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Detrimental consequences of tebuconazole on redox homeostasis and fatty acid profile of honeybee brain

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ABSTRACT

Excessive use of azole fungicides in agriculture poses a potential threat to honeybees and other pollinator insects; however, the detailed effects of these molecules remain largely unclear. Hence, in the present study it was aimed to investigate the acute sublethal effects of tebuconazole on the redox homeostasis and fatty acid composition in the brain of honeybees. Our findings demonstrate that tebuconazole decreased total antioxidant capacity, the ratio of reduced to oxidized glutathione and disturbed the function of key antioxidant defense enzymes along with the induction of lipid peroxidation indicated by increased malondialdehyde levels, while it also altered the fatty acid profile of the brain. The present study highlights the negative impact of tebuconazole on honeybees and contributes to the understanding of potential consequences related to azole exposure on pollinator insects' health, such as the occurrence of colony collapse disorder.

1. Introduction

Honeybees (*Apis mellifera*) are significant pollinators in the ecosystem and hence play a critical role in the maintenance of biodiversity and in sustainable agriculture. There has been an intense decline in the number of honeybees and other pollinator insects throughout the world, which trend is projected to continue (Neov et al., 2021). This finding is of critical importance, since besides other plants, approximately 75% of crops are pollinated by insects, of which honeybees represent one of the most important species under most climatic conditions (Klein et al., 2007). One of the major causes of losses is the so called colony collapse disorder (CCD), which cases have been on the rise since the early 2000s. It is a complex, not yet fully understood disease, which is most likely caused by the combination of numbersome stressors and their synergistic influence on honeybee health, mainly affecting honeybee workers inducing rapid decrement in their numbers (Watson and Anthony Stallins, 2016). CCD can be traced back to the spread of various viral or bacterial diseases, the infection by *Varroa* mites, serious ecologically dangerous factors such as climate change, the fragmentation and disturbance of natural habitats, risk factors associated with modern farming such as the increased exposure to heavy metals and the overuse of plant protection products (Cornman et al., 2012; Gashawbeza et al., 2020; Paxton 2010; vanEngelsdorp et al., 2017). Plant protection products including insecticides, herbicides and fungicides are estimated to be applied globally at 3.5 million tons per year, raising public concern about their potential toxic effects on non-target organisms (Sharma et al., 2019). The better understanding of the molecular consequences caused by agricultural pesticides is of exceptional importance for the conservation of honeybees as well as other exposed insect species (Poquet et al. 2016).

Plant protection products are considered as high risk to pollinator species and are potentially one of the major factors playing a role in the development of CCD in honeybees (Grassl et al., 2018; Magal et al. 2020; Sánchez-Bayo et al., 2016). Although herbicides and fungicides are not applied directly on target insects, we do not have extensive knowledge as to whether they pose an increased risk to pollinator species (Tamburini et al., 2021). Azole type fungicides like triazoles and imidazoles act through the blocking of fungus ergosterol synthesis by inhibiting

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cytochrome P-450 (CYP450)-dependent 14α-demethylation of lanosterol, and the CYP associated biotransformation of further endogenous or exogenous molecules (Belzunces et al. 2012). On the other hand, they were reported to cause negative consequences on the overall health status in various species (Nesnow 2013). Triazoles may have a direct negative impact on cellular metabolic processes, including the antioxidant system (Nesnow et al., 2011; Souders et al., 2019; J.-D. Yang et al., 2018). These agents have been also found to affect bee behavior, foraging effectiveness, pollination, learning as well as the maintenance and development of normal colony functions (Jaffe et al. 2019; Mao et al. 2017). Behavioral disturbances along with decreased cognitive functions may be in strong correlation with the intense oxidative stress induced by triazole exposure (Elhady et al., 2019). Oxidative stress causes also negative effects on the fatty acid metabolism of the brain inducing neurodegenerative disorders; however no information is available in the literature regarding this topic in pollinator insects such as honeybees (Montesinos et al. 2020; Tsaluchidu et al., 2008). It is also worth mentioning, that azole fungicides have been reported to act synergistically with other pesticides such as neonicotinoid insecticides (Schmuck et al. 2003). This synergism is most likely in connection with the inhibition of CYP enzymes, mentioned above, leading to the slower degradation as well as the amplified and prolonged effects of neonicotinoids (Haas and Nauen 2021; Thompson et al., 2014). Since all these detrimental effects can contribute to the development of CCD as well, extensive research of azole-related consequences is of key importance for the conservation of honeybees along with other endangered pollinator insects (Poquet et al. 2016).

Based on the aforementioned findings, our main hypothesis was that tebuconazole-related negative effects may be connected to increased levels of oxidative stress resulting from pesticide exposure. This situation can lead to cellular damage, as well as detrimental metabolic consequences, including alterations in redox and lipid homeostasis. In order to gain information about the abovementioned questions, the main aim of our study was to analyze fatty acid composition and various oxidative parameters in the brain of honeybees, including total antioxidant capacity (TAC), state of the glutathione defense system, the activity of glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase (SOD) as well as xanthine oxidase (XO) and the production of malondialdehyde (MDA) following acute sublethal, field-realistic tebuconazole exposure.

2. Materials and methods

2.1. Collection and treatment of laboratory animals

Adult honeybees have been collected as it was previously described by Williams et al., (2013). In order to ensure the similar overall health status and origin of the animals, one single colony was involved in the study. The colony was not exposed to any chemicals or treatments 3 months prior to the experiment and no sign of disease was observed according to thorough veterinary examination.

Honeybee workers were gathered during the morning hours from frames containing no brood. Animals were randomly distributed into separated groups (3 replicates/treatment group), containing approximately 200 individuals per group. The size of the applied hoarding cages was 30 cm × 20 cm, which have been stored in a room at 25 ± 2 °C and 50–65% relative humidity (RH). Honeybees had *ad libitum* access to 50% sucrose solution as well as to water. In the first 36 h of the study, a short accommodation period has been included, in which the animals received no specific treatment. Following the accommodation time, the feeding solutions were supplemented with tebuconazole (Merck KGaA, Darmstadt, Germany) in the appropriate concentrations and were replaced by fresh solutions in every 8 h. Treatment phase lasted for 48 h, during which time feeding solutions contained tebuconazole in the concentrations of 202.5 μg/mL, 101.25 μg/mL and 50.625 mg/mL ("TebuHigh", "TebuMedium" and "TebuLow" groups, respectively). Considering the daily consumption of sucrose solution to be approximately 40 μL/bee according to previous research studies, the applied doses referred to *per os* lethal dose 50 (LD50)/10 ("TebuHigh": 8.305 μg/bee/day); LD50/20 ("TebuMedium": 4.156 μg/bee/day) and LD50/40 ("TebuLow": 2.076 μg/bee/day), respectively (Jumarie et al. 2017; Helmer et al., 2015; Rondeau and Raine 2022). The applied treatments can be considered as acute sublethal doses (El Hassani et al., 2005; Desneux et al. 2007; Decourtye et al., 2005). Furthermore, the study design also corresponds to a field-realistic dose of tebuconazole exposure (Rondeau and Raine 2022). Mortality was registered in every 12 h before and during treatment and did not exceed 2% in any of the cages (Table S1).

2.2. Preparation of brain homogenates

Following treatment, cages were placed into dry ice and transported to the laboratory, where bee samples were stored at −80 °C until further process. Individual samples for homogenization and measurements were randomly selected from replicates of treatment groups (n = 10). After gently thawing, the head of honeybees has been removed and dissected under stereomicroscope, on ice. Dissected brain samples included the protocerebrum, antennal lobes, optic lobes as well as the suboesophageal ganglion. Samples were homogenized using Potter-Elvehjem tissue homogenizer device, in Tissue Protein Extraction Reagent (T-PER) supplemented with Pierce Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation on 5000×*g* for 10 min, supernatants were aliquoted and stored at −80 °C until further analysis.

For the analysis of the samples, all reagents and kits were purchased from Merck KGaA (Darmstadt, Germany), except otherwise specified. All colorimetric measurements were carried out using a Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Measurement of redox parameters

2.3.1. Total antioxidant capacity

Determination of TAC was assessed using a commercial colorimetric kit (SKU: MAK187). After the preparation of Trolox standards, 100 μL of homogenate or standard was mixed with 100 μL Cu^{2+} working solution. The plate was incubated for 90 min at room temperature and absorbance values were detected at 570 nm.

2.3.2. Glutathione (total, GSSG, GSH) content

Total glutathione as well as GSSG concentrations were determined also by colorimetric tests (SKU: 38185). Standards and samples were pipetted into clear 96-well plates (40 μL/well), supplemented with the provided buffer solution (120 μL). The plate was incubated at 37 °C for 60 min. Thereafter, 20 μL substrate solution was added to each well, followed by 20 μL coenzyme working solution as well as 20 μL enzyme working solution. In case of GSSG measurement masking solution has been also added to the wells. Absorbance values were measured at 412 nm after a 10 min long 37 °C incubation. GSH concentration was calculated according to the instructions of the manufacturer, using total glutathione and GSSG values.

2.3.3. Malondialdehyde (MDA) content

As a marker of lipid peroxidation processes, MDA concentration has been monitored by a tiobarbituric acid reactive substances (TBARS) based colorimetric test (SKU: MAK085). According to the manufacturer's protocol, 300 μL thiobarbituric acid stock solution was mixed with 100 μL brain tissue homogenate supernatants or standard solutions, following by an incubation at 95 °C for 1 h. After cooling down on ice, absorbance was measured at 532 nm using a transparent 96-well plate.

2.3.4. Glucose-6-phosphate dehydrogenase (G6PDH) activity

For G6PDH assay (SKU: MAK015), homogenized samples were

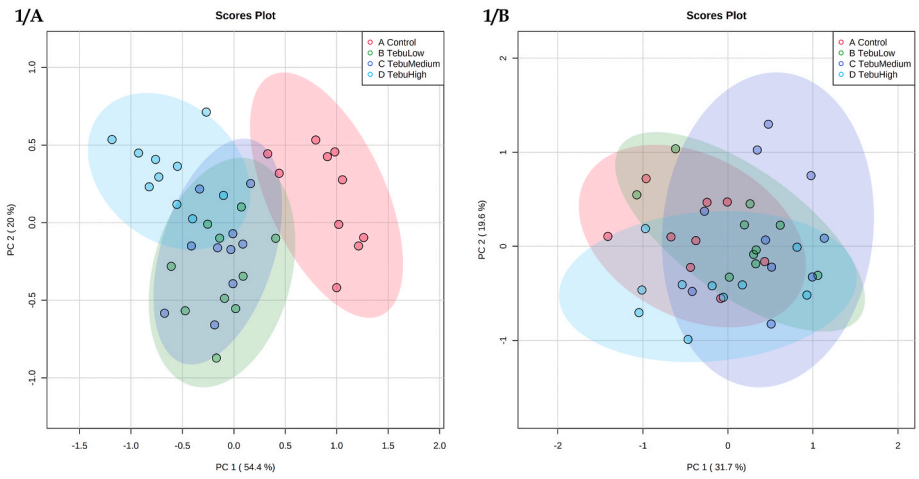

Fig. 1. Principal component analysis (PCA) of the combined data set of redox parameters (Fig. 1/A) and fatty acid profile (Fig. 1/B) in honeybee brains. The 2 major components (PC 1 and PC 2) that accounted for the most variation of the metabolite abundance were used to plot. Each dot in the figure represents a single sample, and different colors indicate the different treatments. "Control" refers to control group with no treatment; "TebuLow", "TebuMedium" and "TebuHigh" refer to 2.076, 4.156 and 8.305 μg/bee/day tebuconazole exposure, respectively.

diluted in assay buffer and preprepared standards and samples were pipetted into clear 96-well plates (50 μL/well). Thereafter, 50 μL master reaction mix was added to all wells (containing 46 μL G6PDH assay buffer, 2 μL G6PDH substrate mix and 2 μL G6PDH developer mix). After 2–3 min incubation at 37 °C, the initial absorbance values were measured at 450 nm and further measurements were taken at 5 min intervals until the absorbance of the most active sample was higher than the highest standard value. The enzyme activity was then determined using the manufacturer's formula.

2.3.5. Superoxide dismutase (SOD) activity

In case of the SOD measurements, samples and blanks were pipetted onto transparent 96-well plates (20 μL/well; SKU: 19160). Subsequently, 200 μL of working solution was pipetted into each well and mixed, then 20 μL enzyme working solution was added to the wells. The absorbance values were read at 450 nm after 20 min incubation at 37 °C and activity values of the samples were calculated according to the formula provided by the manufacturer.

2.3.6. Xanthine oxidase (XO) activity

Measurement of XO enzyme activity happened also with the help of a commercial colorimetric test (SKU: MAK078). 50 μL reaction mix was added to the wells of a transparent microplate, containing 50 μL sample or standard solution. 50 μL reaction mix consisted of 44 μL xanthine oxidase assay buffer, 2 μL substrate mix, 2 μL enzyme mix and 2 μL peroxidase substrate. After 3 min of initial incubation on room temperature, absorbance values were detected at 570 nm in every 5 min until the value of the most active sample was greater than that of the highest standards. XO activity was calculated using the method and equation provided by the manufacturer.

2.3.7. Total protein concentration

To avoid sample preparation-related variances and to ensure equal protein load for all samples, total protein concentration was assayed by Pierce™ Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA; catalog number: 23,225) applying bovine serum albumin as standard solution. After 30 min incubation at 37 °C, absorbance values were detected at 562 nm.

2.4. Measurement of fatty acid concentrations

First, 30 mg of each homogenized sample (10 samples per treatment group; 4 brains per 1 sample) was weighed into individual 100 mL vials followed by the addition of 40 mL of methanol and 6 mL of NaOH (50% w/v). 1.6 mL of nonadecanoic acid (C19:0) was added as an internal standard (500.2 mg/100 mL). The weights of the samples and the internal standard were recorded to the nearest 0.1 mg. The mixture was heated for 1 h at 80 °C. After cooling the solvent was diluted with 50 mL of distilled water and was acidified with 35 mL of HCl (4 M). Chloroform was added (2 × 25 mL) for extraction followed by 0.9% NaCl solution to cause phase separation. The lower phase was then collected and dried with anhydrous Na_2SO_4 and finally was evaporated under nitrogen to dryness. The dry residue was then redissolved in 6 mL of 0.5 M NaOH in methanol and was heated for 5 min at 80 °C. Methyl ester derivatization was performed by adding 6 mL of BF_3-methanol reagent (14%, w/v) and heating at 80 °C for 5 min. Hexane (3 mL) and saturated solution of NaCl (6 mL) were added, and the tube was then vortexed for 1 min and allowed to stand until layer separation was complete (5 min). The top layer was then transferred to gas chromatography (GC) vial for fatty acid analysis by GC-MS.

A Zebron BPX-70 column, 30 m × 0.25 mm x 0.25 μm film thickness, was used (Phenomenex, USA). GC was done with a GCMS-QP2010 SE (Shimadzu Co, Japan). Helium was the carrier gas with a column head pressure of 60.1 KPa. Total flow rate at the split vent was 45.4 mL/min, the flow rate through the column was 1.03 mL/min and the septum purge flow was 3 mL/min. The injector was set at 220 °C using the splitless injection mode, and 1 μL injections were made. The temperature gradient started with a 60 °C initial temperature, a linear increase to 120 °C at 13 °C/min, a slower linear increase to 240 °C at 2 °C/min, and a final 8-min hold in 240 °C. Ion source temperature was 200 °C and interface temperature was set at 240 °C.

2.5. Statistics

Data processing and analysis has been performed using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA). Homogeneity of variance and normal distribution were checked by Levene's test and Shapiro-Wilk test, respectively. Differences between various groups were assessed using one-way analysis of variance (ANOVA) and Dunnett's post hoc tests for pairwise comparisons. Differences between groups were considered significant at $P < 0.05$. Principal component analysis and heatmap were performed with MetaboAnalyst 4.0 (https://www.metaboanalyst.ca). Animal sample number was n = 10/group in all measurements of the study.

3. Results

3.1. Principal component analysis (PCA) and heatmap

For the better overall visualization of the effects of tebuconazole treatment on the redox state and fatty acid profile of honeybee brains, principal component analysis (PCA) has been carried out (Fig. 1/A and

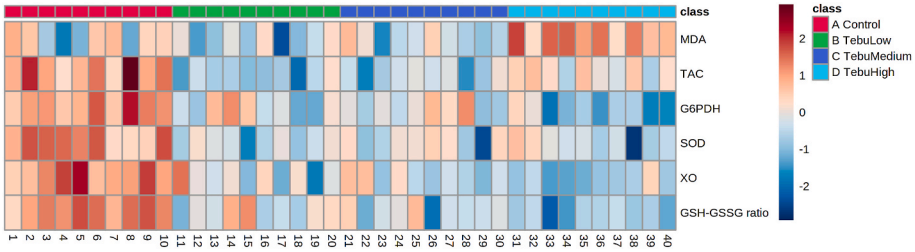

Fig. 2. Heatmap of the measured redox parameters. The heatmap was generated using autoscaled data and used color coding to show relative concentration averages for treatment groups. Samples are presented individually identified by numbers and groups are color-coded according to the treatments. MDA = malondialdehyde; TAC = total antioxidant capacity; G6PDH = glucose-6-phosphate dehydrogenase; SOD = superoxide dismutase; XO = xanthine oxidase; GSH – GSSG ratio = reduced and oxidized glutathione ratio.

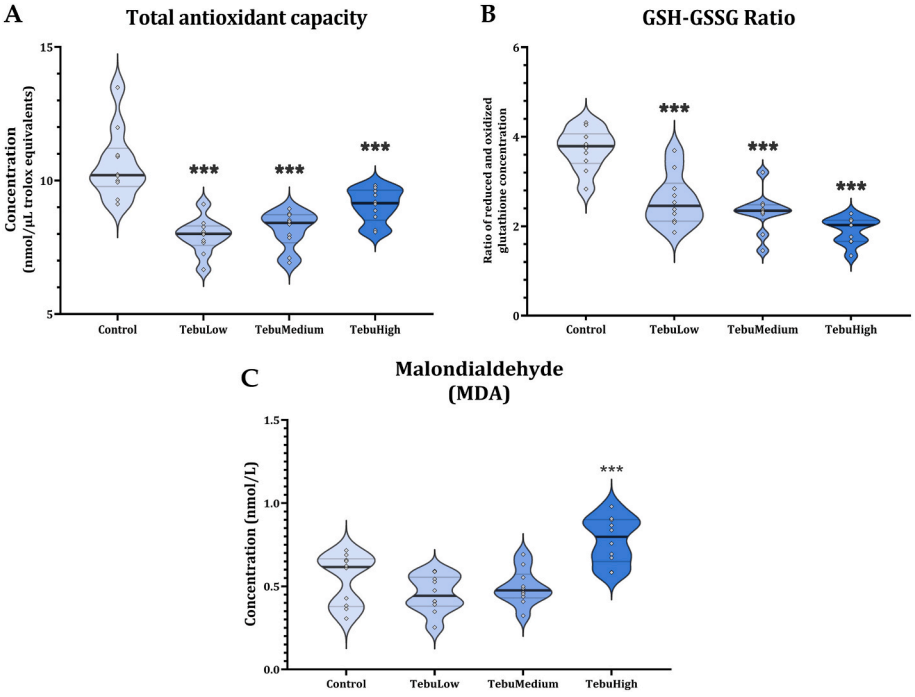

Fig. 3. Redox parameters measured in honeybee brain samples. Total antioxidant capacity, GSH-GSSG ratio, Malondialdehyde concentration: Fig. 3/A, 3/B, 3/C, respectively. Data are visualized using violin plots, where black lines indicate median and grey lines indicate the first (Q1) and third (Q3) quartiles. Individual values are presented as grey squares. "Control" refers to control group with no treatment; "TebuLow", "TebuMedium" and "TebuHigh" refer to 2.076, 4.156 and 8.305 µg/bee/day tebuconazole exposure, respectively. Significant differences between control and tebuconazole exposed groups are indicated with asterisks. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 1/B, respectively). PCA analysis of the redox parameters revealed that the first two components detailed around 64.4% of the total variation in the experimental data (PC1: 54.4%, PC2: 20%). Regarding fatty acid composition, 51.3% of the total variation can be described by PC1 and PC2 (31.7% and 19.5%, respectively). The results suggest that tebuconazole may have an intense dose-dependent impact on the measured redox parameters, while no characteristic treatment-associated pattern was observed in relationship with the fatty acid content of the samples. Redox homeostasis-related data is also presented using a heatmap for the better visualization and overview of the single samples (Fig. 2).

3.2. Redox parameters

Detailed results regarding all measured redox parameters are presented in Table S1/a.

Based on our findings, TAC was significantly decreased after all of "TebuLow", "TebuMedium" and "TebuHigh" treatments compared to the "Control" (P < 0.001; Fig. 3/A). Intense decrease of GSH – GSSG ratio has been observed in "TebuLow", "TebuMedium" and "TebuHigh" groups following treatment period (P < 0.001; Fig. 3/B). MDA concentration was found to be significantly higher (P < 0.001) in the "TebuHigh" group compared to that of the controls, while no significant effect was observed in the "TebuLow" and "TebuMedium" groups (Fig. 3/C).

Regarding G6PDH activity, remarkable, significant decrease has been observed as the effect of tebuconazole treatment in all the exposed groups in comparison with the control animals (P < 0.001; Fig. 4/A). Similarly to G6PDH, significant decrease has been detected concerning SOD activity in the "TebuLow", "TebuMedium" and "TebuHigh" groups compared to control (P < 0.001; Fig. 4/B). Significant decrease of XO activity was described in all the three treated groups compared to the control (P < 0.001; Fig. 4/C).

3.3. Fatty acid composition

Tebuconazole treatment resulted in increased concentration of lauric acid (C12:0) in honeybee brain in the "TebuLow" and "TebuMedium" groups (P = 0.045 and P < 0.001, respectively, Table 1.). "TebuMedium" treatment also caused significant increase of myristic acid (C14:0), pentadecylic acid (C15:0) and palmitic acid (C16:0) concentrations (P = 0.005, P = 0.040 and P = 0.001, respectively). Oleic acid (C18:1; n-9), alpha-linolenic acid (C18:3; n-3), total fatty acid, polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA) concentration increased following "TebuHigh" treatment (P = 0.005, P = 0.033, P = 0.005, P = 0.029 and P = 0.006, respectively). Saturated fatty acid (SFA) content was increased as the result of "TebuMedium" exposure (P = 0.034). Results are summarized and presented in Table 1 and Table S1/b.

4. Discussion

According to numerous studies, oxidative stress is one of the most important pesticide-related molecular consequences in various organs of

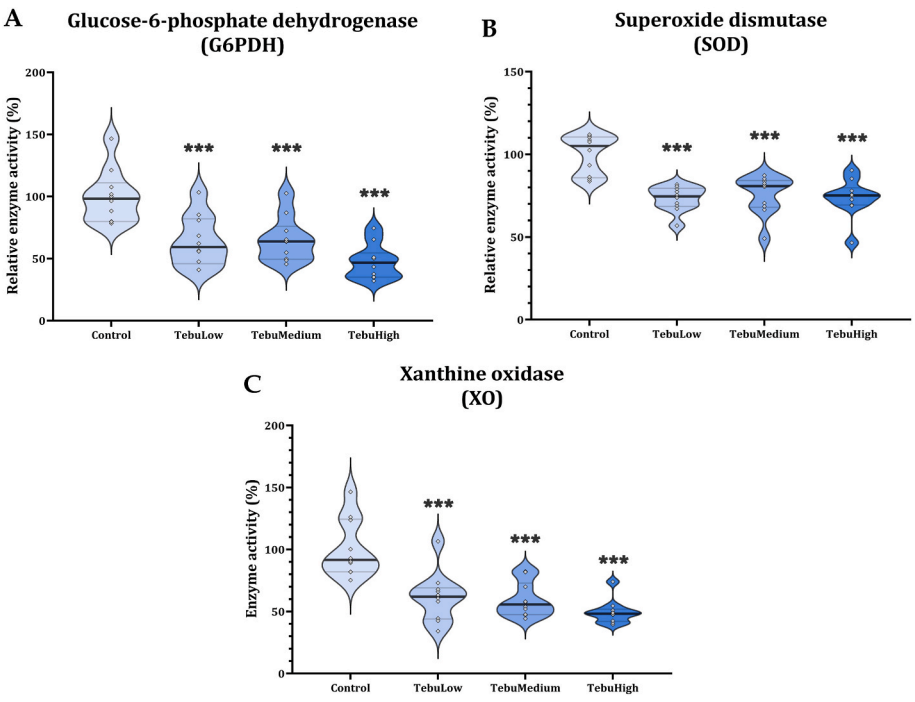

Fig. 4. Redox enzyme activities measured in honeybee brain samples. Glucose-6-phosphate dehydrogenase, Superoxide dismutase, Xanthine oxidase: Fig. 4/A, 4/B, 4/C, respectively. Data are visualized using violin plots, where black lines indicate median and grey lines indicate the first (Q1) and third (Q3) quartiles. Individual values are presented as grey squares. "Control" refers to control group with no treatment; "TebuLow", "TebuMedium" and "TebuHigh" refer to 2.076, 4.156 and 8.305 μg/bee/day tebuconazole exposure, respectively. Significant differences between control and tebuconazole exposed groups are indicated with asterisks. *P < 0.05, **P < 0.01, ***P < 0.001.

non-target species (Mostafalou and Abdollahi 2013; Nesnow et al., 2011; Souders et al., 2019; J.-D. Yang et al., 2018; Dussaubat et al., 2016; Chakrabarti et al., 2015). Due to their excessive use in agriculture and medicine, triazole fungicides have been released in massive amount into the environment (Rondeau and Raine 2022). Although tebuconazole is considered as a relatively low-acute toxic molecule, it may have detrimental effects on pollinator insects including honeybees (Raimets et al., 2022). Besides acute toxicity, tebuconazole can also persist in the environment due to its high stability and relatively low biodegradability, delivering increased ecotoxicological risk (Vieira et al. 2022). Previous studies in various animal species have reported the possible occurrence of developmental toxicity (Kumar et al., 2019), respiratory and osmoregulatory disorders (Macirella et al. 2019), endocrine disruption (S. Li et al., 2019; Yu et al., 2013), alterations in the intermediary and energy metabolism (Sancho et al., 2010) as the effect of tebuconazole exposure. Intense oxidative stress, apoptotic changes and neurotoxicity as important consequences of triazole exposure have been also described (Bruno et al., 2009; S. Li et al., 2020; Perez-Rodriguez et al., 2019; Valadas et al., 2019). Tebuconazole can also been linked to CCD of honeybee colonies. CCD is a phenomenon in which adult honeybee forager workers abandon their hive in high numbers and do not return, leaving the queen, a smaller amount of nurse workers and the brood alone, often resulting in the colony's death (Lu et al., 2020; Watson and Anthony Stallins, 2016). The exact cause of CCD is unknown, but it is thought to be caused by the combination of factors including pesticides, pathogens, and poor nutrition (Cornman et al., 2012; Grassl et al., 2018). Furthermore, besides resulting in intense oxidative stress, tebuconazole has been also shown to interfere with honeybee cognitive abilities, causing changes in behavior and impairing their ability to find food and navigate back to the hive, which can affect normal colony functions negatively (Jaffe et al. 2019; Mao et al. 2017). However, the background and pathogenesis of these observations are

Table 1
Fatty acid profile of honeybee brains. "Control" refers to control group with no treatment; "TebuLow", "TebuMedium" and "TebuHigh" refer to 2.076, 4.156 and 8.305 μg/bee/day tebuconazole exposure, respectively. Data is expressed as mean ± standard error of mean (SEM), as μg fatty acid/g sample. Significant differences between experimental groups (control vs. treated groups) are indicated with asterisks. *P < 0.05.; **P < 0.01.; ***P < 0.001.

	Control Mean ± SEM	TebuLow Mean ± SEM	TebuMedium Mean ± SEM	TebuHigh Mean ± SEM
C8:0	14.42 ± 2.96	18.72 ± 3.22	15.89 ± 1.85	17.35 ± 2.02
C10:0	13.04 ± 1.40	13.31 ± 1.24	15.85 ± 1.08	14.99 ± 1.30
C12:0	26.72 ± 2.15	34.82* ± 1.20	40.24*** ± 3.13	24.25 ± 2.12
C14:0	118.04 ± 5.05	140.10 ± 5.87	154.37** ± 10.57	115.28 ± 6.78
C15:0	58.14 ± 3.52	73.23 ± 4.88	79.46* ± 7.89	56.91 ± 5.77
C16:0	1215.28 ± 50.29	1374.13 ± 30.82	1471.73** ± 40.24	1338.56 ± 58.99
C16:1; n-7	104.80 ± 6.71	112.26 ± 11.39	128.60 ± 6.81	98.82 ± 6.73
C17:0	50.93 ± 1.98	54.67 ± 1.50	55.27 ± 2.04	48.76 ± 2.52
C18:0	1979.33 ± 61.89	2,030.66 ± 41.00	2,049.34 ± 46.91	2,154.78 ± 86.73
C18:1; n-9	7,730.95 ± 471.29	7,846.12 ± 315.63	8,845.31 ± 612.40	10,103.90** ± 519.94
C18:2; n-6	511.57 ± 22.92	524.73 ± 23.57	591.44 ± 25.33	598.553 ± 29.56
C18:3; n-3	2,685.28 ± 98.64	2,711.31 ± 6.49	2,941.07 ± 96.33	3,062.92* ± 115.48
C20:0	87.04 ± 4.24	90.17 ± 4.01	82.08 ± 3.50	97.60 ± 4.62
C22:0	41.63 ± 1.86	42.98 ± 3.80	41.33 ± 2.54	36.55 ± 1.77
C24:0	16.68 ± 1.31	18.18 ± 1.28	21.76 ± 2.42	18.81 ± 1.16
Total fatty acid	14,653.33 ± 657.96	15,086.00 ± 376.57	16,534.00 ± 706.35	17,787.00** ± 790.25
Polyunsaturated fatty acids (PUFA)	3,196.85 ± 116.33	3,236.05 ± 101.91	3,532.51 ± 116.14	3,661.47* ± 143.51
Monounsaturated fatty acids (MUFA)	7,835.75 ± 472.99	7,958.38 ± 316.68	8,973.915 ± 615.33	10,202.76** ± 519.52
Saturated fatty acids (SFA)	3,621.24 ± 108.41	3,890.96 ± 56.89	4,027.32* ± 75.07	3,923.85 ± 161.71

not yet fully understood (Jaffe et al. 2019; Mao et al. 2017). Furthermore, azoles have been shown to cause intense oxidative stress in various species, resulting in DNA, protein, and lipid damage, followed by intense negative metabolic consequences and cell death (Ku et al., 2021; Othmène et al., 2020a; Valadas et al., 2019).

According to our hypothesis, since impairment of the antioxidant defense system contributes to the development of numerous neurodegenerative disorders, redox imbalance and oxidative stress-related negative consequences may be factors of crucial importance in the background of neurotoxicity and cognitive impairment observed by the abovementioned research groups in tebuconazole-exposed bees. Therefore, it is vital to understand whether tebuconazole may have a negative impact on the redox homeostasis of honeybees, possibly contributing to the development of further pathological conditions. It is also important to mention, that in our present study we aimed to test the effects of oral tebuconazole treatment. On one hand, studying *per os* exposure is highly relevant since tebuconazole can be present in the pollen and nectar for a longer period of time even, if spraying was carried out in strict accordance with the current recommendations and regulations. On the other hand, according to most national laws and regulations, the chemical can be legally sprayed during daytime even in case of blooming if it is applied solely and not in combination with neonicotinoids. In this latter case there is a high risk of contact exposure which may result in different effects compared to oral uptake. Although our experiment was designed to monitor the effects of oral exposure, it is also of high importance to carry out further field-realistic experiments in the future about topical effects of tebuconazole, monitoring also the impact of direct spraying.

TAC was significantly diminished by the effect of tebuconazole in every concentration. These findings can be in strong correlation with the intense depletion of the glutathione defense system along with other molecular consequences caused by harmful oxidative damage. Our results are further supported by the fact that similar decrease in TAC has been described in other animal species, such as rat liver, following treatment with tebuconazole (Coremen et al., 2022).

In line with our TAC-related findings, an intense, significant decrease of GSH – GSSG ratio has been observed in the brain of bees following every tebuconazole treatment. The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) serves as one of the most important endogenous antioxidant and xenobiotic detoxifier molecules involved also in the metabolic regulation of the cells. The depletion of the glutathione system, as reflected by the diminished GSH – GSSG ratio, can be indicative of increased oxidative stress (Minich and Brown 2019). With its intense oxygen consumption and rich PUFA content, the brain is especially exposed to oxidative stress (Miceli et al. 2020). Therefore, acting as a free radical scavenger and inhibitor of lipid peroxidation, protein carbonylation and DNA damage, glutathione is of key importance for protecting the central nervous system from free radicals and oxidative stress (Mett and Müller 2021).

According to other studies, tebuconazole, together with further azole fungicides, induced oxidative stress in various animal species. Consequently, it decreased hepatic GSH concentration and increased the activity of major antioxidant enzymes such as glutathione peroxidase (GPx) in rats (Coremen et al., 2022; J.-D. Yang et al., 2018), along with similar effects on kidney and heart (Ben et al., 2020; Othmène et al., 2020b). This effect of azoles was also observed in the liver, gill and muscle of rainbow trout (*Oncorhynchus mykiss*) following propiconazole exposure (Z.-H. Li et al., 2010), in line with mouse Sertoli cells *in vitro* as the effect of ketoconazole, miconazole and prochloraz treatment (Petricca et al., 2022). In these studies, GSH content was significantly decreased and GSSG increased, while glutathione reductase (GR), glutathione S-transferase (GST) and GPx enzyme activities were reported to be also intensely modified.

G6PDH is the primary limiting factor and key enzyme of the pentose phosphate pathway (PPP). It regulates the conversion of glucose-6-phosphate to 6-phosphogluconolactone, which molecule is metabolized in further steps of the cycle whilst NADPH is being produced (Tian et al., 1998). Reduced NADP as an important hydrogen donor can be utilized in various reactions including the regeneration of oxidized glutathione or other molecules of antioxidant character (H.-C. Yang et al., 2019). For this reason, slowdown of PPP observed in our study, has an indirect negative impact on the fight against oxidative attack as well as on the maintenance of physiological redox balance. On the other hand, contradictory observations were described in other animal species regarding the effects of azole fungicides on PPP. For instance, triazole type penconazole treatment increased the activity of G6PDH in goldfish (*Carassius auratus)*, while benzimidazole carbendazim intensely inhibited its function in mouse Leydig cells *in vitro* (Husak et al., 2017; Rajeswary et al., 2007). Although the appropriate function of PPP also seems to be a crucial question, there was yet no data available about the possible effects of tebuconazole in the literature regarding the modulation of PPP according to our knowledge.

We observed similar inhibiting effects of tebuconazole regarding the activity of SOD, following every treatment. SOD is an enzyme that plays a crucial role in the protection of cells from oxidative stress. It functions by converting superoxide, a highly reactive form of oxygen, into hydrogen peroxide, which can then be further broken down into water by other enzymes such as catalase (Yan and Spaulding 2020). Dysregulation of SOD activity has been linked to a variety of cellular disorders and diseases (Saxena et al., 2022). Considering the observed inhibition of SOD activity by tebuconazole treatment, such effects may have negative impact on cellular health and may potentially contribute to the emergence of oxidative damage-related disturbances. The same pattern was described in other studies involving *Cyprinus carpio* and *Oncorhynchus mykiss* fish, where tebuconazole and propiconazole treatment induced the decrement of SOD activity (Z.-H. Li et al., 2010; Toni et al., 2011). On the other hand, since increased SOD activity was observed following azole exposure in other studies, these alterations seem to be dependent on the concentration, time of exposure and the mode of application or the type of the azole fungicide itself (Chang et al., 2020; S. Li et al., 2020; Valadas et al., 2019; J.-D. Yang et al., 2018).

Interestingly, tebuconazole treatment also resulted in the inhibition of the prooxidant XO enzyme. XO catalyzes the conversion of xanthine to uric acid, a product of purine degradation. Meanwhile, since it is an aerobic dehydrogenase, it utilizes oxygen and generates hydrogen peroxide as a byproduct during the reaction (Mehmood et al., 2019). For this reason, taking only the observed inhibitory effect of tebuconazole into account, it could be deemed as a positive outcome of the treatment; however, it is important to note that the relationship between xanthine oxidase and oxidative stress is complex and not fully understood (Furuhashi 2020). Furthermore, in bees and some other insects, uric acid is stored in urocytes of the fat body and serves as a source of energy as well as N-storage to produce N-containing molecules such as amino acids. Therefore, altered production of uric acid can be also of detrimental consequences in insects, but more studies involving further organs and methods are necessary to answer this question (Goh et al., 2013; Weihrauch and O'Donnell, 2021).

According to our results, tebuconazole exposure increased MDA concentration in the brain; however, only in animals exposed to the highest dose of the chemical. The increased level of lipid peroxidation following tebuconazole treatment has been observed by previous studies, although it has been first described in the central nervous system of honeybees in this research. Similar increment has been reported in the liver of rats (Chang et al., 2020; Coremen et al., 2022), mice (Ku et al., 2021), zebrafish (Chang et al., 2020) and *Rhamdia quelen* (Ferreira et al., 2010). In addition, tebuconazole resulted in intense lipid peroxidation in other organs as well, such as in rat kidney (Othmène et al., 2020a), heart (Othmène et al., 2020b), and in the whole body of common carp (Toni et al., 2011). In line with the abovementioned results, tebuconazole-triggered intense oxidative stress may result in mitochondria-mediated apoptosis, being also in connection with the intense lipid peroxidation and elevated MDA levels (Ben et al., 2020). On the other hand, no significant lipid peroxidation was observed in

earthworm *Eisenia fetida* following chronic tebuconazole exposure, while MDA levels significantly increased after short-term treatment (Chen et al., 2018; Zhang et al. 2020). Chronic oral tebuconazole administration also resulted in no MDA elevation in the liver, testis and kidney of rats, while it intensely induced hepatic CYP1A1/2, CYP2B1/2, CYP2E1, and CYP3A enzyme activity (J.-D. Yang et al., 2018). As it was described by various research groups, azole fungicides may act both as inducers as well as inhibitors of CYP enzymes involved in the metabolism of xenobiotics (Knebel et al., 2019; Mao et al. 2011). Since CYP enzymes produce hydrogen peroxide while catalyzing xenobiotic biotransformation reactions, their altered activity may have also a direct or indirect effect on the redox homeostasis of the animals; however, the investigation of this question was not in the scope of our present study (Qi et al., 2013; Veith and Moorthy 2018).

Recent research highlights the importance of lipid and fatty acid homeostasis in maintaining healthy neuronal function along with synaptic plasticity (Montesinos et al. 2020). Therefore, since behavioral changes are described to be an important factor in the development of CCD as mentioned above, and azole fungicides presumably also affect lipid and fatty acid metabolism already in sublethal concentrations, the extensive research of the altered fatty acid profile following tebuconazole exposure is of high importance also in honeybees.

Tebuconazole treatment resulted in increased concentration of lauric acid (C12:0) in honeybee brain in the "TebuLow" and "TebuMedium" groups. This alteration may be in strong connection with tebuconazole-induced cellular defensive mechanisms. Lauric acid has alleviating effects in case of increased ROS and proinflammatory cytokine production (Nishimura et al., 2018). Therefore, these alterations can be also important as the part of the activated antioxidant defense system (Alves et al., 2017; Henry et al., 2002). Lauric acid concentration of the central nervous system is also relevant because it has a positive effect on GPR40-dependent microglial activation and the subsequent neuronal damage in individuals suffering from neurodegenerative diseases such as Alzheimer's (Nishimura et al., 2018). On the other hand, increased amount of lauric acid in the brain is not considered to be always beneficial, since according to some studies it resulted in lower mitochondrial volume, increased oxidative stress-associated DNA damage and atrophy in myocardial cells (Miyagawa et al., 2018).

Following "TebuMedium" treatment, similar change has been observed in the concentration of myristic acid (C14:0). Along with the abovementioned lauric acid, myristic acid is also an important molecule in the scope of neurodegenerative disorder research (Jasbi et al., 2021). These medium-chain fatty acids are considered to have mostly positive effects on brain health and cognitive functions; however, it cannot be excluded that their excessive storage can also have some negative consequences like mentioned above. According to our results it can be also hypothesized that acute tebuconazole-induced free radical production evokes the production and/or storage of medium-chain fatty acids in the brain, facilitating the stabilization of the redox homeostasis under these oxidative conditions.

Tebuconazole treatment caused also higher alpha linolenic acid (C18:3; n-3; ALA) concentrations in the brain of honeybees. ALA as an essential fatty acid is the precursor molecule of longer chain omega-3 fatty acids such as eicosapentaenoic acid (C20:5; n-3; EPA) and docosahexaenoic acid (C22:6; n-3; DHA) (Burdge 2006). For this metabolic process, the role of elongase and Δ5,6-desaturase enzymes is necessary (Barceló-Coblijn and Murphy, 2009). Since the inhibitory action of azole fungicides on desaturase activity of *Candida* cells has been already widely discussed, these agents could have a negative impact on these enzymes also in case of non-target species (Morschhäuser 2016; Vanden Bossche 1997). This suggested desaturase enzyme inhibition of honeybees may presumably cause the slower transformation of ALA into longer chain PUFAs such as EPA and DHA, which can consequently lead to the accumulation of ALA because of its less effective utilization. However, further studies are necessary to underline this hypothesis. Although there are limited and controversial data available, on one hand, ALA is considered to be a beneficial molecule regarding its neuroprotective, anti-inflammatory and antioxidative effects (Reifen et al., 2015). On the other hand, its toxicological hazards have been also highlighted in some studies, which showed that in elevated concentrations it can induce negative metabolic alterations as well as tumorigenic transformation and regressive effects such as macular degeneration (Kim et al., 2014).

Oleic acid (C18:1; n-9) concentration showed a similar pattern compared to ALA, which observation can be also in strong correlation with decreased Δ5,6-desaturase (Park et al., 2018). Since increased oleic acid content is described to cause caspase-3-independent apoptosis by the dephosphorylation of Bad (Bcl2-associated death promoter), it may be a further detrimental outcome of azole exposure (Zhu et al., 2005). In correspondence with this finding, higher oleic acid levels of the brain strongly correlated with the occurrence and the severity of Alzheimer's or Parkinson's disease as well as with dementia (Fabelo et al., 2011; Fraser et al. 2010; Howe et al., 2022; Wang et al., 2014). Tebuconazole-induced higher ALA and oleic acid concentrations have also a significant effect on cellular and mitochondrial membrane fluidity (Dabadie et al., 2006; Zhao et al., 2016), associated with drastic negative changes in several neurodegenerative diseases of other species such as Alzheimer's, Huntington's disease or schizophrenia (Clement et al. 2010; Eckert et al., 2011; Eckmann et al., 2014).

According to our results, tebuconazole resulted in increased concentrations of long chain saturated fatty acids such as pentadecanoic acid (C15:0), palmitic acid (C16:0) and total saturated fatty acids (SFA) following "TebuMedium" treatment. Our findings support the theory that azole type fungicide agents may affect the physiological fatty acid metabolism, which effect was already observed in other organs such as in human liver (Ku et al., 2021). Increased amount of SFAs can result in endoplasmic reticulum stress and mitochondrial dysfunction of the neuronal cells, which leads to significantly higher risk of disturbed cognitive functions (Fraser et al. 2010; Julien et al., 2006; Patil et al., 2008; Sánchez-Alegría and Clorinda,). Further, exposure to tebuconazole in the highest concentration led to the intense increase of total fatty acid content, which may also negatively affect the cognitive performance and neuronal functions and can be the result of azole-induced degenerative consequences (Altenhofen et al., 2017; Fonteh et al., 2014; Moser et al., 2001).

Rapidly increasing pesticide exposure means an emerging ecotoxicological issue in all over the world, including pollinator insects like honeybees. Therefore, it is critical to investigate the possible negative effects of pesticides as they can contribute to the development of CCD suggested by various research groups. In conclusion, the effects of field-realistic tebuconazole concentration on the redox balance and fatty acid profile in the brain of honeybees were investigated in the present study. In the present study, we successfully described the negative effects of tebuconazole exposure on total antioxidant capacity and glutathione metabolism along with declined G6PDH, SOD and XO enzyme activities and increased lipid peroxidation. Furthermore, intense tebuconazole-associated alterations were observed in the fatty acid profile of the brain of honeybees. Our findings were also in line with other studies investigating the development and progression of neurodegenerative disorders, suggesting similarities between these pathological conditions, and highlighting the danger of acute sublethal tebuconazole exposure in honeybee brain, since severe oxidative stress caused by this agent can also result in alterations of fatty acid profile and lead to possible brain damage. It is; however, also worth noting that tebuconazole is often sprayed out together with other pesticides, such as neonicotinoids in tank mixtures, which is suggested to pose even greater risk to honeybee colonies, because of CYP450 inhibition and altered xenobiotic biotransformation related synergistic effects. Although research of these combined effects was not the aim of our present work, the study of this field is also necessary in the future in order to gain better overview about the possible risk connected to the application of modern pesticides.

Author contributions

Máté Mackei: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Project administration, Writing original draft, Writing review and editing, Software, Visualization, Supervision, Validation; Csilla Sebők: Formal analysis, Investigation, Validation; Júlia Vörösházi: Investigation, Validation; Patrik Tráj: Investigation, Validation, Methodology; Fruzsina Mackei: Investigation, Formal analysis; Barnabás Oláh: Conceptualization, Formal analysis, Investigation; Hedvig Fébel: Methodology, Investigation, Funding acquisition, Resources, Data curation, Validation; Zsuzsanna Neogrády: Conceptualization, Methodology, Resources, Writing review and editing, Supervision; Gábor Mátis: Conceptualization, Methodology, Investigation, Funding acquisition, Resources, Writing - Original Draft, Software, Visualization, Supervision.

All authors have read and agreed to the present version of the manuscript.

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Declaration of competing interest

The authors declare no competing interests.

Data availability

All raw datasets of the study are available from the corresponding author upon reasonable request as well as shared on Figshare with the following DOI number: 10.6084/m9.figshare.22293307.

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Appendix A. Supplementary data

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