Original Research Paper

Non-Target Inhibition of Antioxidant Enzymes in Honey Bees (*A. mellifera* and *A. florea*) Upon Pesticide Exposure

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Abstract: Understanding how different insecticides influence bee health is very essential. The aim of this work was to evaluate enzyme kinetics and inhibition by insecticides in *Apis mellifera* and *Apis florea*. Tested enzymes showed variable affinity to substrates. In *A. florea*, glutathione S-transferase (GST) showed significantly higher affinity for its 1-Chloro-2,4-Dinitrobenzene (CDNB) substrate than in *A. mellifera*. Carbosulfan caused the highest inhibition to acetylcholinesterase (AChE) in both tested species in comparison with chlorpyrifos, dimethoate and pirimiphos-methyl. Our results show that exposure to insecticides is harmful to honey bees and results in negative effects on enzyme activity.

Keywords: Insecticide, *Apis mellifera*, *Apis florea*, Detoxifying Enzymes, Enzyme Kinetics

Introduction

Apis mellifera and *Apis florea* are two important plant pollinators and producers of honey in the United Arab Emirates and many other countries. Foraging honey bees are often exposed to insecticides while they are collecting nectar and pollen. In addition, honey bees are affected by Varroa mites, microsporidian parasites like *Nosea apis*, bacterial diseases such as American foulbrood and viral diseases for example deformed wing virus. Most risk assessment studies examining honey bees have focused mainly on the direct acute exposures to pesticides from spray drifts. However, it should be noticed that honey bees are also exposed to sublethal insecticide concentrations in the form of residues in nectar and pollen. The effects of insecticides on beneficial insects and honey bees can be deleterious and possibly lethal (Stapel *et al*., 2000). Many studies have shown that the pollen and nectar collected by honey bees and brought back to the hive contain significant concentrations of residues of different pesticides (including insecticides) (Greenpeace, 2014; Choudhary and Sharma, 2008; Chauzat *et al*., 2006; Pohorecka *et al*., 2012). Nectar insecticide residues are lower than pollen residues (Dively and Kamel, 2012; Choudhary and Sharma, 2008). Sanchez-Bayo and Goka (2014) have shown that pyrethroid and neonicotinoid insecticides pose the highest risk to bees by contact exposure with contaminated pollen. However, in many cases, the synergism of fungicides with the two above-mentioned classes of insecticides can result in much higher risks regardless of the low concentrations of their combined residues (Sanchez-Bayo and Goka, 2014). Additionally, there is concern when using systemic insecticides because these chemicals can translocate from the soil into the pollen and nectar of plants and as a result, they could be ingested by honey bees and other pollinators (Stoner and Eitzer, 2012). Furthermore, studies have shown that foliar-applied treatments and insecticides applied using drip irrigation during flowering can result in high concentrations of pesticide residues in the pollen and nectar of treated plants (Dively and Kamel, 2012). Therefore, it is not surprising that insecticide residues can be detected in honey. A study reported that insecticide residues were found in honey collected by both domesticated and wild bees. It was indicated that more apiary honeys were found to be contaminated with organochlorines and organophosphates at higher concentrations than honeys produced by wild *Apis* species (Khan *et al*., 2004).

It is well-established that upon exposure to various toxins and pollutants, insects have several mechanisms, including induction of detoxifying enzymes, to counteract the toxic effects of pesticides. Detoxifying enzymes, such as esterases, play roles in pesticide synergism and resistance (Ishaaya, 1993). Additionally, increased expression of detoxifying enzymes can provide insects with tolerance to some insecticides, such as pyrethroids (Kostaropoulos *et al*., 2001). In addition, several factors can induce the detoxifying enzymes in

insects (Lee, 1991; Yu, 2004); for example, microsomal epoxidase and GST have been shown to be induced by cruciferous host plants, such as cabbage and their allelochemicals, in diamondback moth larvae (Yu and Hsu, 1993). The induction of detoxifying enzyme activities can provide metabolic resistance against insecticides in many insect species (Li *et al.*, 2007). Studies have shown that GST and oxidase play roles in resistance in selected insecticides. Insecticide resistance primarily occurs due to changes in insect metabolic enzymes or to development of insecticide-insensitive target sites in the insect nervous system (Damayanthi and Karunaratne 2005). Qualitative and/or quantitative changes in GSTs and monooxygenases have been linked to increased insecticide metabolism. In a study, Yu (2004) noted that the decreased toxicity of carbaryl, permethrin and indoxacarb was most likely due to enhanced detoxification of these pesticides by microsomal oxidases and GSTs, which are involved in insecticide metabolism. Unlike the metabolic enzymes, which require high specificity, a high reaction rate and fine tuning by metabolites to carry out their functions, the detoxifying enzymes are slow, have a broad specificity and are essentially unidirectional catalysts that are able to react with a wide spectrum of xenobiotics that cells may encounter (Tang and Chang, 1996). GST enzymes have been reported to play a key role in the detoxification mechanism for insecticides and hence, they may contribute to insecticide resistance in economically important target insect species in diverse agricultural systems (Dou *et al.*, 2006). GST is believed to be crucial because it is a major enzyme involved in phase II detoxification. Furthermore, significant alterations of the total and specific activities of this enzyme were noted during development of the branchiopod *Artemia salina* (Papadopoulos *et al.*, 2004).

In the UAE, most of the *A. mellifera* honey bees are imported from Egypt and they differ from the European honey bee strains by having relatively higher tolerance to summer temperatures. Unlike *A. mellifera* bees that are kept in Langstroth hives, the *A. florea* honey bees live in open nests and small colonies that are not maintained by beekeepers. The aims of this *in vitro* study were (1) to measure enzyme kinetics in *A. mellifera* and *A. florea* and (2) to study enzyme inhibition by insecticides.

Materials and Methods

Insects

A. mellifera honey bees were obtained from the beehives of the Abu-Dhabi Food Control Authority (ADFCA) in Al-Ain, UAE. *A. florea* adult bees were collected from wild colonies in Al-Ain, UAE.

Chemicals

Reduced glutathione (GSH), sodium bicarbonate (NaHCO$_3$), Fast Blue B salt (o-dianisidine, tetrazotized zinc chloride complex) and hydrogen peroxide (30%) [Sigma-Aldrich (St. Louis, MO, USA)]; Acetylthiocholine iodide (ATCh), 5, 5-dithio-bis-(2-nitrobenzoic acid) (DTNB) and 1-naphthyl acetate (1-NA) [Sigma]. 1-Chloro-2,4-Dinitrobenzene (CDNB) and 3,3,5,5-Tetra-Methylbenzidine (TMBZ) [Aldrich (Steinheim, Germany)]. Bovine serum albumin (BSA) and the Bradford dye reagent [Thermo Scientific (Rockford, IL, USA)]; Insecticide analytical standards carbosulfan, chlorpyrifos, dimethoate and pirimiphos-methyl [Fluka/Sigma-Aldrich (St. Louis, MO, USA)].

Insecticide Preparation

All insecticide active ingredients were first dissolved in absolute ethanol then diluted in distilled water to attain the appropriate concentrations.

Enzyme Preparation

Adults of *A. mellifera* and *A. florea* were homogenized manually (with pestle and mortar) in phosphate buffer for the GST, esterase and oxidase enzyme assays. For the AChE assay, heads of *A. mellifera* and *A. florea* adults were homogenized manually in phosphate buffer. All homogenates were centrifuged at 16,500 xg for 10 min at 4°C as described by Smirle (1990). The protein concentrations of insect homogenates were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme Kinetic Parameters

GST

The GST activity toward the CDNB substrate was determined according to Habig *et al.* (1974). Briefly, 250 μL of diluted enzyme preparation, 150 μL of 8.13 mM GSH and 10 μL of CDNB with a range of final concentrations (0.54, 0.27, 0.13, 0.068, 0.034, 0.017 mM) were mixed with 490 μL of 0.1 M potassium phosphate buffer (pH 7.2) and 10 μL CDNB. The non-enzymatic reaction of CDNB and GSH measured without homogenate served as a control. The absorbance was recorded at 340 nm for the CDNB substrate using a spectrophotometer every 2 min for 10 min and converted into nmol CDNB conjugated/min/mg protein with extinction coefficient $\varepsilon_{340nm} = 9.6$ mM^{-1} cm^{-1} (Wu *et al.*, 2008; Dou *et al.*, 2006).

General Esterase

Esterase activity was assayed using 1-NA (1-naphthyl acetate) as a substrate as described by Damayanthi and Karunaratne (2005). A volume of 250 μL of diluted homogenates, 300 μL of 7.8 mM Fast Blue B and 10 μL

of 1-NA with different concentrations (0.1, 0.026, 0.013, 0.0064 and 0.00322 mM) were mixed with 340 µL of 0.1 M potassium phosphate buffer (pH 7.2). The control was a non-enzymatic reaction, which contained no homogenate. The absorbance was recorded at 620 nm for the α-NA substrate using a spectrophotometer every 2 min for 10 min and converted into nmol α-NA conjugated/min/mg protein with extinction coefficient $\varepsilon_{620nm} = 2.222$ mM^{-1} cm^{-1} (He, 2003).

AChE

Acetylcholinesterase activity was measured using acetylthiocholine iodide (ATCh) as a substrate according to Ellman *et al.* (1961). A volume of 100 µL of homogenate, 12 µL of ATCh of different concentrations (1.057, 0.52, 0.26, 0.13, 0.065, 0.037 and 0.016 mM) and 30 µL of 0.33 mM DTNB (Ellman's reagent) were mixed with 758 µL of 0.1 M potassium phosphate buffer (pH 7.2). The control was a non-enzymatic reaction, which contained no homogenate. The absorbance was measured at 414 nm for the ATCh substrate using a spectrophotometer every 2 min for 10 min and converted into nmol ATCh conjugated/min/mg protein with extinction coefficient $\varepsilon_{414nm} = 1.36 \times 10^4$ mM^{-1} cm^{-1} (Ellman *et al.*, 1961).

General Oxidase

Oxidase activity was obtained using 3,3,5,5-Tetra-Methylbenzidine (TMBZ) as a substrate as described by Tiwari *et al.* (2011). The assay was run as follows: 100 µL of the homogenate, 100 µL of TMBZ of different concentrations (0.184, 0.092, 0.045, 0.022, 0.011 and 0.005 mM) and 25 µL of 3% H$_2$O$_2$ were mixed with 675 µL of 0.25 M of diluted sodium acetate buffer (pH 5). The buffer was diluted to avoid the blockage of the substrate position on the enzyme by sodium ions. The control was a non-enzymatic reaction, which contained no homogenate. The absorbance was recorded at 620 nm for the TMBZ substrate using a spectrophotometer every 2 min for 10 min and converted into nmol TMBZ conjugated/min/mg protein with extinction coefficient $\varepsilon_{620nm} = 3.9 \times 10^4$ mM^{-1} cm^{-1} (Josephy *et al.*, 1982).

Enzyme Inhibition

Enzyme inhibition *in vitro* experiments were conducted on AChE and general esterase. Four insecticides were tested: carbosulfan (0.310 nM), chlorpyrifos (0.437 nM), pirimiphos-methyl (0.773 nM) and dimethoate (0.95 µM).

AChE

A volume of 33.3 µL of homogenate was added with 15 µL of an insecticide at the concentrations listed in the esterase inhibition assay. The mixture was incubated for

10 min at room temperature. Then, 199.3 µL of a mixture (10 µL of 0.33 mM DTNB and 189.3 µL of 0.1 M potassium phosphate buffer (pH 7.2)) was added and 4 µL of 1.057 mM ATCh was then added. The whole mixture was incubated for another 45 min at room temperature. The control was a non-enzymatic reaction, which contained no homogenate. The absorbance was recorded at 414 nm for the ATCh substrate using a microplate reader. The change in absorbance was measured as an endpoint reaction (Ellman *et al.*, 1961).

General Esterase

A volume of 83.3 µL of homogenate was added, followed by 15 µL of an insecticide with the following concentrations: (carbosulfan (0.310 nM), chlorpyrifos (0.437 nM), pirimiphos-methyl (0.773 nM) and dimethoate (0.95 µM). The mixture was incubated for 10 min at room temperature, after which 120 µL of a mixture [3.33 µL of 0.1 mM 1-NA +116.6 µL of 0.1 M potassium phosphate buffer (pH 7.2)] was added and 100 µL of 7.8 mM Fast Blue B was then added. The whole mixture was incubated for another 10 min at room temperature. The control was a non-enzymatic reaction, which contained no homogenate. The absorbance was recorded at 620 nm for 1-NA using a microplate reader. The change in absorbance was measured as an endpoint reaction (He, 2003).

Kinetics Parameters of Inhibition (K_m, V_{max} and K_i)

The inhibition kinetics of general esterases and AChE were calculated with carbosulfan, chlorpyrifos and pirimiphos-methyl. The calculations of the kinetics constants (K_m, V_{max} and K_i) of esterase and AChE were performed using double-reciprocal plots, obtained by plotting $1/v_0$ vs $1/[S]$ to determine the intercepts and the slopes of the linear transformation. K_i is the inhibitor constant, which is the concentration required to produce half maximal inhibition. The same esterase and AChE inhibition assay protocols were followed to conduct the experiments in *A. mellifera* and *A. florea* using a microplate reader. The concentration of each insecticide was tested with a range of substrate concentrations of 1-NA (0.1, 0.026, 0.013, 0.0064 and 0.00322 mM) and ATCh (1.057, 0.52, 0.26, 0.13, 0.065, 0.037 and 0.016 mM).

Statistical Analysis

Differences between treatments were tested using analysis of variance *t* test and Analysis of Variance (ANOVA) using SAS software (SAS Institute Inc., Cary, NC, USA). Means were separated in significant ANOVA using Fisher Least Significant Difference (LSD). The significance value was set at $P < 0.05$. Each enzyme assay was conducted at least three times.

Results

Enzyme Kinetics

K_m

The GST of *A. florea* adults showed significantly higher affinity for the enzyme substrate (18.67±0.67 mM) than adults of *A. mellifera* (24.97±0.52 mM) (Table 1). However, the general esterase enzymes of *A. florea* showed a significantly lower substrate affinity (0.63±0.026 mM) compared to *A. mellifera* (0.39±0.020 mM). The oxidase showed no significant differences between *A. mellifera* and *A. florea* in terms of substrate affinity (33.3±1.70 and 26.62±2.57 mM, respectively).

V_{max}

Significant differences were found in the V_{max} values between adults of *A. mellifera* and *A. florea* for GST (195±18.77 and 130±15.53 nmol/min, respectively) and general esterases (693.9±37.53 and 830.5±22.57 pmol/min, respectively) (Table 1). However, the difference between the two species was not significant for the oxidase (49.6±2 and 56.7±1 nmol/min, respectively).

Specific Activity

No significant differences were found in the specific activities between adults of *A. mellifera* and *A. florea* for GST (156.8±31.10 and 117.1±22.46 nmol/min/mg protein, respectively), general esterase (53.89±2.909 and 61.18±2.776 nmol/min/mg protein, respectively) and oxidase (0.0093±0.00040 and 0.0099±0.00003 pmol/min/mg protein, respectively) (Table 1).

Enzyme Inhibition

Carbosulfan, chlorpyrifos and pirimiphos-methyl inhibited AChE and general esterases in both *A. mellifera* and *A. florea* at different levels. In *A. mellifera*, carbosulfan had the highest inhibitory effect (69.76%) on AChE activity followed by chlorpyrifos (60.46%) and pirimiphos-methyl (44.18%) (Table 2). The specific activity of AChE when incubated separately with carbosulfan, pirimiphos-methyl and chlorpyrifos was 13±0.1, 17±0.6 and 24±0.3 pmol/min/mg protein, respectively. However, in *A. florea*, pirimiphos-methyl caused the highest inhibition (80.65%) of AChE followed by carbosulfan (77.42) and chlorpyrifos (70.97). This was demonstrated by the lower specific activity of the enzyme in the presence of pirimiphos-methyl, carbosulfan and chlorpyrifos, which was 12±0.1, 14±0.4 and 18±0.05 pmol/min/mg protein, respectively, compared to the control (43±0.3 pmol/min/mg protein). Similar results were found with general esterase (Table 2). In *A. mellifera*, carbosulfan had the highest significant inhibitory effect on the enzyme (56.82%) followed by pirimiphos-methyl (46.49%) and chlorpyrifos (35.72%).

With carbosulfan, the specific activity was 0.57±0.0120, compared to chlorpyrifos (0.85±0.0186) and pirimiphos-methyl (0.71±0.0058 µmol/min/mg protein). When incubated with one of the three insecticides, the enzyme specific activity was significantly lower than the control (1.327±0.0033). However, in *A. florea*, pirimiphos-methyl had the highest significant inhibitory effect (66.73%), with a specific activity of 0.34±0.0029 µmol/min/mg protein followed by carbosulfan (52.46%; 0.49±0.0033 µmol/min/mg protein) and chlorpyrifos (46.96%; 0.55±0.0152 µmol/min/mg protein) (Table 2).

Kinetics Parameters of Inhibition

AChE

The presence of 0.310 nM carbosulfan in the reaction mixture resulted in an inhibition of AChE activity in *A. mellifera* and *A. florea*. Carbosulfan decreased the V_{max} by 51% and increased the K_m by 61% in *A. mellifera*. However, carbosulfan decreased the V_{max} by 61% and increased the K_m by 79% in *A. florea* compared with control values in the absence of the insecticide (Table 3). The K_i and K_i' of 0.071±0.005 and 0.290±0.017 nM in *A. mellifera* and 0.028±0.002 and 0.197±0.010 nM in *A. florea*, respectively, were calculated for AChE inhibition.

The inclusion of 0.437 nM chlorpyrifos in the reaction for both *A. mellifera* and *A. florea* led to a significant inhibition in AChE activity. Compared with control values, chlorpyrifos decreased the V_{max} by 44%, while the K_m was almost the same in *A. mellifera*. However, the V_{max} decreased by 68% and the K_m increased by 74% in *A. florea* (Table 3). The K_i value of chlorpyrifos was found to be 11.13±0.502 nM in *A. mellifera* AChE while K_i and K_i' values were 0.040±0.002 and 0.200±0.021 nM in *A. florea* AChE, respectively.

Similarly, 0.773 nM of pirimiphos-methyl was incubated with the AChE assay mixture for both *A. mellifera* and *A. florea*. The results show that pirimiphos-methyl caused significant inhibition of AChE activity in both species. In *A. mellifera*, the K_m increased by 54% and the V_{max} decreased by 43%. Moreover, the K_i and K_i' values of *A. mellifera* AChE were 0.273±0.012 and 1.022±0.071 nM, respectively (Table 3). In *A. florea*, the K_m increased by 42% and the V_{max} was decreased by 74%. In addition, the K_i and K_i' values of *A. florea* AChE were 0.141±0.012 and 0.278±0.030 nM, respectively.

General Esterase

Adding 0.310 nM of carbosulfan to the reaction mixture resulted in an inhibition of general esterase activity in *A. mellifera* and *A. florea*. Carbosulfan decreased the V_{max} by 55% and decreased the K_m by 81% in *A. mellifera* esterase, while it decreased the V_{max} by 44% and increased the K_m by 60% in *A. florea*

esterase compared with control values in the absence of the insecticide (Table 3). The K_i and K_i' values of 0.098±0.007 and 0.258±0.011 nM in *A. mellifera*, respectively and 0.092±0.008 and 0.397±0.025 nM in *A. florea*, respectively, were calculated for carbosulfan inhibition of general esterase activity.

For both *A. mellifera* and *A. florea,* adding 0.437 nM of chlorpyrifos to the reaction led to a significant inhibition in

the general esterase activity. Compared with control values, chlorpyrifos decreased both the V_{max} by 52% and the K_m by 89% in *A. mellifera*. However, the V_{max} decreased by 42% and the K_m increased by 60% in *A. florea* (Table 3). The K_i and K_i' values of 0.350±0.029 and 0.408±0.036 nM in *A. mellifera*, respectively and 0.132±0.014 and 0.642±0.052 nM in *A. florea*, respectively, were calculated for general esterase inhibition by chlorpyrifos.

Table 1: Comparison of enzyme kinetics (Mean ± SE) in *A. mellifera* and *A. florea* (*in vitro*)

		A. mellifera (adult)	*A. florea* (adult)
K_m (mM)	GST	24.97±0.52a	18.67±0.67b
	General Esterase	0.39±0.020b	0.63±0.026a
	General Oxidase	33.3±1.70a	26.62±2.57a
V_{max} (nmol/min)	GST	195±18.77a	130±15.53b
	General Esterase	693.9±37.53b	830.5±22.57a
	General Oxidase*	49.6±2a	56.7±1a
Specific activity (nmol/min/mg protein)	GST	156.8±31.10a	117.1±22.46a
	General Esterase	53.89±2.909a	61.18±2.776a
	General Oxidase**	0.0093±0.00040a	0.0099±0.00003a

Mean values within one row followed by the same letter are not significantly different from each other according to *t* test, ($P > 0.05$). GST = Glutathione S-Transferase, AChE = Acetylcholinesterase. K_m = Substrate concentration at which the rate was half of maximum velocity (V_{max}); * Units of V_{max} are in pmol/min. ** Units of specific activity are in pmol/min/mg protein.

Table 2: Enzyme inhibition caused by exposure to insecticides and measured as specific enzyme activity (Mean ± SE) in nmol/min/mg protein of *A. mellifera* and *A. florea* AChE and general esterase after 10 min incubation with insecticides (*in vitro*).

	AChE				General Esterase			
Insecticide	*A. mellifera*	%	*A. florea*	%	*A. mellifera*	%	*A. florea*	%
Control	43±0.3a	0	62±1.6a	0	1.327±0.0033a	0	1.037±0.0033a	0
Carbosulfan (0.310 nM)	13±0.1e	69.76	14±0.4d	77.42	0.573±0.0120e	56.82	0.493±0.0033d	52.46
Chlorpyrifos (0.437 nM)	24±0.3c	44.18	18±0.5c	70.97	0.853±0.0185c	35.72	0.550±0.0152c	46.96
Pirimiphos-methyl (0.773 nM)	17±0.6d	60.46	12±0.1d	80.65	0.710±0.0057d	46.49	0.345±0.0029e	66.73
Dimethoate (0.95 µM)	38±0.3b	11.63	57±0.3b	8.06	1.307±0.0066ab	1.51	0.996±0.0120ab	3.95

Mean values within one column followed by the same letter are not significantly different from each other according to ANOVA, LSD test ($P > 0.05$). % = percentage of enzyme inhibition compared to the control. AChE = Acetylcholinesterase

Table 3: Inhibition kinetics parameters (Mean ± SE) of *A. mellifera* and *A. florea* AChE and general esterase using ATCh / 1-NA, respectively as substrates with the three insecticides (*in vitro*)

		Control	Carbosulfan	Chlorpyrifos	Pirimiphos-methyl
A. mellifera					
AChE	K_m (mM)	0.023±0.002	0.059±0.006	0.026±0.002	0.050±0.005
	V_{max} (nmol/min)	0.265±0.018	0.129±0.004	0.148±0.009	0.151±0.007
	K_i (nM)	--	0.071±0.005	11.13±0.502	0.273±0.012
	K_i' (nM)	--	0.290±0.017	--	1.022±0.071
General Esterase	K_m (mM)	0.037±0.0028	0.007±0.0005	0.004±0.0002	0.008±0.0007
	V_{max} (µmol/min)	5.905±0.4150	2.672±0.1550	2.840±0.2110	1.752±0.0700
	K_i (nM)	--	0.098±0.0070	0.350±0.0290	0.112±0.0060
	K_i' (nM)	--	0.258±0.0110	0.408±0.0360	0.336±0.0230
A. florea					
AChE	K_m (mM)	0.062±0.005	0.290±0.020	0.238±0.021	0.107±0.013
	V_{max} (nmol/min)	0.246±0.010	0.095±0.009	0.079±0.007	0.065±0.002
	K_i (nM)	--	0.028±0.002	0.040±0.002	0.141±0.012
	K_i' (nM)	--	0.197±0.010	0.200±0.021	0.278±0.030
General Esterase	K_m (mM)	0.002±0.0001	0.005±0.0004	0.005±0.0006	0.009±0.0004
	V_{max} (µmol/min)	4.813±0.2850	2.680±0.1870	2.800±0.1530	1.896±0.0970
	K_i (nM)	--	0.092±0.0080	0.132±0.0140	0.067±0.0020
	K_i' (nM)	--	0.397±0.0250	0.642±0.0520	0.505±0.0550

AChE = Acetylcholinesterase. K_m = Substrate concentration at which the rate was half of maximum velocity (V_{max}). K_i = Inhibitor constant, which is the concentration required to produce half maximum inhibition

Similarly, when 0.773 nM of pirimiphos-methyl was added to the reaction it caused inhibition in general esterase activity for both species. In *A. mellifera*, the K_m decreased by 78% and the V_{max} also decreased by 70%. Moreover, the K_i and K_i' values of *A. mellifera* are 0.112±0.006 and 0.336±0.023 nM, respectively (Table 3). In *A. florea*, the K_m increased by 78% and the V_{max} was decreased by 61%. In addition, the K_i and K_i' values for the inhibition of general esterase activity in *A. florea* are 0.067±0.002 and 0.505±0.055 nM, respectively.

Discussion

Enzyme Kinetics

The results of the current study show that the *A. florea* GST has a higher affinity for its substrate (1.3-fold) than the GST of the *A. mellifera* and this is probably a result of species differences or the exclusive feeding of *A. florea* on wild plants unlike *A. mellifera* bees, which receive some artificial feeds such as sugar syrups by beekeepers. Additionally, the results demonstrate that the *A. mellifera* general esterase enzymes has a higher affinity (1.6-fold) for the substrate compared to the general esterase of *A. florea*. In general, the differences in substrate affinities could be related to interspecific differences. Furthermore, insect species have different dietary needs and show differences in body size and this might be the case in the current study where *A. florea* is much smaller than *A. mellifera*. Vanhaelen *et al.* (2004) performed a study on the purification and characterization of Glutathione S-transferases (GSTs) from two syrphid flies (*Syrphus ribesii* and *Myathropa florea*). Their results showed that the specific activity of GST toward CDNB was 1.7-fold higher for *S. ribesii* than *M. florea*. With regard to kinetics, *M. florea* had higher K_m values than those from *S. ribesii*, with values being 2.6, 1.4 and 2-fold higher for GSH, CDNB and DNIB, respectively, indicating differences among species. Also, the V_{max} values were higher for *S. ribesii*, with ratios of 3.2, 5.3 and 1.8 for GSH, CDNB and DNIB, respectively, compared to *M. florea*.

Enzyme Inhibition

In this study, the tested insecticide concentrations were lower than the ones applied on plants in the field. Carbosulfan and pirimiphos-methyl caused higher inhibition in AChE and general esterase enzymes in both *A. mellifera* and *A. florea*. This indicates that these two honey bee species are at risk in the fields treated with carbosulfan and pirimiphos-methyl. These results agree with a published laboratory bioassay showing that carbosulfan was the most toxic insecticide to *A. mellifera* (Husain *et al.*, 2014). Another study showed that carbosulfan is toxic to *A. mellifera* (Akca *et al.*, 2009). In

fact, pesticide damage becomes more aggravated when bees visit different plants during their foraging trips and therefore, they potentially are exposed to mixtures of pesticides. It was reported that honey bees travel more than 5-6 km and even longer distances to collect pollen and nectar (Hagler *et al.*, 2011; Visscher and Seeley 1982).

Enzyme Inhibition Kinetics

In the current study, insecticides were tested to investigate their potential role in inhibiting AChE and general esterase activities. Generally, carbosulfan, chlorpyrifos and pirimiphos-methyl inhibited AChE and esterase. Detoxifying enzymes and target alteration are equally important mechanisms of insecticide degradation (Pasay *et al.*, 2009). AChE is of interest because it is the target enzyme for organophosphate and carbamate insecticides in the central nervous system (Wang *et al.*, 2004) and its role in cholinergic synapses is essential for life. Several studies have been performed on insect AChE to explore the relationship between AChE alteration and insect resistance (especially to carbamates and organophosphates) (Chai *et al.*, 2007). Price (1988) explained that there are many factors that may affect the susceptibility of AChE to specific insecticides, such as cuticle penetration and metabolic processes. Therefore, AChE sensitivity does not necessarily indicate that insects will be sensitive to a particular chemical when it is used as an insecticide. Substrate inhibition at a high concentration is a normal phenomenon for AChE and is most likely due to the binding of excess substrate to the peripheral (allosteric) anionic site to form an enzyme-substrate complex (Hai-Hua *et al.*, 2005). Carbamate insecticides inhibit AChE by adding a carbamyl group on the enzyme, which completely inactivates the enzyme (Leibson and Lifshitz, 2008). Moreover, organophosphate compounds inhibit cholinesterases (Kamanyire and Karalliedde, 2004), leading to the accumulation of acetylcholine. The inhibition of AChE causes overstimulation of nicotinic and muscarinic acetylcholine receptors (Sogorb and Vilanova, 2002).

The poisoning of *A. mellifera* arising from the agricultural use of pesticides has been monitored long time ago. In the United Kingdom the measurement of bee head AChE inhibition was used as an indicator of organophosphate insecticide poisoning (Westlake *et al.*, 1985). In 1978, there were widespread reports of serious poisoning associated with honey bees foraging on oilseed rape to which triazophos (organophosphates) had been applied. Investigation of these incidents showed that AChE inhibition was apparently reversible after triazophos poisoning. Additionally, Suh and Shim (1988) reported that GST (CDNB conjugation) activity was significantly induced by diazinon and moderately induced by permethrin (Suh and Shim, 1988). Westlake *et al.* (1985) reported that lethal insecticide treatment of bees

with most carbamate and organophosphate compounds resulted in significant AChE inhibition. In the current study, pirimiphos-methyl was the most potent inhibitor of the esterase enzymes in both *A. mellifera* and *A. florea*. Furthermore, the presence of carbosulfan, chlorpyrifos and pirimiphos-methyl in the incubation mixture with the enzymes at different substrate (ATCh and 1-NA) concentrations for AChE and esterase, respectively, exhibited mixed inhibition patterns. However, chlorpyrifos exhibited a non-competitive inhibition because the K_m value was not affected. Non-competitive inhibition is considered a mixed inhibition. Generally, this indicates that the insecticide directly binds to the AChE and esterase active site and allosteric site as well, reducing the concentration of free enzyme available for 1-NA/ATCh binding. This potentially allows the insecticide to persist in a honey bee for a longer period of time because of the lower activity of detoxification achieved by AChE and esterase. He (2003) conducted a study using a continuous spectrophotometric assay for the determination of diamondback moth esterase activity. His results illustrated that the 1-NA exhibited a competitive inhibition toward azadirachtin. However, the current study is different from the work of He (2003) because the inhibiting insecticide was a plant extract (azadirachtin). As a result, they obtained competitive inhibition patterns while mixed and non-competitive inhibitions were observed in the present study. In addition, the current study provided the parameters of the inhibition kinetics for AChE and general esterase in *A. mellifera* and *A. florea* in response to exposure to carbosulfan, chlorpyrifos and pirimiphos-methyl.

Conclusion

This study sheds light on four enzymes in *A. mellifera* and *A. florea*. It advances our knowledge and understanding about effects of insecticides on honey bees at low concentrations in the environment. No significant differences occurred in the specific activities of GST, general esterase and oxidase between *A. mellifera* and *A. florea*. Carbosulfan and pirimiphos-methyl caused higher inhibition to AChE in both *A. mellifera* and *A. florea*. Overall, the tested insecticides demonstrated variable inhibition rates and patterns against the four enzymes, which revealed some of the negative effects of insecticides on honey bees. Thus, there is a need for developing new generations of agricultural pesticides that are safer on honey bees. In addition, further studies are warranted to evaluate effects of pesticides on *A. florea* in particular because it is a wild species and its numbers cannot be augmented by beekeepers unlike *A. mellifera*, which is kept in hives maintained by beekeepers.

Author's Contributions

Amal Ahmed Berjawi: Performed the experiments and participated in data analysis and paper writing.

Syed Salman Ashraf: Participated in designing the experiments, in data analysis and in paper writing.

Mohammad Ali Al-Deeb: Conceived and designed the experiments, participated in performing the experiments and in data analysis, provided reagents/materials and wrote the paper.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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