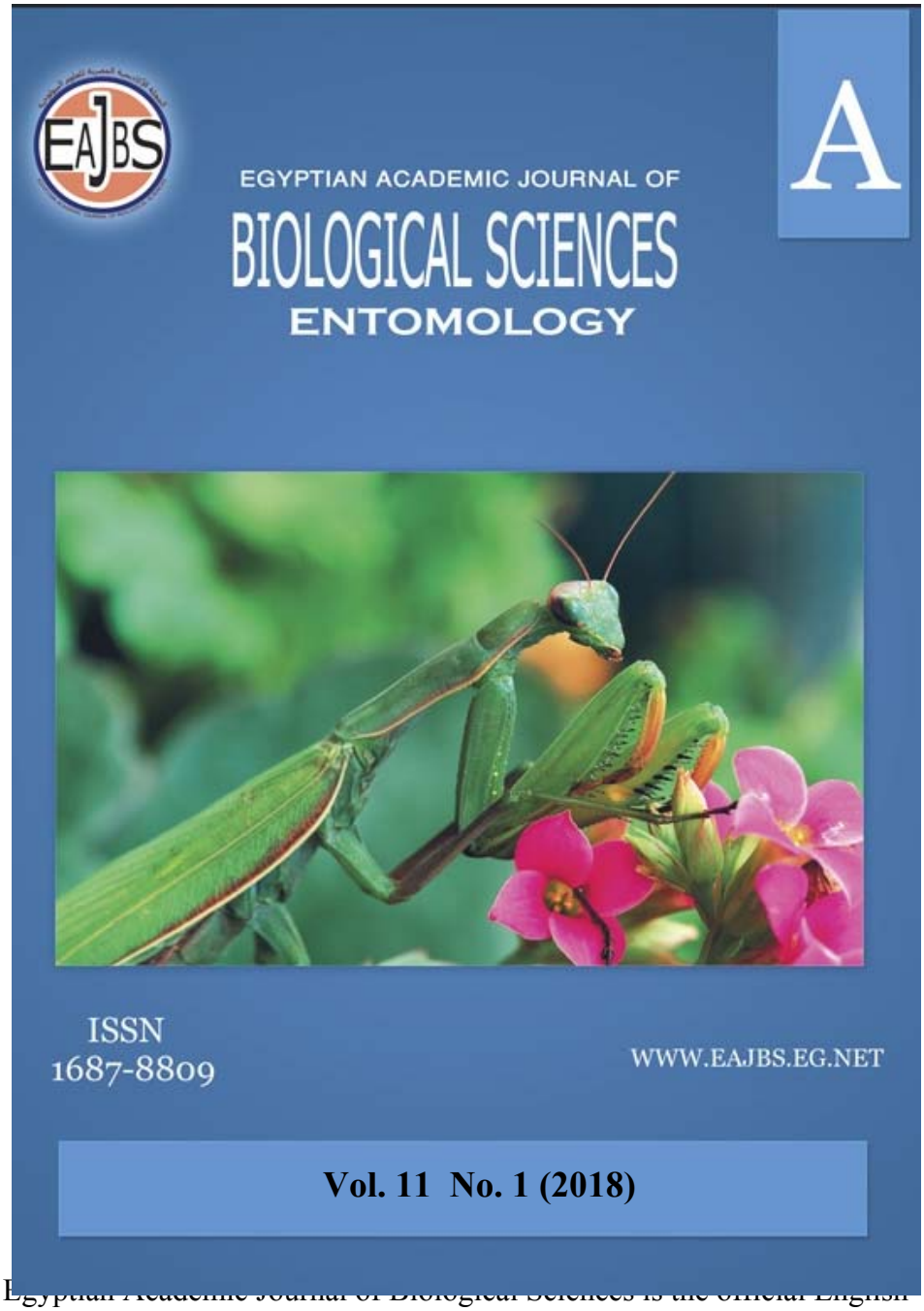

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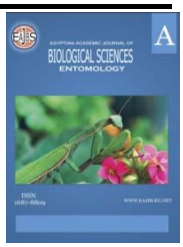

Metabolic Changes Associated With Using Lambda-Cyhalothrin Insecticide and Their Effects on Resistance Development in The Mosquito, *Culex pipiens* (Diptera: Culicidae)

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ABSTRACT

Pyrethroids are the most commonly used insecticides in the vector control programs. This insecticide group is one of the common recommended groups by the World Health Organization (WHO) for mosquito control. Recently, Pyrethroid resistance had rapidly spread worldwide which had its consequences on the effectiveness of control programs and threats public health. In this study, selection of Pyrethroid resistance in field-collected population of *Culex pipiens* was monitored after exposed to 0.05% Lambda-cyhalothrin for multiple generations. Activities of three detoxification enzymes namely; Oxidases, Nonspecific Esterases and Glutathione-S-transferases (GST), that synchronized with the resistance development, were monitored. Enzyme activities showed proportional relationship to Pyrethroid resistance. The results presented in this study will elucidate the Pyrethroid resistance development and its relation to the metabolic mechanisms. This may explain the complexity of resistance mechanisms in vector management and help to mitigate control failure due to insecticide resistance.

INTRODUCTION

Culex pipiens complex, the common and widely distributed mosquito in Middle Eastern region including Egypt, has been incriminated as the main vector of Lymphatic filariasis (LF) and several arboviruses, including; Rift Valley fever (RVF), *St. Louis encephalitis*, West Nile encephalitis, eastern equine encephalitis, Venezuelan equine encephalitis and Japanese encephalitis (Zhang *et al.,* 2012). Worldwide, nearly 1.4 billion people in 73 countries worldwide are currently threatened by LF (Shi *et al.,* 2015). In Egypt, the most common mosquito species in urban and rural areas is *Cx. pipiens* that transmit one of the main important vector borne diseases (Lymphatic filariasis) (Zahran and Abdelgaleil, 2011). This disease causes a major public health problem in 6 Governorates in the Nile Delta, besides Giza and Assiut Governorates in Middle and Upper Egypt (Ramzy *et al.*, 2005).

Chemical control using insecticides are the most important component in the global mosquito vector control effort (Zaim *et al*., 2000; Najera & Zaim, 2001; McCarroll & Hemingway, 2002). Pyrethroids have several advantages over the other

insecticides. Low cost, safety (less toxic to mammals), duration of residual action, rapid knockdown effect besides are the most important privileges of insecticides (references). These advantages besides its combination of both repellent and killing functions have nominated it to be the most recommended class of insecticides used for treated materials. Consequently, Pyrethroid insecticides are presently the most used insecticides for mosquito control all over the world (Zaim *et al*., 2000; Katsuda *et al.,* 2008 and WHOPES, 2011). On the other hand, mosquito-borne diseases are continue to be a big problem, mostly because of the insecticide resistance that has developed in mosquito vectors (Kasai *et al*., 2009; Al-Sarar 2010; Shin *et al*., 2012; Kioulos *et al*., 2014). In Egypt, field-based studies have raised the alarm over the developing mosquito resistance, including *Cx. pipiens*, to the main four groups of insecticides (Pyrethroids, Organophosphorus, Carbamates and Organochlorines) (Zayed *et al.,* 2006; EI-Sheikh, 2011; EL-Sheikh *et al.,* 2014).

Resistance management strategy is the key reference for sound vector control interventions. Monitoring insecticide resistance should be carried out as a routine activity by all vector control implementation agencies regardless of control strategy failure. In this study, susceptibility status of field caught *Cx. pipiens* mosquitoes will be determined. However, the induced Pyrethroid resistance pressure will be monitored after several applications/generations. Change in the resistance behavioral profile will be characterized. Therefore, the ultimate goal of this study is to highlight the threshold of resistance profile development at which control interventions will fail.

MATERIALS AND METHODS

Test Insects:

The reference strain used in this study was *Cx. pipiens* susceptible strain maintained at Naval Medical Research Unit No. 3 (NAMRU 3) insectary follow the method pre-described by Chapman and Barr (1969) under standard conditions (26-28°C, 12h:12h light/dark period, 70-80% relative humidity) with tap water (larvae) and net cages (adults) since 1987 (Zayed *et al.,* 2006). Adults were supplied with a 10% sucrose solution and blood fed on pigeons, while larvae were fed with fish food. This strain has not been exposed to any insecticide or biological control agent for longer than 30 years. Animal use for blood feeding process was conducted according to the guidelines of Institutional Animal Care and Use Committee IACUC (protocol no. 14-01). Mosquito larvae were collected from agriculture area in Giza Governorate and were morphologically identified to species level using the key (Harbach, 1985). Field caught *Cx. pipiens* were reared under the standard insectary conditions.

Set-up of Mosquito Population for Selection:

A population of *Cx. pipiens* was collected from natural habitats (Giza, Egypt) in December, 2015. Mosquitoes were reared in standard insectary conditions. Using the standard method of WHO (1981, 1998 and 2014), 1-3-day-old unfed females were exposed to papers impregnated with diagnostic dose (0.05%) of Lambda-cyhalothrin. Selection of resistant population was performed by exposing individuals obtained from the survivors of the previous exposed generation to 0.05% Lambda-cyhalothrin using the 50% lethal time (LT_{50}). The LT_{50} was determined using the WHO adult kits were procured from the WHO collaborating center, Penang, Malaysia.

Initially, the lethal time (LT) -response curve was established by exposing

females to a wide range of exposure times. Mortality was recorded 24 h post exposure, at least five different exposure times (yielding between 5% and 95% mortality after 24 h) were used to determine LT_{50} values. Three replicates of 25, two to five days age unfed females per time were used. A control group was recorded using 25 females with untreated insecticide papers.

All surviving mosquitoes were put into a cage, fed to obtain eggs for the next generation. The strain was exposed to insecticide stress to induce resistance by selection process for 21 generations. The mean lethal times per generation was determined every 6 generations and modified accordingly. The laboratory susceptible strain of *Cx. pipiens* was used as a reference.

Metabolic resistance:

A-Tested Samples :

Specimens for biochemical tests were collected throughout generations to determine of the changes in the metabolic enzyme activities. Twenty five to 30 mosquito adults of the survivors /generations 1, 6, 12, 18, and 21 were collected and stored at -70°C were analyzed.

B-Enzyme Activity Assays:

Metabolic enzyme activity was measured in individual female mosquitoes according to the procedure developed by Brogdon and McAllister (1998). The microplates used were standard rigid, flat-bottom microtiter plates (Nalge Nunc International, Bridgeport, NJ) and were photometrically read with the use of an ELISA reader (Spectramax 340 PC Molecular Devices, Sunnyvale, CA). Frozen mosquitoes were homogenized individually in a 1.5-ml tube with 1ml potassium phosphate buffer. After centrifugation, 100µl of supernatant was used to test the activity of Oxidases, Nonspecific Esterases and Glutathione-S-transferases (GST) simultaneously. Triplicates were used for each enzyme, and assays were carried out in duplicate.

Oxidases: 200µl TMBZ solution (Dissolve 20 mg 3, 3', 5, 5'-Tetramethyl-Benzidine Dihydronchloride was dissolved in 25 ml methanol. Then 75 ml 0.25 M Sodium Acetate, (pH 5.0) buffer was added to the solution) and 20 µl 3% hydrogen peroxide was added to each well, was incubated for 5 min and then was read at 620 nm, Cytochrome – C was used as positive control.

Nonspecific Esterases: 100µl β-naphthyl acetate (56 mg ß-naphthyl acetate was dissolved in 20 ml acetone, then 80 ml KPO4 was added to the solution) was added to each well, the plate was incubated for 10 min, 100µl Dianisidine solution (100 mg 0-dianisidine tetrazotized was added to 100 ml distilled H2O) was added to each well, the plate was incubated for 2 min, and read at 540 nm, β-naphthyl was used as positive control.

Glutathione-S-transferases: 100 µl reduced glutathione solution (61 mg reduced glutathione was dissolved in 100 ml KPO4 buffer) and 100µlcDNB solution (20 mg 1-chloro-2, 4'-dinitrobenzene (cDNB) was dissolved in 10 ml acetone 90 ml 0.25 M KPO4 buffer was added to the solution) was added to each well, the plate was read using microplate reader at 340 nm immediately T0 and T5 min. The T0 reading was subtracted from the T5 reading. A negative control for each assay was potassium phosphate buffer.

Statistical Analysis:

- The lethal time (LT_{50}) was calculated using probit analysis (SPSS Statistics, 2008). This program tests the linearity of a Time–mortality response, computes the different lethal times (LTs) and their confidence interval (CI) at the chosen probability (here P = 50%).

- To correlate the development resistance to generation number, regression analysis was used. Resistance ratio (RR) between LT_{50}'s of the selected (resistant) and susceptible strains were calculated.

- Based on the LT_{50} values resistance ratio (RR) was determined by the ratio of resistant strain to the ratio of susceptible strain by adopting the method of Brown and Pal (1971). The LT_{50} values were expressed in minutes and the resistance ratio RR were determined as follows:

$$\text{Resistance Ratio (RR-S)} = \frac{LT_{50} \text{ of resistant (Selection pressure) strain}}{LT_{50} \text{ of reference strain (susceptible)}}$$

- One-way analysis of variance (ANOVA) was used to compare between readings, representing enzymatic activities of mosquitoes from different generations under selection pressures.

RESULTS

1-Base Line Susceptibility of Mosquito Populations to Lambda-Cyhalothrin:

The selection pressure of Lambda-cyhalothrin was dynamically monitored in order to identify the mechanism of resistance in the tested population. A resistant strain of *Cx. pipiens* was established after being placed for 21 generations (about 20 months) under Lambda-cyhalothrin insecticide selection pressures. The resistance was gradually increased with succeeding generations of exposure during the selection process (Fig., 1).

After selection of 21 generations, the resistance ratio (RR) increased from 8.294 at generation 1 (LT_{50} = 41.924 min) to 119.144 at generation 21 (LT_{50} = 601.919 min). Subsequent generations were found to be statistically different from each other and from Laboratory(Lab.) strain ($P< 0.05$) (Table, 1).

Table 1. Variation of LT_{50} and RR in susceptible and selected populations of *Cx. pipiens* females under Lambda-cyhalothrin selection pressures.

Generations	n^a	Slope ± SE	LT_{50} (95% CI)	RR^b	χ^{2c}	df	*P*
Lab strain	100	1.812 ± 0.131	5.052 (3.390–6.812$_a$)		10.746	5	= 0.057
Giza population							
F1	100	2.606 ± 0.187	41.924 (25.571–57.574$_b$)	8.294	51.617	6	= 0.000
F6	100	4.309 ± 0.320	134.546 (111.202–153.422$_d$)	26.632	22.271	6	= 0.001
F12	100	8.263 ± 0.566	279.017 (254.989–300.097$_f$)	55.229	26.899	6	= 0.000
F18	100	11.585 ± 0.931	427.252 (398.277–447.384$_i$)	84.570	17.782	6	= 0.007
F21	100	16.244 ± 1.199	601.919 (570.672–625.434$_h$)	119.144	29.592	6	= 0.000

Values followed by the same letter not different, as judged by overlapping 95% CI.

[a]Twenty five mosquitoes per replicates, four replicates per generation.

[b] RR= Resistance Ratio = LT_{50} of Selected strain/LT_{50} of laboratory strain (reference susceptible strain)

[c]Pearson χ^2 goodness-of-fit test on the Probit model ($\alpha = 0.05$).

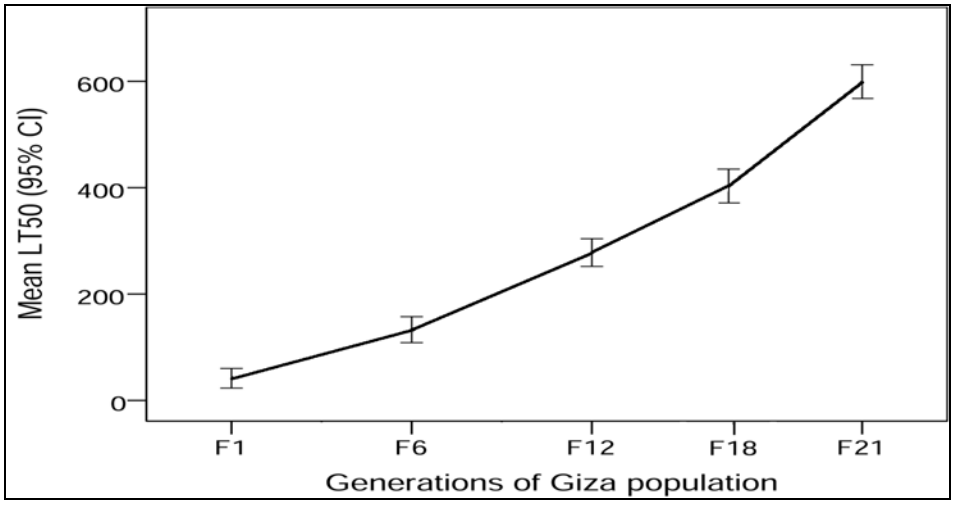

Fig.1. Variation of LT_{50} in selected population of *Cx. pipiens* females under Lambda- cyhalothrin selection pressures.

Metabolic Enzyme Assay:

Metabolic enzyme activities were analyzed in generations FI, F6, F12, F18 and F21 after Lambda-cyhalothrin exposure for 21 generations. Oxidases, Nonspecific Esterases and Glutathione-S-transferases (GSTs) assays were performed using the susceptible reference strain and resistant strain generations of *Cx. pipiens* with sample size ranging from 25 to 30 mosquitoes per generation (Table, 2). The results showed that the Oxidases activities increased significantly with the development of Lambda-cyhalothrin resistance from 1.84 folds in F1 to 4 folds in F21 (Fig., 2).

In the selected strain, Nonspecific Esterases activities increased significantly when compared to reference strain. The enzyme activity increased from 1.74 folds in F1 to 2.8 folds in F21 (Fig., 3). Activities of GSTs recorded 1.45 folds in F1 and also increased to 2 folds in F21 when compared to the reference strain (Fig., 4).

Table 2. Enzyme activity levels for female *Cx. pipiens* of susceptible strain and different successive generations of Giza selected strain as measured by photometric absorbance

S Generation	Enzymes		
	Oxidase	Nonspecific Esterases	Glutathione-S-transferases
Lab strain	0.25 ± 0.05^{a}	1.9 ± 0.38^{a}	0.020 ± 0.001^{a}
F1	0.46 ± 0.09^{b}	3.3 ± 0.56^{b}	0.029 ± 0.006^{b}
F6	0.59 ± 0.08^{c}	3.6 ± 0.36^{bc}	0.031 ± 0.002^{b}
F12	0.72 ± 0.13^{d}	3.9 ± 0.24^{cd}	0.035 ± 0.001^{c}
F18	0.91 ± 0.16^{e}	4.8 ± 0.42^{d}	0.038 ± 0.001^{d}
F21	1.0 ± 0.14^{f}	5.5 ± 0.40^{e}	0.040 ± 0.001^{d}

Data are presented as mean ±standard deviation.

Means followed by the same superscript letter within each column are not significantly different (analysis of variance [ANOVA]; Tukey test; $P > 0.05$).

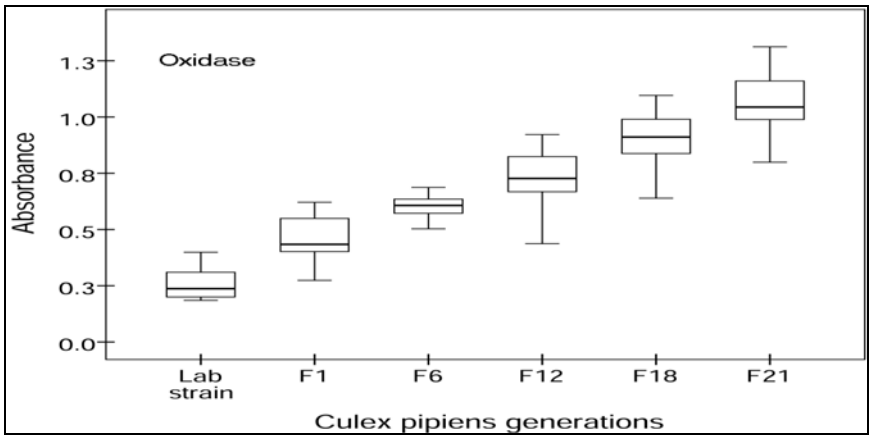

Fig 2. Development of Oxidase enzyme activity of selected Giza *Cx. pipiens* population in successive resistant generations to Lambda-cyhalothrin compared to reference strain.

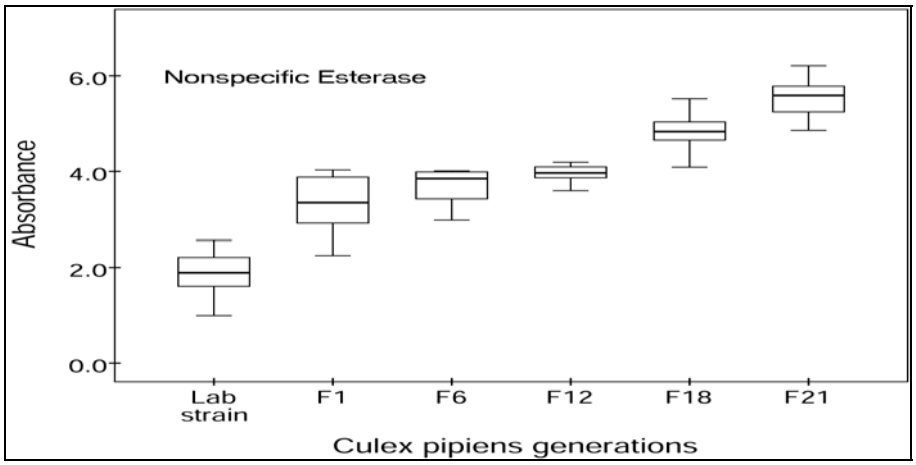

Fig 3. Development of Nonspecific Esterase enzyme activity of selected Giza *Cx. Pipiens* population in successive resistant generations to Lambda-cyhalothrin compared to reference strain.

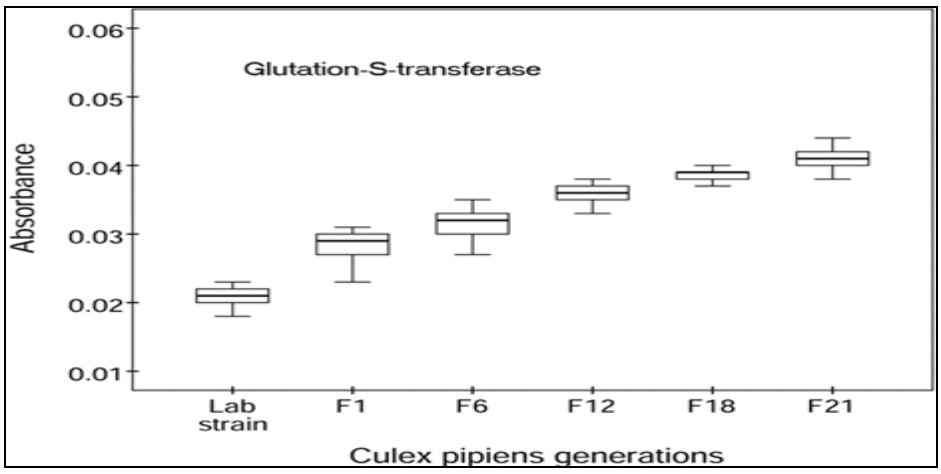

Fig 4. Development of Glutathione-S-transferases enzyme activity of selected Giza *Cx. pipiens* population in successive resistant generations to Lambda-cyhalothrin,compared to the reference strain.

DISCUSSION

The establishment of resistant strains is essential for further understanding of the insecticide resistance development mechanisms among insects, including *Culex* mosquitoes.

During the present study, the resistance of *Cx. pipiens* was intentionally induced and a selection of Lambda-cyhalothrin resistant generation (s) occurred. Resistance increased with the repetitive exposure of succeeding generations to Lambda-cyhalothrin insecticide.

The resistance development to Lambda-cyhalothrin was recorded in larvae of the same species (*Cx. pipiens*) collected from Egypt (EL-Sheikh *et al*., 2014). Also, Shi *et al.* (2015) Shi *et al.,* (2015) reported that, resistance increase to Deltamethrin through subsequent generations of *Cx. pipiens* collected from China. The developing resistance to Lambda-cyhalothrin was studied in other mosquito species such as *Anopheles sinensis* (Zhu *et al*., 2014).

The metabolic resistance mechanism is one of the main decisive factors of mosquito resistance to Pyrethroids (Shi *et al*., 2015). To detect this mechanism in the tested strain, three detoxification enzyme activities were estimated in the *Cx. pipiens* samples. The metabolic resistance resulted from the elevation of the detoxifying enzymes was found to be associated with Lambda-cyhalothrin resistance in the *Cx. pipiens* population. Recent studies have reported the resistance mechanisms as an elevation in detoxifying enzymes in some mosquito species (Somwang *et al.,* 2011 and Pocquet *et al*., 2013). Our results of the biochemical assays showed that, the development of Lambda-cyhalothrin resistance was accompanied by significant increase in activity of Oxidases in the tested field strain. On the other hand, the significant increase in activity of Nonspecific Esterases appeared clearly in the generation 21 in the tested strain. No significant association between GSTs and the level of Lambda-cyhalothrin resistance were found here.

This could be explained by the findings of Brooke *et al*. (2001) who found that, GSTs play only a minor role as a detoxifying enzyme in Pyrethroid-resistant of *An. funestus*. The obtained results were in agreement with those of Zayed *et al.* (2006), who indicated that, Pyrethroid resistance is a result of elevated nonspecific EST activity as they observed in Qalubiya Governorate, Egypt. Also, Akiner and Eksi (2015) indicated that, the Oxidase and Nonspecific ESTEs played an important role for DDT, Malathion and Pyrethroid resistance.

The elevated detoxifying enzymes in the tested population may help the insects to survive the effect of the insecticide. The implication of the three enzymes in promoting Pyrethroid detoxification detected in resistant insects has been reported by many other authors (Martinez-Torres *et al*., 1998; Vulule *et al*., 1999 and EL-Sheikh *et al*., 2014).

The currently applied chemical control programs are mostly depending on using Pyrethroids. Now, the efficacy of these programs is threatened by the rise of resistance in the target populations. Therefore, formulating a new strategy of insecticides used to delay the development of Pyrethroid resistance is of great importance and may be concerned. Based on the present outputs, it is necessary to avoid long-term exposure of mosquito populations to low constant concentrations of Pyrethroids. Where, it can lead to significantly occurrence of increased resistance in mosquito populations.

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ARABIC SUMMARY

التغيرات الأيضية المصاحبة لاستخدام المبيد الحشرى Lambda-cyhalothrin ومدى تأثيرها على تطوير المقاومة في بعوضه *Culex pipiens* (Diptera: Culicidae).

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٢-قسم الحشرات كلية العلوم –جامعة القاهرة

تعد البيرثرويدات (Pyrethroids) من المبيدات الحشرية الأكثر شيوعاً في برامج مكافحة ناقلات الأمراض ، حيث تعتبر هذه المجموعة من المجموعات المعروفة التي أوصت بها منظمة الصحة العالمية (WHO) لمكافحة البعوض. وقد انتشرت بسرعة في الآونة الأخيرة في جميع أنحاء العالم ظاهرة مقاومة النواقل للمبيدات ، مما كان له آثار كبيرة على مدى فعالية برامج مكافحة انتشار الأمراض المنقولة.

في هذه الدراسة ، تم تطوير سلالة مقاومة أنتخبت من سلالة حقلية بعد تعريض بعوضة *Culex pipiens* لتركيز ٠.٠٥٪ من مبيد Lambda-cyhalothrin (Pyrethroid) والتي تم جمعها من محافظة الجيزة علي مدي عدة أجيال (٢١ جيلاً). وأوضحت النتائج أن معدل المقاومة يتزايد من جيل إلى آخر يليه حتى وصل إلى ١١٩ ضعفاً عند الجيل ٢١. كما أن نشاط الإنزيمات الثلاثة المسؤلة عن إزالة السموم وهي : Oxidases ,Nonspecific Esterases و Glutathione S transferases ، سجل متزامناً مع تطوير مقاومة البعوض للمبيد. وعمومًا ، فتفسر النتائج المتحصل عليها في هذه الدراسة تطور مقاومة مبيد Pyrethroid في البعوض وعلاقته بآليات التمثيل الغذائي. وبذلك ، يوضح مدي تعقيد آليات المقاومة في إدارة مكافحة ناقلات الأمراض. وبالتالي، يساهم أيضا فى المساعدة على التخفيف من فشل المكافحة بسبب ظاهرة مقاومة المبيدات الحشرية.