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ORIGINAL ARTICLE

Mixture toxicity of tebuconazole and fluopyram to honeybee (*Apis mellifera* L.): Effects on survival, feeding and antioxidant defenses

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Abstract

Tebuconazole + fluopyram is a new binary mixture fungicide product that is widely used to control many plant fungal pathogens and nematodes in several agricultural crops worldwide, including Egypt. However, there is a lack of information about their toxicological effects on honeybees (*Apis mellifera* L.). In the current study, the lethal and sub-lethal toxic effects of mixture tebuconazole + fluopyram were examined on *A. mellifera* workers. Tebuconazole + fluopyram exhibited low acute toxicity to *A. mellifera* foragers (the 96-h LC_{50} value was 1.389 mg a.i. · ml^{-1}). Sub-lethal effects of tebuconazole + fluopyram on survival, body weight, food consumption and antioxidant defenses of *A. mellifera* were determined by chronic oral exposure of *A. mellifera* workers to sugar syrup which contained two sublethal concentrations of the fungicide, 0.139 mg · ml^{-1} (1/10 of 96-h LC_{50}) and 0.278 mg · ml^{-1} (1/5 of 96-h LC_{50}), along with clear sugar syrup as a control for 18 days. Honeybees exposed to both sublethal concentrations of tebuconazole + fluopyram showed a significant decrease in the bees' survivability and dry body weight. Sugar syrup and pollen consumption by the exposed *A. mellifera* were relatively less than by the controls. Tebuconazole + fluopyram also induced disruptions in the enzymatic antioxidant and detoxification defense systems in bees, indicating the presence of oxidative stress. Fungicide exposure elicited a significant depletion in catalase and superoxide dismutase activities and a significant elevation in glutathione and malondialdehyde levels in bees, indicating lipid peroxidation. This is the first study indicating the harmful impacts of tebuconazole + fluopyram on honeybee health.

Keywords: antioxidant enzymes, chronic toxicity, fungicides, honeybee workers, oxidative stress

Introduction

Bees are the most efficient Hymenopteran insect pollinators, and play a major role in pollinating several wild plants in natural areas and in a wide range of cultivated crops that supply about 35% of food produced for humans and animals worldwide (Klein *et al.* 2007; Cang *et al.* 2023). All bee species, whether honeybees (i.e., *A. mellifera* and *A. cerana*) or wild bees (i.e., *Osmia bicornis* L. and *Bombus* spp), face challenges to their health from complex biotic and abiotic stressors (Johnson

2015). Some of the main stresses include agrochemical toxicants such as fungicides and other pesticides which have led to a widespread decline of bee species worldwide (Delkash-Roudsari *et al.* 2020; Huang *et al.* 2023). Fungicides already contribute >35% of the total chemical pesticide market worldwide (Zubrod *et al.* 2019). Generally, fungicides are harmless to various insect pollinators, especially bee species, therefore, many fungicides are frequently used without restriction in a wide range of crops during the bloom. As a result, forage bees are frequently exposed to these chemicals, which may pose serious risks to bees and colonies (Johnson 2015; Belsky and Joshi 2020; Almasri *et al.* 2021; Rondeau and Raine 2022). Consequently, residues of several fungicides, particularly those belonging to triazole, strobilurins, and pyridinylethylbenzamide, were frequently detected in bees and beehive matrices (Schuhmann *et al.* 2021; Rondeau and Raine 2022). Several toxicological studies confirmed that fungicide exposure can have toxic impacts on survival, reproduction, and brood development (Sgolastra *et al.* 2018; Belsky and Joshi 2020; Almasri *et al.* 2021; Iwasaki and Hogendoorn 2021), navigation, feeding behavior (Schuhmann *et al.* 2021), and physiology and immunity (Iwasaki and Hogendoorn 2021; Tosi *et al.* 2022) of bee species.

Tebuconazole is a broad-spectrum systemic triazole fungicide used extensively in more than 60 agricultural crops to suppress various plant pathogens and to inhibit biosynthesis of sterols in fungal cells (Perez-Rodriguez *et al.* 2019; Li *et al.* 2022). Fluopyram is a relatively new pyridinylethylbenzamide fungicide that is widely applied in several fruit, field, vegetable, and ornamental crop production areas to control various problematic plant diseases by inhibiting succinate dehydrogenase in fungal cells (Matadha *et al.* 2019; Tripathy *et al.* 2022; Herrero-Hernández *et al.* 2023). Recently, fluopyram has also been widely used as an effective nematicide against various plant-parasitic nematodes that can infest different crops (Faske and Hurd 2015; Schleker *et al.* 2022). The exposure to fluopyram has had detrimental impacts on survival, egg hatchability, feeding and locomotion behavior, reproduction, and growth of plant-parasitic nematodes (Li *et al.* 2022). Residues of fluopyram + tebuconazole and their metabolites were frequently found in flowers and several fruits, vegetables and ornamental plants (Matadha *et al.* 2019; Tripathy *et al.* 2022; Shabeer *et al.* 2023). They were also among the most frequently detected systemic fungicides in honeybees' bodies and hive products such as beebread, bee wax, pollen, and honey (Rondeau and Raine 2022; Végh *et al.* 2023).

Tebuconazole had various hurtful effects in different animals (e.g., fish, rats, and earthworms), such as embryonic toxicity, developmental toxicity, endocrine disruption, oxidative stress, immunotoxicity, hepatotoxicity, and carcinogenicity effects (Yang *et al.* 2018; Li *et al.* 2020, 2022). The sub-lethal toxic effects of tebuconazole were reported on the growth and development of *Bombyx mori* larvae and in the histology of silk glands, which was related to increased juvenile hormone and decreased ecdysteroid in larvae (Li *et al.* 2019). Fungicides such as traizoles were able to induce oxidative stress in different animals by stimulating the generation of free radical substrates and reactive oxygen species (ROS) and/or impairing the main components of antioxidant defense systems [e.g., catalase (CAT), glutathione S-transferase (GST), and superoxide dismutase (SOD)], that led to lipid peroxidation, nucleic acids damage, and cellular death (Ben Othmène *et al.* 2022; Cang *et al.* 2023; Yazlovytska *et al.* 2023). Combinations of triazole fungicides with some neonicotinoid or pyrethroid insecticides have been found to synergize the toxicity of these insecticides on bees (Thompson *et al.* 2014; Belsky and Joshi 2020; Schuhmann *et al.* 2021).

Despite the deleterious impacts of tebuconazole and fluopyram alone on various non-target animals, assessing the toxic effects of these compounds, individually or in combination, on bees has not yet been extensively studied. For example, exposure to tebuconazole and tetrachlorantraniliprole + tebuconazole induced neurotoxicity, behavior changes, antioxidant system dysfunction, impaired detoxification metabolism, and immunosuppression in adult *A. mellifera* workers (Cang *et al.* 2023). To our knowledge, no research has been done on the toxicological effects of tebuconazole + fluopyram on bees, especially *A. mellifera*. Therefore, the current study is the first to evaluate: (1) the lethal effects of tebuconazole + fluopyram on forager workers of *A. mellifera*; and (2) the sublethal effects of tebuconazole + fluopyram on the newly emerged workers of *A. mellifera*, including survival, body weight, food ingestion, and antioxidant systems.

Materials and Methods

Honeybees

Carniolan honeybees (*Apis mellifera carnica* Pollman) were obtained from five equally strong and healthy colonies, each containing five standard frames and headed by naturally inseminated sister queens less than 1-year-old. These colonies were maintained in the apiary located at the Faculty of Agriculture, Assiut University, Egypt (Assiut, Egypt, N 27°11'04" and E 31°09'45"). The selected bee colonies were not treated with any chemicals or pesticides.

Fungicide

The commercial binary mixture fungicide formulation Okte Zom® 40% SC (20% tebuconazole + 20% fluopyram; Shukuroglou Ltd., Cyprus) was used.

Acute toxicity assay

The toxicity of tebuconazole + fluopyram mixture fungicide on *A. mellifera* forager workers for 96 h was examined following the standard protocol of OECD (OECD 1998; Huang *et al.* 2023; Mohamed *et al.* 2023). *Apis mellifera* foragers (approximately 21 days old) were gathered from the entrance of five healthy colonies placed in the apiary of Assiut University. Foraging honeybees, which typically initiate external activities at 20 days of age (Winston 1987), were considered the most ecologically relevant life stage (Picard-Nizou *et al.* 1995). Foraging workers were collected according to the method described by Iwasa *et al.* (2004) and Abdu-Allah and Pittendrigh (2018), which typically ensures the acquisition of honeybee workers at approximately 20 days of age. Briefly, five hives were exposed to smoke for 30-60 seconds twice before the collection of worker honey bees, which were shaken from the top super or hive entrance using a brush and placed in a large, clean plastic container. Foragers were taken to the Economic Entomology laboratory in the Faculty of Agriculture, Assiut University and were maintained in an incubator at 35 ± 1°C., 65 ± 5% of relative humidity (RH), and in continuous darkness (Johnson *et al.* 2013; Mohamed *et al.* 2023; Duan *et al.* 2024). Prior to the exposure test, all *A. mellifera* foragers ingested *ad libitum* a 50% (w/v) sugar feeding solution. Preliminary experiments were conducted to determine the concentrations of the tested fungicide which induced a mortality of 10% to 96% in adult honeybees. For the acute toxicity test, for 96 h honeybee foragers fed on 50% sugar feeding solutions which were treated with six serially appropriate concentrations of the formulated tebuconazole + fluopyram (0.960, 1.82, 1.60, 1.92, 2.24, and 2.56 ml a.i. · ml^{-1}). Solutions for the mentioned concentrations of tebuconazole + fluopyram were prepared daily by diluting 120, 160, 200, 240, 280 and 320 µl from a commercial formulation Okte Zom® in 50 ml of a 50% sucrose solution. Each tested concentration was conducted in triplicate by using 10 forager bees per replication (a plastic cage) and 5 ml of each treatment solution was provided to the bees in each plastic cage with a disposable syringe (Mohamed *et al.* 2023; Li *et al.* 2024). Honeybee foragers in the control group were fed a sugar feeding solution devoid of fungicide (Badawy *et al.* 2015; Lv *et al.* 2023; Mohamed *et al.* 2023).

The plastic cages were designed and used according to a previous study of Mohamed *et al.* (2023). Initially, forager workers were anesthetized by cooling them at 4°C for 2–3 minutes in a refrigerator and then gently transferred to the plastic cages, according to Williams *et al.* (2013). Prior to the experiment, forager honeybees inside the plastic cages were deprived of a feeding solution for 1–2 hours, inducing starvation. Following the experimental treatments, the forager honeybees were placed back in the incubator in the plastic cages, under the previously mentioned conditions. The acute bioassay was conducted in duplicate and the same tested concentrations were prepared daily in the same feed solution. Immobile bees were assigned as dead, if they did not respond to touching with a thin paintbrush (Costa *et al.* 2014; Badawy *et al.* 2015). Mortality number was registered every 24 h of exposure, for 96 h. Dead bees were removed daily.

Chronic toxicity assay

For a chronic oral assay, one sealed brood comb from five healthy *A. mellifera* colonies of the apiary of Assiut University were maintained in an incubator (at 35 ± 1°C, 65% ± 5RH, in complete darkness) in screen cages to get newly emerged workers (0 to 24 h old) (OECD 2017; Brodschneider *et al.* 2022; Mohamed *et al.* 2023). After emergence, workers were randomly distributed in 12 plastic cages (30 individuals per cage) and were used in three experimental groups (four plastic cages per treatment). The first experimental group was a negative control treatment, in which *A. mellifera* workers were fed sugar feeding solution (50% w/v) without fungicide (Lv *et al.* 2023; Mohamed *et al.* 2023; Li *et al.* 2024). The second and third groups of *A. mellifera* workers were fed sugar feeding solutions containing two different sublethal concentrations of the fungicide tebuconazole + fluopyram: 0.139 mg a.i. · ml^{-1} (1/10 the 96-h LC_{50}) and 0.278 mg a.i. · ml^{-1} (1/5 the 96-h LC_{50}), respectively for 18 days. The 96-h LC_{50} of tebuconazole + fluopyram was 1.389 mg a.i. · ml^{-1} for *A. mellifera* foragers, which was detected by the above-mentioned acute oral exposure test.

The exposure sugar feeding solutions were prepared and provided to *A. mellifera* workers *ad libitum* at the beginning of the chronic toxicity experiment and renewed every 24-h. Emergent *A. mellifera* workers in all experimental groups were also provided with a protein source so bees were allowed to feed on mix pollen dough that was prepared by mixing (10% w/w) distilled water for 12 days (Mohamed *et al.* 2023). The pollen dough was stored in a freezer at –20°C and saturated before being used. Mortality of *A. mellifera* workers was also assessed daily for 18 days, after which dead bees were removed from the plastic cages. The daily volumes of sugar solution and pollen ingested by *A. mellifera* workers in plastic cages were recorded

for 18 and 12 days, respectively, and then the average food consumption was calculated every day and represented as µg/bee/day. On the 18th day of the exposure, 20 *A. mellifera* workers from each experimental treatment group (five bees per replicate) were gathered and frozen until death and then weighed to determine worker fresh biomass. After that, bee samples were dried at 65°C for 3 days to determine the dry weight.

Biochemical assay

Nine surviving honeybee workers from each treatment group (three bees per replicate "cage") were sampled at 3, 9, and 15 days during the exposure to measure certain antioxidant enzyme activities including CAT, SOD, and GSH, lipid peroxidation, and protein content. For each treatment, three samples were collected and each sample consisted of tissues of the three honeybees per cage. Each sample was quick-frozen and ground into powder using liquid nitrogen, and then homogenized in ice-cold phosphate buffer (PBS) solution (pH 7.4; 10% w/v). The homogenates of each sample were centrifuged at 15 000 rpm for 20 min at 4°C with Triton-X (1% v/v), and the supernatants were gently collected and stored at −80°C for later use in the antioxidant enzymes assay. CAT level was assayed according to Lück (1965) and Tawfik *et al.* (2023) by measuring the depletion of hydrogen peroxide (H_2O_2) concentration spectrophotometry at 240 nm. SOD activity was determined at 550 nm in accordance with Misra and Fridovich (1972) and Tawfik *et al.* (2020), which is based on the ability of the SOD to inhibit the autoxidation of epinephrine in the alkaline medium. GSH levels were measured at 430 nm with 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) as the substrate (Tawfik *et al.* 2020). Lipid peroxidation (LOP) levels were determined by quantitating the thiobarbituric acid level reactive substance as described previously by Ohkawa *et al.* (1979) and Tawfik *et al.* (2020), and the malondialdehyde (MDA) level was measured at 532 nm. Protein content was assayed according to Lowry *et al.* (1951) and Tawfik *et al.* (2023), using bovine serum albumin (BSA) as the standard and measured spectrophotometrically at 595 nm.

Statistical analysis

The honeybee mortality data were analyzed using probit analysis. The LC_{50} and LC_{90} (mg · ml^{-1}) values and their 95% confidence limits were determined. Honeybee survival data were initially checked for normality with the Shapiro–Wilk test and homogeneity of variances with Bartlett's test. The honeybee survival among the tested treatments was analyzed based on a log-rank (Mantel-Cox) test of Kaplan-Meier analysis (Abou-Shaara *et al.* 2018; Mohamed *et al.* 2023). Statistical differences among the means for each treatment group in food consumption (sugar syrup and pollen), a bee's dry body weight, antioxidant enzymes activity, and total protein content were assessed by two-way analysis of variance with treatment, and time as independent variables and testing for their interaction, followed by Duncan's new multiple range tests. Differences were considered statistically significant when $p < 0.05$. All statistical analyses were performed using the SPSS program, version 16 (SPSS Inc., Chicago, USA, 2007).

Results

Acute toxicity determination

The results of acute oral toxicity experiments conducted on honeybee foragers were presented in Table 1. The LC_{50} values of tebuconazole + fluopyram on *A. mellifera* foragers were 2.117, 1.568, and 1.389 mg a.i. · ml^{-1} after 48, 72, and 96 h, respectively (Table 1), so the fungicide is considered low toxicity on *A. mellifera* foragers. The fungicide toxicity on forager honeybees was enhanced over time (Table 1). Mortality of foragers did not reach 30% following 24 h exposure to the tested concentrations of tebuconazole + fluopyram, so the 24-h LC_{50} for the fungicide could be determined accurately (Fig. 1).

Effects on survival, food consumption and body weight

Chronic exposure to the sublethal concentration 1/5 of the 96-h LC_{50} of tebuconazole + fluopyram for 18 days significantly diminished the survival rates of *A. mellifera* workers (Mantel-Cox paired test: $X^2 = 6.497$, df = 2, $p < 0.05$) in comparison to unexposed bees and bees exposed to 1/10 of the 96-h LC_{50} (Fig. 2). Honeybee workers exposed to 1/10 of the

Table 1. Acute oral toxicity of a mixture of tebuconazole + fluopyram fungicide in *Apis mellifera* foragers after 24–96 h of exposure

Time [h]	LC_{50} (95% CL*) [mg a.i. × ml^{-1}]	LC_{90} (95% CL) [mg a.i. × ml^{-1}]	Slope ± SE
24	N.C.**	N.C.	N.C.
48	2.117 ± 0.08 (1.917–2.527)	4.221 ± 0.80 (3.462–8.718)	4.63 ± 1.06
72	1.568 ± 0.06 (1.330–1.788)	2.717 ± 0.05 (2.277–4.244)	5.37 ± 0.19
96	1.389 ± 0.01 (1.272–1.492)*	2.292 ± 0.11 (2.114–2.636)	5.97 ± 0.63

*CL – confidence limits

**N.C. – the 24-h LC_{50} or $_{90}$ values not calculated

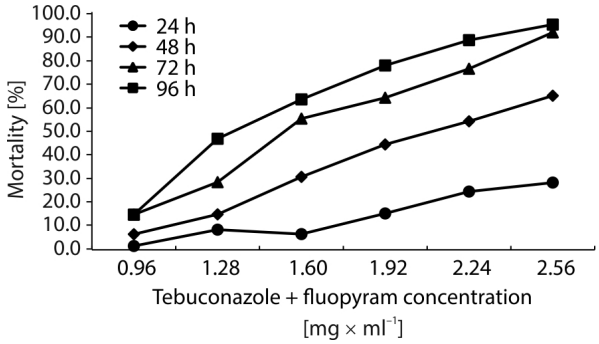

Fig. 1. Mortality of *Apis mellifera* foragers exposed to a mixture of tebuconazole + fluopyram fungicide at 24, 48, 72, and 96 h

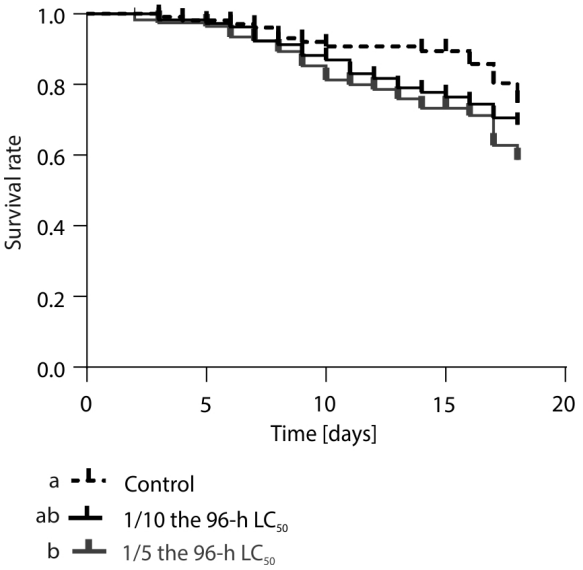

Fig. 2. Effects of tebuconazole + fluopyram on survival of *Apis mellifera* by the Kaplan–Meier survival curves. Newly emerged of A. mellifera orally exposed to sucrose solutions containing no fungicide (0.00 mg · ml^{-1}, control), and different sub-lethal concentrations of tebuconazole + fluopyram (1/10 the 96 h LC_{50} and 1/5 the 96 h LC_{50}) for 18 days. The data showed the proportion of surviving *A. mellifera* exposed to the fungicide. Treatments with different letters behind the curves are significantly different ($p < 0.05$)

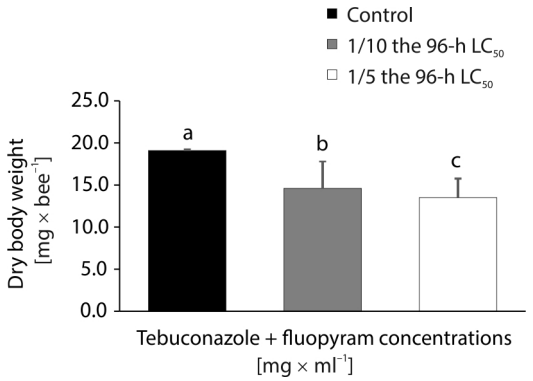

Fig. 3. Effects of exposure to different sub-lethal concentrations of tebuconazole + fluopyram (0.00, 1/10 the 96 h LC_{50} and 1/10 the 96 h LC_{50}) on dry body weight of *Apis mellifera* workers for 18 days. Treatments with different letters denote significant differences ($p < 0.05$)

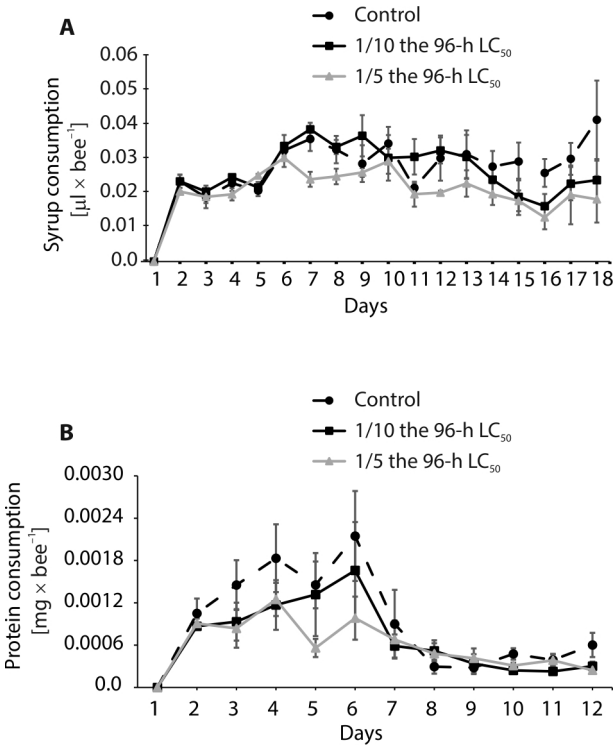

Fig. 4. Effects of exposure to different sub-lethal concentrations of tebuconazole + fluopyram (0.00, 1/10 the 96 h LC_{50} and 1/5 the 96 h LC_{50}) on mean daily syrup consumption (µl · bee^{-1}) – A and pollen consumption (mg · bee^{-1}) – B of *Apis mellifera* workers for 18 and 12 days, respectively. Treatments with different letters denote significant differences ($p < 0.05$)

96-h LC_{50} and 1/5 of the 96-h LC_{50} of tebuconazole + fluopyram for 18 days also exhibited a significant depletion in dry body weight of honeybees by 23.85 and 29.90%, respectively, compared to the control (Fig. 3). Sugar syrup consumption was significantly decreased by both sublethal concentrations of tebuconazole + fluopyram (two-way ANOVA, F = 12.28, df = 2, $p = 0.000$, $p < 0.05$) and exposure time (two-way ANOVA, F = 9.76, df = 17, $p = 0.000$, $p < 0.05$), with no significant interaction (Fig. 4A). Pollen consumption was also significantly decreased by both sublethal concentrations of the mixture fungicide (two-way ANOVA, F = 3.91, df = 2, $p = 0.023$, $p < 0.05$) and exposure time (two-way ANOVA, F = 11.33, df = 11, $p = 0.000$, $p < 0.05$), with no significant interaction (Fig. 4B). The sublethal toxicity impacts on survival, food ingestion and body weight of bees were dosage-dependent.

Biochemical biomarkers

Chronic exposure to tebuconazole + fluopyram at 1/10 of the 96-h LC_{50} and 1/5 of the 96-h LC_{50} for 18 days also induced alterations in the enzymatic antioxidants and detoxification defense systems in honeybee workers on days 3, 9, and 15, suggesting oxidative stress. CAT activity was significantly diminished by both sublethal concentrations of tebuconazole + fluopyram (two-way ANOVA, F = 35.58, df = 2, $p = 0.000$, $p < 0.05$) and exposure time (two-way ANOVA, F = 29.57, df = 2, $p = 0.000$, $p < 0.05$), with their interaction present (two-way ANOVA, F = 4.39, df = 4, $p = 0.012$, $p < 0.05$) (Fig. 5A). SOD activity was significantly decreased by both sublethal concentrations (two-way ANOVA, F = 45.48, df = 2, $p = 0.000$, $p < 0.05$) and exposure time (two-way ANOVA, F = 43.50, df = 2, $p = 0.000$, $p < 0.05$) with no interaction (Fig. 5B). GSH levels were significantly elevated by the sublethal fungicide concentrations (two-way ANOVA, F = 89.09, df = 2, $p = 0.000$, $p < 0.05$) and exposure time (two-way ANOVA, F = 50.23, df = 2, $p = 0.000$, $p < 0.05$), with their interaction (two-way ANOVA, F = 4.76, df = 4, $p = 0.009$, $p < 0.05$) (Fig. 5C). MDA levels were also significantly increased by both sublethal concentrations of the fungicide (two-way ANOVA, F = 46.19, df = 2, $p = 0.000$, $p < 0.05$) and exposure time ((two-way ANOVA, F = 326.17, df = 2, $p = 0.000$, $p < 0.05$), as well as their interaction (two-way ANOVA, F = 5.28, df = 4, $p = 0.0054$, $p < 0.05$) (Fig. 5D).

Discussion

Despite the fact that honeybees are likely to be extensively exposed to mixtures of pesticides in agrosystems, limited research has focused on the acute and chronic toxicity of fungicide mixtures on honeybee health, and most of the research has been restricted to interactions between azole fungicides and insecticides (some neonicotinoids and pyrethroids) (Fisher *et al.* 2017; Almasri *et al.* 2020; Schuhmann *et al.* 2021; Al Naggar *et al.* 2022; Cang *et al.* 2023). Considering the above-mentioned facts, the lethal toxicity of tebuconazole + fluopyram mixture fungicide on adult workers of *Apis mellifera* as well as the sublethal effects of the tested fungicide on the survival, food ingestion, body weight, and antioxidant systems of honeybees, have been investigated in the present study. The LC_{50} of tebuconazole + fluopyram for forager honeybee workers were 2.117 and 1.389 mg · ml^{-1}, respectively, after 48 and 96 h, which indicated that tebuconazole + fluopyram exhibited low acute toxicity to adult

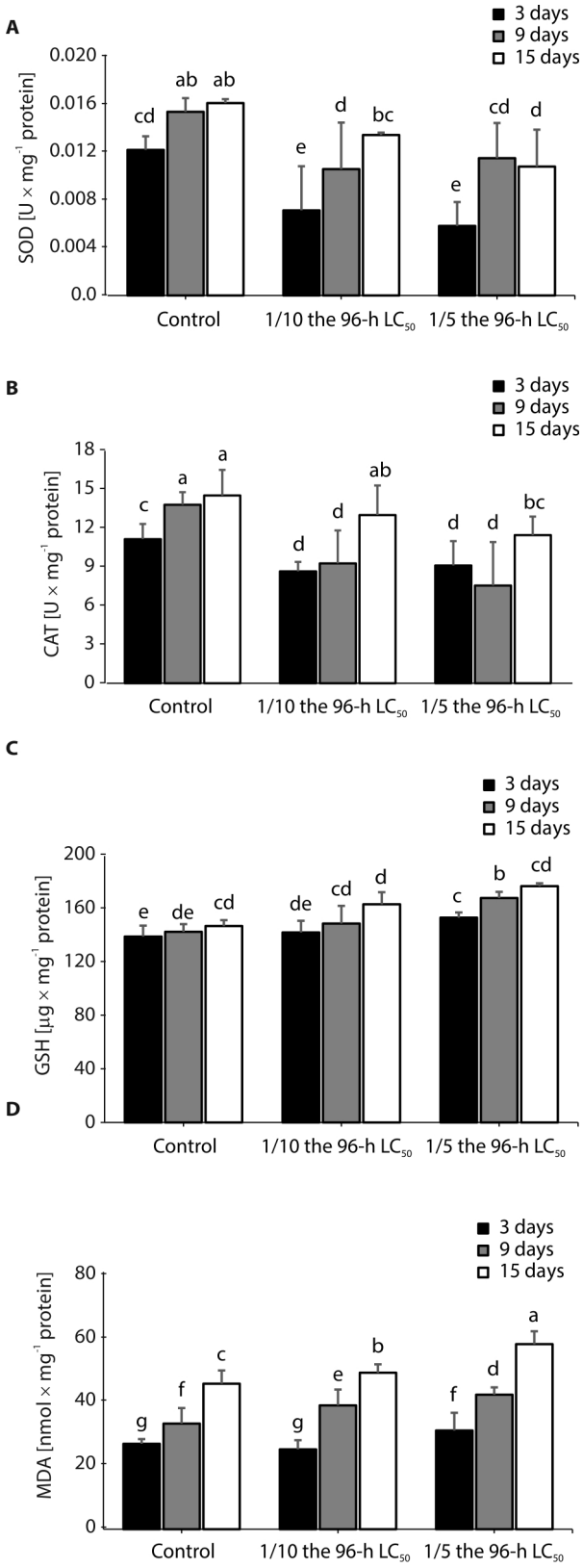

Fig. 5. Effects of different tebuconazole and fluopyram concentrations (0.00, 1/10 the 96 h LC_{50} and 1/5 the 96 h LC_{50}) on the activity of oxidative stress enzymes (SOD – A, CAT – B, GSH – C, MDA – D) in newly emerging honey bees after 3, 9 and 15 days of exposure. Different letters on error bars between control and fungicide groups in each exposure time in oxidative stress parameter denote significant differences ($p < 0.05$)

honeybees. Cang *et al.* (2023) reported that the LC_{50} values of tebuconazole for honeybee workers were 2.770 and 1.841 mg · ml^{-1} at 48 and 96 h, respectively, while the LC_{50} values of tebuconazole + tetrachlorantraniliprole mixture were 0.235 and 0.124 mg · ml^{-1} at 48 and 96 h, respectively, indicating that the combination of tetrachlorantr-aniliprole with tebuconazole exhibited a strong synergistic effect on honeybees. In line with the mentioned results of Cang *et al.* (2023), the added toxicity of fluopyram + tebuconazole on honeybees may be due to the interactions between the fungicide's active ingredients and/or the inert ingredients in the formulated products.

Tebuconazole showed an increase in the toxicity of different neonicotinoids such as imidacloprid, clothianidin, acetamiprid and thiamethoxam or a pyrethroid lambdacyhalothrin to adult honeybees when used in mixture (Thompson *et al.* 2014; Zhu *et al.* 2017b; Wernecke *et al.* 2019; Schuhmann *et al.* 2021). A mix of boscalid+pyraclostrobin (Pristine®) has been found to decrease adult honeybee worker survival and queen production (DeGrandi-Hoffman *et al.* 2013; Fisher *et al.* 2017). Fisher *et al.* (2021) stated that toxicity of boscalid + pyraclostrobin (Pristine®) on honeybees may be due to the combined active ingredients. Everich *et al.* (2009) indicated that the toxicity of the fungicide captan to *A. mellifera* brood development was due to a formulation of inert ingredients other than the fungicide active ingredient alone. In the present study, chronic exposure to tebuconazole + fluopyram at 1/5 of the 96-h LC_{50} significantly decreased the survival of bees compared to the control. Although fungicides were considered harmless to different bee species, including honeybees, chronic exposure of bees to some fungicides (e.g., difenoconazole and tebuconazole) was lethal (Thompson *et al.* 2014; Almasri *et al.* 2020; Rondeau and Raine 2022). Furthermore, many studies indicated that exposure of bees to pesticide combinations caused depletion in survival of bees and most combinations are more toxic on bees than the pesticides alone (Sgolastra *et al.* 2018; Almasri *et al.* 2020; Al Naggar *et al.* 2022; Al Naggar and Wubet 2024).

Apis mellifera workers exposed to a mixture of azole fungicides and some neonicotinoid or pyrethroid insecticides often exhibit increased mortality and reduced food consumption compared to individual pesticide exposure or control groups (Sgolastra *et al.* 2018; Almasri *et al.* 2020; Migdał *et al.* 2024). These studies consistently demonstrate synergistic effects of pesticide combinations, with toxicity increasing proportionally to concentration. This study investigated the effects of tebuconazole + fluopyram on honeybee worker feeding behavior. Honeybees exposed to 1/5 of the 96-h LC_{50} of the fungicide mixture consumed less sugar syrup or pollen than honeybees in the control and those exposed to 1/10 of the 96-h LC_{50}.

Many studies indicated that sugar syrup consumption of bee species is decreased by the presence of various fungicides such as trizoles (tebuconazole, propiconazole, myclobutanil and difenoconazole), singly and in combination with other pesticides. This hoarding behavior may be caused by the repellent properties of different fungicides (Zhu *et al.* 2017b; Azpiazu *et al.* 2019; Pal *et al.* 2022). For example, honeybee workers exposed to the combination of the commercial formulation of imidacloprid with tetraconazole exhibited lower food consumption than the unexposed honeybees which might be due to the repellent impacts of tetraconazole fungicide (Zhu *et al.* 2017b). The fungicide boscalid + pyraclostrobin (Pristine®) has been found to reduce pollen consumption of honeybee workers in the laboratory (Fisher *et al.* 2021). Food ingestion in honeybees and a solitary bee exposed to some pesticides could also be positively correlated with the concentration of the pesticides (Zhu *et al.* 2017b; Azpiazu *et al.* 2019; Almasri *et al.* 2020). For example, honeybees chronically exposed to high concentrations of the pesticide mixtures showed significantly lower food intake than those subjected to low concentrations (Zhu *et al.* 2017b; Almasri *et al.* 2020).

Adults of solitary bees exposed to mixtures of imidacloprid + myclobutanil or imidacloprid + myclobutanil + acetamiprid, showed significantly less pollen and sugar syrup ingestion than the control and adults ingested about 80% less syrup than the control (Azpiazu *et al.* 2019). Bee species such as *A. mellifera*, *B. terrestris*, and *O. bicornis* are especially fond of sugar syrup containing the neonicotinoid insecticides, but the presence of the triazole fungicide myclobutanil in this preferred food source is likely to suppress their feeding (Kessler *et al.* 2015; Azpiazu *et al.* 2019, 2022). In *O. bicornis*, combinations of propiconazole–clothianidin or propiconazole alone altered the hoarding behavior of bees and decreased their syrup consumption in comparison to the control (Sgolastra *et al.* 2018). Tebuconazole also reduced feeding of *Tineola bisselliella* larvae on wool (Sunderland *et al.* 2014). Fluopyram induced damage to feeding of the nematode *Caenorhabditis elegans* (Liu *et al.* 2022). Different agro-pesticides can elicit oxidative stress in bees and other animals by generation and accumulation of ROS, impairment and destruction of the antioxidant systems, and production of lipid peroxide (Pal *et al.* 2022; Ward *et al.* 2022). Exposure to tebuconazole provoked an elevation in ROS production and lipid peroxidation in different fish such as common carp and zebrafish (Toni *et al.* 2011; Kumar *et al.* 2019), the earthworm, *Eisenia fetida* (Chen *et al.* 2018; Li *et al.* 2022), and rats (Yang *et al.* 2018). Fluopyram was

also found to induce oxidative stress in nematodes (Liu *et al.* 2022).

In the current study, CAT and SOD activities were significantly declined in worker honeybees exposed to tebuconazole + fluopyram at 1/10 of the 96-h $LC_{50}$and 1/5 of the 96-h LC_{50} compared to the unexposed group. This decrease in activity of both antioxidant enzymes may be attributed to production and accumulation of ROS (e.g., superoxide anion and hydrogen peroxide) that led to oxidative stress and lipid peroxidation in treated bees. Immoderation of ROS including hydrogen peroxide may be responsible for declined SOD activity, while superoxide anion may reduce CAT activity (Bagnyukova *et al.* 2006). These enzymatic antioxidants are commonly used in toxicological studies as important biomarkers, indicating the formation of oxyradicals (Monteiro *et al.* 2006). When antioxidant defenses in insects and other animals are insufficient to degrade ROS, these ROS may accumulate and react with cellular biomolecules like proteins, nucleic acids, and lipids, to possibly contribute to oxidative damage like lipid peroxidation (Pal *et al.* 2022).

In the present study, a significant elevation was observed in MDA contents and GSH levels in honeybees exposed to both sublethal concentrations of tebuconazole + fluopyram compared to the control. Tebuconazole caused a decrease in AChE (acetylcholinesterase), Caspase, SOD, and CAT activity and an increase in the malonaldehyde level and the activity of α-Amylase, cytochrome P450 and carboxylesterase in honeybees (Cang *et al.* 2023). Winter honeybees exposed to difenoconazole and imidacloprid + difenoconazole ($1\ \mu g \cdot l^{-1}$) showed significant depletion of SOD and CAT activity and significant elevation of GST activity and lipid peroxidation level compared to the control (Pal *et al.* 2022). CAT, GSH, SOD, and GST are the essential ROS scavenging and antioxidant enzymes for xenobiotic detoxification in insect species including honeybees (Corona and Robinson 2006; Rand *et al.* 2015; Tawfik *et al.* 2020; Liu *et al.* 2021). GST and GSH also play a major role in degradation of the main lipid peroxidation products (e.g., MDA), to less toxic substrates, that are generated during oxidative stress (Corona and Robinson 2006; Rand *et al.* 2015; Tsikas 2017; Pal *et al.* 2022; Tawfik *et al.* 2020). GSH content, GST activity, and lipid peroxidation levels in treated bees were positively correlated with each other (Liu *et al.* 2021). In the current study, the modulations in antioxidant parameters in treated honeybees depended on the fungicide concentration.

Elevation in levels of GSH and GST have been found in honeybees exposed to ethiprole pesticide for 14 days, indicating the vital role of these antioxidants in the detoxification of ethiprole in honeybees (Liu *et al.* 2021). Elevation in GST activity was reported when honeybee workers were orally exposed to low concentrations of tebuconazole or tebuconazole + tetrachlorantraniliprole (Cang *et al.* 2023), tetraconazole + imidacloprid (Zhu *et al.* 2017a), imidacloprid and thiamethoxam (Li *et al.* 2017), and glyphosate at $1\ \mu g \cdot l^{-1}$ (Almasri *et al.* 2020). The MDA content was enhanced and lipid peroxidation occurred in honeybees after exposure to tebuconazole or tebuconazole + tetrachlorantraniliprole (Cang *et al.* 2023). Tebuconazole induced an increment in lipid peroxidation levels in different fish such as *Rhamdia quelen*, *Cyprinus carpio*, and *Danio rerio* (Ferreira *et al.* 2010; Toni *et al.* 2011; Chang *et al.* 2020), and rodents such as mice and rats (Ku *et al.* 2021; Coremen *et al.* 2022). Such elevations in the GST activity in animals exposed to pesticides may reflect the activation of an animal's defense system against ROS to scavenge their levels and prevent oxidative damage.

Therefore, SOD, CAT, GSH, and GST have key antioxidant and detoxifying functions in an animal's defense system when exposed to chemical agro-pesticides (Toni *et al.* 2011; Santana *et al.* 2018; Liu *et al.* 2021; Li *et al.* 2022; Migdał *et al.* 2024). These findings confirmed that sublethal concentrations of tebuconazole + fluopyram might induce oxidative stress in honeybees. In the current study, disruption in the oxidative status in honeybees after exposure to both concentrations of tebuconazole + fluopyram might reflect the toxic effects of this fungicide product on survival, feeding behavior, and body mass of honeybees, that may pose a high risk to all individuals of a bee colony. Previous researchers confirmed that tebuconazole elicited oxidative stress in honeybees by impairing honeybee cognitive functions, and altering feeding and locomotion behavior, which can negatively affect honeybee colony functions (Mao *et al.* 2017; Jaffe *et al.* 2019). In adult honeybees, tebuconazole has also been found to distribute the antioxidant systems, increase lipid peroxidation, and change the total fatty acid profile in bees' brains, due to possible brain destruction (Mackei *et al.* 2023).

Conclusions

In conclusion, the 96-h LC_{50} of tebuconazole + fluopyram was $0.139\ mg \cdot ml^{-1}$ to honeybee foragers, which was less toxic. Sublethal exposure to 1/10 of the 96-h LC_{50} and 1/5 of the 96-h LC_{50} of tebuconazole + fluopyram induced a reduction in survival and body weight of the newly emerged honeybees, and altered feeding behavior by decreasing the carbohydrate and protein intake. Tebuconazole + fluopyram also induced oxidative stress in the newly emerged honeybee

workers, possibly through a decline in the activity of the main antioxidant enzymes (SOD and CAT) and elevated levels of the detoxification factor GSH and the MDA concentration. More research is needed to investigate potentially harmful impacts of tebuconazole + fluopyram, alone or in combinations, on immunity and reproductive systems, and the physiological status of wild bees and honeybees under laboratory and colony settings.

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