Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1806-2636
ISSN on-line: 1807-8672
Doi: 10.4025/actascianimsci.v35i4.18683

Influence of agrochemicals fipronil and imidacloprid on the learning behavior of *Apis mellifera* L. honeybees

Marcela Pedraza Carrillo, Thaís de Souza Bovi, Adriana Fava Negrão and Ricardo de Oliveira Orsi*

*Departamento de Produção Animal, Faculdade de Medicina Veterinária Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Distrito de Rubião Júnior, s/n, 18618-970, Botucatu, São Paulo, Brazil. *Author for corresponding. E-mail: orsi@fmvz.unesp.br*

ABSTRACT. Agrochemicals on crop cultivated areas is a source of contamination for bees and may cause physiological and behavioral disorders and mortality. The LD_{50} of the pesticides fipronil and imidacloprid was determined and their effect on the learning behavior of *Apis mellifera* L. honeybee evaluated. LD_{50} was determined by the ingestion of contaminated food with different concentrations of insecticide concentrations: Fipronil (0, 0.8, 0.4, 0.2, 0.1 and 0.05 μg bee^{-1}) and imidacloprid (0, 0.4, 0.2, 0.1, 0.05 and 0.025 μg bee^{-1}). The method of proboscis extension reflection (PER) and learning through citral odor evaluated their responses to food stimulation. LD_{50} obtained were 0.28 ± 0.11 and 0.10 ± 0.04 μg bee^{-1} for fipronil and imidacloprid, respectively. The PER test showed no significant difference ($p < 0.05$) although agrochemicals affected the learning of bees. Insecticides fipronil and imidacloprid are extremely harmful to foraging Africanized *Apis mellifera* bees.

Keywords: beekeeping, toxicity, lethal dose.

Influência dos agroquímicos Fipronil e Imidaclopride no aprendizado de abelhas *Apis mellifera* L.

RESUMO. O uso de agroquímicos nas áreas de cultivo representa uma fonte de contaminação para as abelhas, podendo ocasionar distúrbios fisiológicos, comportamentais e mortalidade. O objetivo do presente trabalho foi determinar a DL_{50} dos agroquímicos Fipronil e Imidaclopride e avaliar o efeito destes no aprendizado de abelhas *Apis mellifera* L. adultas. A DL_{50} foi determinada por meio de teste de ingestão de alimento contaminado, com diferentes concentrações dos inseticidas Fipronil (0; 0,8; 0,4; 0,2; 0,1 e 0,05 μg abelha^{-1}) e Imidaclopride (0; 0,4; 0,2; 0,1; 0,05 e 0,025 μg abelha^{-1}). Para avaliar as respostas ao estímulo do alimento foi utilizado o método de reflexão de extensão da probóscide (REP) e aprendizado, utilizando odor citral. As DL_{50} obtidas foram 0,28 ± 0,11 e 0,10 ± 0,04 μg abelha^{-1} para o Fipronil e Imidaclopride, respectivamente. O teste REP não apresentou diferenças significativas ($p < 0,05$); porém, os agroquímicos afetaram o aprendizado das abelhas. Dessa maneira, conclui-se que os inseticidas Imidaclopride e Fipronil são nocivos às abelhas campeiras de *Apis mellifera*.

Palavras-chave: apicultura, toxicidade, dose letal.

Introduction

As any other group of living beings, bees need chemical substances for growth, reproduction and other vital functions. These chemical substances, named nutrients, may be obtained through nutrition. Plants are the main nutrition source for these insects (EDWARDS; WRATTEN, 2000).

However, the expansion of cultivation areas, especially fields planted with a single plant species, promotes the emergence of pests and diseases which make agriculture even more dependent on agrochemicals (COUTINHO et al., 2005). These products enter the soil-water-plant chain and represent a direct and indirect dangerous source of contamination to bees, which, in their turn, contaminate their products.

Bees poisoning may occur by contact or ingestion during flower visiting. During forage, bees get in touch with different groups of agrochemicals, such as insecticides, fungicides, acaricides and herbicides. However, the effect of insecticides on the pollinator fauna of agro-ecology systems has been well shown and is directly responsible for the decline of bee populations and indirectly for economic losses owing to the population decline of pollinators (RICHARDS; KEVAN, 2002). In the United States, about one third of commercial crops undergo liabilities through the decrease of natural pollinators (PAOLETTI, 1999).

Agrochemical group of insecticides acts directly upon the nervous system and causes over activity of neurons, or rather, those connected to learning and memory, and, as a consequence, may change brain structures with paralysis of leg, wings and digestive tract. Insects stop consuming water and food and, consequently, die from starvation or dehydration (MALASPINA; STORT, 1985). These products damage the return flight to the nest or the information given by the forager to the other worker bees through dance and sound (PHAM-DELÈGUE et al., 2002).

Commercially known as *Regent*, the insecticide fipronil belongs to the pyrazole class used to control agricultural pests. Nevertheless, the agrochemical affects non-target insects and causes the mortality of important pollinators such as *Apis mellifera* L. (GUNASEKARAN et al., 2007). In sub-lethal doses, fipronil may affect the gustatory perception, olfactory learning and motor activity of bees, that are essential functions to forage insects (HASSANI et al., 2005).

Within the neonicotinoid chemical group, the insecticide *Gaucho*, whose active agent is imidacloprid, is highly important. The above pesticide is extremely toxic to bees, which, even in low concentrations, causes high mortality due to the toxicity of its metabolites (FAUCON et al., 2005), whereas sub-lethal doses change foraging and communication (DECOURTYE et al., 2003).

One method to evaluate agrochemical toxicity is through LD_{50} tests, i.e., an acute dose may cause 50% mortality in a population. Generally, LD_{50} tests are done by contact and/or ingestion, and mortality is recorded 24/48h after exposure to the substance (DEVILLERS, 2002).

Other method of toxicity study in bees is through the proboscis extension reflex (PER) test which evaluates the bee´s apprenticeship with olfactory stimulus. During bee foraging, a learning process occurs by floral parameters, such as shape, location, color, and flower odor, associated with food reward (MENZEL; MÜLLER, 1996). In this manner, the proboscis extension reflex (PER) test, based on a temporal association paired to conditioned and unconditioned stimuli, simulates honey-bee-plant interactions. During conditioning, PER is caused by contact with taste receptors of the antennas with a sucrose solution (unconditioned stimulus), and simultaneously providing an odor (conditioned stimulus). When the proboscis extension occurs, the bee is immediately rewarded by receiving a sucrose solution (ROMERO et al., 2008).

Current research determines the lethal dose (DL_{50}) of the insecticide fipronil and imidacloprid and evaluates the effect on learning of the bee *Apis mellifera* L.

Material and methods

Experiment location and honeybees samples

The experiment was conducted at the Beekeeping Production Area, located in Lageado Experimental Farm, Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu, 22°50'30.16"S; 48°25'41.90"W.

Over 20-day-old Africanized honeybees from five hives, standardized with regard to number of brood frames and food, containing a young queen, were used. During the experiments, the hive entrance was closed for ten minutes and forager bees returning from the field were collected. They were placed in a recipient with a screen plastic and transported to the laboratory for the accomplishment of the experiment.

LD_{50} determination

LD50 toxicity was determined according to methodology described by Miranda et al. (2003), with modifications. Ten adult bees over 20-days-old were placed in wooden boxes containing screen sides (25.0 x 15.0 x 10.0 cm) and kept at room temperature. Further, 1 mL of honey was supplied in a feeder with test tube lid containing different doses μg bee^{-1} of the pesticides: 0, 0.8, 0.4, 0.2, 0.1 and 0.05 in the case of fipronil and 0, 0.4, 0.2, 0.1, 0.05 and 0.025 in the case of imidacloprid. The number of bees with behavioral modifications or dead during 30, 60, 90 and 120 minutes was reported. A control group received only honey and the experiments were performed in triplicate. The results obtained were used to calculate LD50, or rather, the concentration that would kill 50% of the bee population.

Pesticides effect on reflection and learning by proboscis extension

Proboscis Extension Reflection (PER) method was employed to evaluate the response to food stimulus (sugar syrup 50%) (SCHEINER et al., 2004). Twenty bees per treatment were used for the experiments, or rather, control (without agrochemical), fipronil and imidacloprid (LD_{50}). The bees were kept individually in plastic tubes, so that only the antennas were free to the outside. The bees remained necessarily without food for a period of three hours prior to tests. The antennas were then stimulated with a solution of sugar syrup and the number of individuals that exhibited PER was recorded. The control group received only a solution of sugar syrup without any agrochemical.

For the performance tests of learning and memory, bee antenna was put in contact with a floral odor citral which stimulated the antennas´ gustatory receptors. After the bees learned and memorized the floral odor, fipronil and imidaclopride were administered at previously obtained LD_{50} concentrations. Reflex or its absence of the proboscis extension after stimulus with floral odor was observed.

All tests were performed in triplicate.

Statistical analysis

Probit analysis with BioStat program was employed for LD_{50} calculation and the evaluation of bee learning was undertaken by analysis of variance with Tukey´s test at 5% significance level to compare means (ZAR, 1996).

Results and discussion

Table 1 shows rates for LD_{50} (μg bee^{-1}) obtained by ingestion test of insecticides fipronil and imidacloprid.

Table 1. Determination of mean lethal dose (LD_{50}) in µg bee^{-1} for fipronil and imidacloprid in *Apis mellifera* honeybees by ingestion test. Results represent average and standard deviation.

Fipronil	Imidacloprid
0.28 ± 0.11	0.10 ± 0.04

Research demonstrated that LD_{50} rates for insecticides under analysis lay below 2 µg bee^{-1} and thus classified as highly toxic to bees, according to Johansen and Mayer (1990).

Fipronil´s high toxicity is due to the mechanism which interferes with the functioning of the neurotransmitter GABA (amino-butyric acid) and which breaks the normal neuronal influx (chloride ions passage), causing excessive excitation, severe paralysis and death (GUNASEKARAN et al., 2007).

Similarly, imidacloprid acts as a competitive agonist of the insects´ nicotinic receptors, triggering severe neurotoxic effects (SUCHAIL et al., 2003). Suchail et al. (2000) registered LD_{50} reported rates of 5 and 25 ng bee^{-1} respectively for ingestion and contact tests.

It has also been reported that honeybees exposed to LD_{50} of the insecticides above showed no changes to the reflection of proboscis extension. However, their learning was affected, or rather, the reaction to the odor stimulant used within experimental conditions was significantly reduced. Hassani et al. (2005) observed changes in motor activity and a significant decrease in olfactory learning of honeybees exposed to a dose of 1 ng bee^{-1} of topically applied Fipronil. Decourtye et al. (2004) found that Imidacloprid reduced the olfactory perception and flight activity in worker bees exposed to sub-lethal doses. Bees exposed to doses of Fipronil by contact and ingestion (0.1 and 0.01 ng bee^{-1}, respectively), showed an impairment on the olfactory learning. Whereas bees treated with 0.1 ng bee^{-1} died after 7 days of exposure, those treated with 0.01 ng bee^{-1} ingested more water than those in other treatments and had their movements impaired (ALIOUANE et al., 2009).

Table 2 shows data related to the proboscis extension reflex test and learning when the insecticides fipronil and imidacloprid were used.

Table 2. Percentage of negative response to proboscis extension reflex test (PER) and learning in control, fipronil and imidacloprid treatments for *A. mellifera*. Results represent averages and standard deviation.

	Control	Fipronil	Imidacloprid
PER	17.94 ± 0.12 a*	23.25 ± 0.33 a	44.71 ± 0.00 a
Learning	6.25 ± 8.80 a	59.00 ± 2.10 b	70.63 ± 15.00 b

*Different lower case letters on the same line indicate statistical difference between the averages ($p \leq 0.05$)

Exposure of bees to agrochemicals causes adverse impacts on their foraging activity and reduces the worker bees´ longevity (MCKENZIE; WINSTON, 1989) leading to physiological changes, individual behavior and cell physiology alterations, due to stress induced by chemicals agents (GREGORC; ELLIS, 2011).

One factor that is linked to pollinators´ loss is Colony Collapse Disorder (CCD), characterized by a great decrease in the number of worker bees. CCD was first reported in the United States in 2006-2007, which lost an average of 45% of its hives (COX-FOSTER et al., 2007). Mullin et al. (2010) have reported a direct association between exposure of bees and agrochemicals, CCD occurrence and pollinators decline. The combination of different effects by pesticides causes high liabilities to bees and is intrinsically linked to the decrease in bee population observed over the past years (ALIOUANE et al., 2009).

The effect of insecticides on the agro-ecosystems pollinator wildlife has been well evidenced, being directly responsible for the reduction of bee populations and indirectly for economic losses as a result of the insects´ population decline (KEVAN, 1999).

Current results indicate the need for further studies that deal with the toxicity of agrochemicals on bees, coupled to the importance of the use of non-toxic pesticides to wildlife pollinators.

Conclusion

Current research found insecticides fipronil and imidacloprid are extremely harmful to foraging Africanized *Apis mellifera* bees.

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Received on September 21, 2012.
Accepted on December 14, 2012.

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