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Effects of Imidacloprid on honey bee queen *Apis mellifera* and detection of the residue in honey and bee bread product

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Abstract

Honey Bees are the most important regulators in global biodiversity maintenance and food security. Overtime there's more evidence has been shown elevated the colony losses for honey bee and recorded a negative effect on the pollination. Pesticides of the neonicotinoid type have been reported with these losses, while a few studies have explored how residual exposure doses effect on the honey bee queen. This study evaluates two different doses of imidacloprid (5 and 200 ppb in sugar syrup or in pollen supplement), on honey bee queen quality and detected the residue of imidacloprid in honey and bee bread samples extracted from treated colonies. There was a decrease in number of queens ovarioles number at high concentration of pesticide treatment (200 ppb) and the spermathecae diameter decreased in 5 and 200 ppb pesticide in sugar syrup treatment with no significant difference. The residue of Imidacloprid were higher in colonies fed with 5 or 200 µg/kg in diet patties compared to residues in colonies fed with 5 or 200 ppb sugar syrup. It is clearly that the highest residue was found in honey which was collected from colonies exposed to treated diet patties than colonies treated by sugar syrup treatment. The results may help elucidate the effect of imidacloprid on the egg laying behaviors of honey bee queens.

Keywords: Honey bee, Honey, Bee bread, Queen, Imidacloprid.

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1. Introduction

The neonicotinoids pesticides like Imidacloprid are agonist's substances of acetylcholine receptor (nAChR). They affect the central nervous system by competing with the naturally occurring neurotransmitter acetylcholine to block the transmission of impulses. Irreversible and selective binding to insect central nervous system causes paralysis and death by over-stimulation and blockage to nAChR [1]. Imidacloprid was the first synthetic neonicotinoid insecticide commercialized in 1991. It becomes poisonous through touch and oral-intake, it is one of the most often used insecticides in the world because to its systemic efficacy and relatively low mammal toxicity [1]. Honey bees essential in pollination and produce, collect a variety of important hive products, such as honey, pollen, wax, royal jelly, and propolis, in addition to providing crucial ecosystem services through pollination. The survival of honey bees and the sustainability of the services they provide are both seriously threatened by recent population decreases. The causes of honey bee declines are thought to be multifactorial and include, inter alia, intensification of modern agricultural practices, spread of infectious and parasitic diseases, decreased genetic diversity, and poor queen quality [2-4]. The effect of neonicotinoids on these studies demonstrated the association between neonicotinoid exposure at the colony level and reduced queen quality; however, it is unclear if the observed changes are caused by secondary effects by exposed workers failing to give developing and adult queens the best care, or the direct effects of neonicotinoids on specific queens. But it is known that queen failure is a major driver of managed honey bee colony losses. However, little information exists regarding the effects of environmental stressors on queens [5-6]. Finally, this study makes evident the importance of conducting risk assessment studies on honey bee colonies over longer periods to reveal the chronic sublethal effects on queen health that can ultimately impair colony performance.

2. Materials and methods

The present investigation was carried out at the Faculty of Agriculture apiary, Cairo University, Giza, Egypt through three successive years during March 2015 to August 2017 to study the biological effect of neonicotinoid pesticide (Imidacloprid) on honey bee queen quality.

Also, determinate the presence of pesticide *residues in honey and bee bread samples*.

2.1. Insecticide used

Commercial formulations available in Egypt were used. Confidor® 200 SC Insecticide, Bayer Crop science (Hawthorn East Victoria Australia), Chemical Name Imidacloprid (Concentration 18.30 %).

2.2. Pesticide Treatments

Eighteen apparently healthy honey bee colonies (local hybrid of *A.m carnica*) of the same strength were selected, queen's full sisters and mated from the apiary, these honey bee colonies were divided in to three treatments. Each colony was consisted of three brood, two honey and pollen combs and was fed after 30 days of the introduction the queens. After that the feeding by Imidacloprid in two concentrations (5 and 200 ppb) continued for 10 weeks.

2.3. Preparation of pesticide concentration

The stock solution was (20% Imidacloprid) kept in deep freeze in -20C^{o} diluted solution of Imidacloprid (0.2%) was prepared by mixed 1ml of stock solution (20%) with distilled water and complete the volume to 100 ml.

2.4. 5ppb Imidacloprid Pesticide treatment

Pesticide sugar syrup feeding solution was Prepared by dissolving 500 g of sucrose in one liter and add 25µl from the diluted solution of Imidacloprid (0.2%) and mixed well; three colonies were fed by this sugar syrup (500ml/three time /week for two and a half months). Pesticide pollen patty: Prepared as follows: 250 g of cracked pollen and 100 ml sugar syrup (50%), and add 25µl from the diluted solution of Imidacloprid (0.2%) and mixed well with chickpeas flour to make a soft pastry (1kg). The soft patty was divided to 50 g/piece and store in freezer. Each colony takes one pollen supplement, every week for two and a half months. Three colonies were fed by this pollen supplement.

2.5. 200 ppb Imidacloprid Pesticide treatment

Pesticide sugar syrup feeding solution was Prepared by dissolving 500 g of sucrose in one liter and add 1µl from the stock solution of Imidacloprid (20%) and mixed well; three colonies were fed by this sugar syrup (500 ml/three time /week for two and a half months). Pesticide pollen patty: prepared as follows: 250 g of cracked pollen and 100 ml sugar syrup (50%), and add 1µl from the stock solution of Imidacloprid (20%) and mixed with chickpeas flour to make a soft pastry (1kg). The soft patty was divided to 50 g/piece and store in freezer. Each colony takes one pollen supplement, every week for two and a half months. Three colonies were fed by this pollen supplement.

2.6. Control treatment

The pollen patty was used without Imidacloprid pesticide, three colonies were fed by this pollen patty and sugar syrup feeding solution (1 lit water + 500 gm of sugar) without Imidacloprid pesticide, three colonies were fed by this sugar syrup for two and a half months.

2.7. Effect of Imidacloprid on honey bee queens

2.7.1. Queen reared

This experiment was carried out in 2015. The treatments were applied on 4 colonies from bee hybrid of *A.m carnica*. The colonies were selected from the treatment groups previously mentioned, one colony from each treatment. These colonies were Queen-less without open brood and containing on one honey and sealed brood frame before grafting 24 hours. The grafting was based on established queen rearing protocols, one day old larvae were grafted to plastic queen cups and reared in queen less colonies until they emerged and newly-emerged queens were collected [7].

2.7.2. Queen anatomy

2.7.2.1. Total number of ovarioles

The right ovary was carefully isolated and placed in a drop of xylene on a glass slide according to [9]. The loosened ovarioles were counted with the aid of binocular magnification according to [8-10].

2.7.2.2. Spermatheca diameter

Diameter of the spermatheca was measured in mm under a stereomicroscope by the utilization of microscopic micrometer slide, according to [11].

2.8. Determination of Imidacloprid insecticide in honey and pollen by liquid chromatography

2.8.1. Imidacloprid solutions

Individual Imidacloprid was prepared at concentration of 1 mg/ml in acetonitrile and stored in amber volumetric flasks at -18c.

2.8.2. Sampling procedure

The Samples were taken from pollen and honey stored from each of all colonies (Before the start of the experiment - after the end of feeding - finally at the end of the experiment). Collected from hives on experimental apiary and stored at 4^{o} C until the analysis. 2g of samples were weighed in 50 ml centrifuge tube and 10 ml 1% acetonitrile and ethyl acetate mixture (8:2v/v) and 200 µl of 20% TEA in acetonitrile was added; the sample was homogenized with vortex mixer for 1min, then centrifuged and supernatant was transferred to strata X-CW cartridge preconditioned with 3ml of methanol and water. The cartridge was washed up by 5ml of water, dried under vacuum for 5min and eluted twice with 3 ml of mixture of acetonitrile and ethyl acetate (8:2 v/v). The eluted was evaporated to dryness and the residue was dissolved in 250 µl of water.

2.8.3. High Performance Liquid Chromatography

HPLC equipment (Agilent technologies 1260 lnfinity ll) was used UV- detector. The column Eslips plus C 18, di.5 Mn and Len. 4.6 * 2.5 mm. The wave length detector at 210,235 and 254 nm corresponding to each pesticide formulation. The mobile phase was mixture acetonitril: methanol (70:30). The flow rate was 1.3 ml/min.

2.9 Statistical Analysis

One-way analysis of variance for the data collected was done using the SAS General Linear model procedure [12].

Mean values were compared using Duncan's multiple rang test [13] when significant differences existed. The significance level was set at 5%.

3. Results and Discussion

3.1. Queen quality

The number of ovarioles and spermathecae diameter has been much speculation about which phenotypic traits serve as reliable indicators of productivity in queen honeybees (Apis mellifera), The obtained results in Table 1 showed the effect of Imidacloprid on the developing of honey bee queens which has predominantly been evaluated in colonies exposed to 5 ppb and 200 ppb of Imidacloprid through sugar syrup or pollen patty. The queens raised in these colonies showed a decrease in number of ovarioles on average at high concentration of Imidacloprid pesticide treatment. In addition, there is no significant difference between control treatment and 5 ppb treatment, on other hand there was a significant difference between control or 5 ppb treatment and 200 ppb treatment in sugar syrup or pollen patty treatment. It is Cleary from observed changes in number of ovarioles that due to the primary effects of Imidacloprid on the queens resulting from exposed workers providing suboptimal care to developing queen. The diameter of the spermathecae decreased from 0.90 ± 0.05 to 0.80 ± 0.05 mm in 5 ppb pesticide in sugar syrup and to 0.63± 0.03 mm in 200 ppb pesticide in sugar syrup with no significant difference. But the treated colonies with 200 ppb of Imidacloprid in pollen patty were found to be significantly different from 5 ppb treatment or control treatment ($P < 0.01$). According to these data, it can be reported that the spermathecae diameters of the queens reared was affected by Imidacloprid pesticide. These findings raise further concern about the impact of systemic neonicotinoids on queen honey bee productivity. The key result emerging from this work is that ingestion of imidacloprid has negative effects on reproductive fitness of honey bee queens. Environmentally realistic levels substantively reduced the fecundity of worker bumble bees. This finding is consistent with those of previous studies, which have shown that the treatment with imidacloprid, the midgut of queens showed modifications in the external musculature and cellular alterations. Such changes could lead to the nonrecovery of the epithelium and subsequently malabsorption of nutrients. Besides, the stomach related cells of queen honey bees exposed to the imidacloprid occur in pyknotic nuclei, proposing a cell death process. The main alterations observed in the ovaries of these reproductive bees treated with commercial imidacloprid were degeneration and desorption of the ovariole content, which probably affected their fertilization and colony development. There were no significant changes in the spermatozoa morphology for both treatments with imidacloprid, but this insecticide may interfere with the development and reproductive success of A. mellifera colonies because it affects the morphology and function of essential organs for the survival of queens [14]. The imidacloprid sub-lethal dose was (0.02 ppm) and decreased viability of sperm by 50%, seven days after treatment. Sperm viability was a downward trend (about 33%) in queens treated with high doses of coumaphos (100 ppm), but there was no significant difference [15].

3.2. Residue Analysis

The present results of a three-year study to determine the fate of Imidacloprid residues in hive matrices (honey and bee bread) on whole honey bee colonies fed with sugar syrup or supplemental pollen diet containing Imidacloprid at 5 and 200 ppb over three seasons of study. Samples of honey and pollen from all treatments were analysed for residue levels of Imidacloprid using liquid chromatography spectrometry

3.2.1. Honey

3.2.1.1. First season

Table 2 summarizes the Imidacloprid residues found in honey after the treatment in first season of the study with 5 and 200 ppb Imidacloprid, In the sugar syrup treatment the residue ranged from 0.19 to 0.76 ppb with mean 0.48±0.29 ppb and in the pollen supplement ,the residues were ranged from 1.17 to 8.70 ppb with mean 4.94±3.77 ppb in 5 ppb concentration treatment, On other hand the residues in the sugar syrup treatment ranged from 1.66 to 4.69 ppb with mean 3.34±1.54 ppb and in the pollen supplement ,the residues were ranged from 10.34 to 12.74 ppb with mean 12.74±2.4 ppb in 200 ppb concentration treatment.

3.2.1.2. Second season

The residue levels were higher in colonies fed with 5 or 200 µg/kg in diet patties compared to residues in colonies fed with 5 or 200 ppb sugar syrup. The samples collected from colonies fed treated diet patties recorded mean 24.36±3.22 and 65.57±7.36 in 5, 200 ppb treatment respectively, in addition the sugar syrup treatment colonies contained residues of Imidacloprid recorded 20.54 ±2.87, 68.83 ±3.47 ppb in 5, 200 ppb treatment respectively. It obvious that the highest levels were found in honey which was collected from colonies exposed to treated diet patties, followed by sugar syrup treatment.

3.2.1.3. Third season

Data in Table 2 showed the Imidacloprid residue in honey sample collected from colonies treated by 5, 200 ppb Imidacloprid in sugar syrup and recorded range from 35.79 to 41.98 under 5 ppb concentration treatment but the range were 88.27 to 104.16 ppb under 200 ppb concentration treatment.

3.2.2. Pollen

3.2.2.1. First season

Date in Table 3 showed the Imidacloprid residues found in pollen after the treatment in first season of the study with 5 and 200 ppb Imidacloprid. In sugar syrup treatment the residue was 33.74 ppb and in the pollen supplement, the residues were 4.54 ppb in 5 ppb concentration treatment, on other hand the residues in the sugar syrup treatment were 148.13 ppb and in the pollen supplement, the residues were 82.92 in 200 ppb concentration treatment.

3.2.2.2. Third season

Table 3 showed the residue levels were higher in colonies fed with 200 ppb of Imidacloprid in sugar syrup compared to residues in colonies fed with 5ppb of Imidacloprid in sugar syrup.

Table 1: Number of ovarioles / ovary in honey bee queens and the mean diameter of the spermathecae of queens reared in exposed colonies with 5 ppb and 200 ppb Imidacloprid pesticide through sugar syrup and/or pollen supplement.

Treatments	Control	Imidacloprid concentration in feeding		P-value
		5 ppb	200 ppb	
No. of Ovarioles	**Sugar syrup**			
	168.0^{a*}±3.78	156.6^{a}±5.60	113.3^{b}±4.70	0.0004
	Pollen supplement			
	169.3^{a}±2.33	161.6^{a}±2.34	109.3^{b}±2.33	<.0001
Diameter of the spermathecae	**Sugar syrup**			
	0.90^{a}±0.05	0.80^{ab}±0.05	0.63^{b}± 0.03	0.0270
	Pollen supplement			
	0.93^{a}±0.03	0.80^{a}±0.05	0.63^{b}± 0.03	0.0077

*Means with different superscripts, within Treatments, differ significantly (P < 0.05).

Table 2: Residues of Imidacloprid in honey samples resulting from feeding with 5 and 200 ppb Imidacloprid diet patties pollen supple or sugar syrup to honey bee colonies in first, second and third seasons.

Treatments	Control ppb	Feeding 5 ppb of Imidacloprid		Feeding 200 ppb of Imidacloprid	
		Sugar syrup	Pollen supplement	Sugar syrup	Pollen supplement
	First season				
Honey samples	ND	0.19	1.17	1.66	15.14
	ND	0.76	8.70	3.66	10.34
	ND	0.48	4.94	4.69	12.74
Mean		0.48^{b}±0.165	4.94^{b}±2.174	3.34^{b}±0.889	12.74^{a}±1.386
	Second season				
Honey samples	13.265	22.695	26.892	67.040	73.574
	11.643	21.629	25.443	66.617	59.086
	11.782	17.283	20.734	72.836	64.055
Mean	12.23^{c}±0.519	20.54^{b}±1.655	24.36^{b}±1.858	68.83^{a}±2.01	65.57^{a}±4.251
	Third season				
Honey samples	6.501	37.416	--	92.551	--
	13.545	41.984	--	104.165	--
	8.615	35.790	--	88.278	--
Mean	9.55^{c}±2.09	38.40^{b}±1.85	--	95.0^{a}±4.75	--

--ND means Non detectable.

Table 3: Residues of Imidacloprid in bee bread samples resulting from feeding with 5and 200 ppb Imidacloprid diet patties pollen supplement or sugar syrup to honey bee colonies in first and third seasons.

Treatments	Control	Feeding 5 ppb of Imidacloprid		Feeding 200 ppb of Imidacloprid	
		Sugar syrup	Pollen supplement	Sugar syrup	Pollen supplement
	First season				
Bee bread samples	ND	33.74	4.54	148.13	82.92
	Third season				
Bee bread samples	14.091	27.766	--	116.899	--
	11.660	19.362	--	144.161	--
	9.335	20.586	--	127.080	--
Mean	11.70^{b} ±1.37	22.57^{b}±2.62	--	129.38^{a} ±7.95	--
SD	2.38	4.54	--	13.78	--

--ND means Non detectable.

The samples collected from colonies fed treated diet patties recorded mean 22.57±4.54 and 129.38±13.78 in 5, 200 ppb of Imidacloprid treatment respectively. The residue levels were higher in colonies fed diet patties with Imidacloprid compared to residues in colonies fed sugar syrup with Imidacloprid and higher in colonies fed 200 ppb concentration compared to residues in colonies fed 5 ppb concentration. Imidacloprid residues became diluted or non-detectable within colonies due to the processing of pollen and honey and the rapid metabolism of the chemical. Due to the processing of bee bread and honey and the rapid metabolism, Imidacloprid residues became diluted or were no longer detectable within colonies. In particular, nearly all honey samples had detectable Imidacloprid residues lower than in beebread, even six weeks after exposure. Furthermore, the residue was diluted by addition of bee-collected un contaminated nectar entering the colonies. The Imidacloprid residue in bees and beebread collected weeks after treated provided evidence that colonies were exposed for at least two to three brood cycles. Residue was higher in beebread than in bees and more consistent in samples from colonies treated to the higher treatment doses. Average residue levels of the positive detections ranged up to 3.7 µg/kg, and the majority of bee and beebread residues exceeded concentrations of Imidacloprid found in bee-collected pollen, honey and bees reported from colony surveys [16-19]. In particular, nearly all honey samples had detectable Imidacloprid residues ranging from 2.3–13.4 µg/kg, after six weeks of exposure [20]. Colonies fed with patties spiked with 100 µg/kg Imidacloprid for six weeks showed residue levels in honey, bees and beebread that were close and more than those dietary doses of Imidacloprid that caused sublethal effects in the laboratory [21]. Imidacloprid concentrations in bees reached average levels up to 3.7 µg/kg and 2.8 µg/kg, respectively, from colonies exposed to 20 and 100 µg/kg doses. Considering the rapid metabolism of Imidacloprid by honey bees, foragers were probably subjected to a chronic dose exposure of Imidacloprid well exceeding levels that might be encountered by feeding on nectar in Imidacloprid seed-treated fields. Used a spiked diet patties placed within colonies to deliver continuous direct exposure over multiple brood cycles to Imidacloprid residues that were generally higher than levels found in bee-collected pollen and nectar under field conditions. The within-hive fate experiment reported that Imidacloprid residues of 100 µg/kg in diet patties or 20 µg/kg in sucrose syrup became diluted or non-detectable because of the processing of beebread and honey and the fast metabolism by bees. Given the weight of evidence presented here, they conclude that chronic exposure to Imidacloprid at the higher range of field doses (20 to 100 µg/kg) in the pollen of certain treated crops could contribute to reduced overwintering success but the most likely encountered field doses of 5 µg/kg, especially relevant for seed-treated crops [20]. Finally, this study makes evident the importance of conducting risk assessment studies on honey bee colonies over longer periods to reveal the chronic sublethal effects on queen health and bee behaviours that can ultimately impair colony performance [22].

4. Conclusion

The present study has demonstrated for the biological effect of neonicotinoid pesticide (Imidacloprid) on honey bee queen quality and the presence of pesticide residues in honey and bee bread products by treated honey bee colonies by two concentration of imidacloprid (200 and 5 ppb in sugar syrup or in pollen patty).the number of ovarioles and spermathecae diameters clearly affected by the high concentration of imidacloprid (200ppb) and there was a systemic effect of imidacloprid on the productivity of honey bee queen. the highest levels of imidacloprid residues were recorded in honey samples collected from colonies treated with imidacloprid diet patties than colonies treated with imidacloprid sugar syrup treatment. The higher treatment concentration (200ppb) produced higher residue content in Bee bread and honey samples. These results may help in the future studies on the chronic sublethal doses of neonicotinoid pesticides on the honey bee queen performance and honey bee behaviour.

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