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Ingestion of Flupyradifurone, Pyraclostrobin and Fluxapyroxad Change *Melipona scutellaris* Behavior and Promote Individuals' Early Mortality

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ABSTRACT

Pesticides are widely used in Brazilian agriculture to protect crops from target organisms. Nevertheless, pesticide use has also been shown to harm beneficial insects, such as bees. Considering the knowledge gap regarding the effects of pesticides on non-*Apis* species and the importance of stingless bees for ecosystems and human food production, the research aimed to evaluate the survival and behavior of *Melipona scutellaris* after ingestion of the active ingredients flupyradifurone, pyraclostrobin, and fluxapyroxad, individually and in combination. Therefore, foragers were collected and assigned to experimental groups according to three diets: flupyradifurone 6 ng/µL (IS), pyraclostrobin 0.1 ng/µL and fluxapyroxad 0.05 ng/µL (FN); flupyradifurone 3 ng/µL, pyraclostrobin 0.05 ng/µL, and fluxapyroxad 0.025 ng/µL (IF). The results showed that active ingredients caused early mortality in *M. scutellaris* foragers. Moreover, just four hours of exposure to IS was sufficient to induce symptoms similar to those caused by neonicotinoids (stumbles, uncoordinated movements, and uncontrolled proboscis extension), whereas exposure to the FN combination induced lethargy in the individuals. The data highlight that even short-term exposure to these active ingredients can cause severe damage to foragers' physiology, compromising their ability to gather resources for the colony and affecting its overall health.

Keywords: biomarkers, insecticides, fungicides, side effects, stingless bees

INTRODUCTION

Pesticides are commercialized as formulated products, potentially containing one or more active ingredients (a.i.) and adjuvants, which are essential to stabilize the molecule and promote greater solubility of the pesticides (Azevedo and Freire, 2018). These substances are classified based on their a.i. and target organism, with possible applications as fungicides, rodenticides, herbicides, and insecticides (US EPA, 2025).

In Brazil, the insecticide flupyradifurone is used against pests present in leafy vegetables, coffee, soy, tomato, citrus, cashew, among others, totaling 65 crops (Ministério da Agricultura e Pecuária [MAPA], 2025). In target insects, the a.i. has a neurotoxic action, acting as an agonist on nicotinic acetylcholine receptors (nAChRs) and impairing signal transmission between neurons in the central and peripheral nervous systems (Nauen et al., 2014). However, studies have demonstrated adverse effects on beneficial insects.

In A*pis mellifera* (Linnaeus, 1758), flupyradifurone induces motor disabilities, cytotoxic symptoms, oxidative stress and reduction on sucrose sensitivity (Hesselbach and Scheiner, 2019; Tosi et al., 2021; Chen et al., 2024). Furthermore, according to Hesselbach et al. (2020) and Guo et al. (2021), the insecticide changes honey bees' life cycle, anticipating the initiation of workers foraging and promoting faster mortality. Although insecticides are widely associated with harmful impacts on bees, pesticides with different target organisms, including fungicides, can also cause lethal and sublethal consequences (Cullen et al., 2019).

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Pyraclostrobin is a fungicide that acts by inhibiting mitochondrial respiration, interrupting ATP production in fungi and affecting energy demand during its growth (Bartlett et al., 2002). Similarly, fluxapyroxad acts as an inhibitor of the enzyme succinate dehydrogenase (SDHI), blocking the ubiquinone binding sites in complex II of the mitochondria and, consequently, impairing fungal metabolic respiration (Avenot and Michailides, 2010). When combined, they encompass more than 70 different cultures (MAPA, 2025). Nevertheless, studies showed that pyraclostrobin a.i. affects the morphophysiology of important organs in *A. mellifera*, as well as compromise the species survival and development (Zaluski et al., 2017; Domingues et al., 2023; Xiong et al., 2023).

Although *A. mellifera* is considered a model species for ecotoxicological testing, worldwide, it is estimated that there are more than 20,000 species of bees (Michener, 2007). In this scenario, Brazil stands out by its diversity. According to Silveira and Almeida (2002), the country harbors about 3,000 species, including stingless bees from the tribe Meliponini. This tribe has more than 250 representants in Brazil (Nogueira, 2023), and some species are suitable for application in ecotoxicology studies, among them *Melipona scutellaris* (Latreille, 1811) (Figure 1), an essential pollinator for native vegetation and an important species for local stingless beekeepers (meliponiculturists) (Wolowski et al., 2019; Real-Luna et al., 2022).

Figure 1: A forager of *M. scutellaris* pollinating *Cosmos sulphureus*. **Source:** Lais Vieira Bello Inoue.

M. scutellaris is found in almost all states of the Brazilian Northeast (Pedro, 2014), a region where more than 71,000 tons of agricultural chemical active ingredients (mainly pesticides) were sold in 2023 (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis [IBAMA], 2025). Considering the forager behavior of the species, including flower visitation of at least five botanical families and the estimated flight range of 2 km, bees from the same colony may be exposed to multiple pesticides, increasing the risk of oral intoxication through contaminated resources (Araujo et al., 2004; Ramalho and Carvalho, 2007).

Given the ecological and economic importance of *M. scutellaris* and the systemic mode of action of the pesticides mentioned, the research aimed to evaluate the effects of the a.i. flupyradifurone, as well as the combination of pyraclostrobin and fluxapyroxad a.i.s, on the survival and behavior of *M. scutellaris* foragers following oral exposure.

MATERIALS AND METHODS

Foragers collection

M. scutellaris foragers were collected in April 2025 from a meliponary located at São Paulo State University (UNESP), Rio Claro campus (22°23′48.9″S, 47°32′38.2″W). The collection was performed following the "Organization for Economic Cooperation and Development" recommendations (OECD, 1998). Individuals from four visually healthy, and non-parental colonies were collected using plastic containers (250 mL) adapted as beecages — with ventilation perforations (1.2 mm) around the container and perforations in the lid (12 mm) for inserting the feeder (2 mL microtube) and removing dead bees (15 mm). The cages were placed at the colonies' entrance to obtain a sample size of 15 bees, and a total of 75 individuals were collected from each of colony. In addition, cages contained only individuals from the same colony.

The sampled bees were conducted to the "Laboratory of Ecotoxicology and Bee Conservation" (LECA) and acclimated in a biochemical oxygen demand incubator under conditions similar to those of stingless bee colonies, including 28±1°C temperature, 70%±5% humidity, and luminosity absence. In Brazil, according to the "National Council for the Control of Animal Experimentation" (CONCEA), invertebrates are exempt from animal ethics committee approval - federal law no. 11,794 (Brazil, 2008).

Active ingredients and oral exposure

The pesticide analytical standards were acquired from Sigma-Aldrich (PESTANAL®): flupyradifurone (CAS No. 951659-40-8; purity: ≥98%), pyraclostrobin (CAS No. 175013-18-0; purity: ≥98%), and fluxapyroxad (CAS No. 907204-31-3; purity: ≥98%). Two stock solutions were prepared: flupyradifurone, using distilled water, and pyraclostrobin and fluxapyroxad, using 60% of distilled water and 40% of acetone. Both solutions underwent serial dilutions using sucrose syrup (1:1 water and sugar).

Experimental groups with four cages each were separated according to the individuals' feed: control (CL), syrup only; acetone control (CA), syrup with acetone; flupyradifurone insecticide (IS): 6ng/μL; pyraclostrobin and fluxapyroxad fungicides (FN): 0.1ng/µL and 0.05ng/µL, respectively and the combination of both solutions, insecticide and fungicide (IF): flupyradifurone 3ng/µL, pyraclostrobin 0.05ng/µL and fluxapyroxad 0.025ng/µL. Doses were chosen based on field exposure research, considering ecotoxicological studies and the worst-case scenario ingestion for flupyradifurone (Pettis et al., 2013; Tosi and Nieh 2019; Rondeau and Raine, 2022). The acetone experimental group was added due to fungicides low solubility in water, concentration did not exceed 1%, as suggested by OECD guidelines (OECD, 1998).

Bees were fed *ad libitum*. Due to the rapid mortality of individuals in the IS and IF experimental groups, observations were conducted at 4-hours intervals. After the closure of these groups, the observation was increased to 24 hours. These periods were intended for counting and removing dead individuals, as well as for transferring bees to clean beecages and refill feeders when necessary.

Throughout the experiment, individuals fed with flupyradifurone (IS and IF) also exhibited symptoms of intoxication, which were registered and classified as: abnormality in the hind legs (1), inability to retract the proboscis (2), abnormality in the hind legs along with inability to retract the proboscis (3) and dorsal position (4).

Behavioral assessments

As a result of the abrupt mortality of IS individuals within the first 4 hours of the assay, the experimental groups were categorized into two periods of exposure: 2 hours for individuals from CL and IS, and 4 hours for all experimental groups CL, CA, IS, FN and IF). Therefore, following the methodology developed by Bernardes et al. (2021) and Batista et al. (2023), after the corresponding exposure period, five individuals from each colony were transferred to arenas—Petri dishes (90 × 20 mm) with lids—and recorded for 10 minutes under artificial light at a temperature of 28±1 °C (n= 20/per experimental group). Videos were recorded in Full HD resolution (1920×1080 pixels) at 30 frames per second (fps) through a smartphone camera (Samsung Galaxy A14).

Recordings were processed using the artificial intelligence-based software Ethoflow® ("Instituto Nacional de Propriedade Industrial" - INPI, Brazil, BR 512020 000737–6). The arenas were evaluated individually, resulting in a total of 28 datasets containing the mean values for tracked distance (cm), mean movement time (s), mean speed (cm/s), maximum speed (cm/s), meandering, and number of stops.

Statistical analysis

All results were subjected to the Shapiro-Wilk normality test. Survival data were analyzed using Kaplan-Meier survival curves. Behavior data of tracked distance (cm), mean movement time (s), mean speed (cm/s), maximum speed (cm/s), meandering, and number of stops obtained from Ethoflow® software, were compared among experimental groups using Kruskal-Wallis non-parametric test for multiple comparisons (Dunn's post-test), with p-values adjusted using the Holm method (alpha = 0.05 Reject Ho if p <= alpha/2). Comparisons between control and treatments were performed considering the exposure time. FN and IF were also compared to CA experimental group. Symptoms registered in the IF and IS experimental groups were analyzed using descriptive statistics of proportion and prevalence. Ethoflow® data sheets were also processed using the program Jupyter Notebook to track individuals' movements.

Analyses were performed using R and RStudio software (Version: 2025.05.1+513). The packages used were "shapiro.test", "survival", "survminer", "kruskal.test", "dunn.test", "dplyr", "tidyr" and "ggplot2".

RESULTS

Chronic exposure and survival probability

The survival assay lasted 784 hours (32.66 days). However, bees fed with flupyradifurone exhibited an earlier onset of total mortality (IS: 48h, and IF: 36h), as shown in the Kaplan-Meier survival probability curve (Figure 2).

According to the global log-rank test, experimental groups demonstrated statistically significant differences (Chisq= 383 on 4 degrees of freedom, p<2e-16). In addition, pairwise comparison evidenced alteration between CL vs. IS (p<0.0005), CL vs. FN (p<0.005) and CL/CA vs. IF (p<0.0005). CL vs. CA and CA vs. FN exhibited no statistical differences, although individuals from CA experimental group survived 408h longer than those in the FN group.

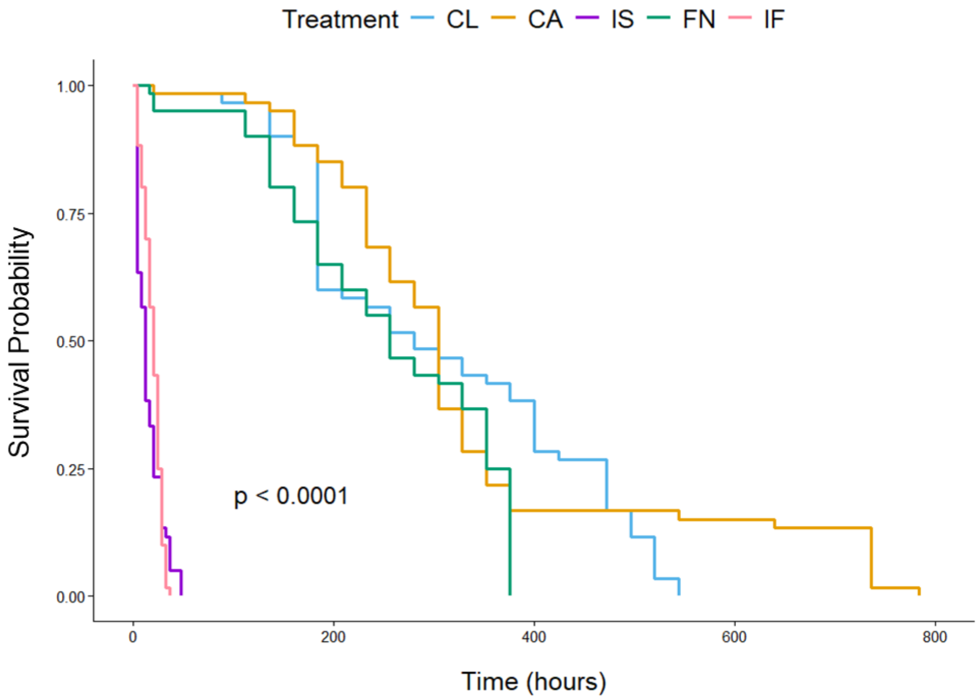

Figure 2: Kaplan-Meier survival probability curve for *M. scutellaris* foragers after oral exposure to pesticides. **CL:** Control; **CA:** Acetone Control; **IS:** Insecticide flupyradifurone at 6ng/μL; **FN:** Fungicides pyraclostrobin at 0.1ng/μL and fluxapyroxad at 0.05ng/μL; **IF:** Insecticide and Fungicide - flupyradifurone at 3ng/μL, pyraclostrobin at 0.05ng/μL and fluxapyroxad at 0.025ng/μL. IS ($p<0.0001$), FN ($p<0.001$) and IF ($p<0.0001$) experimental groups showed statistical difference in comparison to CL group. n=60 individuals per experimental group.

Behavior effects

The videos processed using Ethoflow® software resulted in four raw data sheets per experimental group, and period with >98% of accuracy. Analysis of means for distance (Figure 3A), movement time (Figure 3B), speed (Figure 3C), and meandering (Figure 3E) did not show any significant differences between treated groups and control (4h). Nevertheless, regarding the evaluation of maximum speed results (Figure 3D), FN showed a decreased activity in relation to CL (4h) ($p<0.005$) and CA ($p<0.0005$). Still, FN also demonstrated a higher level of number of stops ($p=0.0000$), as well as IS ($p<0.005$) and CA ($p<0.005$) in comparison to CL (4h) (Figure 3F). CL and IS at 2h showed no statistical differences.

In addition, colony 1 individuals exposed to flupyradifurone for 4 hours showed hyperactivity in comparison to CL (4h), moving extensively throughout the arena (Figure 4). This behavior was not observed in bees from IS after 2 hours of exposure.

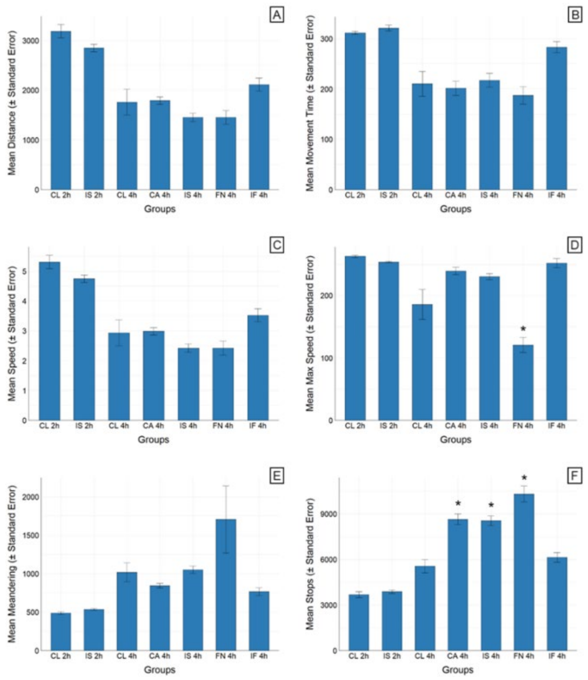

Figure 3: Statistical analysis of behavior parameters. **(A)** Mean distance, **(B)** mean movement time, **(C)** mean speed, **(D)** mean maximum speed, **(E)** mean meandering, and **(F)** mean stops. **CL:** Control; **CA:** Acetone Control; **IS:** Insecticide flupyradifurone at 6ng/μL; **FN:** Fungicides pyraclostrobin at 0.1ng/μL and fluxapyroxad at 0.05ng/μL; **IF:** Insecticide and Fungicide - flupyradifurone at 3ng/μL, pyraclostrobin at 0.05ng/μL and fluxapyroxad at 0.025ng/μL. Asterisks in D, and F indicate statistical difference. n=20 individuals per experimental group.

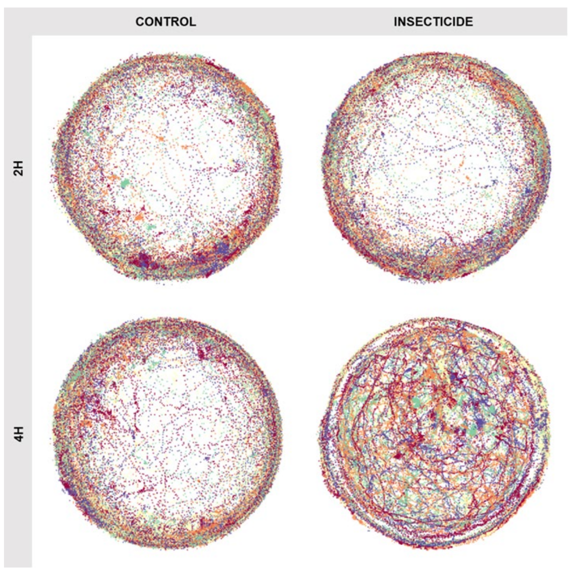

Figure 4: Control and insecticide experimental groups tracking. Visual representation of individuals from the same colony walking for 10 minutes in arenas. Colored dots express bees' course undertaken. n=5 individuals per arena.

Neurotoxic symptoms

Individuals from the IS treatment exhibited three symptoms, abnormality in the hind legs, abnormality in the hind legs along with inability to retract the proboscis, and dorsal position.

The first symptom appeared within the first 8 hours of exposure, and its last observation was at 40 hours. Eight bees presented this abnormality, resulting in a proportion of 13.3% and a mean prevalence of 8.1%. Regarding the abnormality in the hind legs along with inability to retract the proboscis, only five individuals exhibited it (8.3% of proportion); however, the symptom had the most significant mean prevalence (10.5%). The last symptom was observed in ten individuals, and its presence was registered in the first observation (4h) and reported for the last time at 32h, with 16.7% of proportion and a mean prevalence of 5.2%.

Descriptive analysis for the IF group demonstrated the most frequent occurrence of the previously cited symptoms, along with one additional symptom. Accordingly, the following were recorded, abnormality in the hind legs, inability to retract the proboscis, abnormality in the hind legs along with inability to retract the proboscis, and dorsal position.

All symptoms were recorded during the first observation period (4 hours). Abnormality in the hind legs was present in 13 individuals with 21.7% of proportion and a mean prevalence of 6.2%, being last observed at 28h of exposure. Moreover, inability to retract the proboscis affected five bees during the experiment and was visualized the last time at 12h, with a proportion of 8.3% and a mean prevalence of 1.4%. However, both symptoms together demonstrated the most proportion (45%) and mean prevalence (11.9%), with 27 individuals and occurrence until 24h. The last and most severe symptom (dorsal position) impacted 19 individuals during the first six observations (24h) of the assay, causing 31.7% of the proportion and 7.4% of the mean prevalence.

DISCUSSION

The results demonstrated that oral exposure to the active ingredients flupyradifurone, pyraclostrobin and fluxapyroxad, either alone or in combination, impairs *M. scutellaris* foragers health through both lethal and sublethal effects. Our findings highlight that these pesticides can disrupt forager behavior and induce neurotoxic symptoms, suggesting potential impacts on colony performance as well as long-term survival.

Individuals from experimental groups fed with flupyradifurone (IS and IF) exhibited the fastest mortality and the highest values for log-rank test, suggesting lower survival probability rates. The present results reinforce the conclusions of Tosi and Nieh (2019) for *A. mellifera*, indicating that the active ingredient likewise contributes to mortality, although *M. scutellaris* showed greater susceptibility. This interspecific sensitivity was also described by Kline et al. (2025), who reported that *Osmia californica* (Cresson, 1864) and *O. lignaria* (Say, 1837) are more vulnerable to flupyradifurone than *O. cornifrons* (Radoszkowski, 1887), while *A. mellifera* demonstrated greater resistance than all three species. A comparable pattern can be observed for *Partamona helleri* (Friese, 1900) exposed to fungicides and insecticides, either isolated or combined, as stated by Tomé et al. (2017), honey bees showed greater resistance than the neotropical species, including for the synergistic effects of thiophanate-methyl, chlorothalonil, and deltamethrin. Furthermore, similar survival results were described by Inoue et al. (2025) for *M. scutellaris* exposed to the formulated product based on flupyradifurone, with more than 50% of the individuals dying within the first 8 hours of observation.

Although the FN log-rank test assumed statistic differences only for CL, the hazard ratio for FN compared to CA was 81%, highlighting the risk associated with fungicide consumption. Our results are consistent with other studies reporting negative impacts on bees' survival after ingestion of pesticides from strobilurin, and carboxamide chemical groups (Domingues et al., 2020, 2021; Gilbert et al., 2025). Furthermore, strobilurin fungicides have been associated with physiological side effects, including impairments in immune and detoxification responses in honey bees, as well as histopathological changes and cytotoxicity (Duan et al., 2023; Serra et al., 2023). Inoue et al. (2025) also demonstrated that the *M. scutellaris* species was vulnerable to the combination of these fungicides in their formulated product form, inducing histopathological lesions in the midgut, early cellular degeneration in the Malpighian tubules, behavioral changes, and predominantly rapid mortality.

Moreover, treatments containing flupyradifurone (IS and IF) also exhibited harmful poisoning effects, demonstrating dose and time-dependent symptoms, as shown in neurotoxic symptoms topic. Physiological responses of intoxication in bees from the IF group suggested that even at half the dose, the neurotoxic effects of the insecticide could still be observed. Similar effects have been reported for neonicotinoids exposure, including stumbles, uncoordinated movements and uncontrolled proboscis extension (Baines et al., 2017; Tosi and Nieh, 2017; Jacob et al., 2019). As flupyradifurone, neonicotinoids act as nicotinic acetylcholine receptors (nAChRs), and their use has been shown to be harmful to honey bees and non-*Apis* species, leading to its prohibition or restricted utilization in countries from European Union (Tomizawa and Casida, 2003; EFSA, 2018; Raine and Maj Rundlöf, 2024).

The mode of action of the insecticide may also explain the behavioral changes observed in stopping frequency after 4 hours of exposure to flupyradifurone (IS) (Figure 3F). Although the distance traveled and maximum speed did not differ significantly from CL (4 h), the tracking map of single a colony indicated greater individuals' movement (Figure 4). Abnormal activity was not observed in bees from the IS at 2 hours of exposure, indicating the influence of exposure duration. Similar findings were also reported by Tosi et al. (2021), who described hyperactivity and motion coordination deficits for honey bees between 1–30 days after flupyradifurone treatment, and by Castor et al. (2025), who observed reduced mobility seven days flupyradifurone post-exposure in *Scaptotrigona postica* (Latreille, 1807).

Furthermore, the lethargy observed in bees from FN group was also reported by Farder-Gomes et al. (2024) for *M. scutellaris* exposed to pyraclostrobin. While ecotoxicological studies on fluxapyroxad in bees remain limited, the effects of pyraclostrobin are better understood. According to Nicodemo et al. (2020), in foragers honey bees, pyraclostrobin compromises ATP production by affecting thoracic mitochondrial bioenergetics, thereby limiting bees' movement. Considering the distinctive buzz pollination behavior of *M. scutellaris*, this sublethal effect may also reduce fitness during resource collection and interfere with individual communication (Hrncir et al., 2008; Vallejo-Marín, 2022). Additionally, our results for CL (2h) are in agreement with Farder-Gomes et al. (2024) for walked distance and speed, showing similar values in both analyses. Nevertheless, after four hours of exposure, CL individuals demonstrated differences in the evaluated parameters compared to CL (2h) (Figure 3), which could be associated with the stress promoted by the laboratory assay (Lattorff, 2022). Similar results were also found between CL (4h) and CA individuals, with the exception that the CA treatment had a higher mean number of stops.

A previous study from our group demonstrated that oral exposure to flupyradifurone, as well as to the combination of pyraclostrobin and fluxapyroxad commercialized products can cause severe damage to *M. scutellaris* individuals' health and survival (Inoue et al., 2025). In comparison, flupyradifurone exhibited similar mortality for both chemicals (a.i. and formulated product), as well as the neurotoxic symptoms. However, the commercialized formulation induced abnormal behavior for mean speed (cm/s), number of stops and tracked distance (cm) parameters after only 2 hours of exposure. In contrast, the formulated product based on pyraclostrobin and fluxapyroxad was considerably more harmful when compared to their respective a.i.s, still, our results showed that its consumption also compromised the species' safety. Although adjuvants are necessary for pesticides formulations, they have been demonstrated to be toxic for bees and, for the tested a.i.s in this current study, adjuvants may enhance or accelerate the onset of effects (Ciarlo et al., 2012).

In light of all the above, pesticides may produce unpredictable effects across different species of bees and establishing a clear pattern between them remains a significant challenge, even within the Meliponini tribe (Góngora-Gamboa et al., 2025). In this context, toxicological analyses of non-*Apis* species are vital for conservation of biodiversity and for understanding physiological responses across species. Furthermore, *M. scutellaris* is at risk of extinction, emphasizing its vulnerability and the urgency of protective measures for the species (IBAMA, 2018).

CONCLUSION

This study provides evidence that active ingredients of flupyradifurone, pyraclostrobin and fluxapyroxad, isolated or combined, cause early mortality and sublethal effects in foragers of *M. scutellaris*. In addition, the insecticide demonstrated neurotoxicity symptoms in a short-term exposure, even with the dose reduced by half. Given the importance of bee biodiversity for ecosystems and human food production, our finds underscore the importance of expanding the range of bee species evaluated in pesticide risk assessments and toxicological research. Further studies should also investigate long-term effects and potential interactions with other stressors to better predict real-scenarios risks to stingless bee populations.

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Uživanje flupiradifurona, piraklostrobina in fluksapiraksada spremeni vedenje pri *Melipona scutellaris* ter povzroča prezgodnjo smrtnost posameznih osebkov

IZVLEČEK

Pesticidi se v brazilskem kmetijstvu pogosto uporabljajo za zaščito posevkov pred ciljnimi organizmi. Kljub temu pa je bilo dokazano, da njihova uporaba škoduje tudi koristnim žuželkam, kot so čebele. Glede na vrzel v znanju o učinkih pesticidov na žuželke, ki ne pripadajo rodu Apis in na pomen neželatih čebel za ekosisteme ter za pridelavo hrane za ljudi, smo si zadali cilj oceniti preživetje in vedenje vrste *Melipona scutellaris* po zaužitju aktivnih snovi flupiradifurona, piraklostrobina in fluksapiraksada, posamezno in v kombinaciji. V ta namen smo vzorčene pašne čebele razdelili v eksperimentalne skupine glede na tri diete: flupiradifuron 6 ng/μL (IS), piraklostrobin 0.1 ng/μL in fluksapiraksad 0.05 ng/μL (FN); flupiradifuron 3 ng/μL, piraklostrobin 0.05 ng/μL in fluksapiraksad 0.025 ng/μL (IF). Rezultati so pokazali, da so aktivne snovi povzročile prezgodnjo smrtnost pašnih čebel *M. scutellaris*. Poleg tega je že štiriurna izpostavljenost kombinaciji IS zadostovala za pojav simptomov, podobnih tistim, ki jih povzročajo neonikotinoidi (nekoordinirani gibi in nenadzorovano izproženje rilčka), medtem ko je izpostavljenost kombinaciji FN povzročila letargijo. Rezultati kažejo, da lahko že kratkotrajna izpostavljenost tem aktivnim snovem povzroči hude fiziološke poškodbe pri pašnih čebelah, kar oslabi njihovo sposobnost zbiranja prehranskih virov za družino čebel in posledično vpliva na njeno splošno zdravje.

Ključne biomarkerji, insekticidi, fungicidi, stranski učinki, neželate čebele