Determination of fipronil LD_{50} for the brazilian bee *Melipona scutellaris*

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DOI: 10.5073/jka.2012.437.046

To better understand the sensitivity of the models represented by *Apis mellifera* L., 1758 in toxicology studies of insecticides to bees, the aim of this study was to determine the LD_{50} of fipronil by topical application on the stingless bees *Melipona scutellaris* Latreille, 1811. Foraging bees were collected at the nest entrance and in laboratory anesthetized with CO_2 for applying 1.0 µL of fipronil solution on the pronotum. Each group of treatments was made with thirteen bees divided in three cages, while in the control treatments the bees received only acetone. During the assay, the behavior and the number of dead bees were registered. The results showed that the insecticide fipronil applied topically was harmful to *M. scutellaris* and for *A. mellifera*, where the LD_{50} for 48 hours was 0.41 ng a.i./bee or 4.1 ng a.i./ g of bee. Comparing the LD_{50} values here obtained with the stingless bee *M. scutellaris* and those of *A. mellifera* in literature, we can conclude that the native bees are more sensitive to fipronil than the allochtonous bee, suggesting that further studies should be accomplished to determine the real hazard of pesticides to natives bees.

Keywords: stingless bees, pesticides, phenylpyrazole, toxicity, LD_{50}

1. Introduction

The insecticide fipronil (phenylpyrazole - $C_{12}H_4Cl_2F_6N_4OS$) acts on the nervous system of insects by blocking chloride channels through the receptor of gamma-aminobutyric acid and glutamate. Due to its mode of action it is considered a new generation insecticide, being a broad-spectrum systemic insecticide and effective at low application rates. Widely used in Brazil and more than 70 countries it is considered highly toxic to bees, which is why its use is banned in France since 2004[1-10].

Toxicological studies with bees use mostly the model species *Apis mellifera* L., 1758 (Hymenoptera: Apidae) and it has been observed that sublethal doses of fipronil can cause behavioral changes, to these bees, related mainly with tasks such as feeding and foraging, fundamental for the survival of colonies[11-18]. However, native bees exposed to pesticides may be at a different risk, because differences in tolerance among species of bees have been observed by several authors[19-26] and most show that wild bees are more sensitive to the insecticides than the honeybee *A. mellifera*[27,28].

Among the stingless bees within the tribe Meliponini, *Melipona scutellaris* Latreille, 1811 (Hymenoptera: Apidae) is popularly known. It is endemic in northeastern Brazil and distinguished by its ease of domestication and management, honey production and significant potential for replication on a large scale for pollination in greenhouses and open field, beyond its ecological importance as pollinators of native plants in Brazil[29-34].

Seeking to diversify the species of bees to better understand and compare the sensitivity of the models represented by honeybee *A. mellifera*[27], the objective of this study was to determine the topical LD_{50} of the insecticide fipronil for the stingless bees *M. scutellaris*.

2. Material and Methods

Three colonies of *M. scutellaris* from the Universidade Estadual Paulista (UNESP) *campus* Rio Claro, were used in the experiment. The hives were kept in protected room where the bees had free access to the external environment through a plastic tube that connected the nest entrance and the outside. All time, the colonies were surveyed to assess the health, queen laying capacity, foraging activity and food availability. To promote the survival of colonies during a dry season, 60 % of sucrose solution prepared with lemon juice was provided[35].

2.1 Acute toxicity test (LD_{50})

Assays were carried out at the Center for the Study of Social Insects of UNESP with some modifications on the directives of the Organization for Economic Cooperation and Development[36].

To determine the topical LD_{50} of the insecticide fipronil (95% of purity, Bayer CropScience, Brazil) to foragers of *M. scutellaris*, a stock solution (1000 ng a.i./µL acetone) was prepared and next a range of several concentrations between 0.5 to 5.0 ng of a.i/µL acetone. The control treatment received only acetone, after its low toxicity had been assessed in preliminary assay compared with water.

To facilitate the handling and application, the bees were anesthetized with CO_2 (ten seconds). With a repetitive micropipette the volume of 1.0 µL of solution was applied on the thorax of the bees. To ensure the variability among the colonies and to obtain a realistic and reliable value of LD_{50}, the bees from each repetition were taken directly from a single colony. In each treatment (=concentration) we had three distinct groups of bees which originated from different colonies. Thus, each treatment (group of bees from one concentration) consisted of three replicates with ten bees, in total thirty specimens. During the assay, bees were fed *ad libitum* with sucrose-solution (50%), and cages were kept in climatic room at 29±2ºC, relative humidity of 70±5% and darkness.

2.2 Data collection and analysis

Along 72 hours after the application of fipronil on *M. scutellaris*, assessments were made one, four and every twenty-four hours, with registration of all behavior, as well as the number of dead bees. Statistical analysis to determine the LD_{50} value were performed using a log-logistic model from the package "drc" (Analysis of Dose-Response Curves) [39] compiled by the statistical software R[40].

3. Results and discussion

Bioassays performed in order to compare the toxicity of acetone and water showed that this organic solvent was no toxic to *M. scutellaris* foragers. Already, the insecticide fipronil topically applied was considered highly toxic to *M. scutellaris* foragers, with a LD_{50} for 48 hours of 0.41 ng a.i./bee ($CL_{95\%}$ = 0.23 - 0.58; D.F. = 16 and χ^2 = 9.8238, Figure 1). Comparing this result with the LD_{50} of fipronil established for other species of bees, foragers of *M. scutellaris* were more sensitive to fipronil than *A. mellifera* (1.9- 6 ng a.i./bee), *Megachile rotundata* Fabricius, 1787 (4 ng a.i./bee), *Nomia melanderi* Cockerell, 1906 (113 ng a.i./bee) and *Scaptotrigona postica* Latreille, 1807 (0.54 ng a.i./bee)[13,18,37,38,41,42]. Likewise, taking into consideration that the workers of *M. scutellaris* have a mean weight of 0.1g, recalculating the LD_{50} we got a LD_{50} of 4.1 ng a.i./g of bee. In this way, *M. scutellaris* remains more susceptible to fipronil than *A. mellifera* (103 ng a.i./g of bee), *M. rotundata* (132 ng a.i./g of bee) and *N. melanderi* (13,190 ng a.i./g of bee)[41].

The doses of fipronil from 1.5 to 5 ng a.i./bee for 48/72 hours and 5 ng a.i./bee for 24 hours, caused 100 % of mortality, respectively (Figure 2) The dose of 1.5 and 1.0 ng / bee also had high rates of mortality after 48 hours of intoxication, with 96% and 85% of dead bees, respectively.

Still, the bees in the group treated with 5.0 ng of fipronil/bee showed signs of intoxication: after 4 hours this group had bees with their wings vibrating. This same behavior was observed in *A. mellifera* treated with 0.1 ng fipronil/bee and after 11 days of exposure dose of 0.01 ng of fipronil/bee[11]. According to these authors the behavior of vibrating wings is accompanied by the emission of alarm pheromone, which causes attacks among individuals, also observed in this study. After 24 hours of contamination, surviving bees in the groups treated with the higher doses of fipronil (2.0, 2.5 and 5.0 ng a.i./bee) had tremors followed by paralysis and death. These same signals were also observed in honeybee *A. mellifera* treated with sucrose solution contaminated with fipronil 2 g/Kg of diet[12].

These results are consistent among the diversity of bees, which differ in their vulnerability to exposure to insecticides[28]. Several studies[19-26] show differences in tolerance and/or sensitivity between species of bees and pesticides, most of these results show that the species honeybee *A.*

mellifera was more resistant compared to species of stingless bees which corroborates the suggestion that wild bees are a pollinating group at particular risk for exposure to pesticides[27].

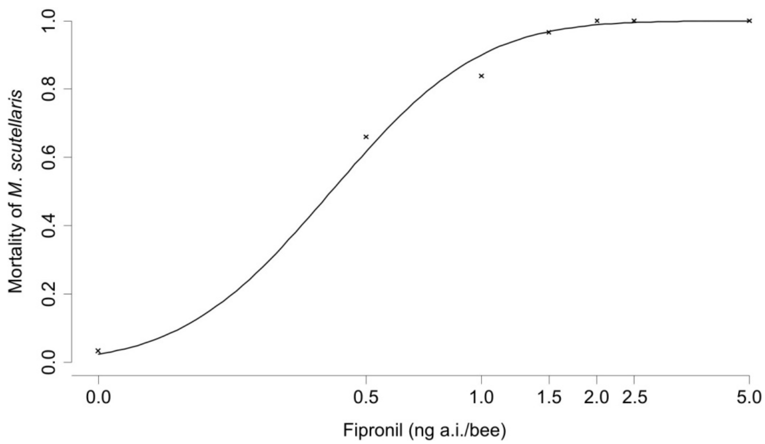

Fig. 1 Acute toxicity (48 hours) by topical application of the insecticide fipronil to foragers of *Melipona scutellaris*.

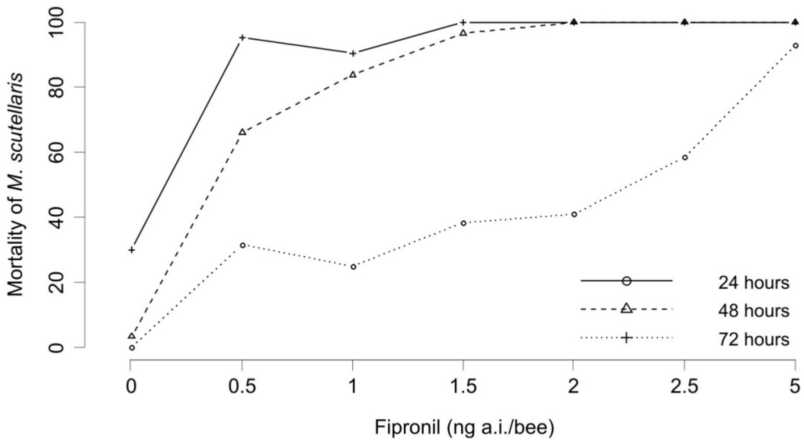

Fig. 2 Mortality evolution of foragers *Melipona scutellaris* when exposed at different doses of fipronil.

The findings also support the idea that ecotoxicological studies on diverse pollinating species can be used to obtain a better understanding of how the sensitivity of a model representative of honeybees (*A. mellifera*) can be compared to other species of bees[43].

The bee species *M. scutellaris* is considered a potential effective pollinator species for production on a large scale as a pollinator in greenhouses and the open field, with ease of maintaining strong hives, which can be easily transported and multiplied[30]. Brazil has a high diversity of bees that interact with numerous plant species[44] and it is believed that 33% of the crops that provide food for the human population depends on pollination by bees[45].

We believed than poisoning by insecticides is one of the causes of high mortalities of bees, especially in areas in southern and southeastern Brazil, where the disappearance of bees caused by insecticides has become a concern. Between 2008 and 2010, about 5000 bee hives of Africanized *A. mellifera* were lost in the central region of São Paulo. Hives of native bees were not included[46].

Since Brazil has a high diversity of native bees, endemic in the tropics and sensitive to low temperatures, studies on the toxicity of insecticides in Brazil should focus on these species[47].

4. Conclusion

The insecticide fipronil was highly toxic to foraging stingless bee *M. scutellaris* under laboratory conditions, with a topical LD_{50} in 48 hours of 0.41 ng a.i./bee (4.1 ng of fipronil/g bee). It is suggested that bees of *M. scutellaris* are more sensitive to fipronil than *A. mellifera* (Africanized and Italian), *M. rotundata*, *N. melanderi* and *S. postica.* Tremor followed by paralysis were the main signs of intoxication observed in the groups treated topically with the highest dose of fipronil.

The LD_{50} results determined in this work are being used to assess behavioral changes through the Proboscis Extension Reflex (PER) and locomotor activity, in particular which doses of fipronil causing sublethal effects in foragers of *M. scutellaris*.

Acknowledgement

This study was supported by CAPES (Brazil), Dutch Ministry of Agriculture, Nature and Food Quality (BO-10-011-113) and GEF.

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