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THIAMETHOXAM-INDUCED NEUROTOXICITY AND GUT DAMAGE IN HONEYBEES (*APIS MELLIFERA*) MOLECULAR AND BEHAVIORAL EVIDENCE OF A SILENT THREAT

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ABSTRACT

Neonicotinoid insecticides are widely used in agriculture for controlling insect pests. Sub-lethal doses of neonicotinoids can damage gut tissues and cause neurophysiological disorders in honey bees. They disrupt the normal function of the Acetylcholinesterase (*AChE*) enzyme which plays a vital role in nerve impulse transmission and honey bee foraging behavior. This study investigated the potentially toxic impact of thiamethoxam exposure on honey bee, (*Apis mellifera*) gut as well as Acetylcholinesterase (*AChE*) and Major Royal Jelly Protein (*MRJP1*) gene expression. Adult honey bees were subjected to thiamethoxam exposure in the laboratory at different concentrations of 0.0097%, 0.0048%, and 0.0024% via feeding bioassay for 3 days. After the thiamethoxam exposure consumption rate, survival rate, and mortality rate of honey bees were assessed. Highest survival was observed at the lowest concentration (0.0024%) followed by a progressive decrease in survival with increasing concentration. Treated group of bees showed abnormal behavioral changes like flight incapacity, ataxia, and proboscis extension. Histological analysis of gut tissue showed epithelial disorientation and vacuolization. Ct values obtained from Real-Time PCR results showed 47.8- and 58-fold upregulation of the *AChE2* and *MRJP1* genes respectively, indicating pesticide-induced cholinergic toxicity and stress response in bees. This study demonstrated the detrimental effects of thiamethoxam which reduces honey bees' survival by inducing neurotoxicity and stress, posing a significant threat to ecosystem stability.

Keywords: Life longevity gene, Cholinergic toxicity, Neonicotinoid, Foraging behavior, Pollinators.

Introduction

About 80 % of crop diversity and 35 % of crop production depends on insect pollination, with the domestic honey bee *Apis mellifera* being the leading pollinator of our agricultural practice (Khalifa *et al*., 2021). Honeybees are the organisms that live in colonies. These colonies are mainly shaped by the three pillars of eusociality which are collaborative brood care, division of labor, and overlapping generations. Honey bees during their life cycle undergo different physiological and morphological changes from the brood (i.e., egg, larva, and pupa) to the adult life (Bruckner *et al*., 2024).

Since the 1980s, the world has experienced a significant decline in honey bee populations due to both anthropogenic factors, which include habitat loss, climate change, and the increased use of pesticides in agriculture (Wagner *et al*., 2021), and biological agents which include viruses and predators and the spreading of *varroa* mites (Shea-Wheller *et al*., 2022; Warner *et al*., 2024). A recent study conducted by Sandstrom *et al.* 2022 examined pesticide distribution in Central California, a highly developed agricultural region in North America. According to this study, pesticides were found in 83 out of 85 sites, with one-third containing more than 10 compounds. The most commonly detected pesticides were the neonicotinoids dinotefuran (found in 49% of sites), imidacloprid (28%), thiamethoxam (27%), and clothianidin (31%) (Paoli and Giurfa, 2024).

Neonicotinoids are the systemic insecticide. They can move throughout the whole plant (Van der Sluijs *et al*., 2013), including pollen and nectars that are carried by honey bees. These pesticides can strongly bind with nACh receptors and also they can be applied by a diverse range of methods including seed coating which is the reason for the extensive adoption of these compounds in agricultural pest control (Hopwood *et al*., 2016). Acetylcholine (ACh), a critical neurotransmitter, plays a pivotal role in transmitting messages across both the central and the peripheral nervous systems (Ali *et al*., 2024). Thiamethoxam functions as a nicotinic acetylcholine receptor agonist, mimicking acetylcholine by binding to the nicotinic receptor site thereby obstructing normal receptor activation. This obstruction results in acetylcholine molecule buildup, which induces paralysis and ultimately leads to mortality. When acetylcholine receptors are abnormally activated, the central nervous system undergoes hyper-excitability which results in the uncontrollably persistent transmission of nerve impulses (Roat *et al*., 2020).

It is crucial to emphasize that neonicotinoid pesticides have severe effects on the expression of the Major Royal Jelly Proteins (*MRJPs*) gene. For instance, it has been demonstrated that imidacloprid exposure can inhibit the expression of major royal jelly proteins (*MRJPs*) in honeybees (Li *et al*., 2019). *MRJPs* are integral to the differential development of queen larvae and worker larvae, thereby facilitating the establishment of a division of labor within the bee colony. Thus, the suppression of *MRJPs* is of significant concern because it is likely to disrupt colony dynamics and contribute to colony collapse (Wang *et al*., 2020).

Not only for the genes neonicotinoids also have hazardous effects on different parts of honey bees' gut tissues. The gut of honey bees is the main site of the interaction of gut microbiota with ingested pesticides. This interaction makes the gut helpful in identifying the effect of pesticides on honey bees (Yang *et al*., 2019). About eight to ten bacterial phylotypes of low taxonomic complexity are mostly inhabited in honey bee gut (Kesnerova *et al*., 2020). Most evidence indicates that the gut microbiota has a direct effect on honey bee health by protecting the host from pathogens, facilitating the digestion of some food components, stimulating the innate immune system, neutralizing dietary toxins, and synthesizing essential nutrients (Almarsi *et al*., 2022). Structural changes in epithelial cells, regenerative cells, and lumen morphology of gut tissues following pesticide exposure may compromise digestive efficiency and increase the susceptibility of honey bees to pathogens.

This article explores the diverse impact of thiamethoxam on honey bees, focusing on the *AChE* gene, *MRJP* gene expression, and gut structure histology. By integrating histological, molecular, and behavioral data, this study aims to elucidate the mechanisms underlying thiamethoxam-induced toxicity, its lethal and sublethal effects on honey bees and contribute to developing strategies to mitigate the risks posed by neonicotinoids to these essential pollinators.

MATERIALS AND METHODS

Pesticide Formulations

In laboratory conditions (25 ± 2°C, 65% relative humidity), the impact of 99.7% pure thiamethoxam (Bayer, Pakistan) on *A. mellifera* was assessed. The inherent toxicity was determined using percentage calculations (Calmorin, 1997). To prepare a 1.25% pesticide stock solution, the following formula was applied:

Percentage = *Part / Whole* × 100 For a 3X stock solution, 45 µl of pesticide was mixed with 3.6 ml of 50% (Sugar: water) sucrose solution using a micropipette.
Subsequent dilutions of the pesticide were prepared in a 50% sucrose solution. Three serial dilutions were made from the stock, yielding desired concentrations of 0.0024%, 0.0048%, and 0.0097%.

Honeybee hive
Honeybee (*A. mellifera*) colonies with a queen were bought from a beekeeper and kept in an apiary. A visual inspection revealed that the queen was in good health. Hive frames were examined for the presence of pupae and larvae. Before or during the study, no chemical treatment was given to the frames. Controlled environmental conditions were maintained to prevent any disease or mite infection in the hive. A 50% (w/v) sucrose solution was provided to the bees as food.

Honey bee collection
Early in the morning, healthy adult worker honeybees were taken from a colony, using a well-ventilated plastic container and were transferred to the Entomology Research Laboratory at Lahore College for Women University in Lahore, Pakistan. These honey bees were given a 50% (w/v) sucrose solution and allowed to acclimatize for two hours at 25 ± 2°C and 45% relative humidity in a container.

Feeding bioassay
The feeding bioassay method developed by Pervez and Manzoor (2020) was applied. The experiment was carried out in a transparent plastic container. The experimental group received three different concentrations of thiamethoxam pesticide solutions i.e. 0.0097%, 0.0048%, and 0.0024% *ad libitum*. For the control group, a 50% (w/v) food source consisting solely of sugar syrup was given. There were 25 bees in each group per replicate in each container. Three replicates for every pesticide concentration were made. Each container was equipped with a plastic feeding plate (2 mL), provided fresh daily. It was noted how much pesticide solution was consumed each day. Honey bees which were unable to move or had uncoordinated movement were taken as dead. Following a 72-hour course of treatment, the oral lethal concentration (LC_{50}) was calculated. The mean mortality rates for each day were noted.
The formula for calculating corrected mortality was as follows (Abbott, 1925).

$$Pr = \frac{(Po - Pc)}{(100 - Pc)} \times 100$$

Pr = corrected mortality, Po = observed mortality, Pc = control mortality.

Calculate Consumption rate
After the feeding bioassay, the amount of liquid remaining in the feeder was noted. The original volume (2 mL) given was deducted from this remaining volume. The consumption per bee was then calculated by adjusting for the number of live honey bees after the experiment both in treated and control groups. Assuming that each bee consumed the same mass of food, the feeding rate was computed by dividing the calculated daily consumption of sugar syrup by the total number of bees (Chakrabarti *et al*., 2020).
The following formula was applied to determine the consumption rate.

$$\text{Consumption rate} = \frac{Initial\ Concentration - Final\ Concentration}{Total\ Number\ of\ Bees}$$

Survival analysis in thiamethoxam-exposed honeybees.
After the experiment, the total number of bees that survived in each replicate cage across all treatment and control groups was calculated. The analysis of Kaplan-Meier log-rank survival was done with GraphPad Prism 8.0.2 (GraphPad Software, San Diego, USA).

Gene Analysis

Quantification by real-time PCR (qPCR)

Quantitative analysis of the AChE 2 gene was done by real-time PCR. The bees survived at the end of the feeding bioassays were used for gene analysis.

RNA isolation

Using a micro-tube pestle and mortar, the bee samples were crushed into a fine paste. The TRIzol method was utilized to isolate the total RNA. For every sample, 1.0 ml of TRIzol (Catalog No. 15596026, Thermo, USA) was added. The homogenized samples were allowed to fully dissociate by incubating them for 10 minutes at room temperature. To get rid of cell debris, the samples were centrifuged. The supernatant was moved to a fresh tube. Add chloroform (0.5 ml) in each sample and vigorously vortexed for 5 minutes. The samples were allowed to incubate at room temperature for 5 minutes, then centrifuged for 15 minutes at 4°C at 12,000 xg.

The upper aqueous phase was transferred into a new tube and add isopropyl alcohol (0.5 ml), was allowed to incubate at room temperature for 10 minutes. Centrifuge at 12,000 xg (Catalog no. 5415 CMEB 1039) and allowed to rest the centrifuged tubes at 4°C and precipitate the RNA. Following the complete removal of the supernatant, add 75% ethanol (1 ml) to wash the RNA pellet. The samples were centrifuged at 12,000 xg at 4°C at for 5 minutes. The resultant RNA pellet was air-dried and resuspended in nuclease-free water (Invitrogen). The RNA concentration and purity were determined by using a Thermo Scientific Nanodrop 8000 spectrophotometer. By using the SuperScript® III first strand cDNA synthesis kit (Catalog No. 18080051, Life Technologies) the obtained RNA was reverse transcribed and amplified in a thermocycler (biorad 1 Cycle 009193). That cDNA was then stored at -20 °C until further analysis by Real-time qPCR.

qPCR reaction

To quantify the expression of the *MRJP1* gene and *AChE* gene Real-Time qPCR was performed by using SYBR green qPCR master mix (Maxima SYBR Green/ROX qPCR Master Mix (2X) in triplicates on One Step real-time PCR (Applied Biosystem qRT PCR Step One ™ 4369074). For normalization of RT qPCR and as a positive control the expression of housekeeping gene GAPDH was also measured. Negative Control (no template) was included and yielded no product.

The thermal cycle was set up with 45 Cycles of 10-minute denaturation at 95°C (15 seconds), annealing at 56°C (1 minute), and extension at 72°C (1 minute). Using the relative quantification method (ΔΔCT), the fold induction was ascertained.

Histological Analysis

The brains of *A. mellifera* from the control group and those exposed to 0.0048% thiamethoxam were histologically processed. The bees were cooled to 4 °C, then the brains were dissected in petri dishes using a sharp blade. The tissues were first fixed in 10% formalin (90 ml distilled water, 10 ml formaldehyde) to prevent shrinkage. Dehydration followed, using a graded ethanol series (70% - 100%), with brains submerged for at least one hour per step at room temperature. The dehydrated brains were then cleared in clove oil. The tissues were then embedded in paraffin blocks, sectioned at 5 µm with a microtome (ERM – 2301), and they were mounted on glass slides. After deparaffinization and rehydration sections were then stained with hematoxylin and eosin, and dehydrated in graded ethyl alcohol, cleared, and then slides were permanently mounted with Canada balsam. Microscopy and imaging were performed at 10x and 40x magnifications using a light microscope (Trinocular E−200, digital microscope Camera – Nikon Japan Eil −12).

Statistical analysis

Graph Pad Prism Software (Version 8.0.2) was utilized to analyze the data. The EPA computer Probit Analysis Program (Version 1.5) evaluated the lethal dose, or LC_{50}, and its corresponding 95% confidence intervals. Mean ± SEM was calculated for each observation value in the mortality

assessment, and Tukey's Post Hoc tests were used to determine a significant difference between the treated and control groups using two-way analysis of variance (ANOVA). The survival rate between treatments was compared using the Kaplan-Meier Log Rank paired tests. For each test, the significance level was set at $p \leq 0.05$. Data were represented graphically through the creation of graphs. Based on CT values, fold changes in the gene's expression were evaluated.

Results

Median lethal concentration of thiamethoxam in a feeding bioassay

LC_{50} values of thiamethoxam at different time intervals observed by Probit analysis were 0.002 µl and 0.001 µl at 48 and 72 hours respectively in a feeding bioassay. **(Table 1)**. Lower LC_{50} value for thiamethoxam indicated higher toxicity. Significant decrease in LC_{50} value was observed over time as ($p < 0.05$)

Lethal toxicity of thiamethoxam on exposed honeybees

The feeding bioassay evaluated the lethal toxicity of thiamethoxam on honey bees at three concentrations (0.0097%, 0.0048%, and 0.0024%) over 72 hours, with a control group for comparison (figure 1). After 24 hours, the highest mortality was observed at 0.0097% (17.67 ± 0.88), followed by 0.0048% (12.67 ± 0.88) and 0.0024% (9 ± 0.57), with no deaths in the control group. Mortality increased for all concentrations over time (dose-dependent, linear regression), with the highest rate recorded at 0.0097% (25 ± 0.00) after 72 hours. A two-way ANOVA indicated significant differences between the treated and control groups ($p < 0.0001$, df = 6 and F = 21.52.

Survival Analysis

In a 72-hour feeding bioassay, honeybee survival rates were significantly reduced following exposure to thiamethoxam at different concentrations (0.0024%, 0.0048%, and 0.0097%) compared to the control group, which exhibited the highest survival (Figure 2). Bees exposed to 0.0024% thiamethoxam showed a 25% survival rate, while those exposed to 0.0048% had a survival rate of 13%. At the highest concentration of 0.0097%, no bees survived. Statistical analysis using the Kaplan-Meier and Log Rank tests ($X^2 = 17.16$, df = 1, $p < 0.0001$) revealed significant differences among the groups, demonstrating a dose-dependent toxicity of thiamethoxam.

Consumption rate

Table 2 shows the consumption of thiamethoxam at different concentrations (0.0097%, 0.0048%, and 0.0024%) compared to control. A gradual decrease in the consumption of pesticides was observed with time.

6-hour feeding bioassay experiment: After 6 hours, the mean consumption rate in the treatment groups were 37.33 ± 0.33, 57 ± 0.577, and 85.67 ± 0.33 at 0.0097% 0.0048%, and 0.0024%, concentrations compared to the control group (353.67 ± 1.86). One - One-way analysis of variance showed significant differences among the treated group compared to control (P = 0.0116).

12-hour exposure: Again, no significant difference among the treated groups (One-way ANOVA with Tukey's Post Hoc comparisons) was observed while a significant difference was observed in the control group that was given sugar syrup as shown in Figure 3. At 12 hours, the trend persisted, with mean consumption rates of 43.67 ± 0.33, 65.67 ± 0.33, and 92.67 ± 0.33 for the respective concentrations, while the control group exhibited significantly higher consumption at 552.67 ± 1.45

Quantitative relative abundance of *AChE* 2 gene transcription level in thiamethoxam exposed honey bee (*A. mellifera*) in a laboratory feeding bioassay.

A real-time PCR was performed to measure the quantitative relative abundance of the *AChE* 2 gene after the bees were exposed to the highest 0.0097% concentration of thiamethoxam through a feeding bioassay. *GAPDH* was used as a reference gene (Figure 4). Relative fold changes in gene transcription level were measured by calculating the Ct value. Results showed that after treating

honeybees with 0.0097% thiamethoxam *AChE2* gene transcriptional level was 47.84 folds up-regulated compared to the control group **(**Figure 5).

The graph in Figure 5, clearly demonstrates that the experimental group has much higher *MRJP1* gene expression which is almost 58 times higher than the control group. This even means that in the experimental condition, (consuming neonicotinoids) increases the levels of *MRJP1* in honeybees by a margin. This can suggest that *MRJP 1* which plays a vital role in honeybee growth and royal jelly synthesis will also have its expression level change in reaction to pesticide presence as part of the body's defense mechanism.

Histological analysis

The histological analysis of the honey bee gut revealed distinct differences between the control and thiamethoxam-treated bees. In the control group, the epithelial cells of the midgut appeared well-preserved, with clear outlines and a homogeneous structure, while the regenerative cells located at the basal region of the epithelium had dense nuclei and normal cytoplasmic features, indicating healthy cellular activity. The nuclei were spherical or oval with no signs of overlapping, and the even dye distribution suggested that the cells were healthy and undamaged. The lumen was clear, with no signs of congestion or cellular debris. In contrast, the thiamethoxam-treated bees showed significant structural changes in their epithelial cells, including disorientation, vacuole formation, reduced thickness, and disrupted cell membranes, which made the cells appear misshapen or broken. The regenerative cells in the treated group exhibited pathological features such as nuclear condensation and cytoplasmic degradation, with a possible reduction in their number. The nuclei were irregular in shape, often condensed or fragmented, indicating apoptosis and cellular stress. Additionally, the lumen of the treated bees contained more cellular fragments and debris, with signs of increased secretion and inflammation, suggesting substantial tissue damage and compromised gut function due to pesticide exposure.

DISCUSSION

The present study is a one being reported in Pakistan and was conducted to assess pesticide toxicity, gut histology, and quantitative alteration in *AChE* and *MRJP* gene expression in honey bees (*A. mellifera)* exposed to insecticide *viz*. neonicotinoid (thiamethoxam). Decline in the population of honey bees is attributed to exposure to various insecticides (Williamson and Geraldine, 2013). The results of the survival analysis clearly demonstrate a dose-dependent toxicity of thiamethoxam on honeybees. Over the three-day feeding bioassay, a significant reduction in survival rates was observed as the concentration of thiamethoxam increased. These results are consistent with Laurino *et al*. (2011) who found that exposure to sub-lethal doses of neonicotinoids, including thiamethoxam, significantly increased mortality in honeybees. Similar to our study, their research demonstrated a clear dose-response relationship, with higher concentrations of neonicotinoids leading to increased mortality rates. Laboratory studies by Tome *et al*. (2020) suggested that thiamethoxam exposure resulted in a significant reduction in honeybee survival, particularly at higher concentrations. They observed that bees exposed to thiamethoxam exhibited signs of poisoning, such as impaired motor function and lethargy, which likely contributed to the increased mortality. The toxic effects of neonicotinoids are heightened even at sublethal doses in prolonged exposure (Lu *et al*., 2020).

The mortality results from the experiment clearly demonstrate the dose-dependent toxicity of thiamethoxam on honeybees over time. The findings from this experiment are in line with Henry *et al.* (2012) and Goulson (2013) research studies which have demonstrated that neonicotinoids can have lethal and sublethal effects on honeybees, affecting not only survival rates but also critical to colony maintenance, such as foraging and navigation. Martinello *et al*. (2020) reported that honey bee mortality in Italy is directly related to pesticide exposure. He noted that approximately 150 different pesticide remains were found in dead honeybees. Di Prisco *et al.* (2013) found that even low doses of thiamethoxam can impair cognitive functions and increase mortality in honeybee colonies over time.

Results revealed that the LC_{50} value of thiamethoxam at 48hr and 72hr had toxic effects (0.002% and 0.001% (10 µg/l and 20 µg/l) on honey bees. The highest concentration of 1.44 ng/µL in food (equivalent to 144 µg/kg) was determined based on the LC_{50} previously established by Tavares *et al*. (2015) and corresponds to 1/10 of the LC_{50} value indicating a high level of toxicity even at lower concentrations. Higher LC_{50} value for thiamethoxam i.e 0.0018% (18 µg/L) for honey bees at 48 hours (Iwasa *et al.*, 2004), 0.002% (20 µg/l) at 48 hours and 0.001% (10 µg/l) at 72 hours (Choudhary *et al.*, 2022) and 0.0005% (5 µg/L) at 72 hours (Laurino *et al*., 2011) have been reported.

The consumption patterns of thiamethoxam at different concentrations in the 6-hour and 12-hour feeding bioassays provide insights into the feeding behavior of honey bees when exposed to this neonicotinoid pesticide. No significant difference in the consumption rate of treated groups, while a significant difference was noted in the control group that consumed sugar syrup in a feeding bioassay. Pesticide prolonged exposure diminishes the bee's ability to discriminate between different concentrations of thiamethoxam, leading to uniform consumption. Trophallaxis also occurs among bees which lowers the rate of consumption. Over time, the toxic effects of the pesticide might impair the bee's sensory or cognitive functions, resulting in less selective feeding behavior (Williamson *et al*., 2014). Honey bees prefer non-contaminated food sources when given a choice (Tison *et al*., 2016) to justify the higher consumption of sugar syrup. These results also align with Kessler *et al*. (2015) studies which reported that bees could detect and avoid food sources with certain levels of neonicotinoid contamination.

AChE is a main biomarker of neurotoxicity detection in honeybees. The observed up-regulation of the *AChE2* gene in honey bees indicates a significant stress response at the molecular level. AChE is a critical enzyme involved in the termination of nerve impulses by hydrolyzing the neurotransmitter acetylcholine. The over-expression of the *AChE2* gene suggests an adaptive response to counteract the effects of thiamethoxam which acts as an agonist on nAChRs. Exposure to neonicotinoids has led to alterations in the expression of genes associated with neural function and detoxification processes (Martelli *et al*., 2020; Suchail *et al*., 2004). The high binding of pesticides to ACh receptors may cause cholinergic neurons to become permanently activated, which may be compensated by the *AChE* level (Tavares *et al*., 2017). Previous research has shown that chronic exposure to neonicotinoids at sub-lethal concentrations can induce oxidative stress, immunosuppression, and alterations in gene expression related to detoxification and neural activity (Di Prisco *et al*., 2013; Christen *et al*., 2016).

MRJP1 is an important gene responsible for royal jelly production that is required for the growth of the queen larva and the well-being of the colony. According to the literature review, neonicotinoids affect honey bee's neurological and physiological competencies and thus change the gene expressions in bees. Such observations have been made in this study where it was established that neonicotinoid inhibits the normal functioning of genes in honey bees which in the long run may be bad for honey bee colonies (Henry *et al.*, 2012). The significance of these findings is that they demonstrate that neonicotinoids could impact colony dynamics indirectly by altering such crucial genes as *MRJP1*. Acute increase in the level of *MRJP1* may be beneficial in the short run, but long-term effects of sub-lethal doses of neonicotinoids could reduce the health and productivity of the colony since the energy required to maintain such levels of *MRJP1* is not easy to come by (Tsvetkov *et al.*, 2017).

The morphological analysis showed that exposure to a sublethal dose of thiamethoxam is cytotoxic to the midgut and to the Malpighian tubules of the bees. In comparison to the control group, the epithelial tissue cells in the pesticide-treated groups had aberrant nuclei and were distorted. The treated cells' cytoplasm was crude and scattered. In treated groups, lesser amounts of regenerative cells were observed. Reduced number of regenerative cells shows a lesser ability of epithelial cells to repair damage (Pervez and Manzoor, 2020). In a morphological study of the midgut of the Africanized honeybee with a dose of 0.428 ng/mL of thiamethoxam per day, showed that thiamethoxam causes cytoplasm vacuolization, increased apocrine secretion, and enhanced cell

elimination in the midgut digestive and regeneration cells of exposed bees. Therefore, intoxication with sublethal levels of thiamethoxam can produce the midgut impairment and shorten the honeybee's lifetime (Oliveira *et al.,* 2013).

Conclusion

To sum up, this study confirmed the high toxicity of thiamethoxam to *A. mellifera*, particularly at higher concentrations and with extended exposure. The results of 3 days of feeding bioassay for mortality rate, consumption rate, survival rate, behavioral analysis, and quantitative alteration in the *AChE* and *MRJP* gene clearly demonstrated the toxicity of thiamethoxam on honey bees. The results showed that *A. mellifera* was a sensitive breed to thiamethoxam. Bees exposed to increasing concentrations of thiamethoxam exhibited progressively lower survival rates and higher mortality. Reduced consumption rates in exposed honey bees indicated that they were avoiding the pesticide. Abnormal behavior (ataxia, moribund, flight incapacity, and proboscis extension) was observed. Significant upregulation of the *AChE* and *MRJP* gene was confirmed by conducting qRT PCR. The histological analysis of the treated honey bee's gut showed morphological damage and cell death in epithelial cells and Malpighian tubules indicating pesticide significant toxic effects of thiamethoxam.

Author Contributions

Tahira Minal, **Mahnoor Pervez**, **Farman Ali,** and **Zerish Kiran Jamil** conceptualized the study and designed the experimental framework. **Nimra Aslam** and **Rabiya Latif** performed laboratory experiments and data collection. **Fawad Khan** provided expert entomological input and contributed to data interpretation with a focus on toxicological analysis. **Nimra Akhtar** assisted in statistical analysis and literature review. **Sadia Amir** supported behavioral testing and helped prepare the final draft. **Mahnoor Pervez** served as the corresponding author, coordinated the overall research process, and supervised manuscript development. All authors critically reviewed, revised, and approved the final version of the manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Conclusion

This study provides compelling molecular and behavioral evidence of the neurotoxic and gut-damaging effects of thiamethoxam in ***A. mellifera***, highlighting its role as a silent threat to honey bee health. The results reveal significant impairment in bee cognition and gut integrity, which could have broader implications for pollination efficiency, colony collapse, and ecosystem stability. These findings underline the urgent need for stricter regulation and careful monitoring of neonicotinoid pesticide usage.

Recommendations

Implement stricter guidelines on the use of thiamethoxam in agricultural practices, especially near apiaries.

Promote the use of bee-friendly pesticides or integrated pest management (IPM) systems.

Encourage beekeepers and farmers to collaborate on reducing pesticide exposure risks.

Raise awareness among stakeholders about the sub-lethal effects of neonicotinoids on pollinators.

Future Research Suggestions

Investigate long-term effects of sub-lethal thiamethoxam exposure on colony-level health and reproduction.

Explore synergistic impacts of thiamethoxam with other environmental stressors (e.g., pathogens, climate change).

Conduct large-scale field studies to validate laboratory findings and assess real-world implications.

Utilize omics-based approaches (genomics, proteomics, metabolomics) to gain deeper insights into the molecular pathways affected.

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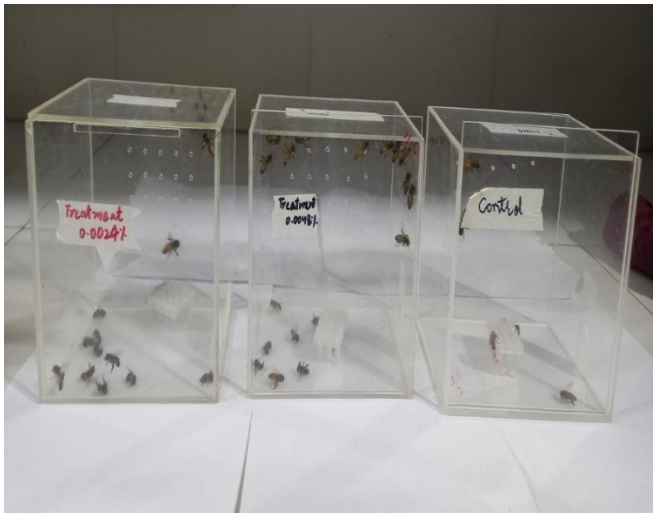
Table 1: Calculated Median Lethal Concentrations (LC_{50}) of Thiamethoxam on honey bee (*A. mellifera*) in feeding bioassay

Chemicals	**Time (hours)**	**LC_{50} µl/bee**	**CI95%**	X^2	**Slope** (± SE)
Thiamethoxam	**72**	0.001	0.00 - 0.002	0.790	2.545 (±1.043)
	48	0.002	0.00 - 0.004	0.431	1.349 (±0.632)



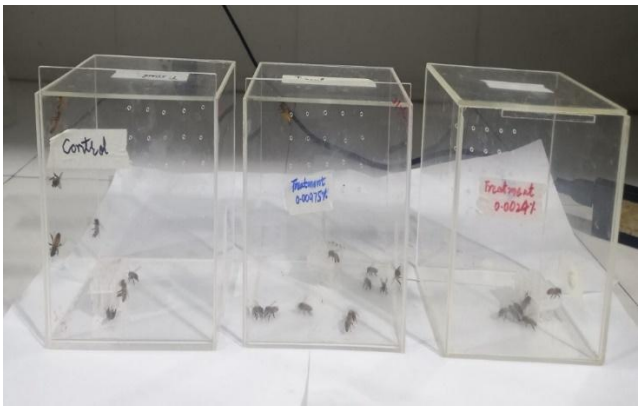


Table 2: Mean (± SEM) of honeybee (*A. mellifera*) at different concentrations of thiamethoxam in feeding bioassay after 6 hours and 12 hours of treatment, n = 25.

Hours	Treatment Groups			
	T1 (0.0097%)	T2 (0.0048%)	T3 (0.024%)	Control (sugarsyrup)
	Mean ± SEM			
6 hours	$37.33^{a} \pm 0.33$	$57^{a} \pm 0.577$	$85.67^{a} \pm 0.33$	$353.67^{b} \pm 1.86$
12 hours	$43.67^{a} \pm 0.33$	$65.67^{a} \pm 0.33$	$92.67^{a} \pm 0.33$	$552.67^{b} \pm 1.45$

Means followed by similar letters in the rows indicate non-significant difference ($p > 0.005$, $df = 15$) by one-way ANOVA test. Comparison of honey bee consumption rate data was made with Tukey's post hoc test (Graph pad prism 8.0.2)

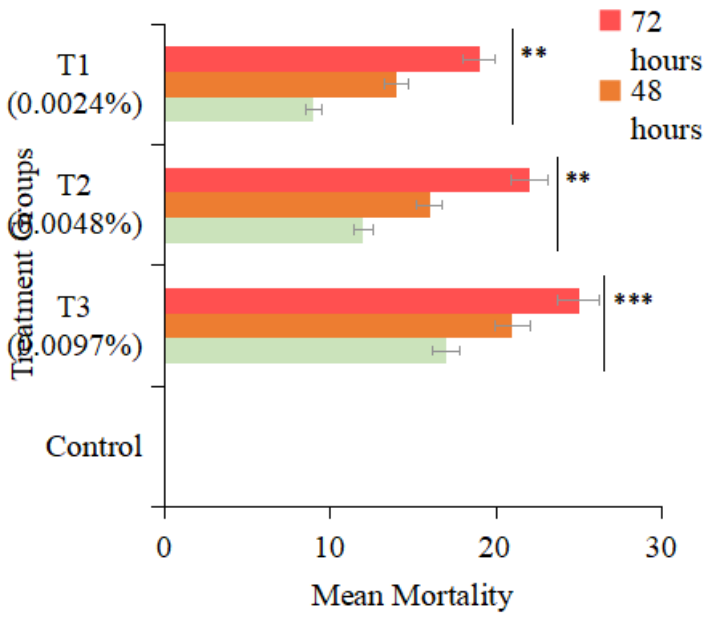


Figure 1: Mean (± SEM) mortality of honeybee (*A. mellifera*) after exposure todifferent concentrations of thiamethoxam in a feeding bioassay, n = 25.

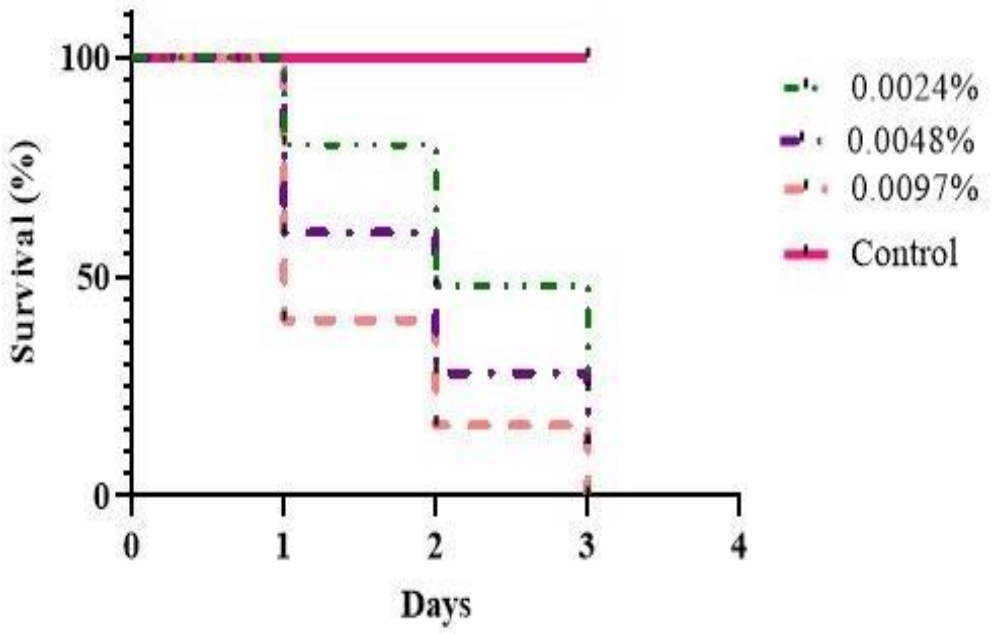


Figure 2: Kaplan - Meier survival curves of pesticide-exposed (0.0097%, 0.0048%, and 0.0024%) honey bees after 72-hour intervals in feeding bioassay compared to control.

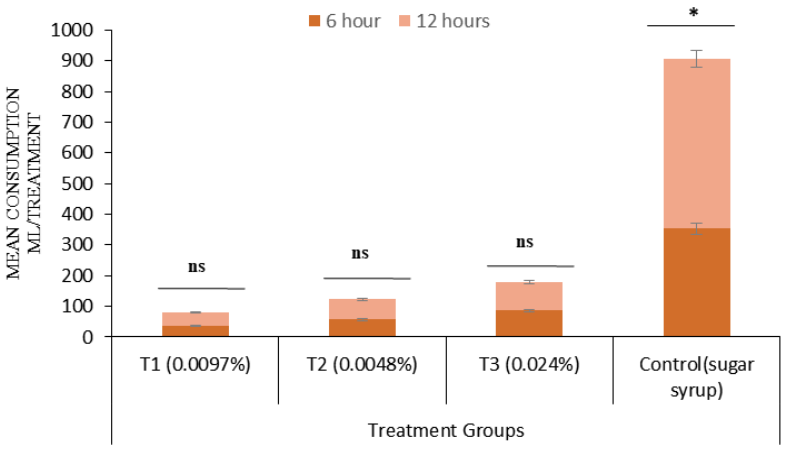


Figure 3: Mean consumption rate of Thiamethoxam and sugar syrup during the (a) 6-hour and (b) 12-hour feeding bioassay experiments. Honey bees were exposed to 0.0097% , 0.0048%, and 0.0024% of thiamethoxam (neonicotinoid). The control group was given a 50% sucrose solution.

**** Indicates statistical significance at p > 0.05.***

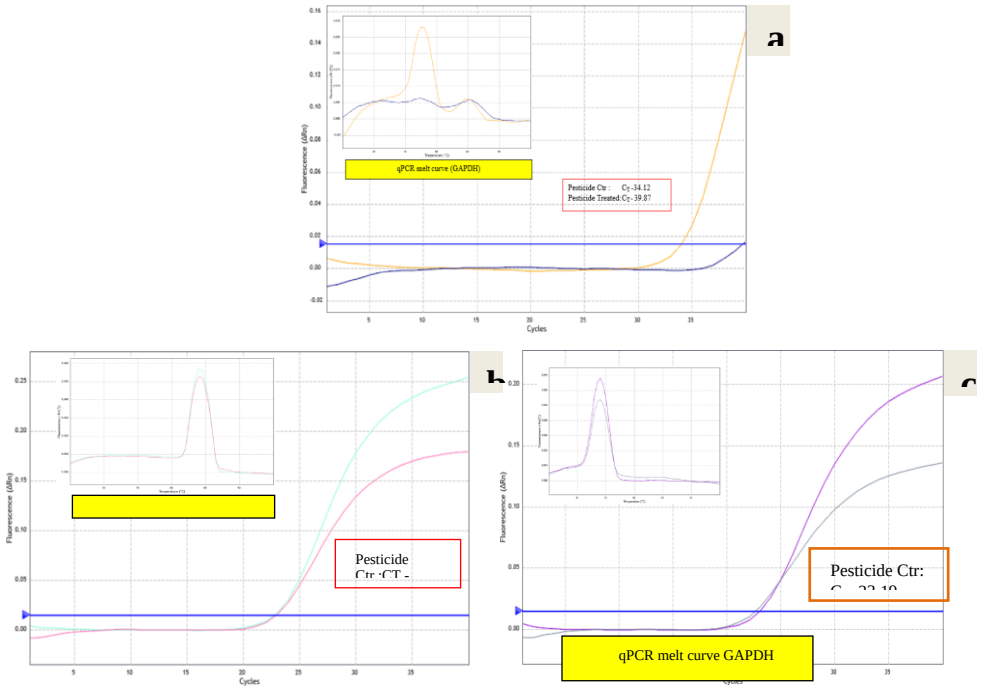


Figure 4: qPCR analysis showing Ct values and melting curves of three different genes. A, Graph showing Ct value of Glyceraldehyde 3-phosphate dehydrogenase(*GAPDH*) gene. b, Graph showing Ct value of *AChE* 2 gene. c, Graph showing Ct value of *MRJP* 1 gene in both treated and control groups of honey bees (*A. mellifera*).

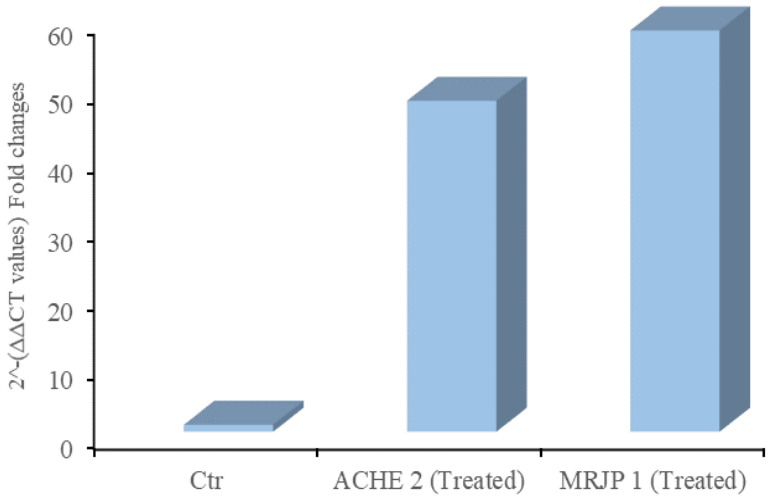


Figure 5: Comparative expression levels of *AChE*2 and *MRJP*1 gene in control and thiamethoxam-treated honey bees.

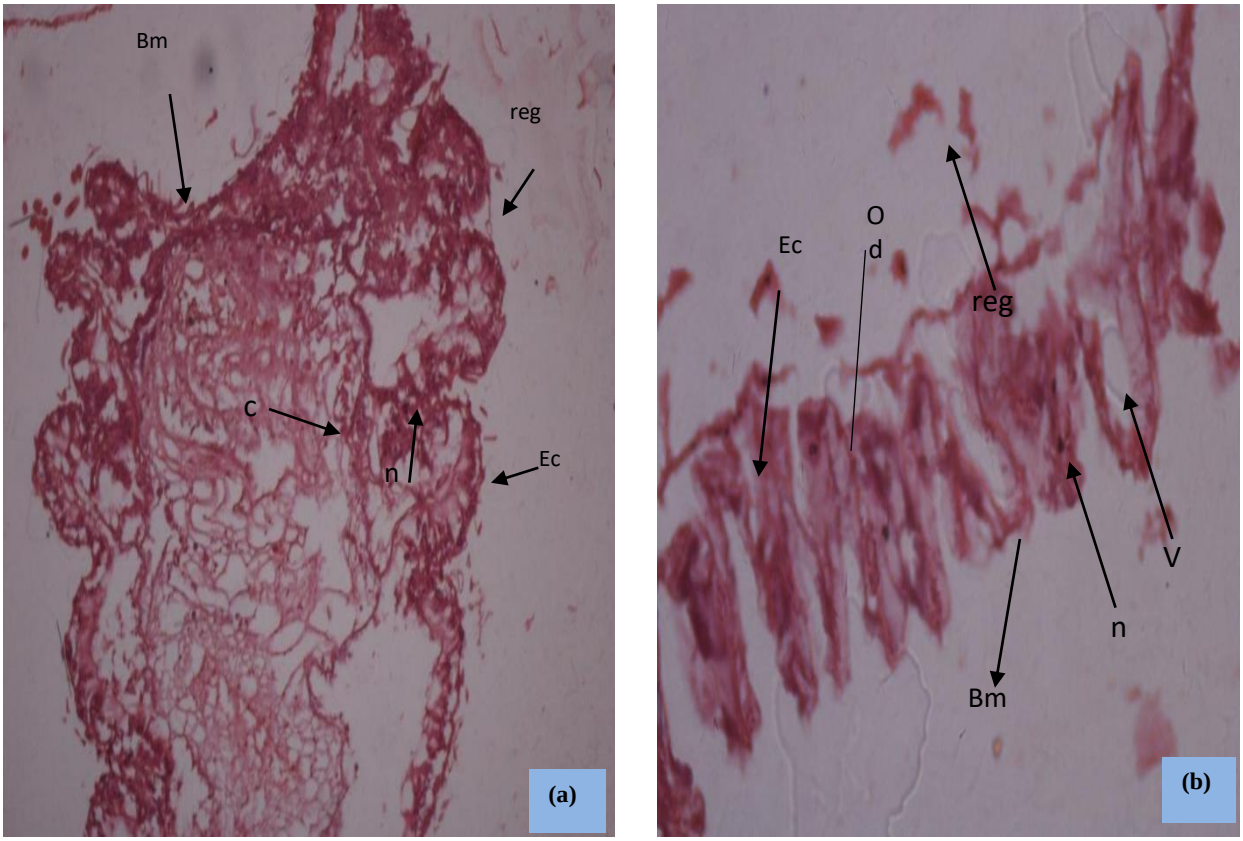


Figure 6: Histological sections of honey bee (*A.mellifera*) midgut (a) midgut of the control group. Reg: regenerative cells, Ec: epithelial cells, n: nucleus, c: cytoplasm, Bm: Basement membrane. (b) Thiamethoxam exposed group midgut. V: vacuole, Bm: basement membrane, Od: edema N: nucleus, reg: regenerative cells.