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Unraveling the acute sublethal effects of acetamiprid on honey bee neurological redox equilibrium

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Understanding the off-target effects of neonicotinoid insecticides, including acetamiprid, which is the most commonly applied agricultural chemical, is crucial as it may be an important factor of negative impact on pollinator insects causing a number of problems such as colony collapse disorder (CCD) of honey bees. While CCD is known as a multifactorial disease, the role of pesticides in this context is not negligible. Therefore, it is essential to gain a deeper comprehension of the mechanisms through which they function. The aim of this research was to study the effects of sublethal acetamiprid doses on honey bees, specifically focusing on the redox homeostasis of the brain. According to our findings, it can be confirmed that acetamiprid detrimentally impacts the redox balance of the brain increasing hydrogen peroxide and malondialdehyde levels, suggesting consequential lipid peroxidation and membrane damage as consequences. Moreover, acetamiprid had negative effects on the glutathione system and total antioxidant capacity, as well as key enzymes involved in the maintenance of redox homeostasis. In summary, it can be concluded that acetamiprid adversely affected the redox balance of the central nervous system of honey bees in our study. Our findings could potentially contribute to a better understanding of pesticide-related consequences and to improvement of bee health.

Keywords Apis mellifera, Neonicotinoids, Oxidative stress, Glutathione, Malondialdehyde, Colony collapse disorder

One of the most significant pollinator species globally, the western honey bee (*Apis mellifera*) is economically important and is in charge of pollinating 87.5% of flowering plants[1]. A number of factors have contributed to the decline in honey bee populations and other pollinating insect species in recent decades[2]. These factors include habitat degradation and shrinkage, crop monoculture, excessive pesticide use, the presence of pathogens and parasites, inadequate forage nutrient content, and the detrimental impacts of climate change[3]. Subsequently in the 2000s, honey bee colonies have been affected by CCD, a serious issue that was initially identified in the United States and then identified in Europe a few years later. The disorder has subsequently expanded throughout the world[4]. Since then, a significant amount of research has been performed regarding the topic, leading to the conclusion that several factors must be taken into account rather than just one[5]. These include parasitic infections, pathogenic bacteria or viruses, pesticide exposure, temperature fluctuations, crowded living arrangements, a lack of pollen and nectar, modification of the sensitive bee microbiome as a result of inadequate foraging, and scarce or contaminated water sources[6]. Regarding parasites, not all infestations have been reported in the impacted colonies; nonetheless, it is important to draw attention to the infection with the *Varroa destructor* mite and the unicellular parasite *Nosema ceranae*, which are suggested to be predisposing factors[7].

Understanding the etiology and causes of CCD requires research on the effects of certain insecticides such as neonicotinoids. Pest control has become increasingly important recently as agriculture has grown quickly. Imidacloprid, the first compound in the neonicotinoid family, was developed as a result of extensive research into safer pesticides during the 1980s[8]. Acute lethal effects of neonicotinoids have already been described in mammals in case of severe overdoses, although its mechanism of action is highly selective for insects, acting on the nicotinic acetylcholine receptors in their central nervous systems[9]. Registered for crops and livestock in 2002, acetamiprid is a more recent member of the chemical family with superior efficacy and a more favorable toxicity profile, making it safer to use than other compounds in the this group[10]. Despite not being a target animal species, honey bees can exhibit severe symptoms when exposed to sublethal concentrations of some

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chemicals[11]. These can include incoordination, distorted olfactory memory, altered flight capability, foraging, and navigation[12]. Behavioural impacts are often not the direct cause of bee deaths, but over time, these negative consequences can accumulate and make colonies more susceptible to other stressors[13]. This is one of the factors presumed to lead to CCD. Additionally, there is a major concern within the One Health concept, that these chemicals may accumulate in the food chain, potentially posing risks to humans[14].

Neonicotinoids exert their negative effects mostly through DNA damage, apoptosis, and mitochondrial dysfunction. Since their major targets are mitochondria, they can have severe effects on health and cognitive functions through this pathway. Neonicotinoids are able to disrupt mitochondrial Ca^{2+} homeostasis, limit mitochondrial respiration, and induce the generation of reactive oxygen species (ROS)[15,16]. Presumably, these changes in redox homeostasis of the central nervous system may lead to the appearance of behavioural modifications and contribute to the emergence of CCD.

Accordingly, numerous studies conducted on species other than honey bees have demonstrated that intense exposure to neonicotinoids can result in elevated oxidative stress; nevertheless, more research on bees is required to elucidate this matter[10,17–21]. Based on definition, oxidative stress occurs in cases of overproduction or accumulation of pro-oxidant substances as well as reactive molecules or if the concentration of available antioxidant compounds decreases[22]. Several studies have demonstrated that neonicotinoid insecticides deplete both enzymatic and non-enzymatic antioxidant mechanisms due to oxidative distress in addition to their effects on target receptors[23–25]. Therefore, when evaluating their effects on the honey bees, it is crucial to fully understand the alterations in the status of the antioxidant defense system, as they were reported to cause lipid peroxidation, oxidative stress-related DNA as well as protein damage in a number of species[20,21,24].

As the above processes are not well understood in bees, but may clearly underlie the adverse effects of neonicotinoids, our main objective was to investigate how acute sublethal per os acetamiprid exposure may affect brain redox homeostasis in order to better understand the biochemical background of these effects.

Materials and methods

Collection and treatment of honey bees

The honey bees involved in the study originated from a single healthy *Apis mellifera carnica* colony of a registered apiary from Veszprém county, Hungary. To ensure reliability and representativeness, the hive was not given any medication or other chemical products for 3 months prior to treatment. The overall health status of the bees was also monitored during this period, and no clinical signs of detectable disease were observed. The selection, collection, and subsequent treatment of the workers involved in the experiment were carried out randomly.

The collection followed the procedure previously described earlier by Williams et al.[26]. Sampling took place in the early morning hours from honey frames containing no brood, to optimally model acetamiprid exposure in foraging bees within field-realistic conditions. Honey bees have been randomly separated into 4 different treatment cages of size 30 cm × 20 cm; 3 replicate cages per group, each containing 50 individuals. This was followed by a 36-h acclimatization phase, during which the bees were only provided with water and 50% sucrose solution ad libitum. After this period, the feeding solutions were supplemented with acetamiprid (Merck KGaA, Darmstadt, Germany, product number: 33674, purity: ≥ 98.0%) in three different concentrations (AcetHigh, AcetMedium, AcetLow), along with a control group. Treatments were carried out at 25 ± 2 °C and 50–65% relative humidity (RH) and lasted for 48 h, during which time the feeding solutions were replaced with fresh ones every 8 h.

Treatment concentrations of acetamiprid were determined based on the results of previous studies[27,28], considering the average daily feed intake of bees, which is approximately 40 µL/bee[29]. The amount of active ingredient in each group was referenced to *per os* lethal dose 50 (LD50) /10 (AcetHigh: 35 mg/L feeding solution), LD50/20 (AcetMedium: 17.5 mg/L feeding solution), and LD50/40 (AcetLow: 8.75 mg/L feeding solution), referring to 8.305, 4.156 and 2.076 µg/bee/day acetamiprid exposure, respectively 50 mg of pure acetamiprid powder was dissolved in 1 mL of acetone, after which 142 µL of this acetamiprid stock solution was added to 200 mL of sugar syrup to achieve the concentrations described in the manuscript. This concentration represents the most concentrated feeding solution (LD50/10), from which further dilutions were prepared to produce the more diluted treatment solutions (LD50/20, LD50/40). Aliquots of each solution were prepared, stored at -20 °C and only thawed and used freshly when the feeding solution was replaced. In previous experiments this model was proved to be suitable for detailed studies of the acute, sublethal effects of acetamiprid[30,31]. Every 12 h before and during treatment, deaths were recorded; none of the cages had a mortality rate greater than 2%. At the end of the treatments, the bees were euthanized by placing the cages into dry ice and transported to the laboratory. The specimens were then stored at -80 °C until sample processing.

Preparation of brain homogenates

After careful thawing, the heads of the specimens were removed, and further dissection was conducted under a stereomicroscope. The dissected brain parts included the protocerebrum, the antenna lobe, the optic lobe, and the subesophageal ganglion. Samples from each group (10 individual brain sample/group) were homogenized using a Potter–Elvehjem homogenizer in 1000 µL Tissue Protein Extraction Reagent (T-PER) solution supplemented with Pierce 1% Protease Inhibitor Cocktail Solution (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the samples were centrifuged at 5000xg for 10 min, and the aliquoted supernatants were stored at -80 °C until further measurements.

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

Laboratory analysis

All colorimetric and fluorimetric measurements mentioned below were measured in triplicates on the plates, the average of which was taken as the result for the statistics.

Xanthine oxidase (XO) activity

XO enzyme activity was measured using a commercial colorimetric test (cat. number: MAK078). Wells of a transparent microplate were filled with 50 µL of reaction mix and 50 µL of either the standard solution or the sample. The reaction mix included xanthine oxidase assay buffer, peroxidase substrate, enzyme mix, and substrate mix. After a 3-min initial incubation period at room temperature, absorbance values were measured at 570 nm every five minutes until the most active sample's value exceeded the highest standard. The XO activity was calculated using the manufacturer's equation.

Hydrogen peroxide (H_2O_2) concentration

H_2O_2 concentration was determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, Waltham, USA; cat. number: A22188). 50 µL of tissue homogenate was added to 50 µL of Amplex Red solution in a 96-well plate. After a 30-min incubation at room temperature protected from light, the concentration of fluorescent resorufin was measured using a Victor X2 2030 microplate reader.

Malondialdehyde (MDA) concentration

A colorimetric test based on thiobarbituric acid reactive substances (TBARS) was employed to determine MDA concentration as a marker of lipid peroxidation processes (cat. number: MAK085). Thiobarbituric acid stock solution (300 µL) was mixed with 100 µL of brain tissue homogenate supernatants or standard solutions, then incubated for 1 h at 95 °C. After cooling on ice, absorbance was measured at 532 nm.

Protein carbonyl (PC) concentration

The concentration of PC was measured by Sandwich ELISA method (Mybiosource Inc., San Diego, CA, USA; cat. number: MBS2600294). The standards or samples were added to PC monoclonal antibodies coated wells. After various incubation times and washes, absorbance values were read at 450 nm.

8-Hydroxy-2'-deoxyguanosine (8-OHdG) concentration

Competitive ELISA detection technique was used for measuring 8-hydroxy-2'-deoxyguanosine concentrations using a pre-coated microtiter plate (Mybiosource Inc., San Diego, CA, USA; cat. number: MBS764814). After incubation and addition of various solutions, the OD of the samples and the standard curve were compared to ascertain the concentration of 8-OHdG in the samples.

Total antioxidant capacity (TAC)

To determine TAC, a commercial colorimetric kit (cat. number: MAK187) was utilized. Trolox standards were prepared, and then 100 µL of Cu^{2+} working solution was mixed with 100 µL of homogenate or standard. After a 90-min incubation period at room temperature, absorbance values were measured at 570 nm.

Glutathione (total, GSSG, GSH) content

Glutathione was measured separately for reduced glutathione (GSH), oxidized glutathione (GSSG) and total glutathione. The concentrations of GSSG and total glutathione were determined using colorimetric tests (cat. number: 38185). Standards and samples (40 µL/well) were pipetted into transparent 96-well plates, followed by the addition of 120 µL of the supplied buffer solution. After a 60-min incubation at 37 °C, substrate solution, coenzyme working solution, and enzyme working solution were added to each well. For the GSSG measurement, masking solution was also added. After a 10-min incubation at 37 °C, absorbance values were measured at 412 nm. GSH concentrations were determined based on the values of GSSG and total glutathione following the manufacturer's instructions.

Glucose-6-phosphate dehydrogenase (G6PDH) activity

Homogenized samples were pipetted into transparent 96-well plates (50 µL/well) after dilution with assay buffer and pre-prepared standards for the G6PDH assay (cat. number: MAK015). A master reaction mix (50 µL) comprising G6PDH assay buffer, G6PDH substrate mix, and G6PDH developer mix was then added to each well. Initial absorbance values were recorded at 450 nm after 2–3 min of incubation at 37 °C. Measurements were taken every 5 min until the absorbance of the most active sample exceeded the highest standard value. The enzyme activity was calculated using the manufacturer's formula.

Superoxide dismutase (SOD) activity

Samples and blanks were added to transparent 96-well plates (20 µL/well; cat. number: 19160) for the SOD assays. Enzyme working solution (20 µL) was pipetted into each well after adding 200 µL of the working solution and mixing. After a 20-min incubation at 37 °C, absorbance values were measured at 450 nm, and the activity values of the samples were determined using the manufacturer's formula.

Total protein concentration

The total protein concentration was measured using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA; cat. number: 23225), with bovine serum albumin (BSA) used as the standard solution to mitigate sample preparation-related variations and ensure an equal protein load for all samples. After a 30-min incubation period at 37 °C, absorbance values at 562 nm were measured. Collected data

regarding protein concentration were used for normalization of the measurements in order to avoid possible homogenization and dilution-related inequalities.

Statistics

GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA) was used for the data processing and analysis. To verify homogeneity of variance and normal distribution, Shapiro–Wilk and Levene's tests were used. One-way analysis of variance (ANOVA) and Dunnett's post hoc tests were utilized to evaluate the differences among the different groups in pairwise comparisons. A $P < 0.05$ threshold was used to determine the significance of differences between groups. The heatmap was created using MetaboAnalyst 4.0 (https://www.metaboanalyst.ca). In every study measurement, there were $n = 10$ animals per group.

Results

XO activity, H_2O_2, MDA, protein carbonyl and 8-OHdG concentrations

Regarding XO activity, a significant decrease was observed in the "AcetLow" ($P = 0.027$) and "AcetMedium" groups ($P = 0.003$), while no significant effect was observed in the "AcetHigh" treatment group compared to the control animals (Fig. 1A). The level of H_2O_2 was significantly elevated in the "AcetMedium" ($P = 0.022$) and "AcetHigh" groups ($P = 0.004$), but no significant changes were observed following the "AcetLow" treatment (Fig. 1B). Based on our measurements, MDA levels were significantly higher following all of the "AcetLow", "AcetMedium", and "AcetHigh" treatments compared to the "Control" group ($P < 0.001$, Fig. 1C). As for protein carbonyl and 8-OHdG, no significant changes were observed (Fig. 1D, E).

G6PDH and SOD activities, total antioxidant capacity

A significant decrease was detected in G6PDH activity in the "AcetLow" ($P = 0.004$), "AcetMedium" ($P < 0.001$), and "AcetHigh" groups ($P = 0.005$, Fig. 2A) in comparison with the controls. However, no significant changes were observed regarding SOD enzyme activity in the exposed groups compared to the control animals (Fig. 2B). A significant increase was measured in total antioxidant capacity in the "AcetHigh" treatment group compared to the "Control" group ($P < 0.001$, Fig. 2C).

GSH, GSSG, total glutathione concentrations, GSH-GSSG ratio

In terms of GSH concentration, a significant decrease was observed only in the "AcetLow" treatment group compared to the "Control" ($P = 0.002$, Fig. 3A), while GSSG concentration was found to be higher in the "AcetLow" ($P = 0.009$), "AcetMedium" ($P = 0.031$), as well as in the "AcetHigh" treatment groups ($P = 0.007$, Fig. 3B). Similarly, the GSH-GSSG ratio was significantly lowered following all of the "AcetLow", "AcetMedium", and "AcetHigh" treatments compared to the "Control" ($P < 0.001$, Fig. 3C). According to our findings, no significant changes were observed in the case of total glutathione concentrations (Fig. 3D).

Additionally, a heatmap is used to present the results, so that individual samples can be properly visualized and analyzed (Suppl. Figure 1).

Discussion

Since agriculture has to meet the need of the rapidly increasing population globally therefore expansion of the production of adequate quantity and quality of food is necessary[32]. However, this expansion presents new challenges for crop production, including the cultivation of monocultures, extreme weather conditions associated with climate change, increasing pest resistance, and alterations in soil quality due to intensive farming practices[33]. The use of pesticides has become essential to address these challenges and sustain the industrial-scale production of food and feed[34].

In the intensive farming, the importance of pollinator species, primarily that of honey bees must be also mentioned. The significant decline in honey bee colonies in recent decades has been attributed to CCD, a phenomenon that has been well-documented[35]. As CCD is recognized as a multifactorial problem, pesticides cannot be solely blamed; however, their impact should not be overlooked. Therefore, it is imperative to enhance our understanding of their contribution to CCD[36].

Compared to earlier products in the neonicotinoid group, acetamiprid, a new-generation neonicotinoid insecticide, is significantly safer to apply and has lower toxicity to the environment[37]. While lethal toxicity of acetamiprid in field conditions is rare, there is limited understanding of its acute sublethal effects and its impact on non-target species such as pollinators[38]. In cases of chronic exposure, neonicotinoids affect various aspects of honey bee behaviour, notably the proboscis extension reflex, homing ability, social communication, and memory, all crucial for locating nectar-rich foraging sites[39]. Previous research findings also support the hypothesis that acetamiprid affects behaviour by inducing oxidative stress and mitochondrial damage; however, this relationship has not been fully elucidated in honey bees[15,16].

When oxidative agents, primarily ROS, become sufficiently abundant to overwhelm the organism's antioxidant defense system, oxidative stress may ensue[22]. A variety of enzymatic and non-enzymatic components work together to maintain stable oxidative status. These parameters can be quantified under laboratory conditions, serving as biomarkers to assess the body's redox homeostasis and evaluate the presence and extent of oxidative stress related to acute acetamiprid exposure[40]. Accordingly, a total of 12 different parameters was monitored in the present study. These parameters serve as appropriate markers for assessing the accumulation of ROS resulting from oxidative stress, as well as enzymes and non-enzymatic components that play pivotal roles in maintaining antioxidant balance. Additionally, we included parameters to detect potential damage to lipids, DNA, and proteins.

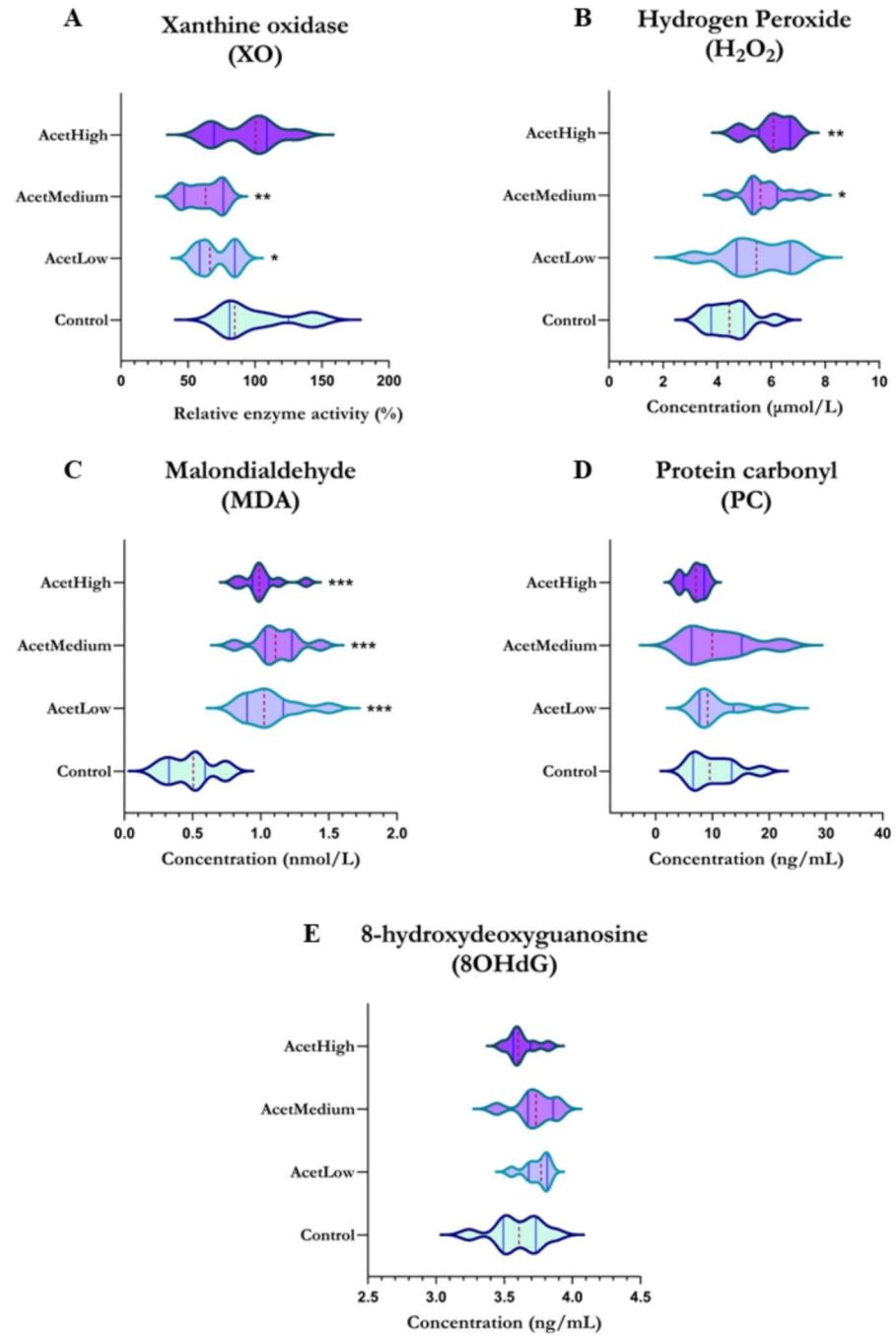

Fig. 1. Redox parameters measured in honey bee brain samples. Concentrations of (**A**) xanthine oxidase, (**B**) hydrogen peroxide, (**C**) malondialdehyde, (**D**) protein carbonyl, (**E**) 8-hydroxydeoxyguanosine, respectively. Data are visualized using violin plots, where dotted lines indicate median and solid lines indicate the first (Q1) and third (Q3) quartiles. "Control" refers to control group with no treatment; "AcetLow", "AcetMedium" and "AcetHigh" refer to 2.076, 4.156 and 8.305 µg/bee/day acetamiprid exposure, respectively. Significant differences between control and acetamiprid exposed groups are indicated with asterisks. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

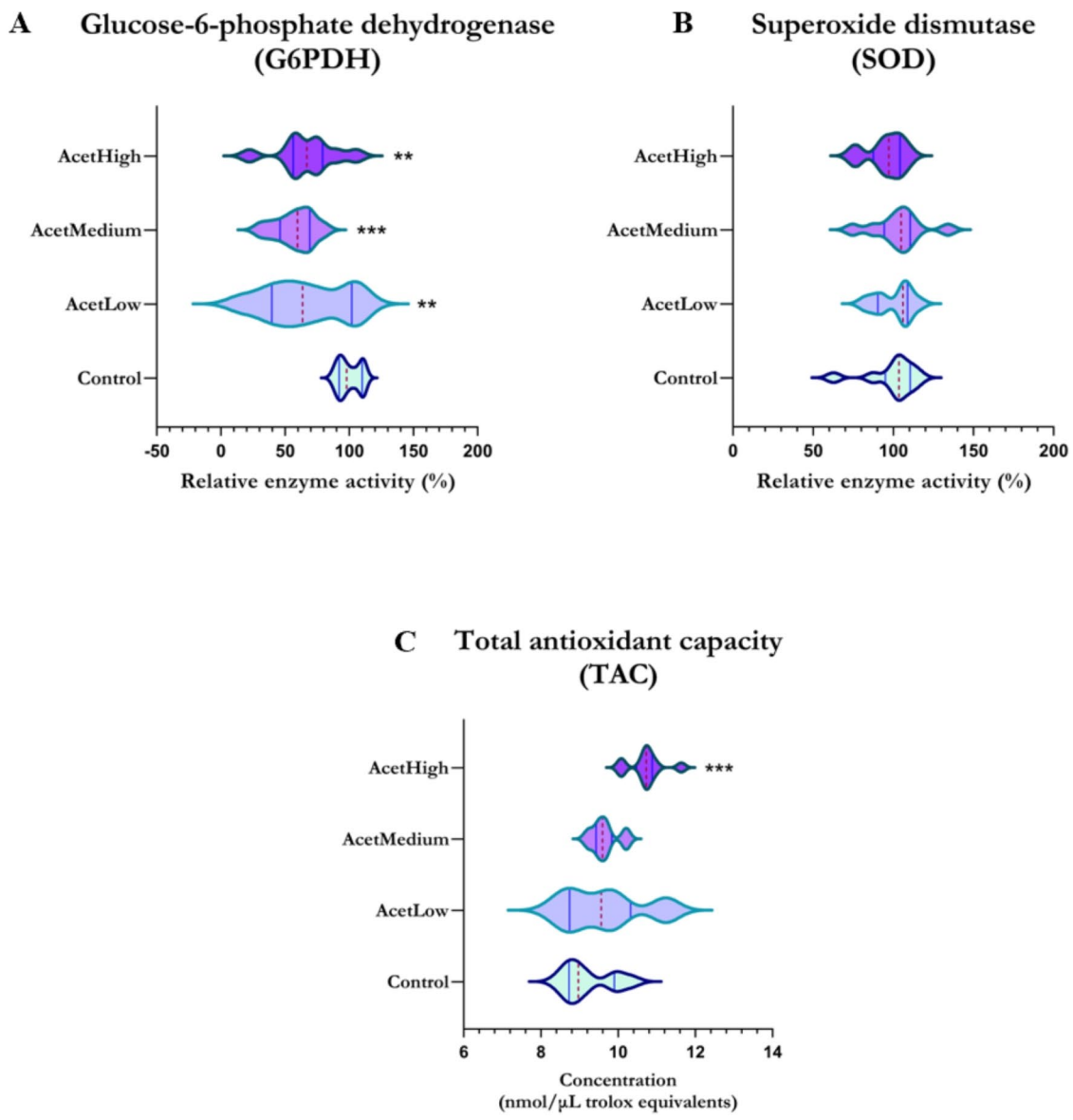

Fig. 2. Redox parameters measured in honey bee brain samples. Activities of (**A**) glucose-6-phosphate dehydrogenase, (**B**) superoxide dismutase, (**C**) total antioxidant capacity, respectively. Data are visualized using violin plots, where dotted lines indicate median and solid lines indicate the first (Q1) and third (Q3) quartiles. "Control" refers to control group with no treatment; "AcetLow", "AcetMedium" and "AcetHigh" refer to 2.076, 4.156 and 8.305 µg/bee/day acetamiprid exposure, respectively. Significant differences between control and acetamiprid exposed groups are indicated with asterisks. $^{**}P < 0.01$, $^{***}P < 0.001$.

XO is a vital enzyme involved in purine metabolism and in maintaining redox homeostasis by converting xanthine and oxygen into hydrogen peroxide and uric acid[41]. Our results showed that the AcetLow and AcetMed treatment doses reduced the enzyme activity, but the background of this change is not fully understood. A possible hypothesis of the observation may be that exposure to acetamiprid induces a protective mechanism to minimize ROS production under oxidative stress, such as via decreased XO gene expression, which may result in a decline in the synthesis of the enzyme. This alteration might be connected to a complex compensation mechanism, also suggested by the TAC parameter, however, further investigation is needed to confirm this hypothesis[42,43].

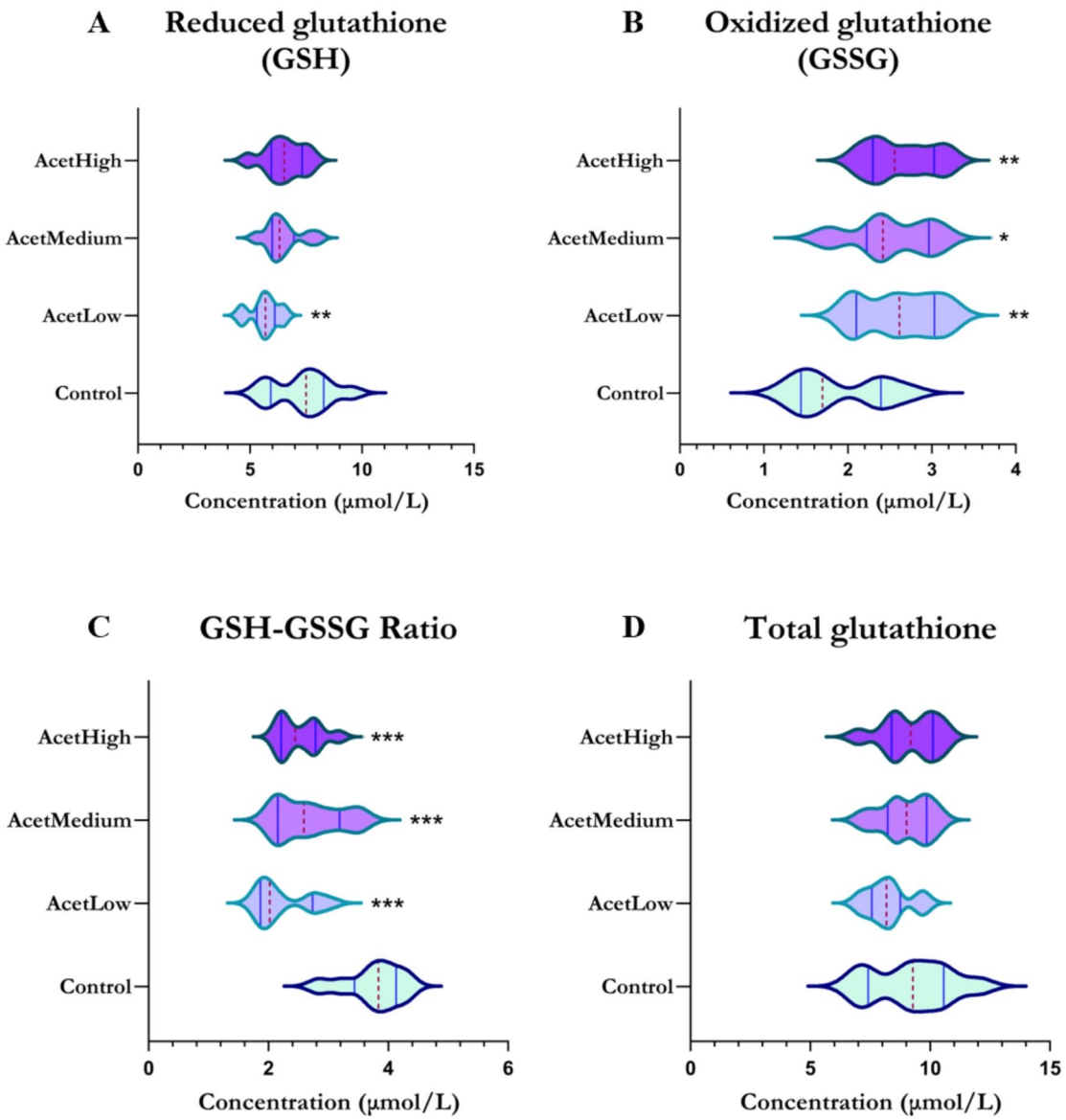

Fig. 3. Redox parameters measured in honey bee brain samples. Concentrations of (**A**) reduced glutathione, (**B**) oxidized glutathione, (**C**) GSH-GSSG ratio, (**D**) total glutathione, respectively. Data are visualized using violin plots, where dotted lines indicate median and solid lines indicate the first (Q1) and third (Q3) quartiles. "Control" refers to control group with no treatment; "AcetLow", "AcetMedium" and "AcetHigh" refer to 2.076, 4.156 and 8.305 µg/bee/day acetamiprid exposure, respectively. Significant differences between control and acetamiprid exposed groups are indicated with asterisks. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Since H_2O_2 is a ROS itself, although a nonradical compound and therefore less reactive than molecules containing unpaired electrons, it serves as a useful marker of oxidative stress in tissues. Additionally, H_2O_2 acts as a messenger molecule involved in a wide range of metabolic processes[44]. When the oxidative balance is disrupted, the scale tips in favour of its production[45]. Our study findings demonstrated that H_2O_2 concentrations increased in both the AcetMed and AcetHigh treatment groups, consistent with earlier findings where an elevation in ROS concentration was also observed in *Eisenia fetida* treated with acetamiprid[43]. In an experiment carried out on *Drosophila melanogaster*, the primary focus was to investigate imidacloprid's mechanism of action, which was found to increase the influx of Ca^{2+} into neurons and subsequently lead to rapid accumulation of ROS in the brain[24]. Although we acknowledge that neonicotinoid pesticides may have a detrimental impact

on non-target insects, an experiment indicated that honey bees may be even more vulnerable to pesticides than *Drosophila melanogaster* fruit flies and *Mamestra brassicae* cabbage moths, based on the results of post-exposure H_2O_2 production[46].

Given that pesticides induce oxidative stress in the body by damaging the mitochondria, the free radicals released can disrupt various molecules, including lipids especially following acute exposure if the organism does not have enough time to effectively activate all compensatory defense mechanisms. One widely measured parameter used to indicate the extent of lipid peroxidation is MDA[47]. MDA is formed from the oxidation of polyunsaturated fatty acids through chemical means, but it can also be enzymatically produced by lipoxygenases, cyclooxygenases, or cytochrome P450s[48]. MDA serves as a signalling molecule in cells in addition to indicating the level of lipid peroxidation[49]. Several previous studies have demonstrated that oxidative stress increases the concentration of MDA, a phenomenon supported by this study, as all treatment groups showed an elevation in MDA concentration compared to the control group, indicating lipid peroxidation[19,50–52].

Monitoring the levels of 8-OHdG is considered a reliable method for assessing DNA damage[53]. Despite detecting several signs of oxidative stress in this study, no changes in the concentration of 8-OHdG were observed, suggesting potential evidence of the absence of DNA damage. In similar experiments, 8-OHdG levels were found to increase in other species, such as Chinese rare minnows (*Gobiocypris rarus*) treated with imidacloprid, but only at high doses, with no such increase observed at lower concentrations[54]. A study on *Eisenia fetida*, which involved the application of the neonicotinoid sulfoxaflor, reported a significant rise in the 8-OHdG concentration only after day 7 in the two treatment groups receiving the highest doses. This suggests that DNA damage may occur only after an extended period, which aligns with our results as well[55]. Similarly, despite the presence of oxidative stress, no alterations were observed in protein carbonyl levels, an indicator of oxidative stress-induced damage to proteins[56].

In comparison to the control group, a significant elevation in the TAC of the AcetHigh treatment group was detected. TAC comprises elements of the non-enzymatic antioxidant system, such as albumin, GSH, ascorbic acid, α-tocopherol, β-carotene, uric acid, bilirubin, and flavonoids, which interact with a number of factors[57]. Our results suggest that although the glutathione defense system is depleted following acetamiprid exposure, concentrations of the aforementioned molecules may increase, resulting in higher TAC and indicating the activation of protective compensatory mechanisms. Similar compensation was also observed in rat liver following clothianidin exposure[58]. Consistent with our findings, changes in TAC after neonicotinoid exposure were noted in some studies involving honey bees, although an increase has not yet been reported in the case of acetamiprid exposure[59,60].

Based on our findings, the glutathione system was significantly altered by acetamiprid treatment. The concentration of GSSG significantly increased in all treatment groups, while GSH significantly decreased in the AcetLow group. Our results align with several studies conducted in other animal species. For instance, in a study involving in vital organs (liver, kidney, brain) of male rats, acetamiprid was also found to exert adverse effects on the glutathione system[61]. Similarly, in a study on *Eisenia andrei* earthworms, which assessed the effects of various doses of acetamiprid, as the acetamiprid concentration increased an initial rise was detected in the concentration of GSH followed by a plateau, which indicates that the GSH concentration was dose-invariant. Consequently, when earthworms were exposed to higher concentrations of acetamiprid, GSH levels were not altered. This phenomenon corresponds with the observations in our study, which may be explained by the depletion of the glutathione system in response to oxidative stress, however, further research is necessary to corroborate our hypothesis[21].

In healthy cells, with normal oxidative status, free glutathione exists predominantly in its reduced form, with a nearly negligible amount of GSSG[62]. GSH plays a pivotal role in cellular defense against oxidative stress by inactivating free radicals while converting from its reduced to oxidized form, thereby resulting in a lower GSH:GSSG ratio[63]. Hence, our observation of a decline in the GSH:GSSG ratio in all three treatment groups is reasonable and consistent with previous research findings[64–66]. Notably, early detection of oxidative stress is crucial in the pathophysiology of various diseases, and the GSH:GSSG ratio appears to be a suitable indicator of oxidative stress in the body[67].

The total glutathione content is determined by the concentrations of oxidized and reduced forms of glutathione. The parameter indicates how an organism responds to negative factors via altering the level of one of its most significant antioxidants[68]. Considering that the reduced form of glutathione decreases under oxidative stress and the oxidized form increases in a simultaneous manner, an equilibration of the concentration of total glutathione, a phenomenon consistent with the observations in this experiment, is implied. Other studies have also found similar results when measuring total glutathione concentrations[69].

The enzyme G6PDH plays a crucial role in the pentose phosphate pathway and is responsible for maintaining appropriate concentrations of NADPH in cells, thereby exhibiting antioxidant activity[70]. Deficiency in G6PD activity and disruption of redox equilibrium can lead to abnormal embryonic development, impaired cell growth and signalling, increased susceptibility to viral infection, and a higher risk of degenerative diseases[71]. Consistent with previous findings, our experiment demonstrated a significant decrease in G6PDH enzyme activity in all three treatment groups compared to the control. In line with our results, a prior study reported that honey bees exposed to imidacloprid, difenoconazole, and glyphosate, either individually or in binary and ternary mixtures, also exhibited reduced G6PDH activity in all treatment groups[72]. Interestingly, contrasting with our findings, a study involving imidacloprid-exposed rats showed an increase in G6PDH activity, suggesting species-specific differences in the effects of neonicotinoid exposure[73].

In this trial, there were no significant differences in the SOD levels between the treatment groups and the control group. In accordance with our results, a study conducted on *Daphnia magna* examining the effects of two newer generation neonicotinoids, guadipyr and cycloxaprid, alongside imidacloprid, on changes in SOD enzyme activities found no significant changes in most treatment groups, except for the highest dose of

imidacloprid[74]. Furthermore, results from studies on several other species indicate that short-term exposure (24–48 h) to neonicotinoid-type agents does not alter SOD activity, while longer treatments, particularly at higher concentrations, may lead to significant changes[19,54,75–77].

On the other hand, as a limitation it is important to note that our results were derived from samples taken at a single point in time. However, in future studies, it may be valuable to measure these parameters at multiple time points to gain a more comprehensive understanding of the observed trends and the progression of the oxidative stress-related effects caused by acetamiprid exposure, as well as monitoring not only the acute effects but the results of chronic exposure.

In conclusion, our study sheds light on the impact of acute sublethal acetamiprid exposure on the redox system of honey bees. It is widely acknowledged that the increased application of pesticides driven by rising agricultural activity may induce several detrimental actions on the environment. Neonicotinoids, the most commonly used pesticides, may have an environmental impact on the development of CCD. While they are clearly not the only causative agents, their contribution to the development of this highly complex phenomenon is not negligible. In order to better understand the aforementioned facts, we examined the effects of short-term exposure to acetamiprid in field-realistic experimental settings, focusing on the alterations in redox balance that could be observed in the bee's nervous system.

In summary, in our experiment H_2O_2 and MDA were increased by acetamiprid, indicating an increase in ROS levels and consequent lipid peroxidation, but no DNA and protein damage (8-OHdG and PC were unchanged) under acute conditions. This particular result further highlights the possibility, that acetamiprid exposure is unlikely to lead to a combined, consistent impairment of all monitored markers, but may lead to increased vulnerability of various lipids, phospholipids and their fatty acids. The glutathione system may have been depleted in response to oxidative stress, as indicated by changes in the concentration and ratio of GSH and GSSG. Similarly, the activity of G6PDH, which regenerates NADPH and thus functions as a member of the enzymatic antioxidant system, was also reduced. The concentrations of other small molecule antioxidants may have been increased by a compensatory mechanism, as reflected in the increased TAC. Our model applied for the study of redox homeostasis in the honey bee nervous system may be a useful tool for the investigation of other factors that could be involved in the development of CCD, as well as for the study of other pesticides, protective agents, and their interactions.

Data availability

All datasets are available through the following link: https://doi.org/https://doi.org/10.6084/m9.figshare.26346565.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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