



Residual effect of imidacloprid and beta-cyfluthrin on Africanized *Apis mellifera* L. (Hymenoptera: Apidae) workers

Raiza ABATI¹, Gabriela LIBARDONI², Gabriela OSOWSKI³, Edgar de SOUZA VISMARA³, Fabiana Martins COSTA-MAIA⁴, Everton Ricardi LOZANO¹, Paulo Fernando ADAMI¹, and Michele POTRICH¹

¹ Programa de Pós-Graduação em Agroecossistemas, Universidade Tecnológica Federal do Paraná, Campus Dois Vizinhos, Estrada para Boa Esperança, Km 04 CEP, Paraná 85660-000 Dois Vizinhos, Brasil

² Programa de Pós-Graduação em Agronomia, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445 Km 380, Campus Universitário Cx. Postal 10.011, CEP 86.057-970, Londrina, Paraná, Brasil

³ Engenharia Florestal, Universidade Tecnológica Federal do Paraná, Campus Dois Vizinhos, Estrada para Boa Esperança, Km 04 CEP, Paraná 85660-000 Dois Vizinhos, Brasil

⁴ Programa de Pós-Graduação em Zootecnia, Universidade Tecnológica Federal do Paraná, Campus Dois Vizinhos, Estrada para Boa Esperança, Km 04 CEP, Paraná 85660-000 Dois Vizinhos, Brasil

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Abstract – Canola has a long period of flowering in the winter season and this, associated with the lack of other flowers and the use of insecticides to control insect pests, means it may have a great impact on bee populations, such as *Apis mellifera* L. (Hymenoptera: Apidae). In this context, the use of insecticides with lower residuals and less harmful effects to bees is fundamental. Thus, the objective of the current work was to evaluate, in a laboratory bioassay, the residual toxicity and effect of imidacloprid, beta-cyfluthrin, and a mixture of both, when applied on canola in the field, on Africanized *A. mellifera* workers. Three commercial products were applied, formulated with imidacloprid, beta-cyfluthrin, and beta-cyfluthrin + imidacloprid. Each plot received one of the three treatments, in the concentration recommended by the manufacturer, at intervals between 14 and 0 days prior to conducting the bioassays. On the day of the bioassay, the bees were exposed to the plant material containing the treatments for a period of two hours, and were transferred to PVC cages, containing food and water. Mortality assessments were then performed at pre-established intervals until a 96 h post-exposure. Residuals on canola leaves of all tested products caused a significant reduction of honeybee worker longevity, which was less accentuated when the interval after treatment application increased. Safer pest management strategies are needed to ensure the preservation of bees.

pollinator / honey bee / insecticide / neonicotinoid / pyrethroid / *Brassica napus*

1. INTRODUCTION

Apis mellifera L. honey bees are social insects belonging to the order Hymenoptera, family Apidae, which have economic importance due to

their production of honey, propolis, royal jelly, apitoxin and also because they are major crop pollinators (Costa-Maia et al. 2010; Giannini et al. 2015; Hung et al. 2018; Imperatriz-Fonseca et al. 2012). These insects need plants for their survival. They pass through several flowers, collecting pollen and nectar, which they transport to their colonies, and as they land on one flower and then another, they deposit pollen on the stigma of the next flower, effecting pollination (Roberto

Corresponding author: M. Potrich,

michelepotrich@utfpr.edu.br

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et al. 2015). However, during the winter period, floral resources are scarce (Sharma et al. 2014).

In this way, canola, which is the main wheat crop rotation option and whose flowering period lasts from 45 to 60 days, may contribute to the maintenance of honey bee colonies in the winter time (Batista et al. 2018). In addition to the benefit for the colonies, the pollination service performed by bees in the cultivation of canola guarantees an increase in the quantity of seeds / grains and also in the quality of the oil (Adamidis et al. 2019; Ali et al. 2011; Bommarco et al. 2012; Durán et al. 2010; Jauker et al. 2012; Rosa et al. 2011). The abundance and diversity of pollinating insects vary by region (Blochtein et al. 2015). *Apis mellifera* honey bees are the most frequent pollinators found in canola culture; Africanized honey bees being the most frequent on this crop in Brazil. In addition to the genus *Apis*, bees belonging to the Apidae, Halictidae, Colletidae, Andrenidae, and Megachilidae families can also be found on this crop (Blochtein et al. 2015; Esquivel et al. 2021; Fuzaro et al. 2019; Rosa et al. 2011).

However, like other crops, canola is affected by insect pests that compromise productivity. Some of these insect pests occur during the vegetative cycle, such as the curcubit beetle *Diatraea speciosa* (Germer, 1824) (Coleoptera: Chrysomelidae), and defoliating caterpillars (Lepidoptera), however, the biggest problem is the aphid *Brevicoryne brassicae* (Linnaeus, 1758) (Hemiptera: Aphididae), especially when the pests occur during the flowering period (Marsaro Júnior et al. 2019, 2017; Nery-silva et al. 2015). In very dry winter years aphids are often the most common insect pests on canola with potential for grain yield reduction demonstrating a need for pest control. The lack of alternative products to control aphids on this crop (Agrofit 2022) leads producers to use synthetic chemical insecticides with a wide spectrum of action and a poor selectivity to natural enemies and pollinators (Marsaro Junior et al. 2017).

Due to their efficacy, imidacloprid (neonicotinoid) and beta-cyfluthrin (pyrethroid) are among the most commonly used insecticides (Agrofit 2022). Despite belonging to different chemical

groups, they act on the central nervous system of insects, with neonicotinoids being modulators of the nicotinic acetylcholine and pyrethroids modulators of the sodium channels (Gallo et al. 2002; Sparks et al. 2020). Although both insecticides are recommended for the control of hemipteran, coleopteran, lepidopteran, and thysanopteran pests they also affect non-target insects such as bees.

The effects of synthetic chemical insecticides on honey bees have been studied, due to the possible influence on Colony Collapse Disorder (CCD) and the mortality of honey bees (Abati et al. 2021; Grigori 2019; Kaplan 2012; MAP 2017). In this sense, research has been carried out on the action of neonicotinoids and pyrethroids in *A. mellifera* with respect to mortality (Aliouane et al. 2009; Baptista et al. 2009; Stanley et al. 2015), behavioral changes (Aliouane et al. 2009; Charreton et al. 2015; Tosi et al. 2017), biochemical and metabolic changes (Catae et al. 2018; Chaimanee et al. 2016; Christen and Fent 2017), and contaminated honey (Gawel et al. 2019). However, there are few studies related to the residual time of products in plants which can cause damage to honey bees (Chen et al. 2017; Gomes et al. 2019). When applied to crops, insecticides can be absorbed and translocated by the plant, or they can be lost through volatilization, degradation or even environmental conditions, such as excessive rain or solar radiation. Despite these important studies, little is known about the residual effect of these products on honey bee survival. The objective of this work was to evaluate, in a laboratory bioassay, the toxicity of the residual effect of commercial insecticide formulated with imidacloprid, beta-cyfluthrin, and their mixture, when applied on canola in the field, on Africanized *A. mellifera* workers.

2. MATERIAL AND METHODS

The experiment was carried out at the Biological Control Laboratory (LABCON), and at the Experimental Apiary of the Apiculture Teaching and Research Unit (UNEPE

– Apicultura) and Teaching and Research Unit Annual Crops (UNEPE Culturas Anuais) of the Universidade Tecnológica Federal do Paraná, Campus Dois Vizinhos (UTFPR – DV).

2.1. Obtaining *Apis mellifera*

To conduct the experiments, worker honey bees of the species Africanized *A. mellifera*, with approximately 48 h of adult life, were obtained from capped brood combs in the apiary. Frames containing one-day eggs were marked and, after 19 days, collected and wrapped in kraft paper, sealed, perforated, and transported to LABCON. The frames were kept for 48 – 50 h in an air-conditioned chamber (30 ± 2 °C, RH $70 \pm 10\%$), in order to simulate the environmental conditions of the colony and thereby obtain homogeneous emergence of the honey bees (Colombo et al. 2020, 2019; Libardoni et al. 2021; Potrich et al. 2020, 2018).

2.2. Treatments

We used commercial products, registered and authorized for sale in Brazil, following the recommendations of the respective manufacturers for the control of aphids in bean culture (Table I), as there were no registered and released products for canola in Brazil. The active ingredients were imidacloprid (neonicotinoid), beta-cyfluthrin (pyrethroid) or imidacloprid + beta-cyfluthrin. Control groups had no treatment.

To determine the residual effect of the insecticides, the experimental area (2000 m²) with canola cultivation was divided into 16 plots (125 m² each). At the time of spraying, the cultivation of canola was in the flowering / grain filling phase, and only the insecticides from the experiment were used in the management. Each plot was sprayed via a CO₂ operated backpack sprayer with a rate of 150 L ha⁻¹ according to the concentration recommended by the manufacturer, at 14, 09, 06, 03, and 0 days prior to conducting the bioassays. The control received no treatment.

On the day of assembly / preparation of the bioassay in the laboratory, the flowers and leaves of the middle and upper third of the canola plants, with 104 days of development, were cut randomly in each plot, using pruning shears, packed in kraft paper bags, identified, and transported to LABCON. The leaves from each day of spraying (14, 09, 06, 03, and 0 day prior to collection) and each product (imidacloprid, beta-cyfluthrin and imidacloprid + beta-cyfluthrin), as well as the leaves without insecticide application (control) were placed in glass Petri dishes (1.5 cm high × 15 cm diameter) properly identified, totaling 16 treatments (one leaf per dish). Worker honey bees of *A. mellifera*, that had emerged within the last 48 h, were anesthetized with CO₂ for up to 120 s, after which groups of 20 bees were allocated to each Petri dish containing the treated leaves, with five dishes per treatment, where the honey bees remained in contact for two hours (adapted methodology Colombo et al. 2020; Potrich et al. 2020). After this period, the honey bees were relocated to PVC cages (20 cm high × 15 cm diameter), with 20 workers

Table I Active ingredients of the products, concentration of the commercial product, and dosage recommended by the manufacturer (Agrofit 2022)

Active ingredient	Concentration g/L	Dosage mL c.p./ha
Imidacloprid	480 g/L	200 mL c.p./ha
Beta-cyfluthrin	50 g/L	100 mL c.p./ha
Imidacloprid + Beta cyfluthrin	100 g/L + 12,5 g/L	750 mL c.p./ha

* Commercial products were dissolved in water, as recommended by the manufacturer

per cage, composing five replicates per treatment (adapted methodology for Colombo et al. 2020; Libardoni et al. 2021).

The cages were closed with voile, and candy paste (confectioner's sugar and honey) and cotton soaked in water were supplied. The cages were placed in an climate-controlled room ($27 \pm 2^\circ\text{C}$, RH $60\% \pm 10\%$ and 12 h photoperiod), where mortality assessments were carried out one, two, three, four, five, six, nine, 12, 15, 18, 21, 24, 30, 36, 42, 48, 60, 72, and 96 h after the transfer of the honey bees to the cages (adapted methodology of Baptista et al. 2009; Colombo et al. 2020; Libardoni et al. 2021).

Due to the knock down effect caused by the insecticides, it was not possible to discriminate dead or alive honey bees within the first hours post-exposure. Indeed, this knock down effect is characterized honey bees remaining at the bottom of the cages with disordered movements and tremors. The confirmation of mortality was carried out only 6 h after the beginning of the experiment, when the movements and tremors had completely stopped. The dead honey bees had a dark color and an exposed / externalized proboscis, characterizing death by poisoning (Libardoni 2017).

The Cox-proportional-hazards model was used to model the survival data. The model's predictors (fixed effects) were the tested products and their residual time. Since the individuals were arranged in cages (replicates), the inclusion of a random cage effect in the model was tested. However, since the inclusion of this random effect did not result in significant differences in the likelihood ratio test, it was decided to use the simplest model with fixed effects only.

The fitted model was submitted to the Wald test to verify the possible occurrence of interaction between the predictors. Next, we plotted the predicted survival proportion at different points in time for the risk groups. Finally, the risk groups (levels of predictors) were pairwise compared using the log-rank test at $p < 0.05$. The complete analysis was performed using the R package software survival test (Kaplan and Meier 1958; Therneau 2020).

3. RESULTS

In all treatments, except for 14-day residual of imidacloprid (Figure 1) and 14-day residual of imidacloprid + beta-cyfluthrin (Figure 3), there was a reduction in the longevity of the honey bees when compared to the control, such that the shorter was the interval between the insecticide application on the canola field and the contact with honey bees, the shorter was the survival time of honey bees.

When exposed to canola leaves treated with imidacloprid on the day of the bioassay, the survival of honey bees 18 h post-exposure was of approximately 17% (Figure 1). The survival of bees was of 25% when exposed 3 days after canola treatment, and of 35% and 78% in the 6 and 9 days post-application groups, respectively. The survival of bees from these groups differed from each other and was significantly lower than in the control group ($p < 0.001$ for all comparisons) (Figure 1). However, approximately 90% of bees were alive in the group exposed to canola leaves treated 14 days before the bioassays (Figure 1), which did not differ from the control ($p = 0.803$).

When exposed to canola leaves treated with beta-cyfluthrin on the day of the bioassay, almost all honey bees were found dead 18 h post-exposure (Figure 2). It was observed that less than 50% of bees were still alive 18 h post-exposure to canola when leaves were treated 14 days before the bioassays (Figure 2). The survival of bees from the 3, 6, 9 and 14-days residual time groups was lower than in the control group ($p < 0.001$ for all comparisons) (Figure 2).

When exposed to canola leaves treated with formulated product with imidacloprid + beta-cyfluthrin on the day of the bioassay, the survival of honey bees 18 h post-exposure was of approximately 35% (Figure 3). For the same time period, a survival of approximately 85% was observed for bees exposed to leaves treated 14 days before the bioassays, a percentage close to the one observed at 96 h post-exposure (70%). The survival of bees from the from the 3, 6 and 9-days residual time groups was lower than in the control group ($p < 0.001$ for all comparisons) and

Imidacloprid residual effect

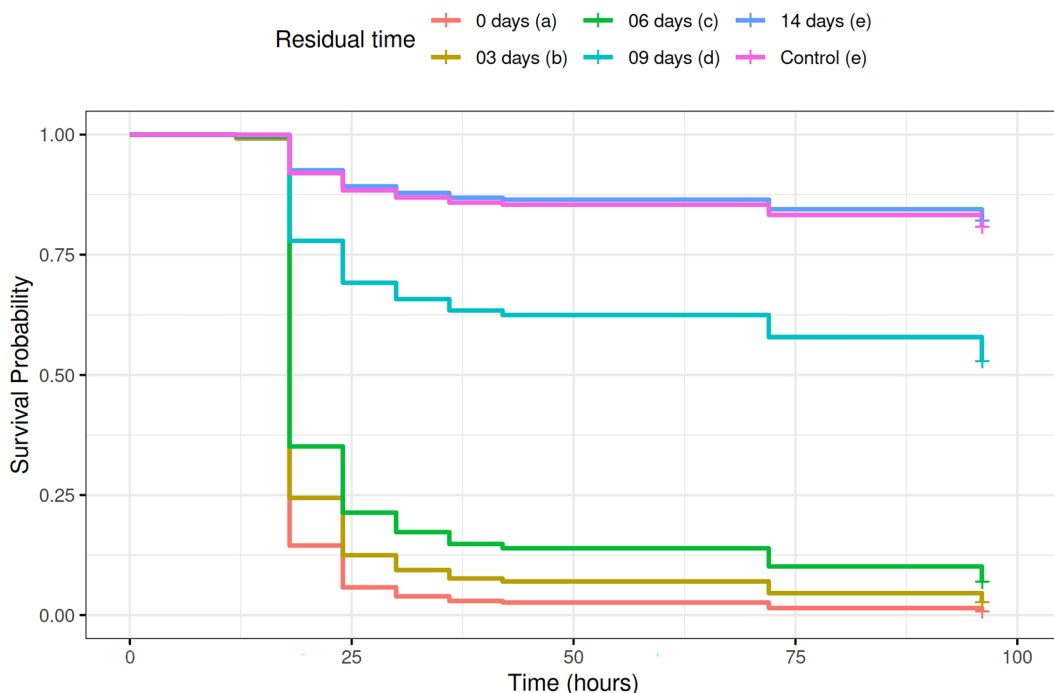


Figure 1. Survival probabilities of *Apis mellifera* bees exposed to canola leaves treated with with imidacloprid 14, 9, 6, 3 or 0 days before the bioassays. Identical letters indicate no significant difference ($p < 0.05$).

differed from each others (0 vs 3 days: $p = 0.027$; 3 vs 6 days: $p < 0.001$; 6 vs 9 days: $p = 0.047$; 9 vs 14 days: $p = 0.015$). Only the survival of bees in the 14-days residual time group did not differ from the control group ($p = 0.101$) (Figure 3).

For a residual time of 0, 3 and 6 days, the combined exposure to imidacloprid and beta-cyfluthrin was less toxic to bees than exposure to imidacloprid or beta-cyfluthrin alone ($p < 0.001$ for all comparisons). For residual times of 9 and 14 days, exposure to beta-cyfluthrin alone was more toxic than imidacloprid and imidacloprid + beta-cyfluthrin ($p < 0.001$ for all comparisons) (Figure 4).

4. DISCUSSION

Death caused by neonicotinoids occurs due to the abnormal prolongation of the activation of acetylcholine receptors, causing continuous and

uncontrolled transmission of nerve impulses, leading to hyperexcitability of the central nervous system of insects (Tomizawa and Casida 2005; Van der Sluijs et al. 2013). Regarding pyrethroids, they increase the time of entry of sodium ions into the cell, by interfering in the opening and closing of neural sodium channels (Gallo et al. 2002). Although they act in different locations in the central nervous system, the symptoms of intoxication are similar in both chemical groups. Among the characteristics presented by adult worker honey bees killed by poisoning with chemical insecticides, the externalization of the oral apparatus (proboscis) and the browning of the integument stand out (Libardoni 2017), which were also verified in the present study.

From the results of this study, it is possible to observe the negative impact caused by insecticides to the environment, since they impair the

Beta-cyfluthrin residual effect

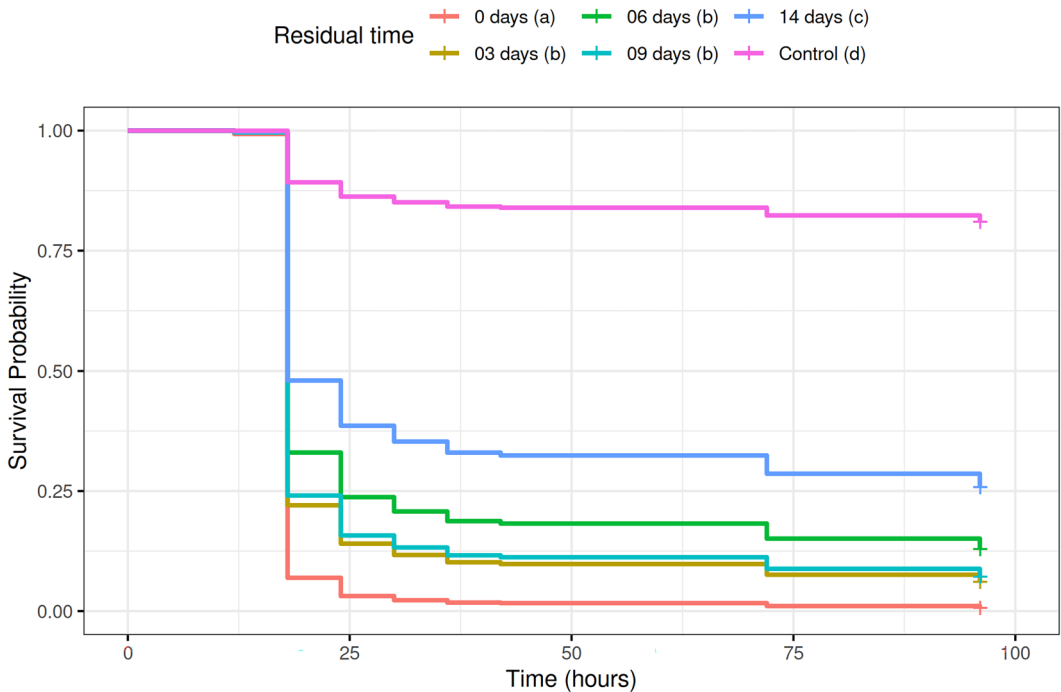


Figure 2. Survival probabilities of *Apis mellifera* bees exposed to canola leaves treated with with beta-cyfluthrin 14, 9, 6, 3 or 0 days before the bioassays. Same letters indicate that there was no significant difference ($p < 0.05$).

survival of Africanized *A. mellifera* honey bees even 14 days after application in the field. In this way, the decision to use insecticides, even before canola flowering, should take these facts into consideration and be avoided whenever possible. Moreover, rain and higher temperatures tend to naturally control aphids, and should also be considered when making decisions about insecticide use (Leite et al. 2005).

Results corroborate other studies, which evaluated the residual effect of imidacloprid in the melon crop (*Cucumis melo* L.) on *A. mellifera* foraging bees; a high toxicity was observed when bees were exposed to the crop 1 and 48 h after imidacloprid application, causing total mortality of the honey bees in the first 24 h (Gomes et al. 2019). Imidacloprid was also found to be toxic to *A. mellifera* honey bees 3, 7 and 14 days after its application on citrus (Chen et al. 2017).

For beta-cyfluthrin, studies are still scarce, however, in research carried out with cyfluthrin, an unresolved isomeric mixture of beta-cyfluthrin (Arena et al. 2020), toxicity was observed in foraging bees after 48 h of confinement with leaves containing residuals.. The three-day residual effect of pyrethroid phenopropatrin in citrus did not allow the survival of *A. mellifera* after 24 h of contact, causing the total mortality of exposed honey bees. At the same time, 60% of *A. mellifera* workers were alive for the residual of seven days, and 85% for the residual of 14 days (Chen et al. 2017).

In the present study, the lower survival of Africanized *A. mellifera* workers in pyrethroid-containing treatments may be related to the fact that it does not act systemically, as is the case with neonicotinoids, which are able to penetrate the plant and translocate via xylem

Imidacloprid + Beta-cyfluthrin residual effect

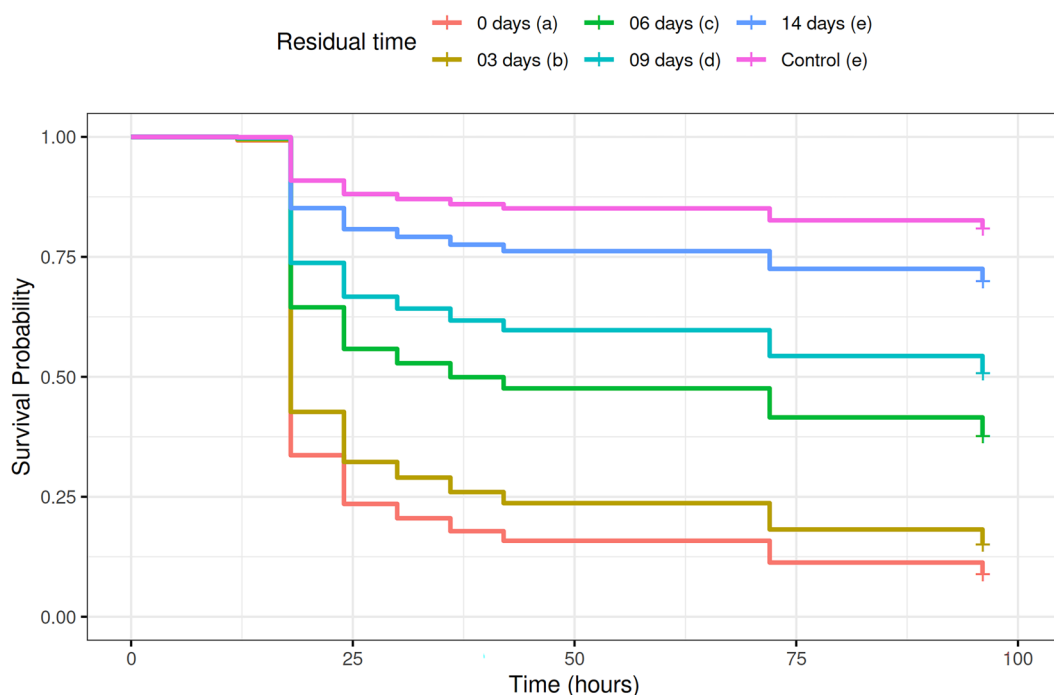


Figure 3. Survival probabilities of *Apis mellifera* bees exposed to canola leaves treated with with imidacloprid + beta-cyfluthrin 14, 9, 6, 3 or 0 days before the bioassays. Same letters indicate that there was no significant difference ($p < 0.05$).

(Aznar-Alemany and Eljarrat 2020; Simon-Delso et al. 2015). In addition, it presents low degradation, which occurs through hydrolysis or oxidation, and is also stable under sunlight and ambient temperatures, which allows a long residual period on the leaf surface (Aznar-Alemany and Eljarrat 2020; de Faria 2009).

Mixing pesticides can cause synergistic interactions, increasing toxicity to bees, and in other cases, there may be an antagonist interaction, where a reduction in toxicity may occur (Robinson et al. 2017; Wang et al. 2020; Zhu et al. 2017). However, when the mixture between pyrethroid and neonicotinoid was evaluated in this study, the lower lethality may be related to the lower concentration of these insecticides in the formulation of commercial products, when compared to the other products tested here (Table 1).

In this sense, the results of the current study confirm that although these insecticides are applied via leaves in the phenological stages of vegetative growth, the residues can remain on the leaves or even, in the case of systemic insecticides such as neonicotinoids, can be translocated to the flowers of the crop itself (Krupke et al. 2012). In addition, the manufacturers' recommendations indicate the realization of two or three applications, with intervals that vary between 10 and 15 days, depending on the product and the incidence of insects (Agrofit 2022). These indications increase the risk to pollinators because they remain for a longer period on the surface of the plants and also, when translocated, accumulate for a longer time in the flowers.

Another relevant factor to be considered is that although the survival of workers of *A. mellifera* in contact with the flowers increases with

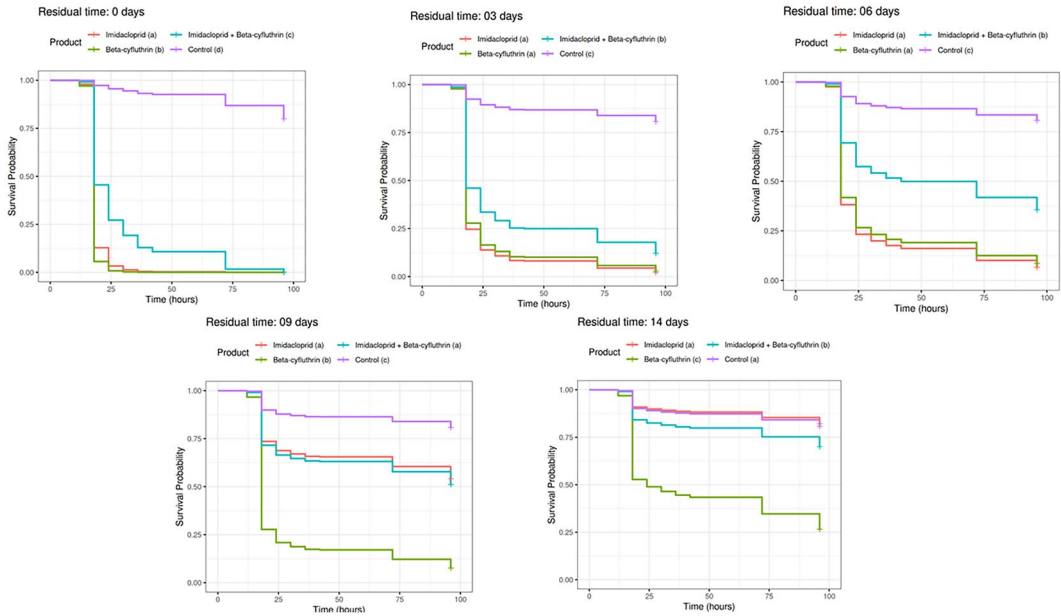


Figure 4. Survival probabilities of *Apis mellifera* bees exposed to canola leaves treated with with beta-cyfluthrin, imidacloprid and beta- cyfluthrin+ imidacloprid 14, 9, 6, 3 or 0 days before the bioassays. Same letters indicate that there was no significant difference ($p < 0.05$).

the longer time since application of the product in the field, in all treatments there was a difference in relation to the control treatment. These results demonstrate that despite losses due to environmental conditions, volatilization, and degradation of products that occur in the field, there is still some presence, even if in exceptionally low concentrations. These results are in line with studies that demonstrated sublethal effects of neonicotinoid and pyrethroid insecticides on this pollinator species.

If the honey bee is in contact with lower (residual) doses of pesticides present in the plant material, immediate death may not occur, but the pesticide may compromise the bee’s cognitive abilities, compromising its return to the colony or, if it manages to return, it carries traces of these products that may affect colonies (Amaro and Godinho 2012; Catae et al. 2018; Tosi and Nieh 2017; Wolff et al. 2008). Among the cognitive skills are learning and memory. For instance, in a learning trial for conditioning olfactory memory, honey bees fed 0.02 ng / mL of imidacloprid,

for 11 days, showed a reduction in learning and memory. In addition, when verifying the genes related to these factors, 130 were expressed negatively (Li et al. 2019). This effect can occur when the honey bees come into contact with low doses of the product, days after it is applied on the crop, as shown in the current work.

Larvae fed with 5 µg / kg of imidacloprid also exhibited early onset of foraging, reduced orientation flights, and decreased foraging flights (Colin et al. 2019). In addition, feeding the larvae with 20 ppb of imidacloprid interfered with the distinct stages of development, decreasing the immune response in the pupal phase, and increasing this response in newly emerged honey bees, which can compromise the health of honey bees (Tesovnik et al. 2019).

Another behavioral trait that can be affected by sublethal doses is flight activity. Forage honey bees fed approximately 5.98 ng / mL of imidacloprid showed a reduction in speed, distance, and flight time (Blanken et al. 2015). In this sense, when the memory and flight ability of the honey

bee are not totally compromised, it returns to the colony with traces of products that will be incorporated into the diet of the other bees, thus being able to compromise other members of the colony.

In addition to interfering in worker honey bees, feeding honey bee colonies with a solution containing imidacloprid compromised the laying of the queens' eggs and also their locomotor activity, even at low concentrations (10 ppb) (Wu-Smart and Spivak 2016). Furthermore, studies demonstrate that this neonicotinoid at 5 ppb, negatively interferes with the quality of semen in drones, mainly in terms of motility and viability (Ciereszko et al. 2017).

There are no data on sublethal effects caused by beta-cyfluthrin, but when evaluating the exposure of honey bees to a solution containing 1 ppm of cyfluphrin, it was observed that it interfered with the locomotion of the honey bees, their self-cleaning activity (grooming), and also in the flapping of wings (Oliver et al. 2015). Other pyrethroids, such as deltamethrin, have sublethal effects related to reduced learning capacity and memory of *A. mellifera* (Decourtye et al. 2005), reduction in the fertility of the queen honey bee, and decreased development of young honey bees to adulthood (Dai et al. 2010); while lambda-cyhalothrin caused cell damage in the midgut, severe damage to the hypopharyngeal glands and also changes in the brain (Arthidoro de Castro et al. 2020). These changes can directly interfere in the behavior and physiology of honey bees, affecting even in the long run, honey bee colonies.

Pyrethroids are more toxic to insects, in general, when compared to neonicotinoids, being efficient in controlling pest insects with less of the active ingredient (de Faria 2009), while neonicotinoids, in turn, have a lower cost and are therefore widely used in agriculture. Thus, neonicotinoids and pyrethroids are among the main chemical groups with residues found in honey bees and their by-products (Johnson et al. 2010; Sanchez-Bayo and Goka 2014; Schmuck and Lewis 2016).

Products containing imidacloprid and imidacloprid + beta-cyfluthrin, tested in this study, leave a residual for approximately 10 days in

canola cultures in the field, while the product containing only beta-cyfluthrin leaves a residual for approximately 14 days. However, these residual times may change due to adverse weather conditions, such as rainfall, higher or lower incidence of UV radiation, among others. In addition, their effects at sublethal doses may alter the behavior and physiology of *A. mellifera*, compromising the colony in the medium and long term, as observed in other studies. These results lead us to seek new management strategies using products that are safer for honey bees, as verified by biological control products (Colombo et al. 2020; Libardoni et al. 2021) and botanical insecticides (Cunha Pereira et al. 2020; da Silva et al. 2020), and that are effective in pest control. This is especially true for canola culture, which has phenological stages, such as flowering and grain filling, that overlap and do not occur homogeneously in the field.

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AUTHORS' CONTRIBUTIONS

Conceptualization/Idea for the article: Raiza Abati, Michele Potrich. *Methodology:* Raiza Abati, Paulo Fernando Adami, Michele Potrich. *Formal analysis and investigation:* Raiza Abati, Gabriela Osowski Vapershski, Gabriela Libardoni, Edgar de Souza Vismara. *Writing—original draft preparation:* Raiza Abati, *Writing—review and editing:* Michele Potrich, Edgar de Souza Vismara Paulo Fernando Adami, Fabiana Martins Costa-Maia, Everton Ricardi Lozano. *Funding acquisition and Resources:* Michele Potrich, Edgar de Souza Vismara, Paulo Fernando Adami, Fabiana Martins Costa-Maia, Everton Ricardi Lozano. *Supervision:* Michele Potrich.

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AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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