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LARVAL SUSCEPTIBILITY OF *CULEX PIPIENS* L. (DIPTERA: CULICIDAE) COLLECTED FROM DIFFERENT LOCALITIES AT SHARKIA GOVERNORATE TO SOME INSECTICIDES

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ABSTRACT

The susceptibility of *Culex pipiens* larvae from different localities at Sharkia Governorate to malathion and lambda-cyhalothrin was studied. The activities of acetylcholinesterase, esterases and glutathione-S-transferase in the tested insect were determined. Bioassay tests indicated that different levels of resistance were found in *Cx. pipiens* against the tested insecticides based on the localities of sampling. For the populations tested with malathion, the resistance ratio ranged from 1–99-fold when compared with the laboratory susceptible strain (SS), while in the populations treated with lambda-cyhalothrin, the resistance ratio ranged between 5–1900-fold. In all cases, the population collected from Diarb Negm (PD) exhibited a high level of resistance to the tested insecticides than the other populations. Activity of acetylcholinesterase in all tested populations was significantly less than that of the SS. On the other hand, both esterases and GST activities were significantly higher in the collected populations as compared with SS. These data concerning the different levels of resistance of the collected populations may be due to different practices of insecticides use either indoor or outdoor against the common pests. This indicates the importance of the adoption IPM programs for such insect under local conditions.

Keywords: ***Culex pipiens*, malathion, Lambda-cyhalothrin, AChE, esterases, GST.**

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INTRODUCTION

Culex pipiens L. was found in tropical areas (Bourguet *et al.*, 1998). Hybrid forms were reported in some parts of Africa, Russia, Australia, North and South America (Azari-Hamidian, 2007). The insect has the major role in human nuisance due to biting (Dehghan *et al.*, 2011). Several human diseases are transmitted by this species of mosquitoes and distributed in tropical and subtropical areas of Africa, Asia, Australia and Pacific Islands (Hayes *et al.*, 2005; Paul *et al.*, 2006 and Cheng *et al.*, 2009).

In Egypt, *Cx. pipiens* is the most common mosquito species in urban and rural areas and causes a health risk and nuisance to humans (Zahran and Abdelgaleil, 2011). The control of mosquitoes depends primarily on continued applications of several insecticides belonging to different chemical groups. (Rozendaal, 1997). Pesticide concentrations that mosquito larvae are exposed at any given time are dependent on the frequency of application and the rate of environmental degradation (Antonio *et al.*, 2008). Therefore, mosquitoes may be frequently exposed to both lethal and sublethal pesticide concentrations, but most studies focus on the effect of lethal pesticide concentrations with little appreciation on the impact of sub-lethal pesticide concentrations despite their potential for the development of resistant strains and alter mosquito population dynamics (Antonio *et al.*, 2008).

Among all the species controlled with insecticides, *Cx. pipiens* is one of a particular interest because it can be considered as an indicator to monitor the nature and persistence of insecticides in the environment: most of its natural breeding sites are indeed localized in various types of drainages that collect filthy waters over large areas. A full set of biochemical and molecular tools has been developed to identify resistance genes in this species. Moreover, its resistance alleles evolve rapidly and can be easily tracked. Besides, over the last 30-40 years, substantial physiological and molecular data have been documented on the evolutionary constraints of insecticide resistance in *Cx. pipiens* (Weill *et al.*, 2005). In particular, it was clearly shown that some resistance genes can be associated with an important fitness cost (Duron *et al.*, 2006 and Berticat *et al.*, 2008). As a result,

the frequency of resistance alleles generally decreases with the end of insecticide treatments (Silvestrini *et al.*, 1998) or in non-treated areas (Lenormand *et al.*, 1999).

In this study, the susceptibility of different populations of *Cx. pipiens* collected from different localities at Sharkia Governorate to malathion and lambda-cyhalothrin was investigated. The frequencies of the associated metabolic and target enzymes were also determined.

MATERIALS AND METHODS

Tested Insect

Mosquito insects were collected as larvae and pupae during summer seasons of 2008, 2009 and 2010 from five different localities at Sharkia Governorate. Theses localities were the 10th of Ramadan (PA), Diarb Negm (PD), Faqous (PF), El-Salhia (PS) and El-Zagazig (PZ). The collected populations were reared in the laboratory for one generation according to the method described by (WHO, 1981). A laboratory susceptible strain (SS) of *Cx. pipiens*, maintained in the laboratory for 15 years (~320 generations) without insecticide exposure, was obtained from Research Institute of Medical Entomology, Mosquito Department, Egyptian Ministry of Health.

Insecticides and Other Reagents Used

Insecticides

Malathion ($C_{10}H_{19}PS_2O_6$), a commercial formulation named Malathion (EC 97% active ingredient) and Lambda-cyhalothrin ($C_{23}H_{19}ClF_3NO_3$), a commercial formulation named Lambda (EC 5% active ingredient) were supplied by a local manufacturer, Kafr El-Zayat for Pesticides and Chemicals Company, Kafr El-Zayat City, Gharbia Governorate, Egypt.

Reagents

All other chemicals used in the study were purchased from Sigma-Aldrich (St. Louis, MO) unless other stated. All commercial reagents, and other chemicals used in this study were of technical grade with the highest purity available.

Larval Bioassay

Larval bioassays were tested by exposing batches of 25 fourth instar larvae to known insecticide concentrations in 100 mL of distilled water in 1 OZ plastic cups

(WHO, 1981). For each tested insecticide, serial concentrations in distilled water were prepared to give mortalities between 10 and 90%. Four replicates for each concentration were used. After 24 h of insecticide-exposure at 25±2°C, the larval mortality was recorded. Four similar batches of 25 fourth instar larvae each were introduced to clean distilled water only to be used as a control.

Larval Homogenate

Samples of larval homogenate were prepared by homogenizing 20 4th instar larvae of each population using a plastic mini pestle in 1.5 mL centrifuge tubes in 250 µL of 0.1 M ice-cold sodium phosphate buffer, pH 7.4, containing 0.02% Triton X-100. The homogenate was then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was then separated in clean 0.5 mL eppendorf tubes and stored at -20°C until used within 15 days for determination of total protein, acetylcholinesterase, esterases, and GST.

Biochemical Determination

Total Protein

Protein concentrations were determined according to the method of Bradford (1976) by incubating 10 µL of homogenate with 300 mL of Bio-Rad protein assay solution for 10 min. Absorption was then measured at 570 nm. Bovine serum albumin was used as the standard.

Acetylcholinesterase

AChE activity in whole larvae was estimated according to the procedure described by Ellman *et al.* (1961). Field and laboratory strains, 20 larvae/patch, were homogenized with 250 µL cold phosphate buffer (0.1 M, pH 7.4) containing 0.02% Triton X-100. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was decanted, kept on ice and used as the crude enzyme preparation. In 10 mL glass test tubes, 10 µL of the crude enzyme was added to 1.5 mL of phosphate buffer (pH, 7.2) containing 0.39 µM of 5,5-dithiobis nitrobenzoic acid (DTNB). The reaction was initiated with the addition of 50 µL of acetylcholine iodide substrate (ATChI) (final conc. in 1560 µL = 7.8 µM). Three hundred µL of the previous mixture was transferred into ELISA plate's well in triplicates. Absorbance was recorded initially after 5 min at 450 ηm in 96-well microplate using Microplate Autoreader,

EL311S (Bio-TEK Instrument, Highland Park, Winooski, VT). Reading was repeated after exactly 30, 60 and 90 sec. The mean absorbance change per 30 sec. (ΔA/30 sec.) was determined. Blank contained the same components except the substrate was used as control. Rates were converted to ηmoL min^{-1} mg^{-1} using the extinction coefficients of 9.25 mM^{-1} 300 μL^{-1} for 2-nitro-5-mercaptobenzoate (Grant *et al.*, 1989).

Esterase

Colorimetric esterase activity assays were determined using the general substrates α- and β-naphthyl acetate as described by Gomori (1953) with modifications. Measurements were performed in 96-well microplates using microplate autoreader. For each reaction mixtures, 480 µL phosphate buffer (0.1 M, pH 7.4) with 0.02% Triton X-100, 20 µL of protein solution, 500 µL of α- or β-NA substrate solutions (final concentration in 1500 µL total volume = 2.5 mM), and 500 µL of Fast Blue B salt solution (consists of 2 parts of 1% Fast Blue B salt and 5 parts of 5% SDS) were mixed in 10 mL glass tubes and incubated at 30°C for 5 min. Thousand five hundred µL total volume of mixture was measured in 5 replicates (300 µL/well) for each sample. The optical density (OD) was measured at 450 ηm during the first 5 min of the reaction, and rates were converted to ηmoL min^{-1} mg^{-1} using the extinction coefficients of 9.25 mM^{-1} 300 μL^{-1} for 1-naphthol (Grant *et al.*, 1989). Activities were corrected for non-enzymatic hydrolysis using reactions without protein as controls.

Glutathion-S-transferase

GST activity assays were done by the modified method of Grant and Matsumura (1988). Twenty five µL of larval homogenate prepared as previously mentioned, 75 µL of chloro-2,4-dinitrobenzen (CDNB), and 75 µL of reduced glutathione (fin. conc. 5 mM) were mixed with 750 µL of phosphate buffer, pH 7.4. Reactions were allowed to take place for 5 min at 37°C, and then terminated by adding 75 µL of trichloroacetic acid to make final assay volume of 1000 µL/test tube. Three replicates were used for each measurement, and activities were corrected for non-enzymatic hydrolysis using reactions without protein as controls. The conjugation of CDNB to glutathione is accompanied by an increase in absorbance at 340 ηm.

The rate of increase was directly proportional to the GST activity in sample. These measurements were done using spectrophotometer (Spectronic 20, Bausch and Lomb, USA) in the same day of getting protein samples from different strains and populations by measuring absorbance at 340 ηm at 30°C for 15 minutes after the addition of glutathione. Rates were converted to ηmol min^{-1} mg^{-1} using the extinction coefficients of 8.5 O.D. mM^{-1} 1000 μL^{-1} for CDNB (Grant *et al.,* 1989).

Statistical Analysis

Mortality data were subjected to probit regression analysis using a Probit polo pc plus software v 3.1 (LeOra Software Inc., Cary, NC) which automatically corrected for control mortality according to the method of Finney (1971) and the lethal concentrations which gave 50% (LC_{50}) and 90% (LC_{90}) mortalities were calculated. Data of acetylcholinesterase, esterases and GST were subjected to SPSS 10.0 for Windows software package for statistical analyses. One-way analysis of variance (ANOVA) was performed and variant groups were determined by means of the Duncan test (Duncan, 1955).

RESULTS AND DISCUSSION

Bioassay

Data presented in Table 1 shows that PD population exhibited the highest degree of resistance (99-fold) followed by the populations of PZ (42-fold), PS (26-fold), PA (7-fold) and the population of PF (1-fold). The population of PF was the most susceptible recording a similar value for the LC_{50} of SS.

The results in Table 2 show the susceptibility levels of the tested populations to lambda-cyhalothrin. It seems clearly that PD population recorded the highest level of resistance to this compound (1900-fold). The other tested populations recorded different levels of resistance, i.e., PS (65-fold), PA (50-fold), PZ (40-fold) and PF (5-fold) as compared with SS.

Vector insects control is a very important part of the current global strategy of insect control, and insecticide application is the most important component in this effort. It appears from the results of Tables 1 and 2 that lambda-cyhalothrin was more effective than malathion and the tested populations developed high levels of resistance to this compound than malathion. The high

Table 1. Susceptibility of the collected populations of *Culex pipiens* larvae to malathion[a]

Populations[b]	LC_{50} (95% CL) ppm	LC_{90} (95% CL) ppm	Slope (SE)	$(X^2/df)^c$	Intercept (SE)	Resistance Ratio[d]
SS	0.012 (0.001 – 0.106)	0.317 (0.053 – 1.072)	0.90 (0.18)	6.89	4.04 (0.26)	1
PA	0.083 (0.065 – 0.102)	0.386 (0.266 – 0.742)	1.91 (0.30)	1.30	1.33 (0.59)	7
PD	1.192 (1.145 – 1.256)	1.518 (1.391 – 1.859)	2.21 (0.61)	0.25	3.21 (0.81)	99
PF	0.012 (0.009 – 0.017)	0.100 (0.059 – 0.251)	1.41 (0.22)	2.54	3.46 (0.27)	1
PS	0.315 (0.231 – 0.406)	2.105 (1.324 – 4.955)	1.55 (0.26)	1.40	1.12 (0.21)	26
PZ	0.504 (0.390 – 0.620)	1.747 (1.246 – 3.386)	2.37 (0.44)	1.41	4.57 (0.84)	42

[a] data are shown from 3 bioassay experiments.
[b] SS: laboratory susceptible strain; PA: population from the 10th of Ramadan; PD: population from Diarb Negm; PF: population from Faqous; PS: population from El-Salhia ; PZ: population from El-Zagazig.
[c] Heterogeneity.
[d] Resistance ratio = LC_{50} of the tested populations/LC_{50} of SS.

Table 2. Susceptibility of the collected populations of *Culex pipiens* larvae to lambda-cyhalothrin[a]

Populations[b]	LC_{50} (95% CL) ppm	LC_{90} (95% CL) ppm	Slope (SE)	$(X^2/df)^c$	Intercept (SE)	Resistance Ratio[d]
SS	0.00002 (0.000019 – 0.00003)	0.00011 (0.00009-0.00016)	1.88 (0.18)	2.32	3.06 (0.27)	1
PA	0.0010 (0.0005 - 0.0017)	0.030 (0.010 – 0.150)	0.90 (0.16)	0.04	5.00 (0.11)	50
PD	0.0380 (0.0309 – 0.0448)	0.100 (0.080 – 0.170)	2.97 (0.54)	2.48	4.31 (0.88)	1900
PF	0.0001 (0.00008 – 0.00020)	0.002 (0.001 – 0.004)	1.15 (0.17)	4.87	6.00 (0.15)	5
PS	0.0013 (0.0009 – 0.0020)	0.017 (0.008 – 0.068)	1.17 (0.22)	2.01	4.86 (0.11)	65
PZ	0.0008 (0.0006 – 0.0010)	0.006 (0.003 – 0.018)	1.46 (0.28)	0.87	5.15 (0.09)	40

[a] data are shown from 3 bioassay experiments.
[b] SS: laboratory susceptible strain; PA: population from the 10th of Ramadan; PD: population from Diarb Negm; PF: population from Faqous; PS: population from El-Salhia ; PZ: population from El-Zagazig.
[c] Heterogeneity.
[d] Resistance ratio = LC_{50} of tested populations/LC_{50} of SS.

levels of resistance to lambda-cyhalothrin than malathion in this insect may be due to less exposure, in nature, to malathion or other organophosphorous insecticides and frequently exposure to pyrethroids either directly for mosquito control or through drift from the pesticides used against the agricultural pests. (Legros *et al.,* 2009 and Muturi *et al.,* 2010).

Malathion, organophosphate pesticide, which act by inhibiting acetylcholinesterase, is one of the commonly used pesticides for controlling mosquitoes. It is applied in wetlands as an ultra low volume (ULV) spray to control adult mosquitoes (Wheeler *et al.,* 2009). Pyrethroids, which account for 25% of the world insecticide market, are currently the most widely used insecticides for the indoor control of mosquitoes and are the only chemicals recommended for the treatment of mosquito nets, the main tool for preventing malaria in Africa. However, mosquito-borne diseases are now resurgent, largely because of insecticide resistance that has developed in mosquito vectors and the anti-parasite drug resistance of parasites (Liu *et al.,* 2006).

In our bioassay, high levels of resistance to the tested insecticides were found particularly in PD population. These resistant populations are difficult to be controlled then they play a role in transmitting different diseases (Tantely *et al.,* 2010).

Biochemical Determination

Fig. 1 shows the activity of acetylcholinesterase in the collected populations as well as the SS. The activity of AChE is significantly low in all collected populations as compared to SS with significant differences among the populations. AChE activities in PD and PS were significantly higher than the other populations, while, both PF and PZ were significantly low in AChE activity. Inhibition of AChE activity was induced by organophosphate insecticides such as fenitrothion which caused inhibition to this enzyme in *Cx. pipiens* strain from Riyadh city, Saudi Arabia (Al-Sarar, 2010). In Sri Lanka, AChE was also found to be sensitive to organophosphate insecticides in Kurunegala and Trincomalee, *An. culicifacies*, field populations (Perera *et al.,* 2008).

Data concerning esterase activity in the collected populations of *Cx. pipiens* are presented in Table 3. All collected populations showed significantly high activity in esterase with α–NA as compared

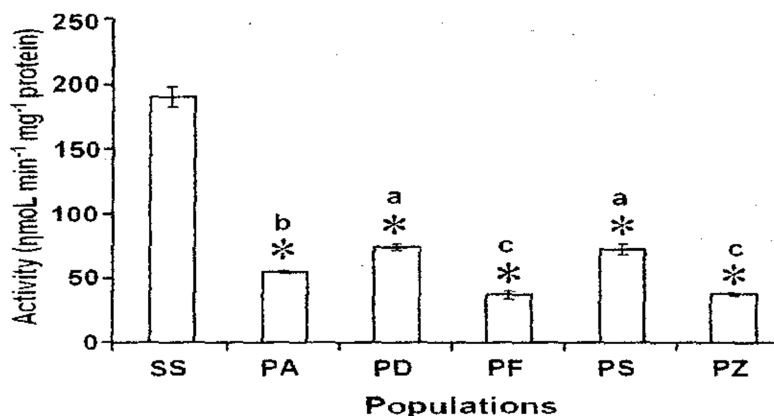

Fig. 1. Acetylcholinesterase activity in collected populations of *Culex pipiens* from different localities at Sharkia Governorate. * indicates that populations are statistically different as compared with laboratory susceptible strain (SS) at P≤0.05. The same letters on bars indicate that there are nonsignificant differences at P≤0.05

Table 3. Esterase activity in collected populations of *Culex pipiens* from different localities at Sharkia Governorate[a]

Populations[b]	Esterases (ηmoL min^{-1} mg^{-1} protein)		Ratio[c]	
	Alfa-NA	Beta-NA	Alfa-NA	Beta-NA
SS	8.6 ± 0.5[d]	7.1 ± 0.3[b]	1.0	1.0
PA	14.9±1.7[b]	7.4 ± 1.1[b]	1.7	1.0
PD	11.7±1.7[bc]	8.0 ± 0.7[b]	1.4	1.1
PF	11.3±1.6[c]	7.5 ± 1.3[b]	1.3	1.1
PS	19.7±1.8[a]	13.3±1.2[a]	2.3	1.9
PZ	12.0±0.7[c]	7.2 ± 0.7[b]	1.4	1.0

[a] Values are shown as means±SD of three determinations. Means followed by different letters within the same column indicate that data are significantly different at P≤0.05.
[b] SS: laboratory susceptible strain; PA: population from the 10th of Ramadan; PD: population from Diarb Negm; PF: population from Faqous; PS: population from El-Salhia ; PZ: population from El-Zagazig.
[c] Ratio is calculated as mean values of collected populations/mean value of SS.

with SS. PS population showed significantly high activity in esterases compared to other populations. When β-NA was used as a substrate, PS esterase activity was significantly increased than other populations or SS. The ratio of esterase activity in PS population with α– and β–NA as compared with SS was 2.3 and 1.9 times, respectively.

Exposure of the mosquito *Cx. quinquefasciatus* to organophosphorus insecticides has commonly selected a resistance mechanism involving overproduction of carboxylesterases, the basis of overproduction is gene amplification (Hemingway and Karunaratne, 1998). The role of these esterases in insecticide resistance is sequestration, a rapid binding of insecticides, preventing them from reaching their target site, acetylcholinesterase, followed by a slow turnover (Small *et al.*, 1998).

Glutathion-S-transferase activity (Table 4) was significantly high in all populations compared with SS. The highest activity was noticed in PA, PD and PF populations recording 2.6, 2.0 and 2.1 as active as the SS, respectively. GSTs are a major family of detoxification enzymes. They catalyze the conjugation of the tripeptide glutathione to electrophilic centers of lipophilic compounds, thereby increasing their solubility and aiding excretion from the cell. Thus, GSTs play a vital role in protecting tissues against oxidative damage and oxidative stress. The GSTs in insects are primarily of interest because of their role in insecticide resistance. They are involved in the *O*-dealkylation or *O*-dearylation of organophosphorous insecticides (Hayes and Wolf, 1988), as a secondary mechanism in the detoxification of organophosphate metabolites (Hemingway *et al.*, 1991) and in the dehydrochlorination of organochlorines (Clark and Shamaan, 1984). Although, GSTs have not been implicated directly in pyrethroid resistance, there are reports of elevated GSTs in pyrethroid resistant wild-caught (Wu *et al.*, 1998) or laboratory-selected insects (Grant and Matsumura, 1988).

The underlying mechanisms of resistance were reported as enhanced oxidative metabolism and possibly elevation of carboxylesterases (Dai and Sun, 1984). In mosquitoes, insecticide resistance is essentially achieved through two mechanisms: increased detoxification and target insensitivity (Nauen, 2007; Whalon *et al.*, 2008 and Tantely *et al.*, 2010).

Table 4. Glutathione-S-transferase activity in collected strains of *Culex pipiens* from different localities at Sharkia Governorate[a]

Populations[b]	GST (µmoL min^{-1} mg^{-1} protein)	Ratio[c]
SS	1800 ± 600[c]	1.0
PA	4700 ± 700[a]	2.6
PD	3600 ± 500[ab]	2.0
PF	3800 ± 300[ab]	2.1
PS	2500 ± 900[b]	1.4
PZ	2500±1000[b]	1.4

[a] Values are shown as means±SD of three determinations. Numbers followed with different letters within the same column indicate that data are significantly different at $P \leq 0.05$.

[b] SS: laboratory susceptible strain; PA: population from the 10th of Ramadan; PD: population from Diarb Negm; PF: population from Faqous; PS: population from El-Salhia ; PZ: population from El-Zagazig.

[c] Ratio is calculated as mean values of strains/mean value of SS.

In conclusion, the collected populations of *Cx. pipiens* showed different degrees of resistance to malathion and lambda-cyhalothrin. These findings attained from bioassay and activities of metabolic and target enzymes show the state of insect in environment and reflect the success of using such chemicals for its control.

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حساسية يرقات بعوض الكيوليكس بيبينز من مناطق مختلفة في محافظة الشرقية لبعض المبيدات الحشرية

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تم دراسة حساسية يرقات بعوض كيوليكس بيبينز والذي تم جمعه من مناطق مختلفة بمحافظة الشرقية تتمثل في مدينة العاشر من رمضان، مدينة ديرب نجم، مدينة الصالحية، مدينة فاقوس ومدينة الزقازيق خلال المواسم الصيفية لأعوام ٢٠٠٨، ٢٠٠٩ و٢٠١٠ بهدف معرفة مدي تحمل الحشرة لمبيدي الملاثيون واللمبادا–سيهالوثرين. كما تم دراسة نشاط إنزيم الأسيتايل كولين استيريز، وإنزيمات الإستيريزس العامة وإنزيم الجلوتاثيون اس ترانسفيريز في كل من السلالة الحقلية والمعملية. أوضحت نتائج تجارب التقييم الحيوي علي مجتمعات البعوض المختبرة أن هناك تباين في مقاومة البعوض للمبيدات المختبرة تبعا لمكان تجميعها. تراوحت المقاومة من ١ – ٩٩ ضعف بالنسبة للملاثيون، وأظهرت العينة التي تم الحصول عليها من منطقة ديرب نجم أعلي درجة مقاومة عنها من باقي العينات سواء بالنسبة للملاثيون أو اللمباد–سيهالوثرين، كما تراوحت درجة المقاومة من ٥ – ١٩٠٠ ضعف ضد المبادا–سيهالوثرين. بالنسبة للمقاييس البيوكيماوية فقد وجد انخفاض ملحوظ في نشاط انزيم الاسيتايل كولين استيريز في كل مجتمعات البعوض المختبرة مقارنة بالسلالة الحساسة، وعلي النقيض من ذلك وجدت زيادة واضحة في نشاط كل من الإستيريزات العامة والجلوتاثيون اس ترانسفيريز في كل مجتمعات البعوض مقارنة بالسلالة الحساسة. وتوضح هذه النتائج أن السلالة الحقلية لمجتمعات البعوض من مناطق مختلفة بمحافظة الشرقية تظهر مقاومة لكل من مبيد الملاثيون واللمبادا–سيهالوثرين وربما يرجع ذلك إلي إستخدام المبيدات المختلفة في مكافحة هذه الحشرات طوال العام وان كان يختلف من مكان عن الآخر أو إلي استخدام المبيدات الزراعية في مكافحة الآفات الزراعية مما يكسب الآفة نوع من المقاومة المشتركة لفعل المبيدات المختلفة أو إلي كليهما. يدل ذلك علي أهمية تبني إستراتيجيات الإدارة المتكاملة لهذه الآفة تحت الظروف المحلية.