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Relationship between Toxicity of Certain Pesticides to the Honey Bee, *Apis mellifera* L. (Hymenoptera: Apidea) Foragers and their Haemolymph Amino Acids.

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ABSTRACT

In this research, certain pesticides, i.e. bio-pesticides and synthetics pesticides proved to be toxic against honey bee foragers. Laboratory evaluation of the pesticides cleared that both group of the insecticides showed highly toxicity to the workers. Both Vertimec®, Dipel2x® and Achook®as a bio-insecticides and the two synthetics pesticides(Sparkle® and Diazinox®)gave the mean number 50 (100%) with each of the recommended, half and quarter doses after 24, 48 and 72 h. On the other hand, Achook® and Dipel2x® (bio-pesticides) have the lowest toxicity among all treatments on the assigned insect. Moreover, the obtained results indicated those, for recommended, half and quarter doses, after 72 h. the mean numbers of mortality were 50 (100%), 47 (94%) and 29.66 (59.33%) for Achook®, and 43.66 (87.33%), 46.33 (92.66%) and 35.33 (70.66%)for Dipel2x®.In addition, the amino acids were evaluated after 24 h. in the heamolymph of all the treated individuals. The results cleared that all five pesticides have negative effect on the contents and amounts of amino acids. 15 amino acids were presented in untreated samples but the most of them disappeared in the recommended doses. For example Dipel2x®had the lowest effect among the other tested pesticides. The mean number of mortality started to be increased after long exposure time of72 h. Although Dipel2x® had a negative effect on bees but it was the only one among the used pesticides, which presented the amino acid Proline. This is considered the most important constituent of honey bee protein. This phenomenon was due to the low toxicity of Dipel2x®.

Key words: *Apis mellifera,* foragers, bio-pesticide, synthetic pesticides, toxicology, heamolymph, amino acids

Introduction

Honey bee, *A. mellifera*, is the most important pollinator of agricultural crops. In addition, honey bee production is very important for human bodies. During the last 10 years, colony populations have decreased significantly in many countries and especially in Egypt. Exposure to pesticides has produced negative effects on individual bees and their colonies for nearly a century. Dead bees either on the hive bottom boards or on the ground in front of the hives, assured the negative effects of chemicals.

Ding bees are very voluminous. In addition, there are also negative effects on queens, drones, developing brood, and bee behavior that eventually result in weakened or dead colonies. Over the decades there has been a succession of insecticides, acaricides, fungicides, and herbicides as new compounds were developed and older chemicals were unused, due to resistance to the target pests.

Honey bee foragers collect pollen and nectar from flowers to sustain the colony and support healthy brood development (Winston, 1987), thus pesticides in the environment potentially could be transmitted to bees' brood through pollen, wax, or brood food contamination (Chauzat *et al*.,2006; Villa *et al*., 2000).

Bee products have the image of being natural, healthy and clean. However bee products are produced today in an environment, polluted by different sources of contamination. (Bogdanov,2006).

The environment around honey bee *A. mellifera* colonies can be contaminated with toxic chemicals from industrial, agricultural and domestic activities. Though honey bees are the non-target organisms for most pesticide applications in the environment, foraging bees can be exposed to pesticides, while collecting pollen andnectar from flowers, resins from various plants and from drinking water from rivers, lakes, ponds, etc... Breathing during flight, if the pesticides are airborne can also effect on the foragers. These pesticides may adverse cases to the colony, as their levels are concentrated further in the waxy nest infrastructure (Gregorc *et al.*,2011).

In surveys of North American honey bee colonies conducted in (2007) and (2008), investigators found 121 different pesticides and metabolites in wax, pollen, bees and corresponding hive samples (Mullin *et al.,*2010).

Many of the pesticides which are exposed to honey bees have insecticidal properties and may be harmful to bees. For example, pesticides are known to lower the developmental rate of queen honey bees, increase the occurrence of queen rejection, and lower queen weight (Nasr *et al*., 2003; Pettis *et al*.,2004), affect forager bee mobility and communicative capacity(Medrzycki *et al*.,2003).

There are many reasons to look at pesticide effects in honey bee workers. Bees have been used as biological monitors for pesticide contamination of geographic regions. (Celli *et al*.,1996).

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This study was conducted to compare and assure the lowest toxicity and the highest toxicity of the used synthetic chemicals and bio-insecticides for honey bees foragers, as well as the determination of the heamolymph amino acid in both treated foragers and check untreated foragers.

Materials and Methods

Honey Bees:

All experiments were conducted at the Apiary in Adco region and extension laboratory in department of plant protection at faculty of agriculture (Saba-Basha) at the University of Alexandria using honey bee colonies derived from crosses between Carniolan and Egyptian strains. Foraging bees were rather at the end of life activity of workers (Michener 1974; Winston 1987; Picardnizou *et al*. 1995) and an extensive literature confirms that foragers are the higher than 20 days bees in typical colonies. No hive treatments to control diseases were conducted prior to our study. Hives were exposed to smoke twice for 30–60 s prior to collection. Honey bees on frames containing honey and pollen were always collected from the top super, and the bees were shaken from the frames into a plastic container. The opening of the container was covered with a solid plastic lid and the bees transported to the laboratory. The bees were maintained at approximately 25 ± 2ºC during transportation to the laboratory. Immediately upon arrival from the field, the bees were kept in experimental cages (10 ×7 × 12 cm^3) in groups of 50 at 25 ± 2ºC and 65 ± 5% relative humidity and fed 50%(w/v) sucrose solution.

Pesticides tested:

The toxicity of Diazinox®, Achook®, Vertimec®, Dipel2x® and Sparkle® were evaluated on honey bees (*A. mellifera* L.) workers by ingestion bioassays which allow honey bees spiked syrup under laboratory conditions. Pesticides were performed at the application rate in the field according the ministry of agriculture (Table,1).Also, the half and quarter concentrations of pesticides were tested. Stock solutions of insecticides were prepared in 50% (w/v) sucrose. Prior to treatment with insecticide, bees (20/cup) were anesthetized by exposure to carbon dioxide gas for no longer than 3 min, and each group of each concentration was composed of three plastic cups covered with a nylon mesh containing 60 honey bees. The amount of solutions was applied on cotton bed attached to the upper surface of the cover and bees were left to feed for 24 h. Bees fed with 50% sucrose solution were used as a control. Experiments were carried out by incubating bees at 25 ± 2ºC, 65 ± 5% relative humidity, and 12:12 (L:D) photoperiod. The bees were considered dead if unable to walk when prodded with a fine hair brush. Mortality percentages were recorded after 24 h, 48 h and 72 h of treatment. The average numbers of bees and their mortality percentages were calculated then data was adjusted by using Abbott's formula Abbott (1925).

Table 1: Pesticides and their rates of application.

Compounds	Common name	Active ingredient (%)	Formulation.	Application Rate/fed.
Diazinox®	Diazinon	60	EC*	300 cm^3/100L
Achook®	Azadirachtin	0.15	EC	750 ml
Vertimec®	Abamectin	1.8	EC	40 cm^3/100L
Dipel2x®	*Bacillus thuringiensis* Subsp. Kurstaki	6.4	WP	200g
Sparkle®	Cypermethrin	25	EC	250 cm^3

*EC = Emulsifiable Concentration and WP=Wet table powder

Haemolymph free amino acids:

To study the effect of the tested compounds on the free amino acids in the haemolymph of treated honey bees' workers, the pooled haemolymph samples (1-2 ml) were taken after 24 h. and analyzed by amino acid analyzer model Dionex ICS-3000 with its reference" Product manual for Dionex Amino Analyzer and Amino Pac PA10. Document No. 031481-12".

Samples preparation:

Preparations were adapted, in order as follows:

1. Samples were hydrolyzed by using 0.1 mg from each sample in 1.0 ml of MHCL.
2. Evaporating to dryness and reconstitute to the same volume with NLeu/azide diluent from section 4.4dilute an aliquot 1.000-2.000x with the NLeu/ azide diluent from section 5.4.
3. Injecting volume 25µl within standard NIST SRM 2389.500x dilution using NLeu/ azide diluent from section 5.4. Then, Column amino Pac PA10 analytical and guard others columns were conducted under column temperature 30ºC.

4. Operating back pressure < 3.000 psi. Afterwords, eluent E1 with 18.2 megohm water, E2 with 250 Mm NAOH and E3 with 1 M Sodium acetate. At last eluent flow rate is 0.25 ml/min.

Results:

A- Effect of certain pesticides on the mortality persentage of honey bees' workers (Apis mellifera):

In this experiment three of bio-pesticides (Achook®, Vertimec® and Dipel2x®) and two of synthetics pesticides (Sparkle® and Diazinox®) were used. These two groups of pesticides were compared by the mean number of the bees' mortality after interval days.

Bio-pesticides are naturally organisms and their products were used successfully for pest control. Most bio-pesticides are currently sold as formulations of *Bacillus thuringiensis* products (i.e. Dipel2x®and Agerin®) that rely on bacterial-produced bio-toxin. Another category of bio-pesticides is that extract from the Neem tree has over 100 compounds with pesticidal properties. The best known from them is Azadirachtin (Achook®). This substance is found in all parts of the tree, but it is much more concentrated in its fruit, especially in its seeds. The active ingredient of Abamectin (Vertimec®) is composed of two molecules, isolated from a set of eight molecules produced by the original soil micro-organism. Abamectin acts at a different target sites, those called GABA receptor protein.

The results in Table 2 showed that, Vertimec®, Sparkle® and Diazinox® caused a highly toxicity for foragers bees compared to Achook® and Dipel2x®. Results indicated, also that the honey bees workers treated with Vertimec®, Sparkle® and Diazinox® dead after first 24 h. due each recommended, half and quarter doses of the aforementioned three compounds. There were detectable effect been observed among the different tested insecticides, in the sense that, were increased the mean number of bees mortality have been increased throughout the period of investigation. It means that the pyrethroid, cypermethrin proved to be the most toxic to honey bee workers *A. mellifera* with almost the same effects azadirachtin and abamectin.

The observed data in Table2 cleared that the highest increase of mortality mean numbers after 24 h. was (100%) by recommend dose of Vertimec®. Followed by Achook®(44%) and Dipel2x® (36%), respectively when bees were treated with the half recommended dose of bio-pesticides on the other hand, the highest mean numbers of mortality was (100%) by using Vertimec®, while no significant difference between Dipel2x®and Achook® (21.33%)and (16%). In respect, using the quarter recommended dose of bio-pesticides Vertimec® ranked the 1st place in activity concerning to the increase of the mean number of mortality recording an increase of (100%),followed by Dipel2x®and Achook®(23.33%) and (27.33%).

After 48h. from application of recommended dose of bio-pesticides the same results as for mean numbers of mortality (100%)occurred by Vertimec® and Achook®.While,Dipel2x®registered themean number of (72%).In case of applying the half recommended dose and after 48hVertimec®ranked the 1strank of activity in increasing the mean numbers of mortality recording an increase of (100%) followed by Dipel2x® and Achook®(73.33%) and (65.33%), successively.

The quarter dose was used for the bio-pesticides Vertimec®that occupied the 1st activity in increasing the mean mortality numbers recording an increase of (100%)followed by both Dipel2x® and Achook® which showed (49.33%) and (44%), subsequently.

Comparatively, after 72h. post application of the recommended dose of evaluated bio-pesticides, Vertimec® and Achook® achieved the mean number of mortality (100%) followed by Dipel2x®(87.33%).The same trends of results were noticed after 72h. by the half recommended dose of the used bio-pesticides data referred the mean number of mortality (100%) for Vertimec® and (94%) for Achook® followed by (92.66%) in Dipel2x®.

After, using the quarter recommended dose of bio-pesticides mean numbers and mortality percentage could be arranged in a descending order as follows: (100 %) > (70.66%)> (59.33%) forVertimec®,Dipel2x®and Achook®, in respect. This means that Vertimec® was the most effective one.

As showen in, the residual effect of the synthetic pesticides (Sparkle® and Diazinox®) against *A. mellifera* were cleared in Table 2 after interval days .The data in Table2 showed that the insecticides (Sparkle® and Diazinox®) were more toxic than the other used pesticides in this study. The same results were obtained after the interval days (24, 48 and 72h.),as a result of applying the recommended, half and quarter doses of insecticide. The Sparkle® and Diazinox® achieved the mean numbers of mortality (100%) after interval days by different concentrations of insecticides.

B- Effect of the pesticides residuals on Apis mellifera workers haemolymph free amino acids after 24h. only of exposure:

The results found in Table 3 shown the analysis of amino acids in the *Apis mellifera* workers haemolymph, by the Amino Acid Analyzer and revealed that only 15 free amino acids presented in the untreated sample, as well as the same 15 free amino acids that were compared with other amino acids presented in treated samples.

Table 2: The effect of pesticides on the average mortality of honey bees' workers *Apis mellifera* L.

Treatments		Adult mortality (%)After Interval days		
		24^{hr}	48^{hr}	72^{hr}
Achook®	A	44	100	100
	B	16	65.33	94
	C	27.33	44	59.33
Vertimec®	A	100	100	100
	B	100	100	100
	C	100	100	100
Dipel2x®	A	36	72	87.33
	B	21.33	73.33	92.66
	C	23.33	49.33	70.66
Sparkle®	A	100	100	100
	B	100	100	100
	C	100	100	100
Diazinox®	A	100	100	100
	B	100	100	100
	C	100	100	100
Control	A	0	4	4
	B	0	4	4
	C	0	4	4

A= recommended dose, B= half recommended dose and C= quarter recommended dose

After 24 h. from the application of the evaluated pesticides, wide range of amino acids with their concentrations in the haemolymph were detected. Some amino acids have been appeared and other disappeared when treated with pesticides. After Azadirachtin application, the predominant amino acid was Histidine111548.4329 µmolar while come next in amount Phenylalanine, Threonine, Aspartic acid and Alanine49382.2258, 24403.9415, 179.8768 and89.6661 µmolar., respectively. When previous results compared with untreated sample; founded another amino acids were disappeared with Azadirachtin treatment like(Arginine, Cysteine, Glutamic acid, Glycine, Leucine, Lysine, Methionine, Proline, Serine and Tyrosine)in Table 3.

In addition, free amino acid amounts appeared after treatment with first bio-insecticide Achook®(Azadirachtin) were almost on par with the same amino acids in untreated samples. For instancesHistidine for 134150.413µmolar and 135190.000, 6873.7464, 161.7441 and 1383.5551 µmolar for Phenylalanine, Threonine , Aspartic acid and Alanine., respectively. It was important to donate that the same amino acid appeared in more amounts in the untreated bees than those treated ones with Achook®, except for the increased amount of Aspartic acid in Azadirachtin treated samples than that of untreated ones.

On the other hand, the highest concentration6596.7429 µmolar of Threonine was related to 24 h. after the treatment with Vertimec®(Abamectin); compared with the comparatively less concentrations such as other amino acids as Phenylalanine, Aspartic acid , Histidine , Cysteine and Leucine (3240.8889, 1010.8467, 564.3953, 326.4381 and 27.6565 µmolar., respectively).

After applied the third bio-insecticide Dipel2x® (B.T.)the 1st ranked amount of amino acids was Threonine with 4079.4169 µmolar; compared with other amino acids appeared in the same treatment byDipel2x®. Then followed by Lysine, Phenylalanine, Proline, Aspartic acid, Arginine and Alanine (968.4188, 516.4490 , 34.1960, 32.0832, 31.5354 and 11.2055 µmolar, in sequence).

As for insecticides Sparkle® (Cypermethrin) the highest amount of Threonine 857.2539 µmolar was evident. While, Glycine recorded 4.4168 µmolar are presenting the lowest amount of amino acid in treated sample. On the contrary, Histidine concentration was 782.3058 µmolar and recorded the second rank of amino acids in the treated bees. Other amino acids with different concentrations appeared after the same treatment like Alanine, Glutamic acid, Arginine, Phenylalanine and Tyrosine with the values of 115.8060, 81.1152, 51.6864, 32.0756 and 11.4241 µmolar, respectively (table,3).

When treatment with the synthetics insecticides Diazinox®(Diazinon) was applied it was noticed that the amino acid, Methionine appeared with amount of 833992.1748µmolar in the treatment samples with Diazinon, but it was higher thanthat of Methionine in the un-treatment samples.Other amino acids were also registered as Cysteine, Alanine, Histidine, Lysine and Glycine with the low concentration of (734.4336, 688.8255, 70.9640,40.7719and 36.1404 µmolar, in respect).It is important to indicate that, Methionine was found only in both Diazinon treatment and un-treatment samples, conversely. In the same time it disappeared in other insecticides treatments.

On the other hand, in the all treatments, except for Vertimec®, the predominant amino acid was Alanine. Aspartic acid was also appeared in all treatments with bio-pesticides, except for the synthetics pesticides (Sparkle® and Diazinox®). Histidine was recorded in all treatments, except for Dipel2x®. Phenylalanine and Threonine were present in all treatments, except for Diazinox®. Leucine was disappeared in all treatments, except for Achook® treatment samples. Methionine also disappeared in all treatment except samples treatment with Diazinox®. Proline was also absent in all treatment, except for Dipel2® treatments. Prolineexisted in higher concentration than other amino acids in drones, and used to some extent in oxidative metabolism under non-flight conditions Barger *et a*l. (1997). As shown in table (2), Dipel2®proved to be the lower toxic to bees. Arginine was the only amino acid found in treated samples with both Dipel2® and Sparkle®. Cysteine was noticed inthe treatments with Vertimec®and Diazinox®. Glycine was speculated only in treatments of Sparkle® and Diazinox®. In addition, the amounts of amino acids varied from treatment to another treatment even if they have the same element of amino acid (table,3).

Table 3: Effect of certain pesticides residues on the amounts of amino acids (µ molars) in bees' workers haemolymph after 24hr.of exposure.

Amino acids in (µ molars)	Side-chain polarity	Control	Achook®	Vertimec®	Dipel2x®	Sparkle®	Diazinox®
*Alanine	Nonpolar	1383.555	89.6661	0	11.2055	115.806	688.8255
*Arginine	Basic polar	3247.436	0	0	31.5354	51.6864	0
*Aspartic acid	acidic polar	161.7441	179.8768	1010.847	32.0832	0	0
*Cysteine	Nonpolar	12116.1	0	326.4381	0	0	734.4336
*Glutamic acid	acidic polar	147130	0	0	0	81.1152	0
*Glycine	Nonpolar	484.077	0	0	0	4.4168	36.1404
*Histidine	Basic polar	134150.4	111548.4	564.3953	0	782.3058	70.964
*Leucine	Nonpolar	1211.68	0	27.6565	0	0	0
*Lysine	Basic polar	2108.885	0	0	968.4188	0	40.7719
*Methionine	Nonpolar	149210	0	0	0	0	833992.2
*Phenylalanine	Nonpolar	135190	49382.23	3240.889	516.449	32.0756	0
*Proline	Nonpolar	6.2626	0	0	34.196	0	0
*Serine	Polar	4897.794	0	0	0	0	0
*Threonine	Polar	6873.746	24403.94	6596.743	4079.417	857.2539	0
*Tyrosine	Polar	45.8804	0	0	0	11.4241	0

Discussion:

Herein this study honey bees were chosen because forager's gather nectar and pollen from flowers treated with insecticides. Afterwards, they die after transferring to their louds colonies and cause polluted brood and honey by their exposure to pesticides. Insecticides can manage the environment pollution; previous studies showed that pesticides caused developmental instability in non-target insects (Hardersen, 1999 ; Frampton, 1999; Chang *et al*., 2007a, 2007b).

These results were observed in Table 2 agreement with those of (Bendahou *et al*.,1999)who indicated that long-term exposure of honey bee to cypermethrin caused significant contaminated damage to colonies. (Singh *et al*., 2007) also studied the toxicity of the synthetic Pyrethroids, Deltamethrin and Lambda-Cyhalothrin against the Italian honey bee,*A. mellifera*L., at different exposure periods. They found that the toxicity of Lambda-Cyhalothrin increased by 4 folds with increasing the exposure time from 10 min to 30 min, while in case of deltamthrin there was non-significant difference in the level of toxicity with the increase in exposure time. In contrast, Abro & Andotra (2003) stated that Endosulfan and Carbaryl were the least toxic. But Chlorpyripos, Neem oil, Quinalphos, and Malathion were moderately toxic. However, Dimethoate was highly toxic and Fenvalerate was extremely toxic to*A. mellifera*. In addition, their results also corroboratedthe findings of Parkash & Kumarswami (1984) as these declared that Endosulfan was the least toxic, Carbaryl conversely ,was moderately toxic, but the Pyrethroids achieved most toxic compound to the foragers of *A. cerana* F.

According to the National Pesticides Telecommunications Network (NPTN)(2013) and United States Environmental Protection Agency (1989), Sparkle® has a highly toxicity to bees and aquatic insects. Previous results indicated that the Sparkle® and Diazinox® were highly toxic to the bees from started the first day than the other tested pesticides.

As a matter fact, the Diazinon was known to be highly toxic to the terrestrial invertebrates, bees and other beneficial insects following acute contact exposure. The acute LD_{50} for bees was 0.22 µg/ one bee,National Pesticide Information Center, (2009).

In general, the toxicity of insecticides to honey bees was increased as theexposure time increased. However, the diazinon, abamectin and cypermethrin showed more increase in toxicity more than other tested insecticides, with the increase and /or the elapse of time after treatments followed by Fenvalerate. These results were in accordance the above mentioned our results, from the view of discuss, (Atallah *et al*., 1979) demonstrated that the mortalities of first generation of carniolan bee workers were increased with the prolongation of the exposure period to residues of certain insecticides. (Sharaf El-Din, 1982), found that the poisoning effect of insecticides on different strains of honey bee workers belonging to different ages increased by prolongation of time after treatment from 24 to 120h. Sharma & Abrol(2005) also studied the contact toxicities of five insecticides, Malathion, Cypermethrin, Deltamethrin and Fenvalerate applied to foragers of honey bee (*A. mellifera*) and *(A.*

cerana F.) they found that the mortality of honey bees increased with increase in concentration and exposure time.

In short discussion it could be mentioned that the amino acids analysis indicated 15 amino acids appeared in untreated samples, whereas five of them only were discovered in Achook® treatments. The samples referred to six, seven, eight amino acids in case of treating bees with Vertimec®,Diazinox®, Dipel2x® and Sparkle®, in respect.

As far as the amino acid are concerned there are 22 different amino acids commonly known in proteins. Proteins are composed either of wholly amino acids together with some types of molecules.

DeGroot (1953) described ten amino acids as essential for growth of honey bees in diet experiments (arginine, histidine, lysine, tryptophane, phenylalanine, methionine, threonine, leucine, isoleucine, and valine).

There are miscellaneous functions that require specific proteins. In addition, proteins provide structural support within the cell and in the cell membrane (Krap, 1979).

Since the amino acids are the main units that constitute proteins, the analysis of the free amino acids might give a clear picture about the effect of the certain pesticides on proteins via the presence, absence, increasing and/or reduction of those amino acids. A decrease in total crude protein content from newly emerged bees to 20 day old bees from 69% to 41% (dry weight) El-Shakaa and Shahein(1987).

But, Salama *et al.* (1994) found that the haemolymph of the potato tuber moth Phthorimaea (Lepidoptera: Gelechiidae) contain 17 free amino acids. This surely owing to the inspect species in haemolymph pools analyzed by paper chromatography stained with ninhydrin reagent the overall concentration of free amino acids in the haemolymph stayed consenat from days 1 to 7 and thereafter decreased generally lower values were found in foragers bees. Crailsheim and Leonhard (1997).

Recommendations:

Most pesticides are lipophilic, so they blend chemically with the hydrocarbons in beeswax and the exoskeletons of honey bees. They also become blended with the lipids in the outer layer of pollen grains. Thus, beeswax and pollens be contaminated.

Therefore, the apiarists should be aware of the chemicals danger in the adjacent fields. Growers should be advised to spray the effective bio-insecticides to prevent negative effects of other pesticides of all types, that cause plant phyto-toxicity in one side and to save bees colonies. Moreover, such bio-insecticides can be used afternoon; these precautions may reduce the negative effects of chemicals on the foragers. But the best way to protect honey bees from damage bypesticides is to keep them from being exposed to chemicals for 48 h. with taking the appropriate measures for hive aeration.

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