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RESEARCH PAPER

Exploring the synergistic toxicity of synthetic pesticides and their impact on development and behavior of Honeybee (*Apis mellifera* L.)

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Abstract

In recent years, there has been an increasing concern regarding the impact of pesticide exposure on pollinators, particularly honeybees (*Apis mellifera* L.). This concern arises from their crucial role in maintaining ecological balance and global food production. Therefore, an extensive investigation has been conducted to explore the intricate relationship between pesticides and the biological development of honey bees in the Rahim Yar Khan region. This study assessed the impact of four pesticides (Emamectin benzoate, Chlorpyrifos, Imidacloprid, and Acetamiprid) on honey bee eggs, larvae, and adult bees under controlled laboratory conditions. The pesticides were applied at concentrations of 10%, 30%, and 50%, as per regional agricultural recommendations. A zero-toxicity control was also included for comparison. Toxicity evaluations were conducted through contact exposure, and Probit and regression analyses were performed using SPSS software to comprehensively assess the toxicity profiles. The study revealed significant adverse effects on the immediate behavioral responses of *A. mellifera* following pesticide exposure. These effects included heightened agitation, narcotic-like symptoms, audible hovering, crawling, ceased food-sharing behavior, and reduced proboscis extension. Chlorpyrifos exhibited the highest toxicity against adult bees, while Emamectin Benzoate had the least toxicity. Regarding honey bee eggs, Chlorpyrifos, Imidacloprid, and Acetamiprid were notably more toxic, whereas Emamectin Benzoate exhibited the least toxicity. The impact on larvae varied across developmental stages and pesticides, with Imidacloprid, Chlorpyrifos, and Acetamiprid causing significant mortality, while Emamectin Benzoate showed lower toxicity. The study highlights that Emamectin Benzoate demonstrates lower toxicity compared to other insecticides. This emphasizes the importance of balancing effective pest management with the preservation of pollinator health. The findings underscore the need for informed and sustainable approaches to pesticide use, taking into consideration the potential repercussions on honeybee development and behavior.

Keywords

Insecticides, biological development, apiculture, honeybee health, pesticide exposure

Introduction

Honeybees play a vital role in pollinating plants, fruits, and vegetables, thus supporting the growth and reproduction of various types of flora (Khalifa et al. 2021). They are an invaluable asset when it comes to supporting economies, nutrition, and healthcare, especially in rural areas, particularly hilly regions. Apiculture, the practice of beekeeping, holds great promise in these regions. The primary product of apiculture, honey, offers numerous advantages such as a long shelf life, concentrated form, and high market value (Khan et al. 2020). Additionally, apiculture has significant commercial potential, requiring low investment and land while serving as an appealing alternative to traditional agriculture. Honeybees not only produce honey but also enhance crop yields through pollination, thereby contributing to food security (Kedar 2020). Furthermore, beekeepers can profit from other valuable bee products such as beeswax, cosmetics, royal jelly, and propolis, all of which offer nourishment and health benefits (Shakeel et al. 2019).

Honey industry in Pakistan has been overlooked by policymakers, including the “Billion Tree Honey Initiative,” which aims to boost annual honey production from 15,000 to 70,000 tons (APP 2022). This program is expected to create approximately 80,000 new employment opportunities and generate earnings of PKR 50 billion from honey production (APP 2022; Kowalczuk et al. 2023). Its primary focus is to enhance honey production by providing training and developing beekeepers’ skills, all while promoting efforts for reforestation (APP 2022).

Meanwhile, bee colonies face various challenges that impact their growth, reproduction, and survival. These include climate change, land use, and management practices. Recognizing and addressing these factors is crucial for ensuring productive pollination (Khalifa et al. 2021). Importantly, pesticide exposure remains a significant contributor to the decline in insect pollinator populations, honeybees included (Rizwan et al. 2020). The primary issue pertains to the persistent accumulation of pesticide residues over time, which can exacerbate the decline of species crucial for pollinating terrestrial ecosystems (Brodschneider et al. 2017). Prolonged exposure of honey bees to pesticides can have a significant impact on their well-being and behavior (Anwar et al. 2022; Abay et al. 2023). Among the various types of pesticides that pose a threat to honey bees, neonicotinoids are particularly hazardous (Fairbrother et al. 2014). These substances are either applied as seed coatings or dispersed onto the soil, eventually infiltrating tree tissues and becoming present in plant pollen and nectar.

Moreover, ingestion of the fungicide Pristine®, containing 25.2% boscalid and 12.8% pyraclostrobin, has been observed to impede honey bee performance in associative learning tests. This observation has led to the hypothesis that Pristine® may disrupt the honey bees’ ability to absorb and regulate carbohydrates, thereby interfering with the crucial post-ingestive feedback mechanisms required for effective learning (Castelli et al. 2023; Desjardins et al. 2023).

Furthermore, pesticide exposure has been linked to a wide range of behavioral changes in bees, particularly in relation to insecticides. These changes include alterations in individual bees’ ability to detect odors and the disorientation of foraging bees, thus impairing their homing abilities. Several of these outcomes have the potential to significantly impact the well-being and survival of bee colonies (Chen et al. 2021; Ali et al. 2023a, b).In recent years, increasing concern has arisen regarding the decline in honey bee populations, largely attributed to pesticide exposure. Given that Rahim Yar Khan is an agricultural region heavily dependent on honey bee pollination services, it is vital to comprehend the potential consequences of pesticides on the biological development of honey bees in this area. The objective of this study is twofold. Firstly, it aims to evaluate the level of pesticide exposure endured by honey bees in Rahim Yar Khan and examine how these chemicals may impact their biological development. This information is crucial in developing effective strategies to mitigate the risks associated with pesticide usage and ensure the sustainable management of honey bee colonies in the region. Secondly, by investigating the influence of pesticides on honey bee development in Rahim Yar Khan, valuable insights can be gained within the broader global context of honey bee population decline. The findings of this study may help identify specific pesticides or application practices that pose a significant danger to honey bees, thus assisting regulatory agencies in formulating policies and guidelines to promote more environmentally friendly and bee-safe agricultural practices not only in Rahim Yar Khan but also in other regions grappling with similar challenges.

Materials and methods

Experimental set up

The toxicity studies on honey bees (*Apis mellifera*) were conducted in a controlled laboratory environment at the Entomology Laboratory, Department of Entomology, Khwaja Fareed University of Engineering and Information Technology (KFUEIT) in Rahim Yar Khan (RYK), Pakistan. The laboratory conditions were maintained at a constant temperature of 30 ± 2 °C and a relative humidity of 65 ± 5%.

Bee hive management

To ensure the well-being of the bees and create controlled conditions for research, a systematic approach was followed. This involved selecting and maintaining hives, monitoring environmental conditions, and executing experimental procedures as depicted in Fig. 1.

Figure 1. Managed bee hives in KFUIET.

Experimental materials

Four commercially available pesticides were selected for the study: Chlorpyrifos (40 EC), Emamectin benzoate (1.9 EC), Imidacloprid (200 SL), and Acetamiprid (20 SP), with specific concentrations detailed in Table 1. Various equipment and materials, including water, syringes, glassware, brushes, micropipettes, and specimens of *A. mellifera*, were utilized for the experiments.

Table 1. Pesticides with their common name, trade name and recommended dose.

Sr. #	Common name	Trade name	Dose/Acre
1	Chlorpyrifos	Lorsban @40 EC	800 ml
2	Emmamectin benzoate	Timew @1.9 EC	200 ml
3	Immidacloprid	Confidar @200 SL	250 ml
4	Acetamiprid	Mospilan @20 SP	125 g

Experimental design

The study used four pesticide formulations, each applied at 10 ml, 30 ml, and 50 ml dosages, matching commercial concentrations. A water-only control treatment was included. Each treatment group consisted of 10 honey bee eggs, 10 larvae, and 10 adult bees.

Collection of bee samples

Adult *A. mellifera* specimens, including workers, larvae, and eggs, were obtained from managed hives at KFUEIT, RYK, from 2022 to 2023. Hives were checked for health, and no interventions were done before sampling. Bees were transported to the lab in sealed containers and provided with a sucrose solution and cold exposure for recovery.

Preparation of pesticide solutions

Three concentrations of each pesticide were prepared using water as the solvent. The solutions were thoroughly mixed to ensure even dispersion of the chemicals.

Pesticide exposure

Three experiments were conducted: contact tests on adult bees, eggs, and larvae. Pesticide solutions were applied using syringes or micropipettes onto respective test groups. Control groups were treated with water only.

Behavioral observations

The behavioral effects of pesticides on bees were observed post-treatment, noting any changes in activity.

Data collection and analysis

Mortality rates and behavioral changes were recorded at specific intervals. Statistical analyses, including probit analysis for LC50 determination and the Abbot (1925) formula for mortality correlation, were performed to assess toxicity levels**.**

Results

Impact of pesticides on behavior of honey bee

This research observed a detrimental impact on the behavioral responses of experimental bees immediately following exposure to various pesticides. The adverse effects were particularly notable in all experimental *A. mellifera* groups except the water control group. Signs of pesticide exposure in *A. mellifera* included heightened agitation, narcosis-like symptoms, audible hovering, and crawling along the bottom of the cages. Notably, the *A. mellifera* lacked food-sharing behavior once contamination in the food solution was detected. Furthermore, experimental bees exposed to different test chemicals displayed reduced proboscis extension compared to unexposed bees (Fig. 2).

Acute toxicity of pesticides

Adult and egg stage

The toxicity of various pesticides on *A. mellifera* adults and eggs through contact assays was summarized in Table 1. Interestingly, the toxicity results did not yield statistically significant effects ($p > 0.01$). Nevertheless, varying degrees of pesticide-induced honeybee mortality were observed. Among the pesticides tested, Chlorpyrifos demonstrated the highest toxicity, as evidenced by its lowest LC50 value (0.16), indicating a relatively smaller concentration required to cause harm. On the other hand, Emamectin Benzoate exhibited the least toxicity, with the highest LC50 value recorded (2.22), indicating a higher tolerance level. Specifically, when examining the impact on honeybee eggs, three pesticides, excluding Emamectin Benzoate, were notably more toxic, as

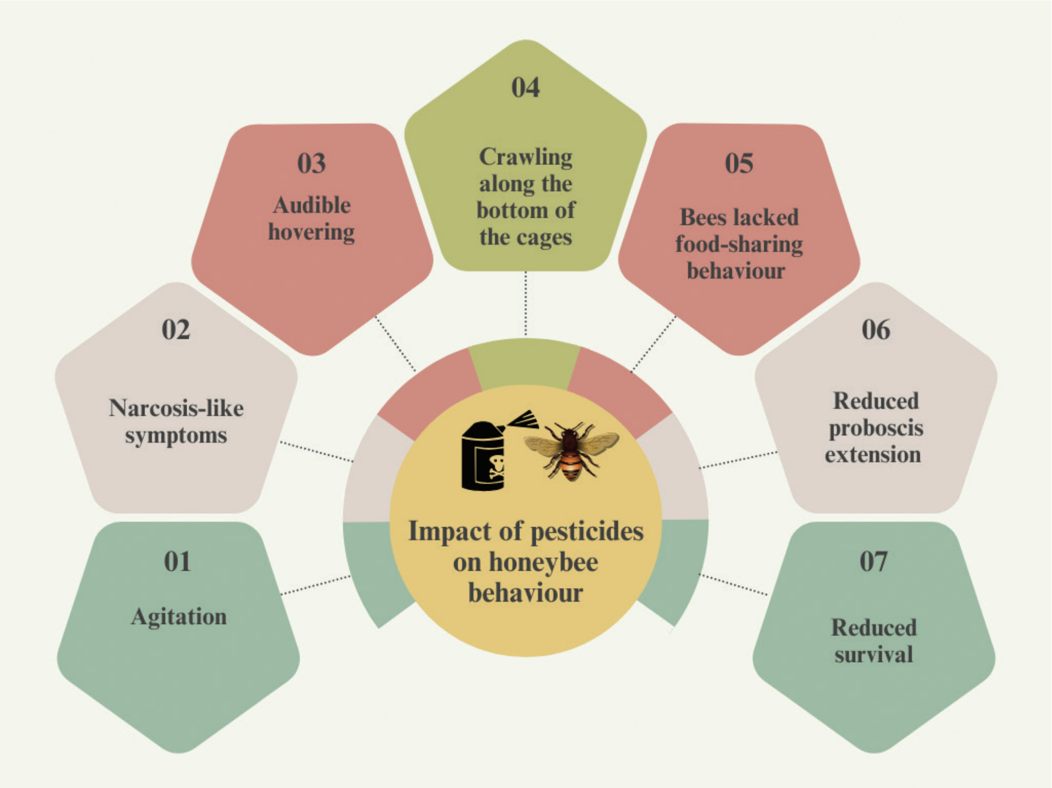

Figure 2. Potential Behavioral response observed towards pesticides.

indicated by their lower LC50 values (-2.33), signifying a heightened sensitivity of honeybee eggs to these compounds. In contrast, Emamectin Benzoate again stood out as the least toxic pesticide for honeybee eggs, with an LC50 value of 2.22.

Larval stage

Contact assays were conducted to assess the toxicity of various pesticides against different larval stages of *A. mellifera*, and the results indicated non-significant toxicity levels ($p > 0.01$), leading to honeybee mortality (Table 2). Among the tested pesticides, Chlorpyrifos and Imidacloprid exhibited the highest toxicity, with an LC50 value of 0.6, specifically affecting 1st instar larvae. Conversely, Emamectin Benzoate showed the lowest toxicity, with an LC50 of 1.69 against the same larval stage. When 2nd instar larvae were tested, Acetamiprid emerged as the most toxic pesticide, with an LC50 of 0.16, while Emamectin Benzoate displayed the least toxicity, with an LC50 of 1.8. For 3rd instar larvae, Imidacloprid and Chlorpyrifos were the most toxic pesticides, whereas Emamectin Benzoate had the lowest toxicity, with an LC50 of 1.49. Notably, Imidacloprid was the most toxic pesticide against 4th and 5th instar larvae, with LC50 values of 0.59, while Emamectin Benzoate exhibited the least toxicity, with LC50 values of 2.16 and 1.22 against 4th and 5th instar larvae, respectively.

Imidacloprid, Chlorpyrifos, and Acetamiprid killed more than 50% of the tested *A. mellifera* stages (adults, eggs, larval instars), while Emamectin Benzoate killed <50% of the population in cases of adults, eggs, 1st, 2nd, and 4th instar larvae. However, it killed >50% of the population against 3rd and 5th instar larvae (Fig. 3). These detrimental effects encompass developmental delays, diminished growth kinetics, morphological irregularities, and augmented mortality rates. The discernible disparities in the magnitudes of these repercussions are contingent upon the specific pesticide variant employed and the corresponding concentration levels used in the experimental protocol.

Table 2. Toxicity of different insecticides on adult and egg of honeybee.

Insecticides	Stage	LC$_{50}$	Linear Regression	Lower Limit 95%	Upper Limit 95%	p-value	R^2
Emamectin Benzoate	Adult	2.22	Y = 1.06 ± 2.64	-16.68	21.05	>0.01	0.9
Imidacloprid		0.87	Y = 3.2 ± 2.19	-16.68	21.05	>0.01	0.9
Chlorpyrifos		0.16	Y = 1.61 ± 4.73	-4.79	14.25	>0.01	0.9
Acetamiprid		0.87	Y = 3.2 ± 2.19	-16.68	21.05	>0.01	0.9
Emamectin Benzoate	Egg	2.22	Y = 1.06 ± 2.64	0.39	4.89	>0.01	0.98
Imidacloprid		-2.33	Y = 0 ± 7.33	7.33	7.33	>0.01	1
Chlorpyrifos							
Acetamiprid							

Table 3. Toxicity of different pesticides against different larval instars of honeybee.

Insecticides	Stage	LC$_{50}$	Linear Regression	Lower Limit 95%	Upper Limit 95%	p-value	R^2
Emamectin Benzoate	1st instar	1.69	Y = 1.20 ± 2.95	2.48	3.43	<0.01	0.99
Imidacloprid		0.6	Y = 2.28 ± 3.62	-9.87	17.16	>0.01	0.9
Chlorpyrifos							
Acetamiprid		0.8	Y = 2.43 ± 3	-10.59	16.58	>0.01	0.91
Emamectin Benzoate	2nd instar	1.8	Y = 1.11 ± 3	-4.25	10.23	>0.01	0.88
Imidacloprid		0.59	Y = 2.28 ± 3.64	-9.87	17.16	>0.01	0.9
Chlorpyrifos		0.63	Y = 1.93 ± 3.78	-12.71	20.27	>0.01	0.81
Acetamiprid		0.16	Y = 1.61 ± 4.73	-4.79	14.25	>0.01	0.9
Emamectin Benzoate	3rd instar	1.49	Y = 1.09 ± 3.37	3.21	3.53	<0.01	0.91
Imidacloprid		0.59	Y = 2.28 ± 3.64	-9.87	17.16	>0.01	0.9
Chlorpyrifos		0.59	Y = 2.28 ± 3.64	-9.87	17.16	>0.01	0.9
Acetamiprid		0.82	Y = 2.43 ± 3	-10.59	16.58	>0.01	0.91
Emamectin Benzoate	4th instar	2.16	Y = 0.66 ± 3.57	0.3	6.84	>0.01	0.93
Imidacloprid		0.59	Y = 2.28 ± 3.64	-9.87	17.16	>0.01	0.9
Chlorpyrifos		0.82	Y = 2.43 ± 3	-10.59	16.58	>0.01	0.91
Acetamiprid		0.82	Y = 2.43 ± 3	-10.59	16.58	>0.01	0.91
Emamectin Benzoate	5th instar	1.22	Y = 3.60 ± 0.58	-40.15	41.33	>0.01	0.71
Imidacloprid		0.59	Y = 2.28 ± 3.64	-9.87	17.16	>0.01	0.9
Chlorpyrifos		1.33	Y = 7.04 ± 4.42	-18.04	9.2	>0.01	0.98
Acetamiprid		1.33	Y = 7.04 ± 4.42	-18.04	9.2	>0.01	0.98

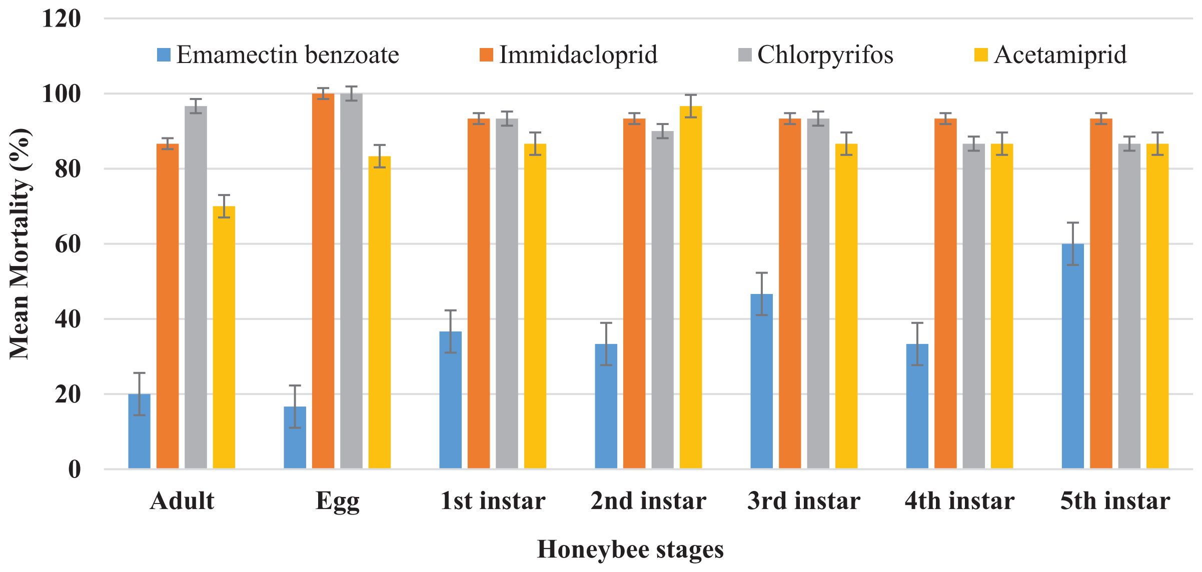

Figure 3. Honeybees (adult, eggs and different larval instars) mortality rates against different pesticides.

Discussion

Honeybees (*A. mellifera*) have assumed a pivotal role within the paradigm of modern agriculture and contribute not only to the production of honey, replete with considerable physiological benefits, but also serve as instrumental pollinators for numerous crop varieties (Papa et al. 2022). It is noteworthy that the utilization of pesticide compounds presents potential hazards. These chemical agents can exhibit toxicity (Hassaan and El Nemr 2020), or they may infiltrate bee colonies, inducing adverse effects on larval and embryonic development (Harwood and Dolezal 2020). Therefore, the present study was designed to evaluate the toxicity of different pesticides against different stages of *A. mellifera*.

Our observations revealed significant distress among experimental *A. mellifera* groups exposed to pesticides, contrasting with the water control group. Common symptoms included agitation, narcotic-like states, audible hovering, and crawling behaviors (Ali et al. 2023b). Pesticide exposure disrupted bee communication and coordination within colonies, leading to heightened agitation and erratic behavior resembling a narcotic-like state (Abay et al. 2023). Additionally, pesticides interfered with sensory perception, causing audible hovering sounds during navigation (Haq et al. 2024).

In the present study, Chlorpyrifos, Imidacloprid, and Acetamiprid exhibited toxicity (mortality > 50%). This observation is consistent with previous findings documented by Cutler et al. (2014), where a similar investigation showed Chlorpyrifos to be profoundly toxic to multiple honey bee variants, causing complete lethality within six hours at substantial concentrations. Correspondingly, Becker (2016) also reported comparable outcomes, examining the toxicological impact of Chlorpyrifos on the worker cohort of *A. mellifera* and categorizing pesticides based on their relative toxicity. Furthermore, Chlorpyrifos administration to larval diets adversely affected survival rates, developmental pace, and mass in nascent honey bees.

Similarly, Imidacloprid demonstrated toxicity towards bees even at sub-lethal concentrations, falling within the range of 1 to 20 μg kg^{-1} or lower (Bonmatin et al. 2005), causing severe deficiencies in essential bioelements and disrupting the equilibrium among these elements. In our study, Acetamiprid showed the second-highest level of toxicity to *A. mellifera*, impacting their behavior, foraging capabilities, and overall health due to its mode of action targeting nicotinic acetylcholine receptors in insects' nervous systems. This elevated toxicity could be influenced by exposure levels, metabolic processes, and interactions with other environmental stressors.

Additionally, our findings align with Mazi et al. (2020), who noted neurotoxic symptoms and initial mortality within minutes of exposure to elevated concentrations of acetamiprid. The calculated LC50 values for acetamiprid after 24 hours were 5.26 ng/μl through topical application and 4.70 μg/μl via oral ingestion. Furthermore, approximately 44.90% of binary to octonary mixtures involving Acetamiprid and seven pesticides showed synergistic impacts on honey bees, emphasizing the need for cautious application in agricultural settings (Ali et al. 2023a).

In contrast, Emamectin benzoate exhibited minimal toxicity towards various beneficial arthropods, including honey bees, their parasitoids, and predators. This reduced toxicity was particularly evident when arthropods were exposed to the pesticide more than 24 hours after application (Lasota and Dybas 1991). The LT50 values associated with emamectin benzoate were notably lower compared to other insecticides assessed in our study, highlighting its comparatively safer profile. However, higher concentrations of Emamectin benzoate led to mortality, indicating the importance of appropriate dosage management to prevent adverse effects.In congruence with the findings of Jansson et al. (1997), the efficacy of emamectin benzoate exhibited variation contingent upon the experimental conditions. Notably, disparate outcomes were documented when emamectin benzoate was subjected to distinct environments, such as controlled laboratory settings versus real-world field conditions. Another study by Reynolds et al. (2017) elucidated that applying emamectin benzoate via topical means resulted in complete insect mortality, achieving a 100 percent kill rate. These factors collectively contribute to the reduced acute toxicity of emamectin benzoate to honey bees, thereby highlighting the significance of comprehending pesticide-specific mechanisms when evaluating their potential harm to pollinators. These findings assume significance in assessing the enduring deleterious effects of these pesticides on the maturation process of honey bees (Dai et al. 2019).

Conclusion

The study investigated the impact of pesticides on honey bees, particularly in the Rahim Yar Khan region, emphasizing their crucial role in ecological balance and global food production. Key aspects examined included bee health, mortality rates, and toxicity thresholds related to contact exposure. Notably, Emamectin Benzoate showed lower toxicity compared to other insecticides, although immediate mortality rates were generally unaffected. These findings underscore the importance of balancing pest management strategies with the preservation of pollinator health. The research contributes significantly to understanding pollinator health and the implications of pesticide use, advocating for collaborative efforts among stakeholders to adopt sustainable agricultural practices that safeguard essential pollinators like honey bees.

Conflict of Interest

All authors declares no conflict of interest.

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