg protein. For measuring Acetyl cholinesterase (AChE) activity in parental and resistant larvae, the methodology described by (Bourguet *et al.,* 1997) with a slight modification were followed. Tested larvae were homogenized in 400 µl of 100 mM phosphate buffer pH 7.8 containing 1% Triton X-100. Homogenate was centrifuged at 10.000 g for 8 min at 4ºC. For AChE assay, two replicates of 100 µl of supernatant were transferred to a fresh microtitre plate. 100 µl of 2 M dithio-bis 2-nitrobenaic acid in 0.1 M phosphate buffer pH 7.0 and 2 mMacetylthiocholine iodide in distillate water.

Glutathione S-transferase (GST) activity

Enzyme activity was estimated following the procedure of (Habig *et al.,* 1974) using 1-chloro-2, 4 dinitrobenzene (CDNB), and GSH as substrates. The change in the absorbance level was measured at r340 nm for 3 min., after every 1 min. in the UV spectrophotometer Absorbance values were converted to units of concentration using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig *et al.,* 1974). Selected larvae were homogenized in 400 µl of 0.1 M phosphate buffer- pH 6.5. The homogenates were centrifuged at 10,000 g for 8 min at 4°C. Routine assays consisted of 1.82 ml substrate buffer (100 mM potassium phosphate buffer pH 6.5), 40 µl from 70 mM 1-chloro-2, 4 dinitrobenzene (CDNB) dissolved in 2ml 95% ethanol; 40 µl 70 mM glutathione reduced in 2ml distal water and 100 µl of the supernatant aliquots of the two larval homogenate. Velocity plots were linear for approx. 3min. Nonenzymatic conjugation (assays conducted without enzyme) was subtracted from all assays.

Proteolytic enzymes assay

The activities of trypsin and aminopeptidase in whole *Cx. pipiens* larvae were determined using synthetic peptide substrates. The cleavage of such substrates results in release of coloured products. These products are p-nitroanilide in case of trypsin and aminopeptidase. The absorption of these coloured products were automatically measured at wave length 410 nm using ELISA reader. For trypsin assay, larvae were homogenized in 400ul 1x PBS and spun at 10,000 g for 8 min. at 4°C, and the supernatant containing enzymes was used in the assays, and 50 µl were added to a microplate well 4 mM α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) in 7% DMF was diluted with 50 mM Trizmahydrochloride (Trishydroxymethylaminomethane hydrochloride, pH 8.5), and 50ul were added to each well (final BA*p*NA concentration was 2mM). After 15 min. at 37ºC absorbance was monitored at 410 nm. The absorbance values of blanks were measured and subscript from the sample the reading. The amount of nitroanilide produced from the enzyme reactions were calculated from standard curves of p-nitroanilide and the results were converted to micromoles/min/ mg of protein. While aminopeptidase assay was measured using larval

homogenate as source of enzyme with 3 mM L-leucine *p*-nitroanilide (L*p*NA). Absorbance values were read after 15 min. at 37°C at 410 nm. The amount of nitroanilide produced from the enzyme reactions were calculated from standard curves of p-nitroanilide and the results were converted to micromoles/min/ mg of protein.

Statistical Analysis

Mortality rates were corrected and analysed by the probit Analyse Program. Probit regression line was plotted and LC90 values were calculated along with their 95% C.L. of resistant and susceptible strains. Resistance ratio was calculated by comparing its LC values with those of unselected counterpart. 95% C.L. of RR_s of lethal concentration ratios were used as a criterion to detect significant differences of susceptibility to chemical and biological insecticide between the populations. C.L. of these ratios include the integer 1, then the two compared LC_s were not significant from each other**.** Biochemical data were subjected to a one –way analysis of variance (ANOVA) to compare the protein content and enzyme production levels within and between populations. All levels of statistical significance were determined at $P < 0.05$. Observed differences in resistance between generations were analysed by independent *t*-test (SPSS version 13.0)

RESULTS

Both field and lab strains were susceptible to all treatments. Susceptibility of lab strain was slightly higher than field strain in all treatments. LC50 values for field strain were 1.585 for Carbaryle, 0.085 for Malathion, and 2.44 using *B.t.i.* For laboratory strain LC50 values were 1.105, for Carbaryl, 0.078 for Malathion, and 2.391 for *B.t.i.* Increasing LC 50 values from 1.455 to 12.309 by raising generation number after treatment with Carbaryl, table 2, indicates increasing in tolerance level. The second generation showed the highest susceptibility level, while the lowest was recorded for generation number 20. The highest resistance ratio (R.R.) was recorded for generation F20 as 8.049 regarding LC50 value and 5.17 using LC90 values. Comparing LC 50 values in (table 3) the parental generation of *Culex pipiens* was the highest susceptible LC 50 = 0.083. The sensitivity decreased as the generations proceeded, reaching the maximum tolerance at generation 20 recording LC 50 = 0.175. The range of relative toxicity using LC 50 values was not high comparing its values from 2nd generation, (2.39). While the difference was significant when using LC 90 values. R.R. started from 2.63 to 1.88 representing the 2nd and last generation. Results in table (4) shows no great variation concerning LC50 values from F0 to F10, as the values ranged from 2.49 to 2.39.

Table 1. Susceptibility of *Cx. Pipiens* larvae of field and laboratory strains to Carbaryl, Malathion and *B.t.i.*

Culex pipiens population	Para meter	Carbaryl	Malathion	*B.t.*H14
Field strain	LC_{50}	1.585 (1.631-1.963)	0.085 (0.078-0.087)	2.440(2.441-2.591)
	LC_{90}	3.336 (2.981-3.937)	0.125 (0.112-0.139)	4.323 (3.826-4.443)
	Slope	1.955	1.299	1.653
	X^2	4.530	3.990	6.170
Laboratory strain	LC_{50}	1.105 (0.983-1.515)	0.078 (0.072-0.075)	2.391 (2.103-2.483)
(susceptible)	LC_{90}	2.089 (1.788-2.555)	0.097 (0.093-0.102)	3.186 (2.900-3.499)
	Slope	1.640	1.125	1.210
	X^2	5.000	5.320	1.281

Table 2. Development of *Culex pipiens* resistance during treatment with Carbaryl.

Generation	LC_{50}	LC_{90}	Slope	X^2	R.R at LC_{50} (C.I)*	R.R at LC_{90} (C.I)*
F0	1.455 (1.30-1.51)	2.205 (2.04-2.55)	1.640	5.000	-	-
F2	3.782 (3.24-4.02)	4.299 (4.19-4.40)	1.161	1.120	2.781 (2.55 -3.02)	1.881 (1.701-2.050)
F4	6.262 (6.13-6.38)	7.663 (7.28-8.06)	2.110	6.090	4.156 (4.01-4.70)	3.244 (3.15-3.55)
F6	7.573 (7.28-7.68)	8.713 (8.41-8.82)	1.23	3.880	5.890 (5.57-6.10)	3.859 (3.44-4.01)
F8	8.135 (8.08-8.18)	8.804 (8.35-8.95)	1.194	2.140	6.090 (5.95-6.32)	4.002 (3.65-4.38)
F10	8.557 (8.44-8.66)	9.161 (8.98-9.33)	1.054	1.10	6.303 (5.96-6.76)	3.650 (3.68-4.38)
F12	8.925 (8.81-9.03)	9.796 (9.47-10.48)	1.075	3.220	6.352 (5.96-6.76)	4.280 (4.09-4.62)
F14	9.128 (9.06-9.58)	9.677 (9.53-9.82)	1.046	5.79	6.497 (6.33-6.89)	4.228 (3.84-4.65)
F16	10.768 (10.63-10.90)	11.566 (11.19-11.94)	1.057	0.350	7.667 (7.19-8.16)	5.053 (4.66-5.45)
F18	10.851 (10.76-10.93)	11.399 (11.25-11.54)	1.039	1.760	7.723 (7.21-8.26)	4.980 (4.51-5.48)
F20	12.309 (12.24-12.37)	13.855 (12.65-14.03)	1.066	5.200	8.049 (7.50-8.62)	5.174 (4.70-5.68)
Susceptible Strain	1.405 (1.30-11.37)					

*C.I. = confidence limits R.R. = resistance ratio

The difference could be recorded after calculating LC90 values which ranged from 3.18 to 2.88 for generations F0, and F10. Raising resistance to the biological insecticide was very difficult, no great change in relative toxicity based on LC50 values. RR values ranged between0.97 to 0.96. Considering LC90 values RR ranged from 0.94 to 0.90. RR values for Carbaryl were high ranged from 2.7 to 8 while for Malathion RR had values 2.01 to 2.8 proving low resistance. From (table 5) it is clear that slight change in protein content was recorded in Carbaryl resistant larvae at generations F12 to F20. While Malathion and *B.t.i.* resistant larvae did not record significant change in values of total protein contents comparing with laboratory colony. In Carbaryl resistant larvae in (table 6) α–esterase activity significantly decreased from susceptible control larvae (p=0.0001). The lowest activity was recorded at F4 (10.388) ml.mol/min/mg with 28% decrease (at r = 6.532 and P = 0.0002) comparing with susceptible larvae. In Malathion resistant larvae, α–esterase activity showed slight decrease in F0, F4, F16, but an increase in enzyme activity by 4%, and 5% was recorded at F8, andF20. Regarding resistant strain against *B.t.i*. the enzyme activity showed increasing level in F0, F2, F4, F6, sudden decrease by 23% in activity was recorded to be (11.38 mM/min/mg) in the last generation, F10.

In Carbaryl pressed larvae, β-esterase activity decreased significantly to about half the activity of susceptible control larvae from F2 to F12, then increased rapidly above control at F16 (4%) and reached (7%) increase at F20as shown in table (7). In Malathion pressed larvae, there were no significant differences in β-esterase level in F0 and F2 then, the activity decreased from the susceptible control to be the lowest at F8 (26% decrease). The activity increased rapidly from F12 to reach maximum increase at F20 (19% increase). In Bacteria resistant larvae, beta-esterase activities increased by (13% to 3%) above control susceptible larvae from F0 to F6. The activity decline gradually to be significantly decreased than control in F8=8.4363mmol/min/mg protein (15% decrease than control), and then the activity increased rapidly at F10= (10.7856 mmol/min/mg protein) to record 11% increase (P=0.0034, t=4.105). Acetylcholinesterase enzyme recorded significant decrease in activity in In Carbaryl resistant colonies from F4 till F14, then it reached normal level as control from F16 to F20. Malathion resistant generations showed sharp decrease in acetylcholinesterase activity by 2 folds. In *B.t.i.* resistant larvae Acetylcholinesterase complete its decreasing level from F0 to F10. Infection by *B.t.i.* inhibit the activity of Acetylcholinesterase although it is a gut poison.

Table 3. Development of *Culex pipiens* resistance during treatment with Malathion

Generation	LC_{50}	LC_{90}	Slope	X^2	R.R at LC_{50} (C.I)*	R.R at LC_{90} (C.I)*
F0	0.083 (0.081-0.085)	0.099 (0.093-0.102)	1.071	6.200		
F2	0.157 (0.147-0.166)	0.256 (0.223-0.293)	1.233	5.090	2.150	2.639
F4	0.146 (0.144-0.147)	0.160 (0.157-0.163)	1.054	3.190	2.000	1.649
F6	0.147 (0.145-0.149)	0.163 (0.159-0.167)	1.080	3.220	2.014	1.680
F8	0.152 (0.150-0.154)	0.168 (0.164-0.172)	1.078	1.220	2.082	1.732
F10	0.154 (0.151-0.158)	0.186 (0.177-0.196)	1.157	1.310	2.110	1.732
F12	0.156 (0.154-0.158)	0.172 (0.168-0.196)	1.180	2.680	2.137	1.773
F14	0.158 (0.156-0.160)	0.177 (0.171-0.183)	1.090	1.540	2.164	1.825
F16	0.167 (0.166-0.169)	0.178 (0.175-0.180)	1.047	1.470	2.287	1.833
F18	0.174 (0.172-0.175)	0.184 (0.181-0.188)	1.047	0.64	2.384	1.897
F20	0.175 (0.174-0.176)	0.185 (0.183-0.187)	1.277	1.990	2.397	1.898

*C.I. = confidence limits

Table 4. Development of *Culex pipiens* resistance during treatment with *Bacillus thuringiensis*- H14

Generation	LC_{50}	LC_{90}	Slope	X^2	R.R at LC_{50} (C.I)*	R.R at LC_{90} (C.I)*
F0	2.491 (2.40-2.58)	3.186 (2.90-3.49)	1.210	1.280		
F2	2.432 (2.36-2.50)	3.00 (2.78-3.04)	1.177	5.570	0.976 (0.97-0.988)	0.942 (0.87-0,96)
F4	2.428 (2.34-2.51)	3.199 (3.00-3.40)	1.239	0.730	0.975 (0.96-0.98)	1.004 (0.97-1.03)
F6	2.427 (2.35-2.50)	3.136 (2.90-3.39)	1.220	1.300	0.974 (0.95-0.99)	0.984 (0.88-0.99)
F8	2.411 (2.37-2.47)	2.970 (2.85-3.11)	1.170	2.591	0.971 (0.96- 0.99)	0.934 (0.89-0.98)
F10	2.395 (2.33-2.45)	2.881 (2.76-3.00)	1.154	1.760	0.961 (0.95-0.97)	0.904 (0.86-0.0.95)

Table 5. Changes of total protein content in resistant *Culex pipiens* larvae against Carbaryl, Malathion and *B.t.i.*

	Protein content	(mg/ml)		
Generation number	Untreated lab. colony	Carbaryl resistant colony	Malathion resistant colony	Bacterial resistant colony
F0	0.2967 ±0.0009	0.2973 ±0.0015	0.2928 ±0.0025	0.2969 ±0.0007
F2	0.2963 ±0.0019	0.2968 ±0.0009	0.2918 ±0.0014	0.2961 ±0.0003
F4	0.2963 ±0.0017	0.2965 ±0.0040	0.2911 ±0.0008	0.2954 ±0.0021
F6	0.2963 ±0.0016	0.2981 ±0.0022	0.2910 ±0.0008	0.2923 ±0.0017
F8	0.2965 ±0.0015	0.2993 ±0.0011	0.2919 ±0.0038	0.2908 ±0.0025
F10	0.2975 ±0.0051	0.3013 ±0.0011	0.2921 ±0.0018	0.2901 ±0.0025
F12	0.2966 ±0.0010	0.3062 ±0.0070	0.2928 ±0.0023	
F14	0.2968±0.0011	0.3068±0.0070	0.2937±0.0022	
F16	0.2916±0.0011	0.3055±0.0073	0.2921±0.0025	
F18	0.2952±0.0011	0.3094 ±0.0078	0.2931 ±0.0021	
F20	0.2964 ±0.0013	0.3131 ±0.0110	0.2936 ±0.0026	

Table 6. Alpha-esterase activity in Carbaryl decrease α esterase (Carbamate) in resistant *Cx. pipiens* Larvae against Carbaryl, Malathion and *B.t.i.*

	α-esterase activity (mMol/min/mg protein)			
Generation time	Control lab colony	Carbaryl res. colony	Malathion res. colony	Bacteria res. colony
F0	14.6588 ±1.25	11.4927 ±0.39	12.3512 ±0.43	16.5710 ± 0.77
F2	14.5511 ±1.29	11.9624 ±0.32	14.8785 ±0.99	16.4709 ± 0.63
F4	14.4216 ±1.24	10.3881 ±0.59	13.7244 ±0.87	15.9503 ± 0.72
F6	14.4230 ±1.24	11.0921 ±0.44	13.o544 ±0.82	15.7183 ± 0.62
F8	14.5746 ±1.23	11.7669 ±0.50	15.1405 ±0.71	14.1814 ± 0.67
F10	14.5946 ±0.53	11.7523 ±0.54	15.5503 ±0.50	11.3801 ± 0.41
F12	14.6176 ±0.52	11.2138 ±0.18	14.6338 ±0.86	-
F14	14.6455 ±0.47	11.7410 ±0.38	14.0831 ±0.78	-
F16	14.6895 ±0.47	11.7196 ±0.51	13.9428 ±0.32	-
F18	14.6691 ±0.57	11.6192 ±0.51	15.0631 ±0.32	-
F20	14.6416 ±0.57	11.5752 ±0.47	15.3263 ±0.52	-

Table 7. Beta-esterase activity in Carbaryl decrease α esterase resistant *Cx. pipiens* Larvae against Carbaryl, Malathion and *B.t.i.*

Generation number	β-esterase activity (mMol/min/mg protein)			
	Control lab colony	Carbaryl res. colony	Malathion res. colony	Bacteria res. Colony
F0	9.4160± 0.71	3.8785 ± 0.30	9.8019 ±0.53	10.6487±0.63
F2	9.6819 ± 0.62	4.0451 ± 0.53	10.2888±0.65	10.6290±0.55
F4	9.6071 ± 0.75	3.9377 ± 0.36	8.0397±1.08	9.9394±0.40
F6	9.6211 ± 0.58	3.9872 ± 0.95	8.0171±1.99	9.2014±0.40
F8	9.4621 ± 0.58	3.9927 ± 0.57	7.0327±0.81	8.4363±0.71
F10	9.6602 ± 0.49	4.0951 ± 0.50	7.2745±0.87	10.7856±0.52
F12	9.6538 ± 0.41	4.3265 ± 0.50	10.2485±0.57	
F14	9.5831± 0.31	5.0920± 0.05	10.5511± 0.85	
F16	9.5922 ± 0.49	9.8540 ± 0.94	10.8117±0.27	
F18	9.652± 0.54	9.940±0.59	10.971±0.17	
F20	9.6416 ± 0.41	10.2791 ± 0.96	11.6907±0.28	

Each point represent means ± SD and (n = 10).

Table 8. Acetylcholinesterase activity in *Cx.pipiens* resistant Larvae to Carbaryl, Malathion and *B.t.i.*

Generation number	AChE activity (µmol/min/mg protein)			
	Control lab colony	Carbaryl res. Colony	Malathion res. Colony	Bt.H14 res. Colony
F0	0.1119±0.010	0.1084±0.011	0.0575±0.007	0.0936 ±0.005
F2	0.1107±0.010	0.1020±0.014	0.0549±0.009	0.0927 ±0.007
F4	0.1100±0.006	0.0900±0.012	0.0605±0.008	0.1011 ±0.006
F6	0.1120±0.013	0.0899±0.009	0.0568±0.009	0.1025 ±0.006
F8	0.1144±0.003	0.0802±0.013	0.0557±0.006	0.1025 ±0.006
F10	0.1145±0.006	0.083±0.017	0.0556±0.009	0.1026±0.007
F12	0.1143±0.002	0.0810±0.011	0.0553±0.006	
F14	0.112±0.007	0.084±0.001	0.0595±0.006	
F16	0.1132±0.007	0.1000±0.007	0.0699±0.010	
F18	0.1133±0.08	0.1014±0.031	0.078±0.013	
F20	0.1133±0.003	0.1024±0.004	0.0827±0.008	

Each point represent mean ± SD and (n = 10).

Table 9. Glutathion S – transferase activity in resistant *Cx. pipiens* Larvae against Carbaryl, Malathion and *B.t.i.*

Generation number	Glutathion S – transferase activity (µmol/min/mg protein)			
	Control lab colony	Carbaryl res. Colony	Malathion res. Colony	Bacteria res. colony
F0	0.9862±0.061	0.8089±0.077	0.7735±0.036	0.7251±0.036
F2	0.9878±0.035	0.9116±0.026	0.8847±0.018	0.8413±0.038
F4	0.9846±0.038	0.9450±0.073	0.9294±0.030	0.9063±0.053
F6	0.983±0.021	0.955±0.017	0.964±0.0311	0.9374±0.033
F8	0.9904±0.034	1.0292±0.103	1.0054±0.050	1.0415±0.063
F10	0.980±0.033	1.029±0.113	1.094±0.004	1.3000±0.036
F12	0.9887±0.064	1.0346±0.064	0.9611±0.076	
F14	0.991±0.001	1.022±0.241	0.991±0.070	
F16	0.9956±0.039	1.0998±0.137	1.0654±0.174	
F18	0.995±0.041	1.198±0.173	1.125±0.140	
F20	0.9948±0.023	1.2361±0.048	1.2852±0.034	

Each point represent means ± SD and (n = 10).

Table 10. Trypsin activity in *Cx. pipiens* resistant Larvae in Carbaryl, Malathion and *B.t.i*

Generation time	Trypsin activity (µmol/min/mg protein)			
	Control lab colony	Carbaryl res. colony	Malathion res. colony	Bacteria res. colony
F0	20.8633±0.545	19.4185±0.431	14.6401±0.782	9.4809±0.875
F2	20.8143±0.626	18.3029±0.446	12.4119±0.531	9.5107±1.297
F4	20.7994±0.577	16.9460±0.672	10.8103±0.860	6.0759±0.999
F6	20.8455±0.580	15.6159±0.407	11.7957±0.755	4.1726±0.869
F8	20.7162±0.730	17.7339±0.451	12.4233±1.013	4.1860±0.868
F10	20.702± 0.420	17.741±0.420	12.43±1.172	2.0974±0.587
F12	20.5018±0.721	17.713±0.441	12.443±0.101	
F14	20.505±0.710	18.651±0.421	12.451±0.111	
F16	20.5014±0.725	19.1534±0.235	13.2366±0.597	
F18	20.531±0.050	19.592±0.551	13.522±0.690	
F20	20.5598±0.585	20.1292±0.438	13.7180±0.621	

Each point represent means ± SD and (n = 10).

Table 11. Aminopeptidase activity in *Cx. pipiens* resistant Larvae in Carbaryl, Malathion and *B.t.i.*

Generation time	Aminopeptidase activity (µmol/min/mg protein)			
	Control lab colony	Carbaryl res. Colony	Malathion res. Colony	Bacteria res. colony
F0	178.3538±2.66	133.8210±5.32	135.48830±6.65	143.9779± 3.78
F2	178.7732±2.57	126.8630±5.72	142.1953±3.60	140.9186± 3.42
F4	179.1750±2.14	144.9567±9.81	138.6622±7.60	131.4775± 2.99
F6	179.3367±2.35	160.1755±2.78	149.4664±7.50	119.5686± 2.00
F8	177.3168±3.10	145.1454±4.11	152.4974±6.77	110.3783± 1.37
F10	187.2213± 3.08	146.44± 3.71	153.9201± 5.67	102.0353±3.57
F12	176.7376±4.02	149.3006±9.33	162.2242±8.364	
F14	177.9763±3.66	149.006±6.32	165.79±6.60	
F16	176.3011±3.07	150.755±6.32	166.46±9.95	
F18	176.3890±4.35	165.241±8.39	169.59±7.44	
F20	176.7130±3.98	165.8514±4.629	170.8790±3.73	

Each point represent means ± SD and (n = 10).

Carbaryl treated larvae for about 20 generations recorded slight decrease in GST activity as shown in table(9),till F6(P=0.3158, t=1.070). Then enzyme activities increased gradually above the control from F8 to F20. In Malathion resistant larvae There was significant decrease in GST activity from F0 to F6, where the decrease in activities between22% to 6%**.** There was no significant difference in activities compared to susceptible control from F8 to F20 (P>0.05, t=0.6165, 0.665, 0.619, 0.6154, 0.814 and 0.8713, respectively). The highest activity of GST was recorded at F20, (activity = 1.23)mmol/min/mg. The same behaviour was recorded after *B.t.i*. treatment, GST activity recorded decrease from F0 to F6 followed by increase than normal at F8, F10. In table (10), Results from microplate assay of BA*p*NA hydrolysis by *Cx. pipiens* larvae extracts demonstrated that activity was reduced in Carbaryl, Malathion and *Bacillus thuringiensis* resistant larvae. The maximum decrease was recorded in *B.t.i*. resistant colonies (P<0.0001) reached. 90% decrease in activity compared with susceptible control) at F10. Aminopeptidase-like proteinase activity significantly decreased in Carbaryl, Malathion and *B. t. i.* resistant colonies, as shown in table (11). Results explain the changes of aminopeptidase activity in *Cx. pipiens* larvae exposed to Carbaryl for along 20 generations, the activity decreased with significant fluctuations, lowest from F0 to F20 with activity at F2 (29% decrease in specific activity, P<0.0001 and t=18.49). At F6, the activity of the enzyme increased rapidly to record 160.175±2.783 followed by decrease at F8, 10, 12, 14, and 16. Sudden decrease in activity was recorded at F18 and 20 as (165.241±8.39 and 165.85±4.62mmol/min/mg). In Malathion resistant larvae maximum decrease occurs at F0 (29%). Activities increased gradually from F8 to F20 recording the highest activity, atF20 (170.8790±3.7395). In *B. t. i.* treated larvae, the activity of aminopeptidase decreased significantly from susceptible control from F0 to F10.

DISCUSSION

The vectors of diseases have developed resistance against many commonly used pesticides all over the world (WHO 1992). Indeed, the continuous development of resistance threatens the long term viability of insecticides based products (Denholm *et al.,* 2002). Organophosphates and carbamates were employed as the main insecticides for mosquito control until pyrethroids were introduced between 1981 and 1985, (Nauen 2007). The proposed work aimed to correlate between the changes in detoxifying and proteolytic enzymes (degradable enzymes) in *Cx.pipiens* larvae, treated with organophosphates, e.g. Malathion, Carbamates and *Bacillus thuringiensis* H-14 as biological insecticides. The change of detoxifying and proteolytic enzymes were studied in resistant and susceptible larvae. Similar studies on the changes in the enzyme activity under the effect of resistance to chemical and biological insecticides used against mosquito larvae and other insects were carried out by (Macoris *et al.,* 2003, *and Zayedet al.,* 2006). In case of artificially selected larvae for Carbaryl, there was an agreement in the results of both diagnostic tests and estimation of resistance ratios. Treatments that were classified as susceptible, showed resistance ratios below three. The susceptibility of *Cx.pipiens* larvae to Malathion was measured also for 20 generations. The resistance increased with succeeding generations of exposure during the selection process. Studying the toxicity to Malathion revealed that the relative toxicity was highest against the parental generation then toxicity response decreased progressively as mosquito generations proceeded. In other words, sensitivity of the larvae toward Malathion decreased as the generations succeeded. Results of resistance ratios of *Cxpipiens*larvae under selection with malathion in comparison to the parent , showed that , at LC50 and LC90 values , a moderate ratio of resistance, during the twenty generations(from 2.0 at F4 to 2.397 at F20) was proceeded.

The susceptibility of *Cx. pipiens* larvae to *Bacillus thuringiensis* was measured every two generations. Larvae subjected to laboratory selection at LC50 for successive generations showed that no big change of LC50 values, the highest number was 2.491 at F0 to reach 2.395 at F10. On the other hand, the selected generations from F2 to F10 showed marked stability in their susceptibility to *B.t.*H-14 , this agree with many previous work explain the beginning of resistance at the tenth generation and only 2.78-fold increase in tolerance.*B.t.i* was induced in *Cx.pipiens* as a result of 10 generations of selection**.** In resistant Carbaryl larvae, the resistance ratio increased at a faster rate compared with Malathion or B.t.i. Our populations were highly resistant to Carbaryl (R>3) followed by Malathion (RR<3) while low level of resistance was recorded for *B.t.i* (RR= 0.9 – 0.97). Low resistance to *B.t.i* was recorded by many researchers (Vasqnez *et al.,*2009, Loke *et al.,* 2010, Kamgang *et al.,* 2011). To define the underlined mechanisms involved in using some chemical and biological insecticides in *Cx.pipiens* larvae, activity of detoxifying enzymes were measured because it is sometimes difficult to measure the effects of these pollutants, especially if the poisoning process is at low level. Some measurable biochemical changes may be useful in detecting insecticide resistance as short term biomarkers such as the inhibition of acetylcholine esterase. However, this would not indicate the frequency and / or long term effect of chronic poisoning**.** The enzymes involved in insecticides detoxification may be qualitatively and / or quantitatively changed to give resistance. Increased metabolic detoxification is one of the most common mechanisms on insecticides resistance (Hemingway, *et al.,* 2004). There were no significant difference could be recorded between the protein of susceptible or resistant generations testing Malathion, Carbaryl or *B.t.i.* New biochemical assay developed for understanding insecticides resistance in field and laboratory populations to overcome the limitation of bioassays. Biochemical assays can help to detect specific resistance mechanisms with the frequency of resistance genes in populations (Julien *et al.,* 2013). The Study of enzymes involved in the detoxification mechanism against insecticides will help to introduce appropriate control measures such as use of chemical or biological insecticides. The Technique and the methods of microplate assays which used in this work were agreed with (Enayati*et al.,* 2005, Zayed *et al.,* 2006, Sukhoruchenko*et al.,* 2008).

α and β–esterases, showed slight significant decrease than normal in case of carbamate (Carbaryl) resistant generations while in case of malathion, a significant increase in activity was recorded from generations 8 to 20 . Increasing in activity of α-esterase was also recorded in *B.t.*i resistant populations especially from (F0 to F6). Measuring β-esterase as indicator for resistance formation showed significant decrease in activity in the early selected colonies (f0 to f14). And from (F0 to F10) using organophosphorus compound (Malathion) then activity increased in late populations, reaching higher activity than normal at (F16 to F20). β-esterase activity fluctuated in *B.t.i* resistant larvae between increase in early selected population (F0 –F2), followed by decrease to normal level (F4 to F6), sudden increase than normal was recorded at (F10). (Macoris *et al.,* 2003) observed that high level of α and β esterase activity in the field populations of *Ae. aegypti*were accompanied by higher resistance ratios. Our results proved that these enzymes are good biomarkers in detecting resistance to organophosphates. The results were compatible with findings of (Hemingway, 2000, Cui *et al.,* 2007, Yangyang Liu *et al.,* 2011,). (Cheikh *et al.,* 2008), detected lower level of esterase activity in Malathion resistant *Ae. stephensi* and *arabiensis*and recorded decrease in esterase activity in Malathion resistant strain of *Plodiainterpunctella*. These findings proved the interfere of esterase activity in organophosphates and carbamate resistant. (Julien *et al.,* 2013) stated that overproduction of detoxifying esterases are one of the mechanisms of raising resistance in mosquitoes. Using laboratory selection to induce resistance was recommended by (McCarrol and Hemingway 2002, Curtis 2001) concluded that results obtained from lab. resistant strains were applicable than measuring field parameters. Measuring ChE activity in susceptible and resistant strains is an important factor in measuring resistance level, (Yanyang*et al.,* 2011). Our data in table (8), proved that the variation in enzyme activity between susceptible and Carbaryl resistant populations was insignificant. This may explain the high resistance values for such insecticides resistance ratio (RR). In contrary, Malathion resistant populations showed significant difference in chemical activity than susceptible one. This proved that in measuring

chemical insecticides resistance, variation of activity could be used as bioindicator for detecting OP resistance. (Raymond *et al.,* 2001) found that resistance to organophosphates was metabolic type and induce production of high level of detoxifying esterases. Using the biological insecticide *B.t.i.*, slight difference was detecting in raising resistant populations. Generally we can consider that raising resistant populations, decrease the enzyme activity. No reference data correlate between *B.t.i.* and ChE activity. Regarding GST, Carbaryl and Malathion resistant strains showed high enzyme activity. The same significant elevation of GST activity was recorded for B.t.i resistant generations (table 9). Over production of this enzyme, the proposed metabolic mechanism of resistance depends on overproduction of detoxifying enzymes in resistant *Cx. pipiens* strains (Julien *et al.,* 2013). As detoxifying enzyme, Glutathion S – transferase can conjugate xenobiotic compounds as insecticides and herbicides with the electrophilic sites of glutathion (GSH, -glutamyl cysteninyl-glycine). (Hemingway and Ranson 2000), confirmed that two enzyme systems were involved in OP and pyrethroid resistance, GSTs and monooxygenases. Activity of trypsin was mildly decreased in carbamate and OP resistant generations, but sharp decrease was detected in B.t.i resistant strains. *B.t.i.*as gut toxin, may interfere with these enzymes. Trypsin is important in remodelling proteomics in insect phases (Herrero *et al.,* 2005). Serine proteases degraded (polyhedron) protein but not *B.t.i.* crystal protein Cry1A (Seo*et al.,* 2005). This may explain inactivation of such enzyme in *B.t.i*. resistant populations. The low level of *B.t.i.* resistance was noticed by (Paris *et al.,* 2011 andTetreau *et al.,* 2012). Amino peptidase activity recorded a significant decrease in carbamate, OP and *B.t.i* resistant populations. In bacteria pressed larvae, the activity of amino peptidase decreased significantly from susceptible control from f0 to f6. Increasing aminopeptidase activity has been recorded with *B.t.i*. resistance in some insects, (Loseva *et al.,* 2002). In *B.t.i.* resistant strain of *Plodiainterpunctella*, amino peptidase like m-RNA expression levels were slightly increased, (Zhu *et al.,* 2000). However aminopeptidase activities in neonates and third instar larvae of *Manducasexta*pierisbarassica, *Mamstera brassica* and *Agrotisipsilon* were inversely correlated with increased resistance during larval development

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