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IMPACTS OF MULTIPLE APPLICATIONS WITH BIOFLY (*Beauveria bassiana*) AND SPINTOR® (SPINOSAD) ON HONEY BEE (*Apis mellifera*) LARVAE

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ABSTRACT

Biofly (*Beauveria bassiana*) and SpinTor® (Spinosad) are increasingly applied as biopesticides throughout Egypt to control various agricultural pests. We investigated, in a previous study, the acute toxicities of the two biopesticides among others and their effect on acetylcholinesterase (AChE) activity of honey bee (*Apis mellifera* L.) workers. In this study, we are focusing on the acute toxicities and the potential side effects of multiple applications with biofly (*Beauveria bassiana*) and SpinTor® (Spinosad) biopesticides on AChE activity of larvae of honey bee workers. Chosen groups of young worker larvae, in a brood comb of honey bee colony, were fed once, twice or three times at 1-day intervals on sugar syrup 1:1 (w/v) containing different concentrations of the two biopesticides. The mortality percentages of treated worker larvae were determined after 24 h of one application, or two or three daily applications, and the lethal concentrations that caused 50% mortality (LC_{50}) were estimated to determine the acute toxicity of Biofly and Spinosad to worker larvae. Also, the impacts on AChE activity of larvae were determined *in vivo* after 24 and 96 h of single application or two daily applications. According to the LC_{50} values, Spinosad showed higher toxic actions to worker larvae comparable to Biofly. Also, the acute toxicity (after 24 h) of three daily applications of Biofly (1905 mg L^{-1}) or Spinosad (12.04 mg L^{-1}) was higher than the corresponding value of two daily applications (3847 and 21.45 mg L^{-1}, respectively).The same trend, the acute toxicity of two daily applications was higher than that of single application (5113 and 51.29 mg L^{-1}, respectively). Therefore, there were lethal cumulative effects of Biofly and Spinosad on worker larvae. Furthermore, our findings indicated that the average of AChE activities in larvae fed twice on sugar syrup with Biofly or Spinosad was significantly ($p > 0.05$) higher than that in larvae fed once after 24 and 96 h. Also, Biofly when found in sugar syrup at tested concentrations has activator effects after 24 h of application, and inhibitory effects after 96 h of application on AChE activity in worker larvae fed once or twice. In addition, Spinosad showed activator effect only after 24 h of single application, and inhibitory effects after 24 h of two daily applications and after 96 h of one or two daily applications on AChE activity in worker larvae.

Keywords: Honey bee, *Apis mellifera* L.,larvae, biopesticides, Biofly, *Beauveria bassiana,* SpinTor®, Spinosad*,* toxicity, Acetylcholinesterase.

INTRODUCTION

Honey bees are a vital part of our agricultural system. The increased use of pesticides, reduction in the number of wild colonies, and the increased value of both bees and the crops they pollinate have all added to the importance of protecting bees from pesticides (Krupke *et al.,* 2012). Honey bees, as domesticated pollinators, may be constantly exposed to pesticides such as biopesticides whenever their colonies are sited in agricultural areas (Weick and Thorn 2002), and also when their pests are controlled inside bee hives.Unfortunately, even pesticides approved for organic agriculture can

cause significant harm to honey bees. Colony Collapse Disorder (CCD) appears to be a multifactorial syndrome and multiple causes have been proposed such as pests, pathogens, chemical pesticides, GM crops (Cox-Foster and VanEngelsdorp 2009; VanEngelsdorp *et al.,* 2009; VanEngelsdorp and Meixner 2010). However, negative effects of these compounds on the honey bee (*Apis mellifera* L.), the most important pollinator for cultivated ecosystem, remained poorly investigated (Eid *et al.,* 2011).Biopesticides such as Biofly [(*Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales)] and SpinTor® (Spinosad) are increasingly produced and applied throughout Egypt in pest management programs to control various agricultural pests.

The entomopathogenic fungus *Beauveria bassiana* is a promising biological control agent of tomato spider mite (Bugeme *et al.*, 2008) and several greenhouse pests. Furthermore, *Beauveria bassiana* can be vectored effectively by bumble bees to control some greenhouse pests (Kevan *et al.,* 2005; Al-mazra'awi *et al.,* 2006a; Kapongo *et al.* 2008).For outdoor crops, honey bees have been shown capable to deliver *B. bassiana* for *Lygus lineolaris* (Al-mazra'awi*et al.,* 2006b). Furthermore, the use of entomopathogenic fungi such as *B. bassiana* has been considered promising alternative to chemical miticides (Chandler *et al.* 2000 and 2001) used for Varroa mites control (Meikle *et al.*, 2007 and 2008 a, b).Meikle *et al.* (2008 a, b) did not found measurable negative impact on colony health or survivorship after application with a formulation containing *B. bassiana* conidia. Also, this fungus has been reported to be extremely virulent to alfalfa leafcutter bees, resulting in >87% mortality after 10 days. It likely has potential to harm all bees, and should be avoided as a pest control option where pollinators are present (EPA, 1999). Furthermore, recent study indicated that Biofly is non-toxic to adult honey bee workers with LC_{50} of 49,766 mg L^{-1} (Eid*et al.,* 2011).

Spinosad, the active ingredient in SpinTor®, is a fast-acting, somewhat broad-spectrum biopesticide that acts on the insect through ingestion (the primary mode), or by direct contact with a spray droplet or a newly treated surface (Larson, 1997). The active ingredients in Spinosad, spinosyn A and spinosynD, are complex organic compounds made by soil microbes. There are a number of products currently on the market that contain Spinosad, and more are being developed every year. Spinosad is a powerful neurotoxin against certain arthropods, especially lepidopteran larvae, coleopterans, some dipterans, thysanoptera and hymenoptera (Bret *et al.,* 1997; Mayes *et al.,* 2003). Spinosad is considered to be a selective pesticide since it exhibits little or no effect on most beneficial insects. The risk of impact to the environment was considered as acceptable, as the U.S. Environmental Protection Agency (EPA) categorized Spinosad as a 'reduced risk product' in 1997 but it is highly toxic to bees in laboratory tests (EPA, 1997 a, b; Cleveland *et al.,* 2001). Glasshouse and semifield studies have demonstrated that dried residues are not acutely toxic, and although pollen and nectar from sprayed plants may have transient effects on brood development, the residues do not overtly affect hive viability of honey bee. Field studies have demonstrated that Spinosad has low risk to adult honeybees and has little or no effect on hive activity and brood development

(Mayes *et al.,* 2003;Miles*et al.,* 2011). An evaluation by Morandin *et al.* (2005) showed detrimental effects of sublethal doses of Spinosad when bumble bee larvae were fed pollen contaminated with Spinosad. Furthermore, recent reports indicated that Spinosad is highly toxic to adult honey bee workers with LC_{50} of 7.34 mg L^{-1} (Rabea *et al.,* 2010) and 11.60 mg L^{-1} (Eid *et al.,* 2011). It has been approved for use in organic agriculture by numerous certification bodies worldwide (Racke, 2007). Spinosad is currently being registered in Egypt as a safer alternative to synthetic pesticides (Aboul-Enein *et al*., 2012).

Mader (2009) listed *Beauveria bassiana* and Spinosad in highly toxic category to honey bee. He showed that bees are poisoned by insecticides when they absorb toxins through their exoskeleton, drink tainted nectar or contaminated water), or when insecticidal dusts become trapped in their pollen-collecting hairs. In addition to directly killing adult bees, insecticides may be carried back to the nest in contaminated pollen or nectar and fed to developing brood. Where this brood food or vegetation is contaminated, larval mortality may occur. Finally, rather than directly killing bees, some insecticides have detrimental sub-lethal effects. Biomarkers have been used to reveal the exposure of organisms to various chemicals in the environment (Hyne and Maher, 2003).

Little has been reported on the side effects of biopesticides on honey bees and little information based on the enzymatic aspects of the host after exposure to biopesticides has been given in literature (Eid *et al.,* 2011). In order to further assess their toxic effects to honey bee larvae, this study are focusing on the acute toxicities and the potential side effects of multiple applications of Biofly (*B. bassiana*), and SpinTor® (Spinosad) on acetylcholinesterase (AChE) activity of worker larvae. Our experiments demonstrate a method for testing biopesticide effects on worker larvae in their cells under field conditions by adding certain amounts of sugar syrup with tested concentrations of biopesticide onto their food (worker jelly for young larvae or modified worker jelly for old ones). We hypothesized that at tested concentrations, mortality and AChE activity of worker larvae would be affected by Biofly and Spinosad.

MATERIALS AND METHODS

Chemicals

The two biopesticides tested were Biofly (*Beauveria bassiana*) containing $30x10^6$ conidia/cm^3 (supplied by El-Nasr Co. for Fertilizaers and Biopesticides, El-Sadat city, Egypt), and SpinTor® 24% SC (Spinosad) (supplied by Dow Agro Sciences Co., England). Acetylthiocholine iodide (ATChI), 5, 50-dithio-bis (2-nitrobenzoic) acid (DTNB) and Folin-Ciocalteu phenol regent were purchased from Sigma-Aldrich Chemical Co., USA.

Honey bees, *Apis mellifera*

Local hybrid (derived from Italian bee *Apis mellifera ligustica* and Carniolan bee *Apis mellifera carnica* and other races existed in Egypt) honey bee colony (free of obvious diseases) from an apiary located in Ezbet

Haggag at El-Beheira Governorate were used. Application of worker larvae was conducted in the field on March. Open brood comb (in the experimental colony) filled with young larvae (about one-day old) was used for this experiment.

Acute Toxicity Assessment

The acute toxicities of the two biopesticides; Biofly (*Beauveria bassiana*) and SpinTor® (Spinosad) were evaluated on larvae of honey bee workers by oral administration under field conditions after 24 h of one application, two and three daily applications. Stock solutions of biopesticides were prepared in sugar syrup 1:1 (w/v). The application was made through feeding on sugar syrup containing different concentrations of the tested biopesticides. Tested concentrations were 93.75, 187.5, 375, 750 and 1500 mg L^{-1} for Biofly; and 1.25, 2.5, 5, 10 and 20 mg L^{-1} for Spinosad. Three groups of 10 worker larvae were subjected to each concentration of each biopesticide (one application, or two or three daily applications) and control. Each treated worker larva was fed by adding 20 µl sugar syrup 1:1 (w/v) containing the biopesticide on its worker jelly or modified worker jelly. Each untreated worker larva was fed sugar syrup alone as control. Worker larva was considered dead if it has been removed by adult workers.The numbers of dead larvae were recorded after 24hours of one application, two and three daily applications to calculate mortality percentages and the LC_{50} of each biopesticide according to Finney (1971).

Preparation of Bee Extract

All steps were carried out between 0 and 4 °C. All treatments were replicate three times. Three groups of three worker larvae for each concentration of tested biobesticides (one application or two daily applications) and control were collected from their cells by forceps and put in an Eppendorf Tube and frozen in an Ice box. Then, they transported to the laboratory and each group (containing 3 larvae) was immediately homogenized using hand glass Homogenizer under cooling with 0.1 M phosphate buffer (pH 7.0). The homogenates were then centrifuged at 5,000 rpm for 20 min. at 0°C. The supernatants were used as enzyme source for assay of AChE activity of worker larvae.

Total Protein Assay

This assay was accomplished following the method of Lowry *et al.*, (1951).

Acetylcholinesterase (AChE) Activity Assay

The AChE activity assay (in vivo) was carried out following the method of Ellman *et al.* (1961) using acetylthiocholine as a substrate utilizing spectrophotometric procedure. Tested concentrations were 93.75, 187.5, 375, 750 and 1500 mg L^{-1} for Biofly; and 1.25, 2.5, 5, 10 and 20 mg L^{-1} for Spinosad. Three groups of 10 worker larvae were subjected to each concentration of each biopesticide (one application or two daily applications) and control. Each worker larva (of one application) was applied in its cell with 20 µL sugar syrup 1:1 (w/v) with one concentration of Biofly or Spinosad. Each worker larva (of two applications) was applied as mentioned above and another time after 24 h. Controls were applied with the sugar syrup alone. The activity, specifically attributable to AChE in worker larvae was determined

by using DTNB (dithionitrobenzoic) after 24 and 96 h of one or two feedings on sugar syrup 1:1 (w/v) with one concentration of Biofly or Spinosad. The supernatants were used as enzyme source for assay of AChE activity. Enzyme (150 µL), 100 µL DTNB (0.01 M), and 30µLATChI (0.075 M) were added to 2.8 mL 0.1 M phosphate buffer (pH 8.0). The mixture was incubated at 37 °C for 15 min. Absorbance measurements were conducted at a wavelength of 412 nm using the Jenway 6305 spectrophotometer. The specific activity of AChE was expressed as nmoles of acetylthiocholine iodide hydrolyzed/mg protein/min. Inhibition or activation percentages of the activities against control were considered in the enzymatic assay.

Statistical Analysis

The mortality data was recorded after 24 h. of one application, and two or three daily applications. All the LC_{50} were calculated according to (Finney 1971). Data of effects of biopesticides on AChE activity experiment was subjected to two-way analysis of variance (ANOVA). The experiment of AChE activity of worker larvae was conducted in factorial (2x5). The experimental design was the completely randomized design with three replicates. Comparisons among means were made using the Least Significant Difference test (L.S.D.) at 5% level of probability with the aid of the SAS program (SAS Institute, 2000) version 8.0.

RESULTS

Acute Toxicity of Tested Biopesticides to Larvae of Honey Bee Workers

The results of acute toxicity assay of Biofly and Spinosad on worker larvae by oral administration under field conditions after 24 h of one application, or two or three daily applications are summarized in Tables (1and 2, in respect). On the basis of LC_{50} values, Spinosad showed higher toxic actions to worker larvae when compared with Biofly. Also, the acute toxicity (after 24 h) of three daily applications of Biofly (1905mg L^{-1}) or Spinosad (12.04 mg L^{-1}) was higher than the corresponding value of two daily applications (3847 and 21.45 mg L^{-1}, in respect). In the same pattern, the acute toxicity of two daily applications was higher than that of single application (5113 and 51.29 mg L^{-1}, in respect). Therefore, there were lethal cumulative effects of Biofly and Spinosad on worker larvae.

Table1: Mortality of worker larvae and acute toxicityof Biofly after 24 h of one application (I), or two (II) or three daily applications (III).

Concentration (mg L^{-1})	Mortality (%)		
	I	II	III
93.75	3.33	10.00	13.33
187.5	6.67	16.67	20.00
375	10.00	23.33	26.67
750	16.67	30.00	36.67
1500	30.00	36.67	46.67
LC_{50} (mg L^{-1})	5113	3847	1905
Slope ± SE	1.08±0.35	0.76±0.28	0.85±0.27
χ^2 *	0.17	0.09	0.01

*** Chi square**

Table 2: Mortality of worker larvae and acute toxicityof Spinosad after 24 h of one application (I), or two (II) or three daily applications (III).

Concentration (mg L^{-1})	Mortality (%)		
	I	II	III
1.25	10.00	20.00	26.67
2.5	16.67	26.67	33.33
5	23.33	36.67	40.00
10	30.00	43.33	46.67
20	36.67	46.67	56.67
LC_{50} (mg L^{-1})	51.29	21.45	12.04
Slope ± SE	0.76±0.28	0.65±0.25	0.64±0.25
χ^2 *	0.09	0.24	0.04

*** Chi square**

Impacts of Tested Biopesticides on AChE Activity in Larvae of Honey Bee Workers

The *in vivo* specific activity and inhibition or activation of AChE activity in worker larvae after 24 and 96 hours of single feeding or two daily feedings on sugar syrup 1:1 (w/v) with different concentrations of biopesticides were calculated and presented in Tables 3 and 4 (Biofly), and 5 and 6 (Spinosad), in respect. Data of specific activity are presented in units of nmoles of acetylthiocholine iodide hydrolyzed/mg protein/min., while those of inhibition or activation are expressed as percentages.

Data of Biofly (after 24 h) are summarized and presented in Table 3. Mean values of AChE activity in worker larvae fed twice were larger than those in larvae fed once on sugar syrup 1:1 (w/v) with 0, 93.75, 187.5, 375, 750 and 1500 mg L^{-1} of Biofly, in respect. Also, the average of AChE activities in larvae fed twice (80.55 nmoles ATChI hydrolyzed/mg protein/min.) was significantly ($p > 0.05$) higher than that in worker larvae fed once (21.17). When data of one and two applications were summed to estimate the effects of concentrations, all treatments resulted in various increases in AChE activity compared with the control. The means of AChE activities in worker larvae fed on sugar syrup 1:1 (w/v) with 93.75, 187.5, 375, 750 or 1500 mg L^{-1} of Biofly were 66.53, 62.14, 40.35, 47.54 and 50.24 nmoles ATChI hydrolyzed/mg protein/min., in respect, compared with 38.34for untreated larvae. Statistical analysis showed that the AChE activities in treated larvae were significantly ($p > 0.05$) higher than that in untreated larvae, except in those treated with 375 mg L^{-1}. In regard to the inhibition or activation of the enzyme activity, the low concentrations (93.75 and 187.5 mg L^{-1}) caused high degrees of activation in AChE activity of larvae fed once, while the high concentrations (375, 750 and 1500 mg L^{-1}) caused different degrees of inhibition. On the other hand, when worker larvae were fed twice, all concentrations resulted in different degrees of activation. Also, the average of activations in AChE activity ofworker larvae fed twice (75.01 %) was significantly ($p > 0.05$) higher than that of larvae fed once (24.70 %). When data of one and two applications were summed, the low concentrations (93.75 and 187.5 mg L^{-1}) caused significant ($p > 0.05$) activations in AChE

activity of larvae (178.44 and 115.67 %, respectively), while the other concentrations caused insignificant activations. Therefore, Biofly when found in sugar syrup at concentration of 93.75, 187.5, 375, 750 or 1500 mg L^{-1} has an activator effect on AChE activity in worker larvae after 24 h of application.

Table 3: *In vivo* effects on AChE activity in larvae of honey bee workers after 24 hours of one feeding or two daily feedings on sugar syrup with Biofly.

Concentration (mg L^{-1})	Biofly- 24 h			
	nmoles ATChI hydrolyzed /mg protein/min.		Inhibition (%)	
	Mean±SD	Average	Mean±SD	Average
One feeding				
0.0	12.10±2.02	21.17 b*	0.00±0.00	-24.70 a
93.75	52.22±4.57		-331.73±37.80	
187.5	35.56±3.85		-193.98±31.81	
375	10.91±0.49		9.80±4.09	
750	9.28±0.68		23.27±5.64	
1500	6.95±0.98		42.58±8.13	
Two feedings				
0.0	64.59±3.30	80.55 a	0.00±0.00	-75.01 b
93.75	80.83±0.98		-25.15±1.52	
187.5	88.73±1.10		-37.37±1.70	
375	69.80±0.97		-8.06±1.50	
750	85.80±0.47		-32.84±0.72	
1500	93.53±1.18		-44.80±1.82	
Total	Mean		Mean	
0.0	38.34 e	50.86	0.00 a	-49.86
93.75	66.53 a		-178.44 c	
187.5	62.14 b		-115.67 b	
375	40.35 e		-0.87 a	
750	47.54 d		-4.79 a	
1500	50.24 c		-1.11 a	

*** Means in the same column followed by the same letter(s) are not significantly different according to L.S.D test at 0.05 level of probability.**
(-) before mean indicate that there is activation in AChE activity.

Data of Biofly (after 96 h) are summarized and presented in Table 4. Mean values of AChE activity in larvae fed twice were larger than those in larvae fed once on sugar syrup 1:1 (w/v) with 0, 93.75, 187.5, 375, 750 and 1500 mg L^{-1} of Biofly, in respect. Also, the average of AChE activities in larvae fed twice (34.93nmolesATChI hydrolyzed/mg protein/min.) was significantly ($p > 0.05$) higher than that in larvae fed once (11.35). When data of one and two applications were summed to estimate the effects of concentrations, all treatments resulted in various decreases in AChE activity compared with the control. The means of AChE activities in larvae fed on sugar syrup 1:1 (w/v) with 93.75, 187.5, 375, 750 or 1500 mg L^{-1} of Biofly were 22.18, 13.13, 11.39, 9.22and 11.78 nmoles ATChI hydrolyzed/mg protein/min., in respect, compared with 71.14 for untreated larvae. Statistical analysis showed that the AChE activities in treated larvae were significantly ($p > 0.05$) lower than that in untreated larvae. In regard to the inhibition or activation of the enzyme activity, all concentrations caused deferent degrees

of inhibition in AChE activity of larvae fed once or twice. Also, the average of inhibitions in AChE activity of larvae fed twice (72.61 %) was significantly ($p > 0.05$) higher than that of larvae fed once (23.01 %). When data of one and two applications were summed, all concentrations caused significant ($p > 0.05$) inhibitions in AChE activity of larvae. Therefore, Biofly when found in sugar syrup at concentration of 93.75, 187.5, 375, 750 or 1500 mg L^{-1} has an inhibitory effect on AChE activity in worker larvae after 96 h of application.

Table 4: *In vivo* effects on AChE activity in larvae of honey bee workers after 96 hours of one feeding or two daily feedings on sugar syrup with Biofly.

Concentration (mg L^{-1})	Biofly- 96 h			
	nmoles ATChI hydrolyzed /mg protein/min.		Inhibition (%)	
	Mean±SD	Average	Mean±SD	Average
One feeding				
0.0	14.74±3.22	11.35 b	0.00±0.00	23.01 b
93.75	13.14±0.55		10.85±3.74	
187.5	12.69±0.99		13.90±6.73	
375	9.65±0.23		34.57±1.56	
750	9.44±1.41		35.96±9.59	
1500	8.44±0.32		42.76±2.15	
Two feedings				
0.0	127.53±15.99	34.93 a	0.00±0.00	72.61 a
93.75	31.21±1.79		75.53±1.41	
187.5	13.58±2.97		89.36±2.33	
375	13.14±1.20		89.69±0.94	
750	9.01±1.10		92.94±0.86	
1500	15.12±1.86		88.14±1.46	
Total	Mean		Mean	
0.0	71.14 a	23.14	0.00 d	47.81
93.75	22.18 b		43.19 c	
187.5	13.13 c		51.63 b	
375	11.39 c		62.13 a	
750	9.22 c		64.45 a	
1500	11.78 c		65.45 a	

*** Means in the same column followed by the same letter(s) are not significantly different according to L.S.D test at 0.05 level of probability.**
(-) before mean indicate that there is activation in AChE activity.

Data of Spinosad (after 24 h) are summarized and presented in Table 5. Mean values of AChE activity in larvae fed twice were larger than those in larvae fed once on sugar syrup 1:1 (w/v) with 0, 1.25, 2.5, 5, 10 and 20 mg L^{-1} of Spinosad, in respect. Also, the average of AChE activities in larvae fed twice (63.18nmolesATChI hydrolyzed/mg protein/min.) was significantly ($p > 0.05$) higher than that in larvae fed once (18.52). When data of one and two applications were summed to estimate the effects of concentrations, all treatments resulted in various increases in AChE activity compared with the control, except in those treated with 10 mg L^{-1}. The means of AChE activities in larvae fed on sugar syrup 1:1 (w/v) with 1.25, 2.5, 5, 10 or 20 mg L^{-1} of Spinosad were 45.21, 39.49, 30.53, 44.75 and 46.77nmolesATChI hydrolyzed/mg protein/min., in respect, compared with 38.34for untreated larvae. Statistical analysis showed that the AChE activities in treated larvae were significantly ($p > 0.05$) higher than that in untreated larvae, except in the cases of2.5 mg L^{-1}(with insignificant increase) and 5 mg

L^{-1}(with significant decrease). In regard to the inhibition or activation of the enzyme activity, the low concentration (1.25 mg L^{-1}) caused slight degree of inhibition in AChE activity of larvae fed once, while the higher concentrations (2.5, 5, 10 and 20 mg L^{-1}) caused different degrees of activation. On the other hand, when larvae were fed twice, the low concentrations (1.25 and 2.5 mg L^{-1}) caused slight degrees of activation in AChE activity, while the higher concentrations (5, 10 and 20 mg L^{-1}) caused different degrees of inhibition.
Also, the average of inhibitions in AChE activity of larvae fed twice (2.18 %), while the average of activations in AChE activity of larvae fed once (53.10 %). When data of one and two applications were summed, all concentrations caused significant ($p > 0.05$) activations in AChE activity of larvae, except in the cases of 2.5 mg L^{-1} (with insignificant activation) and 5 mg L^{-1} (with insignificant inhibition). Therefore, generally Spinosad when found in sugar syrup at concentration of 1.25, 2.5, 5, 10 or20 mg L^{-1} has an activator effect on AChE activity in worker larvae after 24 h of application.

Table 5: *In vivo* effects on AChE activity in larvae of honey bee workers after 24 hours of one feeding or two daily feedings on sugar syrup with Spinosad.

Concentration (mg L-1)	Spinosad-24 h			
	nmoles ATChI hydrolyzed /mg protein/min.		Inhibition (%)	
	Mean±SD	Average	Mean±SD	Average
One feeding				
0.0	12.10±2.02	18.52 b	0.00±0.00	-53.10 b
1.25	11.10±0.65		8.22±5.41	
2.5	12.73±15.18		-5.23±3.57	
5	15.18±0.41		-25.47±3.41	
10	27.50±0.71		-127.39±5.86	
20	32.50±0.70		-168.74±5.80	
Two feedings				
0.0	64.59±3.30	63.18 a	0.00±0.00	2.18 a
1.25	79.33±0.92		-22.81±1.43	
2.5	66.25±1.37		-2.57±2.11	
5	45.89±2.58		28.95±3.99	
10	62.00±1.14		4.01±1.76	
20	61.03±1.41		5.52±2.18	
Total	Mean		Mean	
0.0	38.34 c	40.85	0.00 ab	-25.46
1.25	45.21 ab		-7.30 c	
2.5	39.49 c		-3.90 bc	
5	30.53 d		1.74 a	
10	44.75 b		-61.69 d	
20	46.77 a		-81.61 e	

*** Means in the same column followed by the same letter(s) are not significantly different according to L.S.D test at 0.05 level of probability.**
(-) before mean indicate that there is activation in AChE activity.

Data of Spinosad (after 96 h) are summarized and presented in Table 6. Mean values of AChE activity in larvae fed twice were larger than those in larvae fed once on sugar syrup 1:1 (w/v) with 0, 1.25, 2.5, 5, 10 and 20 mg L^{-1} of Spinosad, in respect. Also, the average of AChE activities in larvae fed twice (46.09 nmoles ATChI hydrolyzed/mg protein/min.) was significantly ($p > 0.05$) higher than that in larvae fed once (13.99). When data

of one and two applications were summed to estimate the effects of concentrations, all treatments resulted in various decreases in AChE activity compared with the control. The means of AChE activities in larvae fed on sugar syrup 1:1 (w/v) with 1.25, 2.5, 5, 10 or 20 mg L^{-1} of Spinosad were 27.58, 20.65, 21.58, 23.51 and 15.78 nmoles ATChI hydrolyzed/mg protein/min., in respect, compared with 71.14for untreated larvae. Statistical analysis showed that the AChE activities in treated larvae were significantly ($p > 0.05$) lower than that in untreated larvae. In regard to the inhibition or activation of AChE activity, the low concentrations (1.25, 2.5 and 5 mg L^{-1}) caused various degrees of inhibition in AChE activity of larvae fed once, while the higher concentrations (10 and 20 mg L^{-1}) caused various degrees of activation. On the other hand, when larvae were fed twice, all concentrations caused various degrees of inhibition in AChE activity. Also, the average of inhibitions in AChE activity of larvae fed twice (63.86 %) was significantly higher than that in those fed once (5.12 %). When data of one and two applications were summed, all concentrations caused significant ($p > 0.05$) inhibitions in AChE activity of larvae. Therefore, generally Spinosad when found in sugar syrup at concentration of 1.25, 2. 5, 5, 10 or 20 mg L^{-1} has an inhibitory effect on AChE activity in worker larvae after 96 h of application.

Table 6:*In vivo* effects on AChE activity in larvae of honey bee workers after 96 hours of one feeding or two daily feedings on sugar syrup with Spinosad.

Concentration (mg L-1)	Spinosad-96 h			
	nmoles ATChI hydrolyzed /mg protein/min.		Inhibition (%)	
	Mean±SD	Average	Mean±SD	Average
One feeding				
0.0	14.74±3.22	13.99 b	0.00±0.00	5.12 b
1.25	7.55±0.65		48.75±4.43	
2.5	9.82±2.09		33.39±14.20	
5	12.70±1.13		13.86±7.69	
10	15.29±0.37		-3.70±2.54	
20	23.82±3.07		-61.56±20.83	
Two feedings				
0.0	127.53±15.99	46.09 a	0.00±0.00	63.86 a
1.25	47.61±8.27		62.67±6.49	
2.5	31.48±4.01		75.31±3.14	
5	30.47±3.50		76.11±2.75	
10	31.74±10.42		75.11±8.17	
20	7.75±2.20		93.92±1.73	
Total	Mean		Mean	
0.0	71.14 a	30.04	0.00 e	34.49
1.25	27.58 b		55.71 a	
2.5	20.65bc		54.35 ab	
5	21.58 bc		44.99 bc	
10	23.51 b		35.71 c	
20	15.78 c		16.18 d	

*** Means in the same column followed by the same letter(s) are not significantly different according to L.S.D test at 0.05 level of probability.**
(-) before mean indicate that there is activation in AChE activity.

When the AChE activities (after 24 h) of worker larvae treated with field application rates of Biofly and Spinosad (1500 and 20 mg L^{-1}, in respect) were compared, AChE activity of worker larvae treated twice with Biofly and

Spinosad (93.53 and 61.03 nmoles ATChI hydrolyzed/mg protein/min, respectively) were higher than those treated once (6.95 and 32.50 nmoles ATChI hydrolyzed/mg protein/min., respectively). Also, Biofly caused an inhibition of 44.80 % in AChE activity, while Spinosad caused an activation of 168.74% when worker larvae fed once. On contrary, Biofly caused an activation of 42.58 % in AChE activity, while Spinosad caused an inhibition of 5.52 % when worker larvae fed twice.On the other hand, comparing the AChE activities (after 96 h) of worker larvae treated with field application rates of the two biopesticides indicated that AChE activity of worker larvae treated twice with Biofly and Spinosad (15.12 and 23.82 nmoles ATChI hydrolyzed/mg protein/min., respectively) were higher than those treated once (8.44 and 7.75 nmoles ATChI hydrolyzed/mg protein/min., respectively) as observed after 24 h of application, but with lower values. Also, Biofly caused an inhibition of 42.76 % in AChE activity, while Spinosad caused an activation of 61.56 % when worker larvae fed once. When worker larvae fed twice, the two biopesticides caused inhibitions of 88.14 for Biofly and for Spinosad 93.92 % in AChE activity.

DISCUSSION

Biopesticides are now considered as the safest insecticides and environmentally-friendly substitutes of broad-spectrum, synthetic insecticides (Afify *et al.,* 2009). Currently the development of biological or non-chemical pesticides as replacements for conventional pesticides is an important strategy (Akdeniz and Özmen, 2011). Biopesticides such as Biofly [(*Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales)] and SpinTor® (Spinosad) are increasingly produced and applied throughout Egypt in pest management programs to control various agricultural pests. Larval bees feeding on exogenous pollen and exposed to pesticides during development may result in lethal or sublethal effects during the adult stage. Exposure in earlier life stages could affect development, resulting in negative impacts that would only be evident if studies were of long enough duration to monitor adult behaviour following larval exposure(Morandin *et al*., 2005). Studies based on toxicity and enzymatic aspects of worker larvae after multiple exposures to biopesticides remained poorly investigated. *B. bassiana* has been isolated from approximately 0.2% of varroa mites collected from a number of apiaries in southern France (Meikle *et al.* 2006) and reported in southern Spain (García-Fernández*et al.,* 2008). Steenberg *et al.* (2010) recorded *B. bassiana* on varroa mites, *Varroa destructor*, from capped worker brood cells of honey bee in Denmark. In addition, Spinosad has been used all over the world for more than ten years in a wide range of crops without a recorded incident to pollinators. The feedback from field experiments is of particular importance as it reflects the potential impact of products on honey bees' inrealistic exposure conditions, which include interactions with other factors. Therefore, the current study was carried out to focus on the acute toxicities and the potential side effects of multiple applications with Biofly (*B. bassiana*), and SpinTor® (Spinosad) on acetylcholinesterase (AChE) activity of worker larvae under field conditions.

Our findings of Biofly indicate that it is considered safe to worker larvae according to our results of toxicity after one, or two or three daily applications. This result confirmed the findings of Meikle *et al.* (2008 a, b and 2009) who found that successive applications of formulation containing *B. bassiana* conidia did not have measurable negative impact on sealed brood, colony health or survivorship. The fungi invade their host insects through the external cuticle. Spores of the fungi attach to the cuticle, germinate and penetrate the host. They then proliferate in the host haemocoel as walled hyphal bodies or wall-less amoeboid protoplasts. The host insects die as a result of several modes of action, including depletion of nutrients, physical obstruction or invasion of organs, and toxinosis (Hajek 1997; Butt and Goettel 2000).Also, Vandenberg (1990) reported that *B. bassiana* reduced honey bee longevity at concentrations of 9×10^7 and 9×10^8 spores/bee when sprayed on the bees or mixed in 50% sucrose syrup and fed to the bees. Goettel and Jaronski (1997) found that no infection was detected in the brood when treated with the equivalent of 5×10^{13} conidia of *B. bassiana* per hectare of canola three times at 5-day intervals. One of the most important abiotic factors for the entomopathogenic fungi is temperature since it affects its metabolism by altering the production processes of enzymes, toxins, spore germination, development of the germinative tube, penetration, colonization, and reproduction (Alves, 1998). Summer temperatures in the colony brood area are usually maintained between 32 and 36 ºC which are too high for germination and development of *B. bassiana* (Le Conte *et al.,* 1990; Ekesi *et al.,* 1999), while in the rest of the beehive vary from 28 to 33 ºC. In winter, in temperate regions, temperature is maintained between 20 and 30 ºC (Chandler *et al*., 2001). The optimal temperature for growth of *B. bassiana* is between 20 and 25 ºC, in which vegetative growth is inhibited and usually ceases at 37 ºC (Inglis *et al*., 2006).An entomopathogenic fungus may be less efficacious because of poor germination at the high temperatures inside brood cells. But, the temperatures encountered in this warm area of a nest are not detrimental to the fungus while colonizing its host mite (Steenberg *et al.,* 2010).However, the use of an entomopathogenic fungus inside hives involves some risk.

In regard to Spinosad, our results were in accordance with those obtained by Mayes *et al.* (2003), Miles (2003), EPA (1997 a, b), Rabea *et al.* (2010) and Eid *et al.* (2011) who found high toxic effect of it to adult honey bees in acute oral and contact toxicity studies. These previous studies, under laboratory conditions, showed that it caused high mortality percentages of adult honey bee workers with LC_{50}'s ranged from 7.34 mg L^{-1} (Rabea *et al.,* 2010) to 11.60 mg L^{-1} (Eid *et al.,* 2011). On contrary, field studies indicated that dry residues of Spinosad were safe to foraging worker honey bees, with no adverse effects seen on mortality or brood queen (Mayes *et al.*, 2003; Miles, 2003). But, this study illustrated the potential adverse effects of multiple daily applications with Spinosad to worker larvae in their cells under field conditions. Three daily applications with Spinosad resulted in LC_{50} of 12.04 mg L^{-1} almost as obtained, after one application, in adult workers in our previous study (Eid *et al.,* 2011). This may due to the dilution in tested concentrations of Spinosad by worker jelly or modified worker jelly fed to

worker larvae inside their cells. Spinosad is toxic to bees when wet, but is relatively safe for them once it dries, so it should be used when foragers are not active (Boucher, 1999). It is both a nerve poison and a stomach poison, so it kills insects that it contacts and those that consume it with their food. Furthermore, it activates the nervous system of the insect, causing loss of muscle control. Continuous activation of motor neurons causes insects to die of exhaustion within 1-2 days (Larson, 1997). Moreover, Spinosad causes activation of the nicotinic acetylcholine receptors and alters the function of GABA-gated chlorine channels (Salgado, 1998; Miles, 2003). Over-activation of the acetylcholine receptors is the primary cause of death, initially resulting in involuntarymuscle contractions and tremors and, after long periods of exposure, paralysis (Salgado, 1998).In other words, Spinosad overstimulates nerve cells by prolonging electrical impulses across synapses by acting like acetylcholine (but attaching at some novel action site as yet unidentified). Thus, acetylcholinesterase does not stop the impulse and nerve stimulation, as is supposed to happen. This in turn over-activates receptor sites in muscles producing contractions, tremors and paralysis from which the insect does not recover. Feeding stops within minutes and death occurs within 48 hours (Boucher, 1999). Sublethal exposures may also adversely affect colony function (Currie, 1999). So, any biopesticide increases bee mortality or/and has sublethal effects such as inhibiting or activating AChE activity of worker larvae may also adversely affects colony strength and performance.

CONCLUSIONS

The present study was carried out to investigate the acute toxicities and the potential side effects of multiple applications with two biopesticides; Biofly (*Beauveria bassiana*) and SpinTor® (Spinosad) on AChE activity of larvae of honey bee (*Apis mellifera* L.) workers. The mortality percentages of treated worker larvae were determined after 24 h of one application, or two or three daily applications under field conditions, and the lethal concentrations that caused 50% mortality (LC_{50}) were estimated to determine the acute toxicity of Biofly and Spinosad to worker larvae. Also, the impacts on AChE activity of larvae were determined *in vivo* after 24 and 96 h of single application or two daily applications. Our results, on the basis of LC_{50} values, indicated that Spinosad showed higher toxic actions to worker larvae when compared with Biofly. Also, the acute toxicity (after 24 h) of three daily applications of Biofly (1905mg L^{-1}) or Spinosad (12.04 mg L^{-1}) was higher than the corresponding value of two daily applications (3847 and 21.45 mg L^{-1}, in respect). In the same pattern, the acute toxicity of two daily applications was higher than that of single application (5113 and 51.29 mg L^{-1}, in respect). Therefore, there were lethal cumulative effects of Biofly and Spinosad on worker larvae. Furthermore, our findings indicated that the average of AChE activities in larvae fed twice on sugar syrup with Biofly or Spinosad was significantly ($p > 0.05$) higher than that in larvae fed once after 24 and 96 h. Also, Biofly when found in sugar syrup at tested concentrations has activator effects after 24 h of application, and inhibitory effects after 96 h of application on AChE activity in worker larvae fed once or twice. In addition, Spinosad

showed activator effect only after 24 h of single application, and inhibitory effects after 24 h of two daily applications and after 96 h of one or two daily applications on AChE activity in worker larvae. Our findings of Biofly indicate that it is considered safe to worker larvae according to our results of toxicity after one, or two or three daily applications. However, the use of an entomopathogenic fungus inside hives involves some risk. This study illustrated the potential adverse effects of multiple daily applications with Spinosad to worker larvae in their cells under field conditions. Three daily applications with Spinosad resulted in LC_{50} of 12.04 mg L^{-1} almost as obtained, after one application, in adult workers in our previous study. This may due to the dilution in tested concentrations of Spinosad by worker jelly or modified worker jelly fed to worker larvae inside their cells. Thus, any biopesticide increases bee mortality or/and has sublethal effects such as inhibiting or activating AChE activity of worker larvae may also adversely affects colony strength and performance. Nevertheless, new research studies are required to ensure its impacts on nurse and field honey bee workers.

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تــأثير المعــاملات المتعــددة بمبيــدي البيــوفلاى(Beauveria bassiana) ،سبينتور ((Spinosad على يرقات شغالات نحل العسل

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مبيدى البيوفلاى (*Beauveria bassiana*)، وسبينتور(Spinosad) من المبيدات الحيوية للآفات التى تطبق بزيادة مضطردة فى مصر لمكافحة آفات زراعية مختلفة. وفى دراسة سابقة تم دراسة السمية الحادة لهما ضمن مبيدات حيوية أخرى وكذلك تأثيراتهما على نشاط إنزيم الأسيتيل كولين إستيريز (AChE) لشغالات نحل العسل (*Apis mellifera* L.). ونركز فى هذه الدراسة على السميات الحادة والتأثيرات السلبية المحتملة للمعاملات المتعددة بهذين المبيدين علىنشاط إنزيم الأسيتيل كولين إستيريز (AChE)ليرقات شغالات نحل العسل. وتم تغذية مجاميع مختارة من يرقات صغيرة للشغالات فى أحد أقراص الحضنة لطائفة نحل عسل مرة واحدة أو مرتين أو ثلاث مرات بفاصل زمنى 1 يوم على محلول سكرى 1:1 (وزن/ حجم) يحتوى على تركيزات مختلفة من المبيدين الحيويين تحت الدراسة.وتم تحديد النسب المئوية لموت اليرقات المعاملة بعد 24 ساعة من معاملة واحدة، أو إثنين أو ثلاث معاملات يومية وتم تقدير التركيزات القاتلة ل 50% من اليرقات (LC_{50})لتحديد السميات الحادة لمبيدى البيوفلاى وسبينتور ليرقات شغالات نحل العسل. وتم تحديد نشاط إنزيم الأسيتيل كولين إستيريز (AChE) حيويا لليرقات بعد 24 و 96 ساعة من المعاملة الفردية أو معاملتين يومية. وأشارت النتائج - على أساس قيم -LC_{50} إلى أن سبينوساد أظهر سمية أعلى ليرقات الشغالات وذلك عند مقارنته مع البيوفلاى. وكذلك فإن السمية الحادة (بعد 24 ساعة) للثلاث معاملات اليومية بالبيوفلاى (1905 مجم/ لتر) أو بالسبينوساد (12.04 مجم/ لتر) كانت أعلى من القيمة المناظرة للمعاملتين اليوميتين (3847 و 21.45 مجم/ لتر على الترتيب). وعلى نفس المنوال فإن السمية الحادة للمعاملتين اليوميتين كانت أعلى من تلك الخاصة بالمعاملة الفردية (5113 و 51.29 مجم/ لتر على الترتيب). وبذلك يوجد تأثير مميت تراكمى على يرقات شغالات نحل العسل. وعلاوة على ذلك فقد أشارت النتائج إلى أن متوسط نشاطات AChE فى اليرقات المغذاة مرتين على محلول سكرى يحتوى على البيوفلاى أو السبينوساد كان أعلى معنويا ($p > 0.05$) من ذلك المتحصل عليه بعد التغذية مرة واحدة، وذلك بعد 24، 96 ساعة. وأيضا فإن البيوفلاى عندما وجد فى المحلول السكرى بالتركيزات المختبرة يكون له تأثيرات منشطة بعد 24 ساعة من المعاملة، وتأثيرات مثبطة بعد 96 ساعة من المعاملة على نشاط AChE فى يرقات الشغالات سواء تم تغذيتها مرة أو مرتين. كما أظهرت النتائج أن السبينوساد عندما وجد فى المحلول السكرى بالتركيزات المختبرة يكون له تأثيرات منشطة فقط بعد 24 ساعة من المعاملة الفردية، وتأثيرات مثبطة بعد 24 ساعة من المعاملة المزدوجة، وبعد 96 ساعة من المعاملة الفردية أو المزدوجة على نشاط AChE فى يرقات الشغالات.

Keywords: نحل العسل، يرقات، المبيدات الحيوية للآفات، *Beauveria bassiana*، Biofly، SpinTor®، Spinosad، السمية، الأسيتيل كولين إستيريز.

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