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Insecticide Residue Containing a Mixture of Active Ingredients Induces Lethal and Sub-lethal Effects in Honey Bees

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Abstract

Understanding the effects of insecticides on bees is essential for proper management and to support pollinator conservation. To evaluate the lethal and sublethal effects of an insecticide formulated as a mixture, Acetamiprid + Pyriproxyfen, on *Apis mellifera* (Hymenoptera: Apidae), through residual contact on melon leaves as a function of different exposure times. Bees were exposed to two commercial doses of Acetamiprid + Pyriproxyfen, a minimum of 200 ml/ha (0.08 g a.i./L of Acetamiprid + 0.04 g a.i./L of Pyriproxyfen) and a maximum of 300 ml/ha (0.12 g a.i./L of Acetamiprid + 0.06 g a.i./L of Pyriproxyfen). Distilled water was used as an absolute control, and the insecticide Thiamethoxam (0.3 g a.i./L) was used as a positive control. After exposure, mortality and motor disturbances were evaluated over 48 hours. The flight capacity of the surviving bees was assessed. Residual contact with Acetamiprid + Pyriproxyfen caused bee mortality rates higher than those observed in the absolute control and lower than in the positive control. However, no significant differences were found between bees exposed to the minimum dose at 1 hour post-exposure (38.9%) and those exposed to the maximum dose at 1, 2, and 3 hours post-exposure (49.2%, 45.8%, and 32.5%, respectively). Regardless of dose and exposure time, residual contact resulted in a median lethal time (LT_{50}) of 85.4 hours. Bees exposed to the insecticide exhibited reduced flight capacity, reaching only the lowest levels of the flight tower. Exposure to Acetamiprid + Pyriproxyfen resulted in a significant mortality rate and a reduction in the flight capacity of honey bees.

Introduction

Bees are the primary pollinating agents, playing a crucial role in food production and contributing significantly to the global economy (Ricketts et al., 2008; Khalifa et al., 2021). Among bee species, *Apis mellifera* (Hymenoptera: Apidae) stands out as the primary pollinator in agricultural areas due to its abundance across diverse ecosystems, generalist foraging behavior, and large colony size (Pires et al., 2016; Klein et al., 2020). However, in recent years, the decline in bee populations has become a global concern (Lunardi et al., 2017; Castilhos et al., 2019), attributed to multiple factors, among which the intensive use of insecticides for pest control in crops is particularly significant (Pires et al., 2016; Farruggia et al., 2022).

One of the main routes of bee exposure to insecticides is through contact with product residues on plant surfaces or during foraging activities (Heard et al., 2017; Serra et al., 2021; Staroň et al., 2024). In addition to causing mortality, insecticide exposure can lead to various physiological, motor, and behavioral disorders in bees, such as tremors, paralysis, reduced flight capacity, impaired learning, repellency, and memory loss (Farruggia et al., 2022; Tosi et al., 2022; Costa et al., 2024).

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In the main melon-growing regions (*Cucumis melo* L.), the presence of *A. mellifera* is essential for pollination and fruit development (Kiill et al., 2015). Therefore, the introduction of bee colonies into these areas during the flowering period of melon crops is a crucial management practice, ensuring fruit production that meets market expectations, especially for export (Klein et al., 2020). However, to control pests in melon cultivation, insecticides such as the formulated mixture of Acetamiprid + Pyriproxyfen are commonly used. This formulation is recommended for managing pests like aphids (*Aphis gossypii*) and whiteflies (*Bemisia tabaci* biotype B) (Agrofit, 2025).

Acetamiprid, a cyano-substituted neonicotinoid, is often considered to pose lower acute risk to bees (Iwasa et al., 2004; Manjon et al., 2018; Schuhmann et al., 2022). However, studies have shown that sublethal exposure can lead to adverse effects, including reduced longevity, impaired memory and learning performance, disrupted foraging behavior, and difficulty returning to the colony (Thany et al., 2015; Shi et al., 2019, 2020). Moreover, its toxicity may intensify over time, becoming lethal under prolonged exposure to low doses (Bommuraj et al., 2021). Pyriproxyfen, although exhibiting low acute toxicity to adult bees, can reduce survival under continuous exposure and compromise larval development and flight capacity (Fourrier et al., 2015; Gomes et al., 2020).

Acetamiprid exhibits synergistic interactions when combined with other pesticides, especially fungicides, which may inhibit detoxification enzymes in bees (Manzer et al., 2024; Schuhmann et al., 2025). One study found that 44.9% of Acetamiprid combinations with seven other pesticides exhibited synergistic effects on bees (Wang et al., 2020). However, knowledge remains limited regarding the specific effects of the Acetamiprid and Pyriproxyfen mixture, particularly through residual contact with treated plant surfaces.

Therefore, the objective of this study was to evaluate the lethal and sublethal effects of an insecticide formulated from a mixture of Acetamiprid and Pyriproxyfen on *A. mellifera* through residual contact on melon leaves at different exposure times following spraying.

Material and Methods

The experiment was conducted at the Entomology Laboratory of the Academic Unit of Agricultural Sciences (UAGRA), part of the Center for Agri-Food Science and Technology (CCTA) at the Federal University of Campina Grande (UFCG), Pombal campus, Paraíba, Brazil. For the study, adult worker bees of *Apis mellifera* were used, sourced from colonies maintained at the UAGRA/CCTA/UFCG apiary.

The evaluated product was a commercial insecticide formulated with the active ingredients Acetamiprid and Pyriproxyfen. It was applied at two concentrations: the minimum recommended dose of 200 mL/ha (equivalent to 0.08 g a.i./L of Acetamiprid and 0.04 g a.i./L of Pyriproxyfen), and the maximum recommended dose of 300 mL/ha (0.12 g a.i./L of Acetamiprid and 0.06 g a.i./L of Pyriproxyfen), as indicated by the manufacturer for pest control in melon crops. Distilled water was used as the absolute control, while Thiamethoxam, applied at the maximum registered dose of 600 g/ha (0.3 g a.i./L), served as the positive control for pest management in melon cultivation.

The residual toxicity bioassay was conducted using a completely randomized design in a 4 × 3 factorial scheme. The factors included four treatments — two doses of the insecticide Acetamiprid + Pyriproxyfen, one positive control (Thiamethoxam), and one absolute control — evaluated at three exposure times (1, 2, and 3 hours post-application). Each treatment was replicated ten times, with each experimental unit consisting of ten adult *A. mellifera* worker bees.

Yellow melon plants (*Cucumis melo*, cultivar Iracema) were cultivated in a greenhouse. The plants were grown in 1.0 kg pots filled with a substrate composed of soil and well-composted cattle manure in a 2:1 ratio, and irrigated twice daily. Once the plants developed at least six fully expanded leaves, ten plants were selected for each treatment group. The selected plants were sprayed with the respective treatments using a handheld sprayer to simulate field-application conditions.

The plants were divided into two main groups: Group 1 received the minimum recommended dose of the product, while Group 2 received the maximum dose. Within each group, three subgroups were established based on the drying time intervals of the Acetamiprid + Pyriproxyfen insecticide: 1 hour, 2 hours, and 3 hours post-application. To ensure proper drying of the sprayed products, the plants were transferred to a well-ventilated, shaded area.

Leaves from each plant group were cut at the petiole and placed into experimental arenas — plastic containers measuring 15 cm in diameter and 15 cm in height, with the top partially covered by anti-aphid mesh to ensure adequate air circulation. Each arena also contained a portion of cotton soaked in distilled water and an artificial diet composed of honey and refined sugar (Pasta Cândi).

Following the described procedures, adult worker bees of *A. mellifera* were released into the experimental arenas to allow contact with insecticide residues on the treated leaves. Mortality and behavioral responses — including signs such as prostration, tremors, paralysis, and collective behavior — were assessed at 1, 2, 3, 4, 5, 6, 12, 24, and 48 hours after the onset of exposure. The evaluation protocol followed the methodology described by Costa et al. (2014).

Flight activity was evaluated using all *A. mellifera* worker bees that survived the 48-hour exposure to residues of Acetamiprid + Pyriproxyfen. The assessment was conducted using a flight tower, following the methodology described by Gomes et al. (2020). The tower consisted of a wooden structure measuring 35.0 × 35.0 × 115 cm, with transparent plastic panels on the sides. The interior was open, allowing bees to fly freely from the base to a light source positioned at the top of the tower.

Each surviving bee was individually placed at the base of the flight tower and given 60 seconds to initiate flight. Observations were recorded regarding whether the flight occurred and the maximum height reached by each bee. The experiment was conducted in a dark room maintained at an average ambient temperature of 26 ± 2 °C and relative humidity of 65 ± 10%. The only light source in the room was a lamp positioned at the top of the flight tower, used to stimulate the bees' positive phototactic response.

The flight tower used in the experiment was divided into five height levels to assess the bees' flight performance. Levels: (1) corresponded to the base of the tower; (2) ranged from 1.0 to 30 cm; (3) from 31 to 60 cm; (4) from 61 to 90 cm; and (5) extended from 91 to 115 cm, representing the top of the tower.

Mortality rates were calculated for each treatment and corrected using Abbott's formula (Abbott, 1925). The corrected data were then subjected to the non-parametric Kruskal-Wallis test (Kruskal & Wallis, 1952) at a 5% significance level, followed by pairwise comparisons using the Wilcoxon test. Survival data of adult bees were analyzed using the Survival package (Therneau & Lumley, 2010) in R, and fitted to a Weibull distribution model. Survival percentages were calculated using the following formula , where μ = lethal time, α = 1/score of the chosen model, t = time (hours), and $f(x)$ = survival (%). Treatments with similar effects in terms of toxicity and mortality rate were grouped using contrast analysis. The median lethal time (LT_{50}) was calculated for each group.

For flight capacity, the Kruskal-Wallis test (Kruskal & Wallis, 1952) was applied at a 5% significance level, followed by the Wilcoxon test for pairwise comparisons. Additionally, the proportions of bees that were able to fly versus those that were not compared using the chi-square test, with expected values based on the absolute control group. All statistical analyses were performed using R software (R Development core team, 2025).

Results

The evaluated doses of the insecticide Acetamiprid + Pyriproxyfen caused significantly higher mortality rates than the untreated control, but lower than those observed with the positive control (Thiamethoxam). Among the Acetamiprid + Pyriproxyfen treatments, no significant differences were found in bee mortality between the minimum dose (0.08 g a.i./L of Acetamiprid + 0.04 g a.i./L of Pyriproxyfen) applied 1 hour after spraying, which resulted in 38.9% mortality, and the maximum dose (0.12 g a.i./L of Acetamiprid + 0.06 g a.i./L of Pyriproxyfen) applied at 1, 2, and 3 hours post-application, which caused mortality rates of 49.2%, 45.8%, and 32.5%, respectively. The minimum dose applied at 2 and 3 hours after spraying showed no significant differences and resulted in the lowest mortality rates, 16.3% and 16.5%, respectively. Regardless of dose and exposure time, Acetamiprid + Pyriproxyfen was less lethal than the positive control, Thiamethoxam, which caused 100% mortality by the end of the evaluation period (Table 1).

Bees exposed to insecticide residues were observed clustering at the center of the arenas, exhibiting signs of stress and increased motor activity. Despite their heightened movement, they were unable to climb the walls or reach the top of the arenas.

Residues from Acetamiprid + Pyriproxyfen treatments resulted in higher median lethal times (LT_{50}) compared to the positive control, Thiamethoxam, regardless of dose or exposure duration. This indicates a slower mortality rate induced by Acetamiprid + Pyriproxyfen. The residual contact,

Table 1: Mortality of *Apis mellifera* exposed to insecticide residues at different intervals after spraying

Treatment	Dose (g a.i./L)	Time (hours)	Mortality (%)*
Absolute control	Distilled water	1h	0.0 a
		2h	0.0 a
		3h	0.0 a
Acetamiprid + Pyriproxyfen	0.08 Acetamiprid + 0.04 Pyriproxyfen	1h	38.9 d
		2h	16.3 bc
		3h	16.5 b
Acetamiprid + Pyriproxyfen	0.12 Acetamiprid + 0.06 Pyriproxyfen	1h	49.2 d
		2h	45.8 d
		3h	32.5 cd
Thiamethoxam (positive control)	0.3	1h	100 e
		2h	100 e
		3h	96.9 e

*Mortality corrected using Abbott's formula (Abbott, 1925). Means followed by the same letter do not differ significantly according to the Wilcoxon test (Kruskal & Wallis, 1952) at a 5% significance level.

independent of dose and drying time, yielded an LT_{50} of 85.4 hours. In contrast, Thiamethoxam caused rapid mortality, with LT_{50} values of 3.3 hours at 1 hour post-application and 6.1 hours at 2 and 3 hours post-application (Fig. 1).

Flight capacity was evaluated using surviving bees from the absolute control, the Acetamiprid + Pyriproxyfen treatments, and the positive control, with all individuals exposed to insecticide residues 3 hours after spraying. The percentage of bees that successfully flew in the flight tower, under both minimum and maximum doses of the Acetamiprid + Pyriproxyfen mixture, at all evaluated intervals (1 h, 2 h, and 3 h), differed significantly from the absolute control. The absolute control group showed approximately 80% of individuals capable of flying or climbing, and even those that did not fly exhibited no signs of motor impairment (Fig. 2).

Treatments with Acetamiprid + Pyriproxyfen showed significant differences in the proportion of bees that were able to fly between the minimum and maximum doses. In the positive control group (Thiamethoxam), 100% of the surviving individuals were unable to fly and remained debilitated at the base of the flight tower, exhibiting symptoms such as tremors, prostration, and paralysis. It was also observed that, for both doses of Acetamiprid + Pyriproxyfen and across all evaluated time intervals, the majority of bees that managed to fly reached

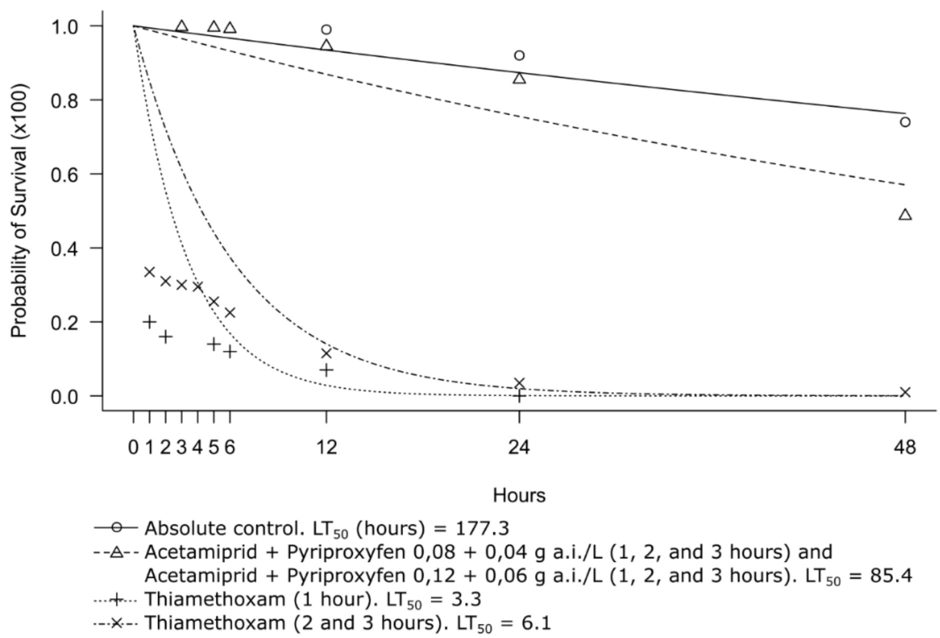

Fig 1. Survival (%) of *Apis mellifera* after contact with melon (*Cucumis melo* L.) leaves sprayed with insecticides (Acetamiprid + Pyriproxyfen, Thiamethoxam) and distilled water (absolute control), and median lethal times (LT_{50}) in hours.

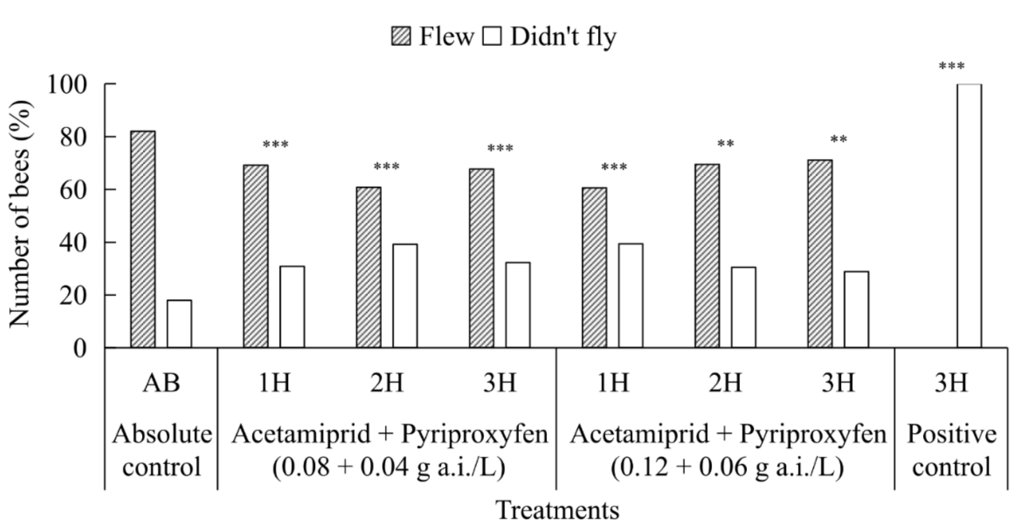

Fig 2. Flight capacity of *Apis mellifera* after residual exposure to Acetamiprid + Pyriproxyfen. Proportions of bees that flew and those that did not were compared using the chi-square test at a 5% significance level (ns = not significant; $^{**}p < 0.01$; $^{***}p < 0.001$).

only the lower height range of 1–30 cm. This indicates that Acetamiprid + Pyriproxyfen compromised the bees' flight altitude, as the number of individuals reaching the maximum height (91–115 cm) was significantly lower compared to the absolute control group at all time points (Fig. 3).

Discussion

Acetamiprid + Pyriproxyfen, regardless of the evaluated dose, was significantly more toxic than the absolute control, but less toxic than the positive control insecticide, Thiamethoxam. Thiamethoxam is widely recognized for its high toxicity to honey bees exposed to its residues (Costa et al., 2014; Choudhary et al., 2022). In a study assessing the toxicity of nine insecticides, Costa et al. (2014) reported mortality rates of 60% for Acetamiprid and 34.7% for Pyriproxyfen. In comparison, Thiamethoxam was markedly more lethal, causing 100% mortality in honey bees, which aligns with the findings of the present study.

The lower mortality observed in comparison to the positive control is likely due to structural differences between Acetamiprid and Thiamethoxam, despite both belonging to the neonicotinoid class. Acetamiprid contains a cyano-substituted moiety, whereas Thiamethoxam features a nitro group

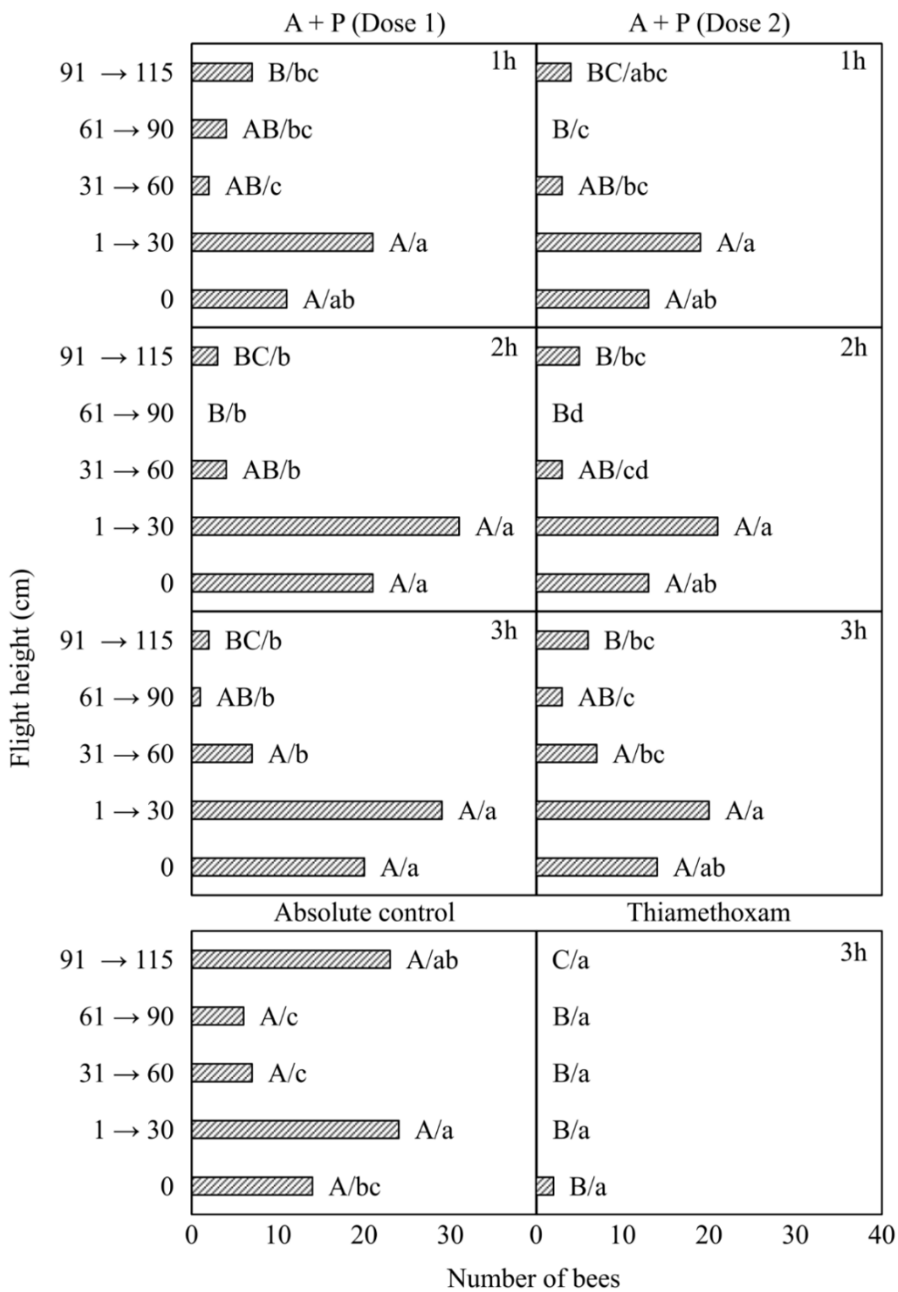

Fig 3. Number of *Apis mellifera* reaching each height level in the flight tower during the flight test. Uppercase letters compare the number of bees at each height level across treatments, while lowercase letters compare the number of bees among height levels within each treatment, according to the Wilcoxon test at a 95% confidence level. A + P (Dose 1) = 0.08 g a.i./L of Acetamiprid + 0.04 g a.i./L of Pyriproxyfen; A + P (Dose 2) = 0.12 g a.i./L of Acetamiprid + 0.06 g a.i./L of Pyriproxyfen.

(N-nitroguanidine), which is associated with higher toxicity to honey bees. A study by Iwasa et al. (2004) demonstrated that neonicotinoids with nitro substitutions, such as Thiamethoxam, exhibited significantly greater toxicity, with an LD_{50} of 29.9 µg/bee. In contrast, cyano-substituted compounds like Acetamiprid showed markedly lower toxicity. Additionally, Acetamiprid may be considered less harmful to *Apis mellifera* compared to other neonicotinoids due to the bees' ability to detoxify these molecules through enzymatic activity of cytochrome P450 in neural tissues (Manjon et al., 2018).

Flight activity following exposure to Acetamiprid + Pyriproxyfen revealed moderate sublethal effects on worker bee locomotion, indicating motor impairment when compared to other neurotoxic compounds (Shi et al., 2019; Choudhary et al., 2022; Barroso et al., 2025). These results are attributed to the individual mechanisms of action of the insecticides. Acetamiprid acts on the insect central nervous system as an acetylcholine agonist, binding to receptor sites and disrupting neural transmission (Iwasa et al., 2004; Simon-Delso et al., 2015). This interference can lead to symptoms such as hyperexcitation, paralysis, disorientation, and death in bees (Thany et al., 2015; Tosi et al., 2017; Shi et al., 2019; Domingues et al., 2022).

Pyriproxyfen, on the other hand, is an insect growth regulator that functions as a juvenile hormone analog. Studies have shown that exposure to Pyriproxyfen can reduce pupation periods, affecting the developmental stages of adult bees (Fourrier et al., 2015), and impair social behavior performance (Litsey et al., 2021). Despite these effects, Pyriproxyfen exhibits low toxicity when bees are exposed via residual contact (Baptista et al., 2009; Costa et al., 2014).

Despite the moderate lethal effects observed at the tested doses, sublethal impacts may compromise foraging activity, thereby affecting food collection and the bees' ability to return to the hive (Goulson, 2013). Several studies have highlighted that sublethal doses of insecticides can impair learning ability, reduce mobility, cause disorientation, and hinder flight performance (Thany et al., 2015; Silva et al., 2015; Tosi et al., 2017; Shi et al., 2019, 2020; Barroso et al., 2025), ultimately disrupting the foraging efficiency of honey bees. These are the first reported results on the residual effects of the formulated mixture of Acetamiprid + Pyriproxyfen at the recommended doses for melon crops and across different post-spraying exposure intervals. The findings may contribute to future field-based research across various economically important crops.

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Authors' contributions

I.D.S.: Data curation, investigation, visualization, writing-original draft.
D.M.T.S.L.: Data curation, investigation, validation, visualization, writing-original draft.
A.B.A.A.: Writing-original draft, metodology, validation, data analysis, writing-review & editing.
E. M. C.: Conceptualization, metodology, investigation, project administration, resources, validation, data analysis, writing-review & editing.
T.A.L.C.: Data analysis, metodology, visualization, writing-original draft.
E.L.A.: metodology, investigation, writing-review & editing.
E.K.S.S.: Data curation, investigation, visualization.
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