Original Article

TOXICITY OF INSECTICIDES WITH DIFFERENT MODES OF ACTION TO *APIS MELLIFERA* LARVAE (HYMENOPTERA, APIDAE)

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Abstract

Pesticides are one of the elements that lead to the decline in honeybee populations. Honey bee while foraging collect nectar and pollen from various crops, can be exposed to pesticide residues, which may be transported into their colonies and consumed by the larvae. The objective of this study was to evaluate acute toxicity of fipronil and lambda-cyhalothrin, two commonly detected pesticides within beehives, on the larvae of *Apis mellifera*. The testes chemicals were given to three-day old larvae. Treatments were administered using a micropipette at the bottom of each chosen comb cell. Each treatment included sixty larvae per dosage and the experiment was repeated three times. Larvae were given only acetone in the control treatment. Fipronil and lambda-cyhalothrin were toxic to the larvae of *A. mellifera* with LD_{50} values of 0.163 and 0.83 µg/larvae, respectively after seven days of exposure. The maximum mortality of 81.35% was recorded with a dose of 0.38 µg/larvae in Fipronil, whereas in lambda-cyhalothrin, it was 94.23% with a dose of 1.70 µg/larvae.

Keywords: Fipronil, honey bee larvae, Lambda-cyhalothrin, lethal-dose mortality

INTRODUCTION

During the last decade, there has been an increase in pollinator declines, and pesticides have been identified as a major cause worldwide (Goulson et al., 2015; Woodcock et al., 2017). The pesticide usage pattern in India is alarming because most of which are insecticides (51%), which affect insect pollinators, followed by herbicides (19%), fungicides and bactericides (33%), and other types such as rodenticides and nematicides (FAO, 2018). Managed honeybee colonies are periodically moved in the vicinity of agricultural fields to increase honey production and improve crop pollination. Since insect pollinators collect nectar and pollen from many crops, they can be exposed to pesticide residues from contact with pollen or foraging for nectar, which can then be transported to the colony. Contamination is not only observed in adults but also in larvae and beehive products, potentially leading to adverse effects to the bees (Doublet et al., 2014; Sharma et al., 2022). Honey bee larvae are orally exposed when they consume pollen and nectar; therefore, the potential for chronic toxicity occurs at the brood stage, early life stages are more sensitive to specific contaminants compared to adult stages (Atkins, 1986; Rortais, et al., 2005). Few toxicity studies evaluate the risk of pesticides to honey bee larvae, but approaches to larvae exposure need to be considered for honey bee larvae (Fantke et al., 2018).

Therefore, we chose fipronil and lambda-cyhalothrin, the two most frequently detected pesticides in the hive at concentrations found in pollen and bee bread. Fipronil belongs to the phenyl pyrazole group ($C_{12}H_4C_{l2}F_6N_4OS$) and is the first product of this group to be introduced for pest control and widely used in India against, thrips, termites, beetles, caterpillars that infest crops such as potato, corn, soy and onion. Lambda-cyhalothrin, another class of insecticide, comes under pyrethroids and is used on various crops, such as almonds, apples and cherries (Epstein et al., 2000). Pyrethroid insecticides have been

widely detected at the global scale (Tang et al., 2018) and are often associated with honey bee deaths (Zhou et al., 2011).

In 1981, an in vitro rearing methodology was proposed as a potential risk assessment tool for testing the toxicity of pesticides to worker bee larvae (Wittmann & Engels, 1981), and since then several in vitro rearing protocols with acceptable survival success have been developed (Vandenberg & Shimanuki, 1987; Peng et al., 1992; Oomen et al., 1998). The most recommended method was by Oomen et al. (1998), an in-hive method in which experimental bees comprise free-flying colonies are artificially contaminated by affixing a one-litre syrup feeder to the hive for twenty-four hours. The method is not reproducible, particularly if the test product is stored in the combs and not immediately dispensed to the brood by nurse bees. In addition, the method provides no quantitative data because it is impossible to measure the product consumed by larvae, and it cannot be used as a laboratory test.

Another method is the vitro method developed by Aupinel et al. (2005, 2007) and adopted by OECD (2016), later revised in 2021, in which 3rd larval stage is shifted to laboratory conditions in culture plates and fed artificial diets. Acute exposure was performed on the 4th day after grafting. Despite standardization, current in vitro rearing methods suffer variable survival rates, and OECD guidelines specify a minimum of 70% survival to adult emergence in the untreated controls for the test to be considered valid**.** In the present study, a method that mimics realistic exposure scenarios of honey bee larvae is used, in which the larva is left in the hive, marked the area with pins (60 larvae) and treated with different concentrations of test chemicals, therefore, simulating realistic exposure scenarios for honey bee larvae. All other protocols followed are in accordance with OECD (2015) requirements for larval testing.

Keeping in view the harmful effects of pesticides to honey bees and other pollinators, the present study evaluates the effects of fipronil and lambda-cyhalothrin on larvae of *Apis mellifera* colonies kept in field conditions.

MATERIALS AND METHODS

Selection of Colonies and Maintenance

Colonies of *A. mellifera*, previously checked for their health status (a healthy queen, abundant honey and pollen stores and regular brood pattern) were selected from the apiary of the Department of Entomology, Dr. Y. S Parmar University of Horticulture and Forestry (Himachal Pradesh, India). At this time onion, coriander and raddish were flowering at the apiary. The treatment started six days after the queen closed, so the age of the larvae was three days. The queens were caged for 24 hours using a queen excluder in order to have same age eggs, which were thereafter released in the hive. To obtain larvae in a sufficient quantity for toxicological bioassays, an empty honeycomb was introduced into a hive to allow the queen to lay eggs. Each colony contained a young normal egg-laying queen and a working population of six to seven brood frames and was supervised during the experiments to ensure the good condition of the colony.

Chemicals

Technical-grade chemicals were used in all experiments (>95% purity) sourced from sigma-Aldrich, and acetone was purchased from Merck Life Sciences. Treatments were administered using a 0 to 100 µl Eppendorf micropipette and a single 2 µl injection of the test solution at the bottom of each chosen comb cell. Larvae were given only acetone in the control treatment.

Range-finding test and exposure to fipronil and lambda-cyhalothrin

Experiments were conducted in 2020 and 2021. In order to determine the LD_{50} range, a preliminary trial was conducted with concentrations of the test chemicals with a mortality rate of 20-80% (Tab. 1). As both test chemicals had low water solubility, organic solvent e.g. acetone @1% was used to prepare test solutions instead of water according to the guidelines of OECD Guidance document, 214 (OECD, 2016). In the control treatment, larvae received a solvent (acetone) equal to that administered in insecticidal treatment.

Three colonies were used to test the different concentrations of each insecticides (n=60 larvae).

Table 1.

Insecticides and doses evaluated

Serial No.	Insecticide	Doses (Adult) Oral toxicity	Source
1	Fipronil (96.7% Purity)	0.005 µg, 0.01 µg 0.18 µg, 0.22 µg 0.30 µg, 0.38 µg	Sigma-Aldrich
2	Lambda-cyhalothrin (96.7% Purity)	0.28 µg, 0.55 µg 0.85 µg, 1.10 µg 1.50 µg, 1.70 µg	Sigma-Aldrich

Combs with treated and untreated larvae were tested for survival up to capping. The presence of standard (i.e., pearly white) larvae was taken as evidence of indication of survival. The larval mortality was calculated in % by comparing the number of bees that died during larval stage (day 3 to day 8) to the number of larvae on Day 3 when dosing started. The larval mortality was inferred from empty cells, larva that was immobile or did not react to the contact of the paintbrush or larvae with slight colour changes (i.e., brownish) was noted as dead (Gashout H.A, 2009)

Data and statistical analysis

Six doses of each insecticide that resulted in larval mortality in the range of 20 to 80% or close to this range were selected for calculating LD_{50} values. The mortality due to each insecticide was corrected using Abbott's correction (Abbott, 1925) as follows:

$$\text{Corrected \% mortality in treatment} = \frac{\text{\% mortality in treatment - \% mortality in control}}{\text{100 - \% mortality in control}} \times 100$$

Corrected mortality data and its fiducial limits were calculated by probit regression analysis (Finney, 1971) to calculate LD_{50} values using OPSTAT software (Sheron et al., 1998).

RESULTS

Control toxicity

The average observed mortality was 1.66% in control in fipronil, whereas in lambda-cyhalothrin it was 13.33%, which is within the acceptable range observed for control mortality as reported by OECD (2016) using the in-vitro larval rearing method where cumulative larval mortality from day 3 to day 8 should be less than 15% across all replicates.

Lethal toxicity of fipronil to bee larvae

The data on the larval mortality of *A. mellifera* after the exposure period to establish the acute oral LD_{50} (µg) when fed with different doses of fipronil is summarized in Tab. 2. The maximum mortality of 81.35 % was recorded with a dose of 0.38 µg/larvae. The larval mortality of *A. mellifera* exceeding 50% was recorded with the doses of 0.22, 0.30 and 0.38 µg/larva, whereas the doses of 0.05, 0.10 and 0.18 µg/larvae resulted in 18.64, 35.59 and 47.45% mortality, respectively. Probit analysis of the data revealed that 0.163 µg/larva were required to kill 50% of the test population after seven days of exposure and % fiducial limit of 0.113 and 0.234 µg/larva. The chi square test showed that the data was homogeneous (χ^2 cal=0.673; χ^2 tab=12.59) at p=0.05 and 6 degrees of freedom. Probit kill followed a linear relationship with log dose as Y=1.8692x+2.7337.

Lethal toxicity of lambda-cyhalothrin to bee larvae

The data on larval mortality of *A. mellifera* after exposure period in order to establish the acute oral LD_{50} (µg) when fed with different doses of lambda-cyhalothrin is summarized in Tab 3. The bioassay was carried out by exposing the larvae of *A. mellifera* to lambda-cyhalothrin at doses ranging from 0.280 to 1.700 µg/bee. The maximum mortality of 94.23% was recorded

Table 2.

Comparison between the larval observed mortality (OM %) and corrected mortality (CM %) for each tested concentration (Conc µg/larva) of fipronil using a probit analysis

Dose x 1000 (µg/larva)	Log (Dose) (X)	No of larvae treated (n)	No of dead larvae	Observed mortality (%)	Corrected mortality (%)	Empirical Probit (Y)	Expected probit
0.005	0.699	60	12	20.00	18.64	-0.891	-0.958
0.01	1.000	60	22	36.66	35.59	-0.369	-0.395
0.18	1.255	60	29	48.33	47.45	-0.064	0.082
0.22	1.342	60	36	60.00	59.32	0.236	0.245
0.30	1.477	60	40	66.66	66.09	0.415	0.497
0.38	1.580	60	49	81.66	81.35	0.891	0.689
0.00	0.00	60	1	1.66	-	-	-

Regression equation Y = 1.8692x + 2.7337
Heterogeneity test
χ2 cal = 0.673
χ2 tab = 12.592
LD_{50}(µg) = 0.163
Fiducial limit (µg) = 0.113 and 0.234

Table 3.

Comparison between the larval observed mortality (OM %) and corrected mortality (CM %) for each tested concentration (Conc µg/larva) of lambda-cyhalothrin using a probit analysis

Dose x 1000 (µg/larva)	Log (Dose) (X)	No of larva treated (n)	No of larva bees	Observed mortality (%)	Corrected mortality (%)	Empirical Probit (Y)	Expected probit
0.28	1.447	60	14	23.33	11.53	-1.198	-1.521
0.55	1.740	60	18	30.00	19.23	-0.869	-0.581
0.85	1.929	60	35	58.33	51.92	0.048	0.024
1.10	2.041	60	40	66.66	61.53	0.293	0.383
1.50	2.176	60	46	76.66	73.07	0.615	0.815
1.70	2.230	60	57	95.00	94.23	1.574	0.989
0.00	0.00	60	8	13.33	-	-	-

Regression equation Y = 3.2087x - 1.1693
Heterogeneity test
χ2 cal = 0.022
χ2 tab = 9.488
LD_{50}(µg) = 0.83
Fiducial limit (µg) = 0.667 and 1.047

with a dose of 1.70 µg/larvae. Probit analysis of the data revealed that 0.83 µg was required to kill 50% of the test population after seven days of exposure. A chi-square test showed that the data was homogeneous (χ^2 cal=0.022; χ^2 tab=9.48) at p=0.05 and 6 degrees of freedom. Probit kill followed a linear relationship with log dose as Y=3.2087x - 1.1693.

DISCUSSION

Few studies have evaluated how insecticides mainly belonging to phenyl pyrazole group (Fipronil) and pyrethroids (Lambda-cyhalothrin) affect the developmental phase of honey bee larvae. The results of this study demonstrate that fipronil and lambda-cyhalothrin negatively impact larval development. The results also showed that the impact on larval development is dose-dependent. This finding agrees with the funnel Hypothesis (Warne MS, 1995) which states that toxicity will tend towards concentration. The LD_{50} obtained was 0.163 µg/larva. The results of the current experiments contradict with those of OECD (2017), which indicated that the LD_{50} value of fipronil was 0.0218 µg/larva. Genetic variations and the approach used by OECD (2017) may cause the differences between our findings.

Other aspects of brood growth may also vary among colonies (Collins, 2004). Additionally, lineage-specificchangesinsusceptibilitytostressors and brood temperature-related vulnerability to toxicants are taken into account (Medrzycki et al., 2010; Jensenet al., 2009). Furthermore, there have been no investigations of GABA receptors in *A. mellifera* larvae; most of the studies on GABA have been conducted on *Drosophila* sp. larvae (Enell et al., 2007). Dzitoyeva et al. (2005) stated that GABA receptors in *Drosophila* are necessary for optimal larval development, given that fipronil, a phenyl pyrazole pesticide, blocks aminobutyric acid (GABA) receptors and central nervous system excitement ultimately results in the death of the insect (Law, 2008). Similarly, Silva et al. (2015) conducted bioassay investigations of fipronil on honey bee larvae and reported necrosis in the head and thorax when bees were subjected to 5 ng and 20 ng a.i /larvae. Even though few larvae survived toxicity tests in our research, developmental failure from larva to pupa was observed and the presence of empty cells was probably the result of hygienic behaviour of house cleaning bees as they threw the affected abnormal larvae.

These findings are supported by Locke (1998) and Martins & Bitondi (2012) who investigated the effect of fipronil on bee fat tissue during postembryonic development and found that fipronil inhibited the synthesis of hexamerins, a protein involved in the transport of hormones, making it essential for metamorphosis. Comparing our result of LD_{50} for larvae (0.83 µg/larvae) with those for adults of *A. mellifera* (0.909 µg/bee - Gough et al., 1984; European Food Safety-Authority, 2013), we noticed that larvae seemed more susceptible to lambda-cyhalothrin than adults. The present investigations are supported by the works of Micheletti and Soares (1993), Cruz-Landim (2009) and Suchail et al. (2001, 2004), who observed that honey bee larvae do not defecate until the pupal stage and then excrete a waste pellet known as meconium (Winston, 1987), and that metabolites may accumulate and thus increase the toxicity of the parent substance in larval stage. Concentrations of pesticides and metabolites inside brood tissues may result in constant pesticide stress (Wu, 2011). Regular avoidance behaviour and excretion of toxic wastes might contribute to decreased toxicity in the field (Desneux, 2007; Atkins, 1986).

It was also found that when larvae survived, they did not develop into pupae. This work was supported by the findings of Tuteja et al. (2022), who reported that lambda-cyhalothrin was highly toxic for 5-6 day old larvae, reduced capping stage to 6.67% and no larva survived until emergence at 12.5 ppm. Dai et al. (2010) similarly found that pyrethroids could reduce capping rate and extend the duration of the immature stage by causing delayed larval development. The observed mortality in the fipronil control group was 1.66%, while the lambda-cyhalothrin control group was 13.33%, both of which were well within the standards of the OECD 239 Guidance Document (2016).

In the present study, we established that

exposure of honeybee larvae to fipronil and lambda-cyhalothrin at concentrations comparable to those reported in honeybee products might result in larval mortality and developmental failure. The mortality might have been caused by the solvent acetone and the high temperatures during the experiments. On the basis of LD50 toxicity, fipronil and lambda-cyhalothrin proved toxic for larvae of *A. mellifera*. Considering the larval toxicity of fipronil and lambda-cyhalothrin in hives, the application during crop bloom is harmful to pollinators and should be limited to minimize its impact on pollinators. Studies on bee colonies should complement our findings in the next stage. The distribution and accumulation of fipronil, lambda-cyhalothrin and their metabolites in honey bees at various developmental stages must be investigated at colony level, as honey bees are eusocial insects. It can be evaluated through semi-field (Tier 2) as described by Schur et al., (2002) and field studies (Tier 3).

Ethics approval and consent to participate

All the methods were performed in accordance with the relevant guidelines/regulations/ legislation.

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