Effect of Sub-lethal Doses of Clothianidin and Spinosad insecticides on Honeybee Larvae.

S. A. Abdel–Kader*, M. F. Abdel–Lateef, A.E. Abdelmonem and A.D. Yousif.

Plant protection department, faculty of agricultural, AL-Azhar University.

*Corresponding author E-mail: saadatta1980@azhar.edu.eg (S. Abdel–Kader)

ABSTRACT

In this study, we have compared the effects of sub-lethal concentrations of clothianidin (Supertox 48% SC) and Spinoza (Tracer 24% SC) on mortality percent, brood lock, C-shape and foraging behavior of first Carniola and Italian hybrids honeybee larvae in hive. The ingestion food was contaminated with the tested insecticides at concentrations of 0.00, 0.001, 0.005, 0.01, 0.05 and 0.1 ppm. Results revealed that the two tested pesticides at hive-residue levels triggered increase in larval mortality according to untreated larvae after 7 days of exposure. Moreover, hybrids Carniola and Italian honeybees' larvae were more sensitive to Spinosad compared with 88.89% and 75.00% mortality after 7 days, respectively, while the corresponding values with clothianidin were 72.22% and 50.00% for both hybrids honeybee' larvae, respectively.

Keywords: Honeybee, Insecticide, Sub-Lethal, Dosage, Carniola, Italian, Clothianidin, Spinosad.

INTRODUCTION

The insecticides are important for ensuring both crop quality and quantity in today's integrated crop management for sustainable agricultural production. Application use of insecticides is one of the most effective practices to control pests. However, what is concerning us most is how residual levels of sub-lethal dosages of those insecticides being used resulted in detrimental effect of non-target pollination species of honeybee development, foraging behavior and colony conditions. Either wild or domesticated honeybee, *Apis mellifera*, has been recognized and used as a major pollinator in the agricultural system and by beekeepers to produce valuable products such as honey, royal jelly and pollen (Kevan, 1999). However, honeybees rely on flower plants while foraging and collecting its food sources of nectar and pollen and thus at-risk endangering exposing to various levels of chemical residues of pesticides while they collect nectar and pollen (Peach *et al*., 1993). In addition, the workers may take the pesticide-contaminated nectar and pollen back to their hive. This will expose the larvae, drones and queen to these pesticides, and eventually poison them and causes high mortality. Recently, one hundred and twenty one different pesticides and metabolites were identified in the hive with an average of seven pesticides per pollen sample, including miticides, insecticides, fungicides, herbicides, and insect growth regulators (Mullin *et al*., 2010 and Johnson *et al*., 2010). Among insecticides, Spinosad is a novel insect control agent derived by fermentation of the actinomycete bacterium, *Saccharopolyspora spinosa.* The active ingredient is composed of two metabolites, spinosyn A and spinosyn D (Thompson *et al*., 1997). Spinosad controls many caterpillar pests in vines, pome fruit and vegetables (including tomatoes and peppers), thrips in tomatoes, peppers and ornamental cultivation and dipterous leaf miners in vegetables and ornamentals (Miles, 2003). Application rates vary between 25 to 150 gm of active ingredient per hectare (a.i. g / ha) and 4.8 to 36 gm of active ingredient per hectoliter (a.i. gm /hL^{-1}) depending on the crop and target pest (Miles, 2003). The mode of action of Spinosad is completely novel, making it a useful resistance management tool. A novel mechanism of activity on the nicotinic acetylcholine receptors was identified as the primary cause of death (Salgado, 1997). Spinosad has additional effects on gamma-aminobutyric acid or GABA receptors, although it has not been shown that these effects contribute to insecticidal activity. Moreover, neonicotinoids are neurotoxins that act as agonists of the nicotinic acetylcholine receptor by disrupting the neuronal cholinergic signal transduction, leading to abnormal behavior, immobility and death of target insect pests (Matsuda *et al*., 2001; Tomizawa and Casida, 2005 and Elbert *et al*., 2008). Frequently, non-target insects, like honey bees, come into contact with these insecticides (Pisa *et al*., 2015). Neonicotinoids are referred to as ''systemic" as they are absorbed by plants and spread to all tissues through their vascular system (Elbert *et al*., 2008). Thus, pollen, nectar and guttation fluids can contain neonicotinoids (Desneux *et al*.,

2007; Cresswell, 2011; Blacquière *et al*., 2012; Goulson, 2013; Van der Sluijs *et al*., 2013 and EASAC, 2015). Honey bee larvae require proteins and energy sources for their growth and development. These larvae rely on the proteins and carbohydrates that are contained in pollen and nectar (Babendreier *et al*., 2004) stored in the hive by forager workers. Studies have detected the presence of various pesticides, in samples of pollen and nectar (Rortais *et al*., 2005; Chauzat *et al*., 2006; Mullin *et al*., 2010 and Krupke *et al*., 2012) that may be used by nurse bees to feed the larvae.

The aim of this work is to evaluate the effect of sub-lethal concentrations of clothianidin and spinosad, on Carniola and Italian hybrids honeybees in hive.

MATERIALS AND METHODS

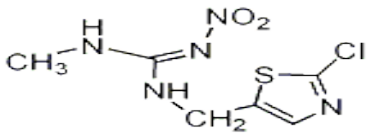
This study was carried out in laboratories of Department of Plant Protection, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt, during the period from June 2018 to July 2020. Ten honey bee colonies five F1 hybrid Carniola and five F1 hybrid Italian of the same strength were selected, each colony consisted of four brood combs, two honey and pollen combs and headed by the same age of new mated queens.

Insecticides used:

Commercial formulations available in Egypt were used. The following pesticides were used:

The neonicotinoid:

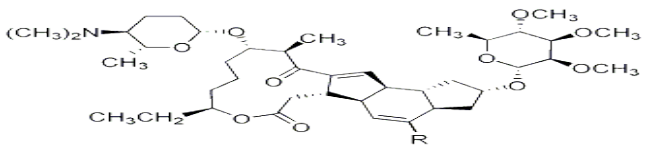
Clothianidin (Super tox-1 48%SC).



(*E*)-1-(2-chloro-1, 3-thiazol-5-ylmethyl) -3-methyl-2-nitroguanidine (IUPAC).

The spinosyn:

Spinosad. (Tracer, 24% SC).



spinosyn A, R = H-
spinosyn D, R = CH_3-

a mixture of 50–95% (2*R*, 3a*S*, 5a*R*, 5b*S*, 9*S*, 13*S*, 14*R*, 16a*S*, 16b*R*)-2-(6-deoxy-2, 3, 4-tri-*O*-methyl-*α*-L-mannopyranosyloxy)-13- (4 dimethylamino-2, 3, 4, 6-tetradeoxy-β-D-erythropyranosyloxy)-9-ethyl-2, 3, 3a, 5a, 5b, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16a, 16b-hexadecahydro-14-methyl-1*H*-*as*-indaceno[3, 2-d]oxacyclododecine-7, 15-dione and 50–5% (2*S*, 3a*R*, 5a*S*, 5b*S*, 9*S*, 13*S*, 14*R*, 16a*S*, 16b*S*)-2-(6-deoxy-2, 3, 4-tri-*O*-methyl-*α*-L-mannopyranosyloxy)-13-(4-dimethylamino-2, 3, 4, 6-tetradeoxy-β-D-erythropyranosyloxy)-9-ethyl-2, 3, 3a, 5a, 5b, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16a, 16b-hexadecahydro-4,14-dimethyl-1*H*-*as*-indaceno[3,2-d]oxacyclododecine-7,15-dione. (IUPAC).

Determination of acute oral toxicity of the tested insecticides on honey bee larvae:

Eggs of the same age were obtained from healthy colonies where queens were confined on a comb in excluder cages for 30 hours. The exclusion cage was placed close to combs containing brood. At 2d maximum 30 hours, after encaging, the queen was released from the cage, after checking the presence of fresh laid eggs. It was reducing the isolation time in order to minimize the variability in size and age between larvae. The comb containing the eggs was left in the cage, near the brood, during the incubation stage and until hatching (1 D) (OECD TG 213, 1998).

These cages permitted worker bees to move freely from the encaged comb to other parts of the colony in order to stimulate egg laying and feeding of the larvae. After removing the queen, the comb was left in its cage in the colony for three days. Then the frame was removed from the hive and brought into the laboratory (Aupinel *et al*., 2010).

The diet was composed of the three following diets, adapted to the needs of the larvae at different stages of development:

Diet A (1 D): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight of glucose and 12% weight of fructose.

Diet B (3 D): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose.

Diet C (from 4 D to 6 D): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose.

In order to avoid bias due to possible heterogeneity of the larvae, newly hatched larvae that have not yet formed a C shapes were selected. All larvae were fed once a day were taken to avoid touching and drowning the larvae when feeding them. Food was placed next to the larva, along the wall of the cell. Larvae from each of the three colonies were selected and treated with 30 µL of the diet C containing the test solution at the suitable concentration. Each treatment (containing the diet) was administered with a different micropipette tip to avoid contamination. At 6 and 7 D, mortalities were counted and the test was terminated. On day 4 (D 4) of the test, a single dose of the test chemical was administered to the larvae with the diet in a range of five increasing concentrations. The experimental unit was the individual cell containing a larva.

A minimum of twelve larvae from each of three colonies were allocated.

Control (12 larvae × 3 colonies = 36 larvae).

Five treatments, 5 increasing test concentrations (each containing of 12 larvae × 3 colonies = 36 larvae). During the test, the temperature in the hive was 32 °C.

Mortalities were recorded on D 6, and D 7 of the test. (OECD TG 213, 1998).

Mortality is expressed in percentage of the initial population after an adjustment according to the Abbott,s formula (Abbott, 1925)

$M = (P - T / S) X 100$ raw mortality

$M = (\%P - \% T) / 100 - \%T) x 100$ percent mortality

M= adjusted mortality expressed in percent of the initial population,

P= number of dead larvae in the treated group.

T= number of dead larvae in the control group.

% P= mortality percentage due to the treatment.

% T= control mortality percentage.

RESULTS AND DISCUSSION

Five concentrations of each insecticide were applied on first Carniola and Italian hybrids honey bee larvae in the hive at the fourth stage to study the toxicity of sub lethal concentration on mortality percentage.

Data in Tables (2 & 3) and Fig. (1 & 2) show the mortality after 6 and 7 days from treatment with different concentrations. The mortality percentages at 0.001, 0.005, 0.01, 0.05 and 0.1 ppm, after 7 days from the egg laying with clothianidin were 47.22%, 58.33%, 66.67%, 61.11% and 72.22% for first Carniola hybrid honeybee larvae comparing with control, respectively. While the corresponding values for first Italian hybrid honey bee larvae at the same concentrations were 16.67%, 22.22%, 30.56%, 47.22% and 50.00%, respectively, compared with 0.00% mortality in the control treatment. Results also indicated that first hybrid Carniola honey bee larvae were more sensitive to clothianidin than first Italian hybrid honeybee larvae. Our results suggest that chronic dietary feed at the levels of clothianidin have remarkable impacts on honey bee larval survivorship. These results are in agreement with several authors. Mogren *et al.*, (2019) Indicate that nutritional stress for the duration of the larval period does carry through to the adult stage and can be measured as increased susceptibility to sublethal concentrations of clothianidin. When reared in pollen supplemented colonies, field-realistic concentrations of clothianidin (10-40 μgL^{-1}) did not affect survival of adults in cage experiments. However, when reared in pollen stressed colonies, mortality of adult bees was greater at 40 μgL^{-1} than control, a concentration encountered in the pollen of untreated forage adjacent to seed-treated corn fields.

Tosi *et al*. (2017) reported that, colony level supplementation with 648, 159 g of pollen (calculated across the field season) was sufficient to help mitigate oral clothianidin as a secondary stressor. Morfin *et al*. (2019) indicate that, when larvae were exposed to three sublethal doses of clothianidin and evaluated 25–36 days, later for hygienic and foraging behaviors as adult bees, the medium and highest sublethal doses of clothianidin significantly reduced hygienic and foraging activity. The greatest effects were on the proportion of adult bees foraging and carrying pollen. These results show that exposure of larvae to clothianidin results in negative effects extending into the adulthood of bees, possibly compromising the colony's fitness by impairing pathogen control mechanisms and by reducing pollen collection. They concluded that exposure of larvae to clothianidin results in negative effects extending into the adulthood of bees, possibly compromising the colony's fitness by impairing pathogen control mechanisms and by reducing pollen collection.

On the contrary, Doublet *et al*. (2015) found no increase in mortality of developing bees after feeding larvae for 5 days with the neonicotinoid insecticide thiacloprid at a dose 1.73 × 104 times lower than the oral LD_{50}.

Data in Tables (4 &5) and Figs. (3 & 4) show the mortality after 6 and 7 days from treatment with different concentrations of Spinosad. The mortality percentages at 0.00 1, 0.005, 0.01, 0.05 and 0.1 ppm concentrations after 7 days from the egg laying with Spinosad, were 27.78%, 44.44%, 63.89%, 77.78% and 88.89% for first hybrid Carniola honeybee larvae, respectively. While the previous concentrations recorded 16.67%, 30.56%, 44.44%, 65.89% and 75.00%, for first hybrid Italian honeybee larvae, respectively, compared with untreated. Results also indicated that first hybrid Carniola honeybee larvae were more sensitivite to clothianidin than first hybrid Italian honeybee larvae.

Similarly, Tomé *et al*. (2015) reported that, the lethal and sublethal doses of the neonicotinoid imidacloprid and the bioinsecticide Spinosad were assessed in the stingless bee species *Melipona quadrifasciata*, an important native pollinator in the Neotropical region. The adult stingless bee workers exhibited high oral insecticide susceptibility, with LD_{50}s of 23.54 and 12.07 ng a.i./bee^{-1} for imidacloprid and Spinosad, respectively. Imidacloprid also impaired worker respiration and overall group activity and flight, while, Spinosad significantly impaired only worker flight despite exhibiting higher oral toxicity to adult workers than imidacloprid. These findings indicate that the hazardous nature is not only of imidacloprid but also the bioinsecticide Spinosad to adult workers of the native pollinator *M. quadrifasciata*. Therefore, bioinsecticides should not be exempted from risk assessment analysis due to their lethal and sublethal components. Both imidacloprid and Spinosad were highly toxic to the adult workers of M. quadrifasciata, with LD_{50}s in the range of 12.07 and 23.54 ng ingested per bee for Spinosad and imidacloprid, respectively. Although imidacloprid is broadly recognized as very toxic to bees, usually with LD50s in the range of 3.8 to over 81.0 ng/ bee^{-1} (Decourtye *et al*., 2004 a & b; Cresswell, 2011 and Blacquière *et al*., 2012), the results with Spinosad provide some evidence of deleterious effects on bees (Miles, 2003; Morandin *et al*., 2005; Besard *et al*., 2011; Biondi *et al*., 2012 and Gradish *et al*., 2012 a &b). Surprisingly, Spinosad exhibited higher acute toxicity than imidacloprid, suggesting its potential impact on M. quadrifasciata. The apparently higher susceptibility of stingless bees to Spinosad, compared with the honeybee and bumblebee (Mayes *et al*., 2003; Bailey *et al*., 2005 and Morandin *et al*., 2005), should also be a matter of concern in future insecticide impact assessments in warmer climates.

Honey bee larva can consume 160 ml of brood food before its pupation (Aupinel *et al.,* 2005), it is quite possible that honey bee larvae were affected by the residue of imidacloprid. Yang *et al.* (2012) tested the doses of 0.0004, 0.004, 0.04 and 0.4 $nglarva^{-1}$, which corresponds to expose the larvae to an imidacloprid concentration of approximately 0.0025, 0.025, 0.25 and 2.5 mg/L, respectively, which represents the level that is very likely present in an agro-environment. This is strong evidence. It indicates that a honeybee larva could remain exposed to the residual imidacloprid in an agro-environment. (Bortolotti *et al.,* 2003) Indicate that because honeybee larvae do not consume raw nectar or pollen, we presumed that they were protected from the contamination of a bee colony, or at least that they were protected by the repellent effect of imidacloprid on the forager and the detoxification abilities of a nectar-collecting forager and a larva food-preparing nurse bee. Nevertheless, because the detoxification gene is deficient in a honeybee (Claudianos *et al.,* 2006), this protection may break down under the synergy of other stresses, such as malnutrition, disease and the intoxication by insecticides of adult workers, and result in colony disorder.

Our results agree with Davis *et al.* (1988) who evaluated the effects of insecticides on *A. mellifera* larval development concluded that these substances could alter normal patterns of development. They found that the growth of *A. mellifera* larvae exposed to dimethoate (0.313 *a.i.* lgm/gm royal jelly) was stimulated in comparison with non-treated larvae. The same study found that certain larvae treated with the insecticide lost their typical C-shape and were either dorsally or dorsolateral elongated and the number of larvae that failed to spin cocoons was greater in the treated larvae groups. On contrary, our results disagree with Zhu *et al*. (2014) who observed that larvae seem to be more tolerant to thiametoxam rather than adults, and Yang *et al*. (2012) who observed that larvae were more tolerant to the imidacloprid when compared with adult of *A. mellifera*. However, even with the discrepancy in the sensitivity of the honeybee during its development, these authors highlight that the

toxic effect of this insecticide at low doses may be harmful and affect the larvae.

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Table 1: some characteristics of Clothianidin and Spinosad commercial formulations used in the tests.

Groups	Neonicotinoids	spinosyn
Chemical name	Clothianidin	Spinosad
Trade name	Super tox-1 48%SC	Tracer 24% SC
Field concentration*	50 ml 100 L^{-1}. water	20 ml 100 L^{-1}. water
Crops	Cotton, vegetables, citrus, grapes, ornamentals.	Cotton, vegetables, fruits.
Highest tested concentration	480 ppm	48 ppm

*According to the Recommendations of Ministry of Agriculture and Land Reclamation (2018), Agriculture pesticide committee (APC).

Table 2: Mortality of honeybees Carniola hybrid larvae after the ingestion of food contaminated with clothianidin.

Concentratios (ppm)	number of larvae	6 day	%Mortality	7 day	%Mortality	Insecticide ingested by larva (μglarva^{-1})
0.00	36.00	00.00	0	00.00	00.00	00.00
0.001	36.00	26.00	27.78	19.00	47.22	0.00003
0.005	36.00	20.00	44.44	15.00	58.33	0.00015
0.01	36.00	17.00	52.78	12.00	66.67	0.0003
0.05	36.00	15.00	58.33	14.00	61.11	0.0015
0.1	36.00	12.00	66.67	10.00	72.22	0.003

Table 3: Mortality of honeybees Italian hybrid larvae after the ingestion of food contaminated with clothianidin.

Concentration (ppm)	number of larvae	6day	%Mortality	7 day	%Mortality	Insecticide ingested by larva (μglarva^{-1})
0.00	36.00	00.00	00.00	00.00	00.00	00.00
0.001	36.00	32.00	11.11	30.00	16.67	0.00003
0.005	36.00	30.00	16.67	28.00	22.22	0.00015
0.01	36.00	27.00	25.00	25.00	30.56	0.0003
0.05	36.00	23.00	36.11	19.00	47.22	0.0015
0.1	36.00	20.00	44.44	18.00	50.00	0.003

Table 4: Mortality of honeybees Carniola hybrid larvae after the ingestion of food contaminated with Spinosad.

Concentration (ppm)	number of larvae	6 day	%Mortality	7 day	%Mortality	Insecticide ingested by larva (μglarva^{-1})
0.00	36.00	36	00.00	36.00	00.00	0.00
0.001	36.00	30	16.67	26.00	27.78	0.00003
0.005	36.00	27	25.00	20.00	44.44	0.00015
0.01	36.00	23	36.11	13.00	63.89	0.0003
0.05	36.00	19	47.22	08.00	77.78	0.0015
0.1	36.00	15	58.33	04.00	88.89	0.003

Table 5: Mortality of honeybee's Italian hybrid larvae after the ingestion of food contaminated with Spinosad.

Concentration (ppm)	number of larvae	6 day	%Mortality	7 day	%Mortality	Insecticide ingested by larva (μglarva^{-1})
0.00	36.00	36.00	00.00	36.00	00.00	0.00
0.001	36.00	30.00	16.67	30.00	16.67	0.00003
0.005	36.00	33.00	08.33	25.00	30.56	0.00015
0.01	36.00	31.00	13.89	20.00	44.44	0.0003
0.05	36.00	26.00	27.78	13.00	63.89	0.0015
0.1	36.00	18.00	50.00	09.00	75.00	0.003

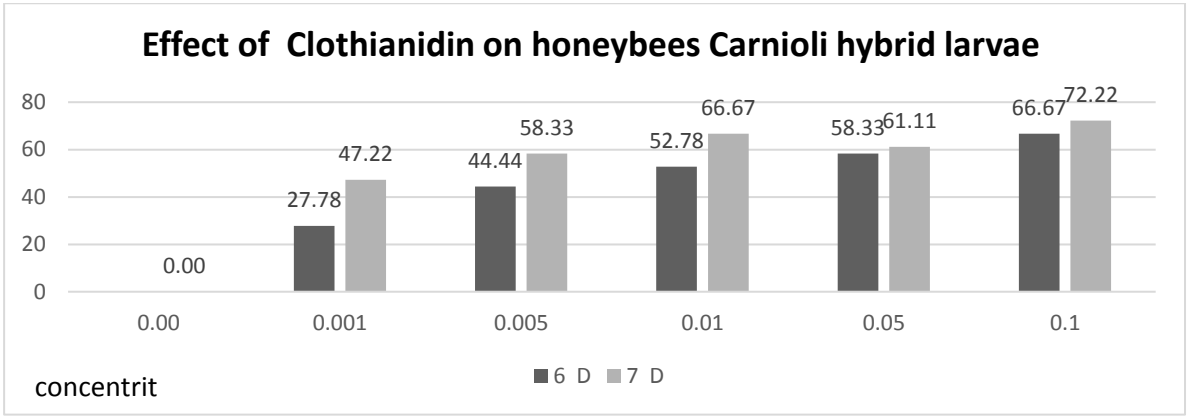

Figure 1: Mortality of honeybees Carniola hybrid larvae after the ingestion of food contaminated with clothianidin.

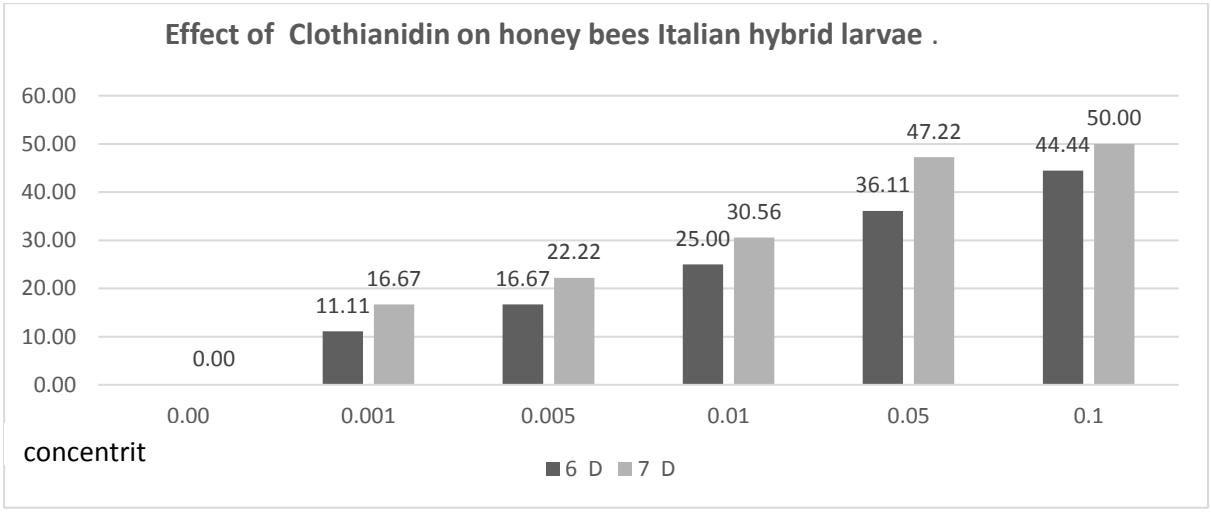

Figure 2: Mortality of honeybees Italian hybrid larvae after the ingestion of food contaminated with clothianidin.

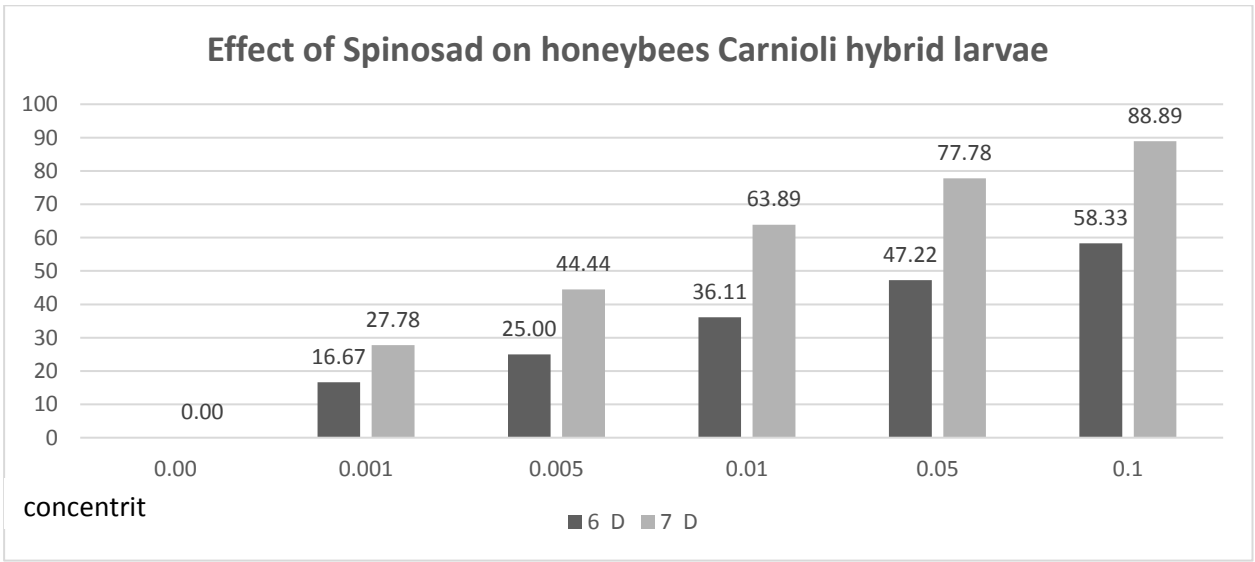

Figure 3: Mortality of honeybees Carniola hybrid larvae after the ingestion of food contaminated with Spinosad.

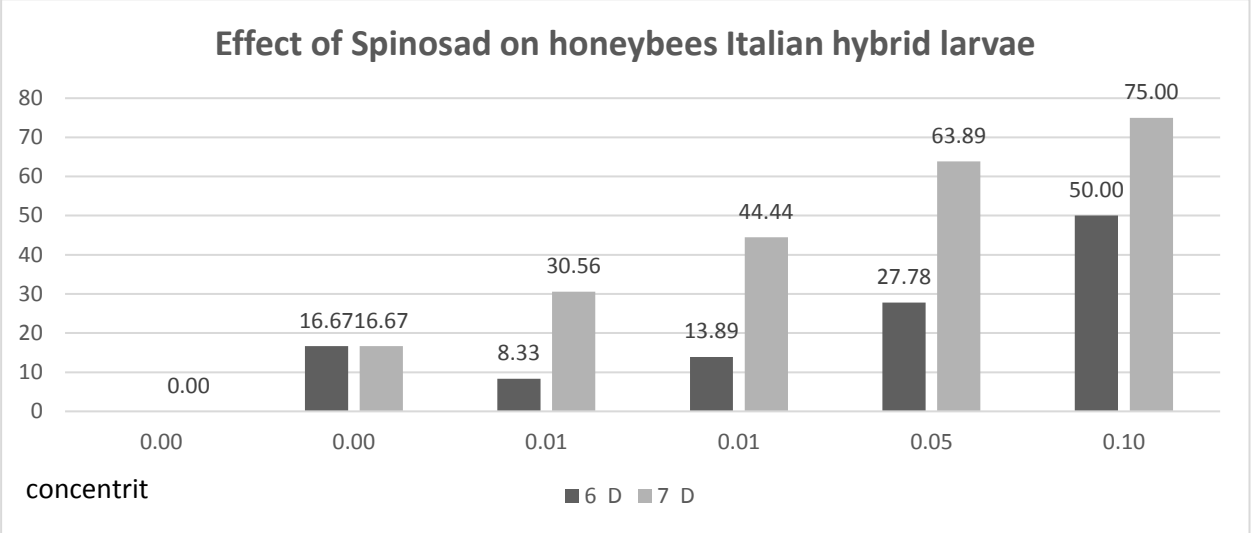

Figure 4: Mortality of honeybee's Italian hybrid larvae after the ingestion of food contaminated with Spinosad.

تأثير الجرعات غير المميتة من مبيد كلوثيانيدين و مبيد اسبينوسادعلى يرقات نحل العسل

سعد عطا شعبان عبد القادر *، محمود فتح الله عبد اللطيف، عبد الله الحسين عبد المنعم ، عادل دياب محمد يوسف.

قسم وقاية النبات، شعبة المبيدات، كلية الزراعة، جامعة الأزهر، القاهرة، مصر.

* البريد الإلكتروني للباحث الرئيسي saadatta1980@azhar.edu.eg

تهدف هذه الدراسة لمقارنة تأثير التركيزات غير المميتة من مبيدى الكلوثياندين و الاسبينوساد على نسبة الموت ,الحضنه المغلقة وشكل (C) وسلوك النحل في يرقات هجين الجيل الأول كرنيولى و هجين الجيل الأول إيطالي فى الخلية .استخدمت طريقة الخلط مع الغذاء بالمبيدات المختبرة عند التركيزات الأتية 0.00 , 0.001 , 0.005 , 0.01 , 0.05 و 0.1 جزء في المليون لكل من المبيدين, بينت النتائج أن كل من المبيدين المختبرين قد أحدث زيادة في نسبة الموت في اليرقات عند مقارنتها بالغير معامل وعلاوة على ذلك فإن يرقات هجين أول كرنيولى و هجين أول إيطالي كانا أكثر حساسية لمبيد الاسبينوساد من مبيد الكلوثياندين حيث أعطت نسبة موت 88.89% و 75.00% على التوالي , بينما أعطى مبيد الكلوثياندين نسبة موت 72.22% و 50.00% على التوالي عند نفس الوقت.

الكلمات الاسترشادية: نحل العسل , مبيدات الحشرات, التاثير غير المميت, الجرعة, كرينيولى.