Original article

Acute toxicity of sublethal concentrations of thiacloprid and clothianidin to immune response and oxidative status of honey bees

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Abstract – The use of insecticides, especially neonicotinoids (NEOCs), poses a significant threat to honey bees, thus prompting the EU to ban all outdoor uses of clothianidin, imidacloprid, and thiamethoxam. However, due to the persistence of NEOCs, as well as the fact that certain NEOCs are in use in some areas of the world, exposure to them is still an on-going issue. In the present study, we used laboratory acute toxicity test to examine the effects of sublethal concentrations (resulting in consumed doses approximately 50, 20, and 5 times below LD_{50}) of thiacloprid and clothianidin on selected parameters of antioxidative defense (superoxide dismutase, catalase, glutathione *S*-transferase activities), oxidative status (reduced glutathione, protein thiols, malondialdehyde), neurotoxicity (acetylcholinesterase activity), and immune response (prophenoloxidase and phenoloxidase activities) in honey bees. Results indicated that both neonicotinoids' sublethal concentrations promote oxidative stress in honey bees and affect their immune defense. NEOCs impair detoxification abilities through reduced glutathione *S*-transferase activity, as well as disturb immune response due to decreased prophenoloxidase and phenoloxidase activities, which can explain the high toxicity of these substances to honey bees.

1. INTRODUCTION

The honey bee (*Apis mellifera* L.) is one of the most important pollinator species worldwide in both natural and agroecosystems. Through pollination, as an important ecosystem service, they provide biodiversity and have a great impact on global economy (Potts et al. 2010; Khalifa et al. 2021). Over the past decades, losses of managed honey bees' colonies in Europe and North America had been reported (van Engelsdorp and Meixner 2010; Potts et al. 2010). It is strongly suggested the causes of this are multifactorial, and numerous harmful factors have been suspected as possible reasons for the observed losses. Malnutrition, climate changes, pollution, apicultural practice, pests, and pathogens, as well as use of agrochemicals, are the most prominent risk factors (van Engelsdorp and Meixner 2010; Jacques et al. 2017). The use of insecticides poses a significant threat, especially widespread use of neonicotinoids (NEOCs) (Blacquière et al. 2012; Tosi et al. 2017; Woodcock et al. 2017).

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NEOCs are a class of insecticides with high toxicity for insects. They had been commercialized in the 1990s and became the most used insecticides for agricultural pest management (Jeschke et al. 2011). High persistence of NEOCs, with a half-life ranging from few months to years, depending on the compound and conditions, water solubility, and high potential for spreading over to adjacent soils and plants, caused their accumulation and environmental contamination (see the review article by Goulson 2013). Consequently, NEOCs were found in different matrices such as soil, plants, nectar and bee pollen many years after application, not only in cultivated fields, but also in wildflowers, ecological focus areas, and organic fields (Liu et al. 2010; Mullin et al. 2010; Krupke et al. 2012; López-Fernández et al. 2015; Botías et al. 2016; Blacquière and van der Steen 2017; Humann-Guilleminot et al. 2019; Wintermantel et al. 2020). Thus, many of the non-target insects are exposed to NEOCs, usually manifesting sublethal effects (Blacquière et al. 2012; Brandt et al. 2016, 2017, 2020). Acting as agonists of nicotinic acetylcholine receptor, NEOCs interfere with neuronal cholinergic signal transduction, thus causing abnormal behavior, paralysis, and eventually death of an insect (Tomizawa and Casida 2005; Brandt et al. 2020).

Honey bees are consequently exposed to pollution-contaminated food resources since they cover large areas through their foraging activity (Krupke et al. 2012; Ruschioni et al. 2013). Harmful sublethal effects of NEOCs on the memory (Christen et al. 2016), susceptibility to infections (Alaux et al. 2010), reproduction (Williams et al. 2015), and development (Wu et al. 2011) of honey bees have been reported. Accordingly, there had been concerns about the usage of NEOCs. The European Food Safety Authority (EFSA) determined that NEOCs were particularly harmful to bees and in 2013, the EU placed a moratorium on the use of three NEOCs–clothianidin, imidacloprid, and thiamethoxam–for the protection of bee-attractive crops (European Commission 2013). According to the new assessments based on a survey of 1500 studies in 2018, the EU banned all outdoor uses of those NEOCs due to concerns about the adverse effects on pollinators (Blake 2018), but they were still allowed to be used in greenhouses. Subsequently, in January 2020, non-renewal of approval of the neonicotinoid pesticide thiacloprid came into force (European Commission 2020). This was in accordance with scientific advice by the EFSA that thiacloprid presents health and environmental concerns. However, in other areas of the world, such as North America and non-EU countries, NEOCs are still in use due to estimated economic and societal benefits (Blake 2018). Relevant regulatory authorities (European Food Safety Authority 2014) emphasize the importance of assessing the impact of environmental pollution on bee health, pointing out the importance of colony monitoring, field, and laboratory tests, as well as the evaluation of the effects of sublethal doses of various pollutants for bees (European Food Safety Authority 2015). All of that makes the study of the sublethal effects of NEOCs on non-target insects still immensely relevant, particularly in regard to honey bee (Alaux et al. 2010; Blacquière et al. 2012; Di Prisco et al. 2013; Brandt et al. 2016, 2017, 2020).

The immune defense of honey bees can be affected by NEOCs' sublethal concentrations. One of important components of innate immune insect defense are phenoloxidase (POx) and prophenoloxidase (proPOx). Both enzymes are important components in the defense against wounding and infection in insects (Pham and Schneider 2008). POx catalyzes two reactions in melanogenesis, as a major innate immune defense mechanism of insects and determinant factor for outcome of an infection (Nakhleh et al. 2017). POx is synthesized in hemocytes as zymogen, proPOx, which is activated by a specific proteolytic cleavage. At the same time, this is a limiting factor in melanization (Pham and Schneider 2008). POx does not seem to be an indicator of resistance but rather of host condition, and it has been reported that individuals in better conditions produce higher levels of proPOX and/or POx (González-Santoyo and Córdoba-Aguilar 2012). Due to the complexity of obtaining bee hemolymph, these hemocyte enzymes are mainly analyzed in honey bee tissue

homogenates, where a decrease in POx and pro-POx activities were usually detected after exposure to NEOCs (Badawy et al. 2015; Zhu et al. 2017).

Furthermore, it is known that exposure to NEOCs is accompanied by the adverse changes in biochemical levels in honey bees, even in sublethal doses (Boily et al. 2013; Gauthier et al. 2018). Thus, an increase in activity of acetylcholinesterase (AChE), a key enzyme involved in the termination of impulse transmission, has been reported in numerous studies and suggested as a potential biomarker of honey bee exposure to NEOCs (Boily et al. 2013; Samson-Robert et al. 2015). Many studies demonstrated that most of NEOCs promote oxidative stress in honey bees (Carvalho et al. 2013; Balieira et al. 2018; Gauthier et al. 2018). This stress leads to damage in the biomolecules and causes changes in the antioxidant defense system (ADS) in both enzymatic and non-enzymatic components. Increasing level of malondialdehyde (MDA), as a marker of lipid peroxidation, is often reported in honey bees after an exposure to NEOCs (Balieira et al. 2018; Gauthier et al. 2018), while activity of key antioxidative enzymes of honey bee, such as superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferase (GST), have also been measured, as usual (Badiou-Bénéteau et al. 2012; Carvalho et al. 2013; Balieira et al. 2018).

To better explain the exposure of honey bees to environmentally realistic concentrations of NEOCs, we tested two NEOCs, clothianidin and thiacloprid, at sublethal concentrations in honey bees, following an acute oral exposure. Taking into account the harmful influence of NEOCs on the honey bee, as well as the importance of revealing consequential changes in the immune response and antioxidant defense in these insects, we determined the activities of AChE, phenoloxidase, prophenoloxidase, SOD, CAT, and GST enzymes, as well as content of known biochemical indicators of oxidative status (reduced glutathione, protein thiols, and MDA) in honey bees, following pesticides exposure.

2. MATERIALS AND METHODS

2.1. Honey bee sample collection

Honey bee workers were collected from a beehive of the Faculty of Sciences at the University of Novi Sad (45.2473° N, 19.8539° E) in July 2015. The colony was managed by experienced beekeeper in according to good beekeeper practice (FAO et al. 2021). The bee colony was strong (approximately 20,000 bees), with no clinical signs of infectious diseases. No chemical treatments against *Varroa destructor* or other diseases were applied during and before the experiment at least 6 months. To reduce the biological variation of the sample, worker honey bees of the same queen and approximately of the same age were used. Adult worker bees were collected from the top super, from frames containing honey and pollen, to ensure sampling of older bees in according to Medrzycki et al. 2013. After collecting the bees in plastic jars, they were immediately transferred to the laboratory.

2.2. Acute toxicity test of pesticides

In the laboratory, honey bees were anesthetized via exposure to carbon dioxide gas for no longer than 3 min and transferred into 1000-mL glass jars covered with nylon mesh, each containing 30 bees. Food was given *ad libitum* through an inverted plastic container with the small hole in the center of the lid, placed on top of the nylon mesh. Treated groups of honey bees were left to feed with 1 mol/L sucrose solution containing three sublethal concentrations of thiacloprid and clothianidin during 48 h, in controlled conditions at 25 °C, 65% RH, and in the dark. Control bees were fed with the sucrose solution without the addition of pesticides. Sublethal pesticide concentrations were chosen to give consumed doses as approximately 50-, 20-, and fivefold range ($LD_{50}/50$, $LD_{50}/20$, and $LD_{50}/5$) of median lethal dose for thiacloprid (17.94 µg per honey bee) and clothianidin (3.68 ng per honey bee), determined by the acute oral toxicity test for honey bees (US EPA 1991, OPP

Pesticide Ecotoxicity Database). The treatments represent concentrations of 2.69 μg/mL (T1), 6.72 μg/mL (T2), and 26.91 μg/mL (T3) of thiacloprid in feeding solution and 0.55 ng/mL (C1), 1.38 ng/mL (C2), and 5.52 ng/mL (C3) for clothianidin. Chosen concentration match to often reported clothianidin residues that were usually over to 2 ng/g, but maximum measurements were reported in the study Humann-Guilleminot et al. (2019) that were up to 18 ng/g in soil and 6.7 ng/g in plant. Reported measurement was published after the EU restricted the use of three NEOCs, including clothianidin (European Commission 2013). On the other hand, manufacturers usually label thiacloprid (LD_{50} is 17.3 μg/honey bee) as safe for bees and without restrictions on use during the flowering. Even though reported thiacloprid residues in bee pollen were up to 1000 μg/kg (López-Fernández et al. 2015), we chose even higher concentration, because local exposure of honey bees could be higher considering permitted pest treatments of thiacloprid during the bloom (Siede et al. 2017). Each pesticide concentration, as well as control, was tested with three biological replicates of 30 randomly selected bees. The total number of experimental bees was 270 for each tested pesticide, with the addition of 90 control bees.

After 48 h, the bee mortality was recorded, the volume of food consumed was determined as a difference in mass of feeding solution before and after conducted treatment, taking into account the density, and expressed as μL per honey bee. From each of three replicates of control and treatment groups, ten surviving bees were taken for hemolymph isolation. According to the method by Hartfelder et al. (2013), honey bee workers were first calmed at 4 °C for a few minutes. Then, the bee was held with forceps, and hemolymph was isolated from dorsal vascular vessel, by incision between the 5th and 6th abdominal segment with sterile needle and hemolymph was collected with micropipette (volume was approx. 1–5 μL per honey bee). These bees were no longer used for any analysis. The hemolymph samples were immediately frozen using dry ice and stored at −70 °C for further analysis. The remaining surviving bees were

collected and transferred into plastic vials which were immediately frozen in dry ice and stored at −20 °C for further homogenization and analysis.

2.3. Sample preparation for biochemical analysis

Homogenization of whole bodies of five intact worker bees per each of three replicates of control and treatment groups was performed in ice-cold 50 mmol/L Tris–HCl buffer pH 7.4 to obtain 10% w/v homogenates. Crude homogenates were centrifuged at 10,000 g (4 °C) for 10 min, and the supernatants were further aliquoted and frozen until enzymatic assays and redox status assessment (Kojić et al. 2019). In this way, for each analyzed parameter, three biological replicates were provided for each experimental group (control and treatments), and further analyses of the samples were performed in a technical duplicate or triplicate.

2.4. Measurement of acetylcholinesterase activity

Acetylcholinesterase (AChE) enzymatic activity was measured spectrophotometrically using the colorimetric method (Ellman et al. 1961), optimized for microplate assay. The Ellman method is based on acetylthiocholine hydrolysis by acetylcholinesterase to give thiocholine and acetate. Thiocholine further reacts with DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) giving yellow color nitrobenzoate, which shows an increase in intensity measured on spectrophotometer at 412 nm during 10 min. The reaction mixture was prepared mixing 100 mM phosphate buffer pH 8.0, 75 mM acetylthiocholine iodide, and 10 mM DTNB in 150:2:5 ratio, and reaction was started by addition of 50 μL of 10% w/v honey bee homogenate, in the final volume of 300 μL. The activity was calculated based on an extinction coefficient of 13.6 mM/cm, in which one unit of AChE is the amount of enzyme that catalyzes the production of 1 μmol of thiocholine per minute, expressed per milligram of protein.

2.5. Measurement of MDA, GSH and -SH groups concentration

MDA levels in bee homogenates were measured according to the method of Slater (1984), which is based on the principle that malondialdehyde, the specific product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex with maximum absorption at 532 nm. Samples of 10% w/v honey bee homogenate (up to 0.5 mL) was mixed with 1 mL of a mixture of 0.375% w/v TBA and 15% w/v trichloroacetic acid and heated on a water bath for 15 min at 90 °C. After cooling the reaction mixture on ice and centrifugation at 1000 g for 10 min, supernatant was further used for spectrophotometric determination of thiobarbituric acid reactive substance (TBARS) concentration. Concentrations in the sample being calculated from a standard curve using MDA as a standard and expressed in nanomole per milligram of protein.

Concentrations of reduced glutathione (GSH) and free thiol (-SH) groups were determined by the Ellman method (Ellman 1959), optimized for microplate assay. Proteins in the samples of 10% w/v honey bee homogenates were precipitated with 4% sulphosalicylic acid. Supernatant was further used for GSH determination, and the pellet, after washing twice with 2% sulphosalicylic acid and resuspension in 6 M guanidine-HCl pH 6.0, was used for determination of protein thiol groups. Using DTNB as substrate, reduced glutathione in supernatant and the free -SH groups in resuspended pellet formed TNB (2-nitro-5-thiobenzoic acid), a product of reaction which is further monitored at 412 nm. Concentrations in the sample being calculated from a standard curve using GSH as a standard and expressed in nanomole per milligram of protein.

2.6. Measurement of antioxidative enzymes activity

The activity of superoxide dismutase was measured by inhibition of cytochrome c reduction by superoxide radical produced in the xanthine-xanthine oxidase reaction at 550 nm (McCord and Fridovich 1968). SOD catalyzes the dismutation reaction of superoxide anion radical, so inhibition of cytochrome c reduction is proportional to the SOD activity present in the sample. The SOD activity required to reduce the rate of cytochrome c reduction by 50%, with a change in absorbance of 0.025/min in initial (uninhibited) xanthine-xanthine oxidase mixture, is defined as the unit of activity of this enzyme, which is further expressed per milligram of protein.

Catalase activity was measured as the rate of hydrogen peroxide decomposition at 240 nm, based on the method by Aebi (1984). The procedure involved adjusting the absorbance of the hydrogen peroxide solution in 50 mM phosphate buffer pH 7.0 to obtaining the range of 0.52 to 0.55. The decrease in absorbance at 240 nm was monitored for 3 min, after addition of 10% w/v honey bee homogenates, which was proportional to the catalase activity in the sample and further expressed per milligram of protein.

Glutathione *S*-transferase activity was determined by following the formation of the product of the reaction between reduced glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, described by Habig et al. (1974). GST activity is measured as the rate of the formation of CDNB-glutathione conjugate, followed by a change in absorption at 340 nm for 3 min, and further expressed per milligram of protein. Protein concentration was determined using the Bradford method (1976), with bovine serum albumin as a protein standard.

2.7. Measurement of prophenoloxidase and phenoloxidase activity

Phenoloxidase activity in its zymogenic (proPOx) and active form (POx) was determined in honey bee hemolymph by protocol published by Laughton and Siva-Jothy (2011). The method is based on the conversion of DOPA (3,4-dihydroxyphenylalanine) as a substrate, by phenoloxidase, resulting in the

formation of a red-brown product dopachrome, whose absorbance is measured at 492 nm. The reaction mixture for determining POx activity contained hemolymph sample (40 µL), distilled water (50 µL), and 16 mmol/L L-DOPA (110 µL), which started the reaction. To determine the activity of proPOx, an additional step of activation, incubation of the zymogenic form of the enzyme with chymotrypsin, is required. Therefore, the reaction mixture for proPOx contained 0.5 mg/mL chymotrypsin (50 µL) instead of distilled water. Specific activity of enzymes was expressed as units of enzyme activity per milligram of protein.

2.8. Data analysis

Percent corrected mortality was calculated for each treatment using Abbott's equation (Abbott 1925). Statistical analysis was performed using Statistica version 13 (StatSoft, Inc., Tulsa, OK, USA) software. All the data are expressed as mean ± SD (standard deviation). Results are tested on normality and homogeneity of variance suggesting that required assumptions for one-way analysis of variance (ANOVA) are met; therefore, statistical differences between each treatment and control values of the analyzed parameters were determined by ANOVA, followed by post hoc Tukey test at $p < 0.05$ (*).

3. RESULTS

3.1. Survival and pesticides consumption

The results regarding the toxicity of thiacloprid and clothianidin, followed acute exposure, on survival and food consumption are summarized in Table I. Three sublethal concentrations in the feeding solution for thiacloprid of 2.69, 6.72, and 26.9 µg/mL (T1, T2, and T3), resulted in consumed doses of 0.295, 0.674, and 1.956 µg/honey bee, respectively. Consumed doses were approximately 60, 25, and 10 times lesser than LD_{50} (17.94 µg/honey bee). For clothianidin, feeding with three sublethal concentrations of 0.55, 1.38, and 5.52 ng/mL (C1, C2, and C3) resulted in consumed doses of 0.072, 0.191, and 0.664 ng/honey bee, respectively. LD_{50} for clothianidin is 3.68 ng/

Table I Concentration of NEOCs in feeding solution (µg/mL), consumed dose of feeding solution (µL/honey bee), and pesticide consumption expressed as µg per honey bee (mean ± standard deviation) and part of LD_{50} dose of thiacloprid and clothianidin, as well as mortality of honey bees after 48 h in oral test. The oral acute toxicity (LD_{50}) for thiacloprid is 17.94 µg/bee and for clothianidin 3.68 ng/bee

Group	Concentration of NEOCs in feeding solution (µg/mL)	Consumption of feeding solution (µL/honey bee)[a]	Pesticides consumption (µg/honey bee)	Pesticides consumption expressed as part of LD_{50}	Mortality (%)[b]
	Thiacloprid				
T1	2.69	109.6 ± 7.7	0.295 ± 0.021	LD_{50}/60	5.81
T2	6.72	100.3 ± 5.2	0.674 ± 0.035	LD_{50}/25	9.23
T3	26.91	72.7 ± 2.5	1.956 ± 0.066	LD_{50}/10	8.13
	Clothianidin				
C1	$0.55 \cdot 10^{-3}$	130.5 ± 16.9	$0.072 ± 0.009 \cdot 10^{-3}$	LD_{50}/50	1.15
C2	$1.38 \cdot 10^{-3}$	138.1 ± 9.3	$0.191 ± 0.013 \cdot 10^{-3}$	LD_{50}/20	4.65
C3	$5.52 \cdot 10^{-3}$	120.3 ± 5.8	$0.664 ± 0.032 \cdot 10^{-3}$	LD_{50}/5	8.13

[a]There were no statistically significant differences between control (105.7 ± 22.9 µL/honey bee) and treatment groups, but the consumption of feeding solution with thiacloprid was statistically significantly ($p \leq 0.01$) lower compared to the intake of feeding solution with clothianidin

[b]Mortality of control (4.4%) was used for Abbott's correction of the mortality of treatment groups

honey bee, and the tested doses were $LD_{50}/50$, $LD_{50}/20$, and $LD_{50}/5$. There was uneven consumption of feeding solutions; thiacloprid consumption was significantly ($p \leq 0.01$) lower than clothianidin consumption. Mortality of treatment groups was corrected for the mortality rate of the control (4.4%) using Abbott's formula (Abbott 1925). Mortality is higher than control for all treatments with NEOCs, in the ranges from 1.15 to 8.13% for clothianidin and 5.81 to 9.23% for thiacloprid.

3.2. Acetylcholinesterase activity

After the exposure of the honey bees to thiacloprid and clothianidin for 48 h, the activity of AChE increased in all treated groups compared to control (24.3 mU/mg protein). Statistically significant ($p < 0.05$) increase in AChE activity was observed only for the middle dose of thiacloprid (30.3 mU/mg protein), as well as the lowest (31.4 mU/mg protein) and the highest (30.0 mU/mg protein) doses of clothianidin (Figure 1).

3.3. Parameters of redox status

To estimate the effects of honey bees' exposure to thiacloprid and clothianidin on their redox status, we measured levels of malondialdehyde (MDA), reduced glutathione (GSH), and protein thiol (-SH) groups. Results are shown in Table II.

Exposure of honey bees to the highest concentration of both NEOCs (T3, C3) induced a significant ($p < 0.05$) increase in MDA level (1.39 and 1.49 nmol/mg protein, respectively) in comparison with control (0.84 nmol/mg protein), indicating a high level of lipid peroxidation in honey bees in these groups. GSH level significantly ($p < 0.05$) increased after exposure to the lowest concentration of thiacloprid (T1) to 14.5 nmol/mg protein and the highest concentrations of both NEOCs (T3, C3) to the levels of 13.6 and 16.1 nmol/mg protein, respectively, in comparison with control (11.1 nmol/mg protein). Treatment with thiacloprid had no effect on protein thiol groups level; however, statistically significant ($p < 0.05$) increase, was observed after exposure to lower concentrations of clothianidin (C1, C2) to the values of 175 and
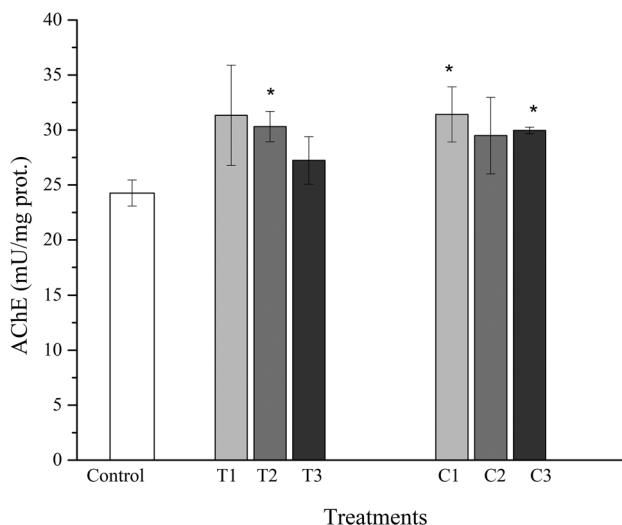

Figure 1. Activity of acetylcholinesterase (AChE) in honey bees exposed to different concentrations of thiacloprid (T1, 2.69 µg/mL ($LD_{50}/60$); T2, 6.72 µg/mL ($LD_{50}/25$); T3, 26.91 µg/mL ($LD_{50}/10$)) or clothianidin (C1, 0.55 ng/mL ($LD_{50}/50$); C2, 1.38 ng/mL ($LD_{50}/20$); C3, 5.52 ng/mL ($LD_{50}/5$)) for 48 h and control group (C). The oral acute toxicity (LD_{50}) for thiacloprid is 17.94 µg/bee and for clothianidin 3.68 ng/bee. Results are expressed as mean ± standard deviation of three biological replicas. Asterisk (*) indicates a statistically significant difference between control and individual treatments, according to Tukey test for $p < 0.05$.

Table II Parameters related to redox status in honey bees exposed to different concentrations of thiacloprid (T1, 2.69 µg/mL (LD_{50}/60); T2, 6.72 µg/mL (LD_{50}/25); T3, 26.91 µg/mL (LD_{50}/10)) or clothianidin (C1, 0.55 ng/mL (LD_{50}/50); C2, 1.38 ng/mL (LD_{50}/20); C3, 5.52 ng/mL (LD_{50}/5)) for 48 h and control group (C)

Group	Malondialdehyde MDA (nmol/ mg prot.)	Reduced glutathione GSH (nmol/mg prot.)	Protein thiol groups -SH (nmol/mg prot.)
Control	0.84 ± 0.19	11.1 ± 0.66	140 ± 9.84
Thiacloprid			
T1	0.82 ± 0.12	$14.5 \pm 1.77*$	165 ± 10.8
T2	0.67 ± 0.14	10.2 ± 1.80	145 ± 16.4
T3	$1.39 \pm 0.15*$	$13.6 \pm 1.42*$	137 ± 15.8
Clothianidin			
C1	0.90 ± 0.14	11.1 ± 0.42	$175 \pm 10.2*$
C2	0.85 ± 0.25	13.1 ± 1.28	$185 \pm 6.39*$
C3	$1.49 \pm 0.22*$	$16.1 \pm 2.10*$	170 ± 16.9

Results are expressed as mean $\pm$ standard deviation of three biological replicas. Asterisk (*) indicates a statistically significant difference comparing to the control, according to Tukey test for $p < 0.05$

185 nmol/mg protein, respectively, in comparison with the control (140 nmol/mg protein).

3.4. Activity of antioxidative enzymes

Thiacloprid had no effect on activities of SOD and CAT, while all sublethal thiacloprid concentration (T1, T2, T3) significantly ($p < 0.05$) reduced GST activity (142, 145, and 129 U/mg protein, respectively) in honey bees, compared to control (196 U/mg protein) (Fig. 2). Clothianidin significantly ($p < 0.05$) affected activity of all measured enzymes compared to control (Figure 2) by increasing the SOD activity in C1 and C3 groups (127 and 113 U/mg protein, respectively, compared to 95.3 U/mg protein in control) and CAT activity in C1 and C2 groups (223 and 217 U/mg protein, respectively, compared to 166 U/mg protein in control), while the activity of GST was reduced (148 U/mg protein), but only after exposure to the highest concentration (C3), compared to control (196 U/mg protein).

3.5. Activity of prophenoloxidase and phenoloxidase

Treatment with both NEOCs induced a statistically significant ($p < 0.05$) decrease in activity of proPOx and POx in honey bees, except for the lowest concentration of thiacloprid (T1) which did not affect these enzymes (Figure 3). Activity of POx was more affected than proPOx. Medium and the highest concentrations of thiacloprid (T2, T3) reduced the activity of proPOx by about 50%, and the activity of POx by about 66% and 87%, respectively, in comparison with control (122 mU/mg protein for proPOx and 87.1 mU/mg protein for POx). Clothianidin induced a reduction of proPOx by about 50% and POx by about 60% in all treatments.

4. DISCUSSION

In the present study, we examined the effects of three sublethal concentrations of two neonicotinoids, thiacloprid, and clothianidin, on selected parameters of oxidative status and AChE activity, as well as on proPOx and POx activities, as they are important enzymes involved in the immune response. Results indicated that both neonicotinoids' sublethal concentrations promote oxidative stress in honey bees and affect their immune defense inducing increased level of MDA, AChE activity, and reducing activities of
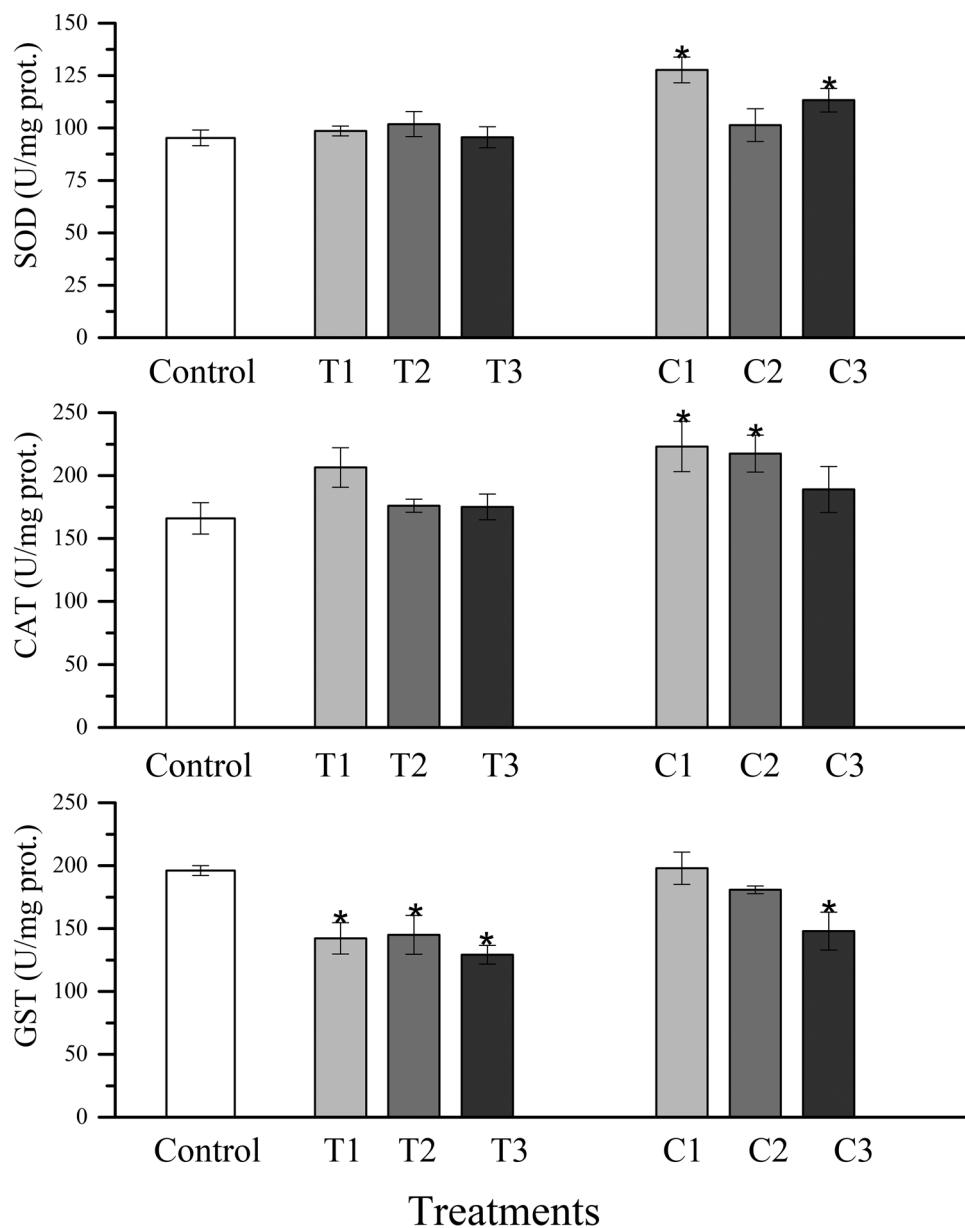
Figure 2. Activity of antioxidative enzymes–superoxide dismutase (SOD), catalase (CAT), and glutathione *S*-transferase (GST)–in honey bees exposed to different concentrations of thiacloprid (T1, 2.69 μg/mL ($LD_{50}/60$); T2, 6.72 μg/mL ($LD_{50}/25$); T3, 26.91 μg/mL ($LD_{50}/10$)) or clothianidin (C1, 0.55 ng/mL ($LD_{50}/50$); C2, 1.38 ng/mL ($LD_{50}/20$); C3, 5.52 ng/mL ($LD_{50}/5$)) for 48 h and control group (C). The oral acute toxicity (LD_{50}) for thiacloprid is 17.94 μg/bee and for clothianidin 3.68 ng/bee. Results are expressed as mean ± standard deviation of three biological replicas. Asterisk (*) indicates a statistically significant difference between control and individual treatments, according to Tukey test for $p < 0.05$.
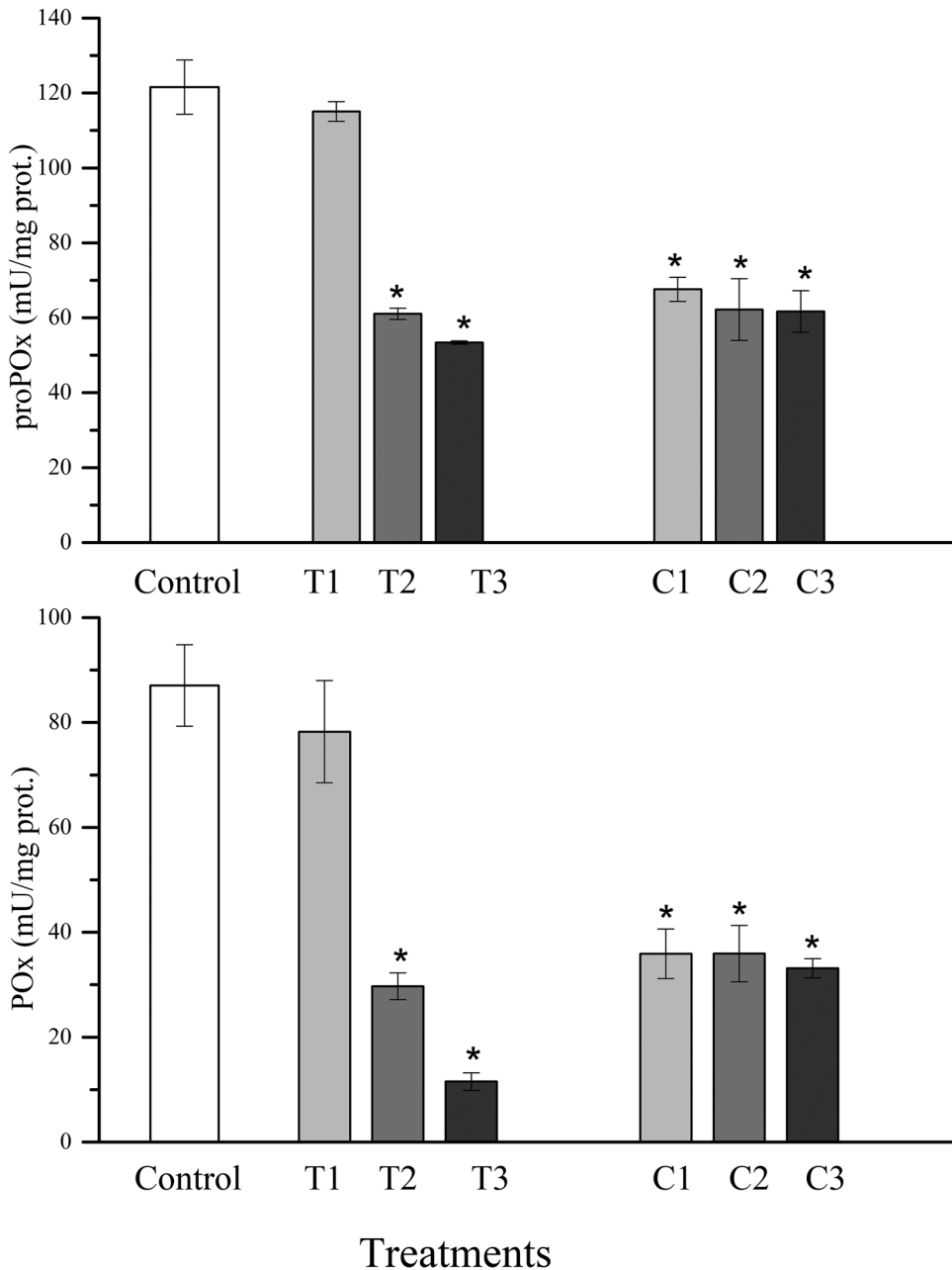
Figure 3. Activity of prophenoloxidase (proPOx) and phenoloxidase (POx) in honey bees exposed to different concentrations of thiacloprid (T1, 2.69 µg/mL ($LD_{50}/60$); T2, 6.72 µg/mL ($LD_{50}/25$); T3, 26.91 µg/mL ($LD_{50}/10$)) or clothianidin (C1, 0.55 ng/mL ($LD_{50}/50$); C2, 1.38 ng/mL ($LD_{50}/20$); C3, 5.52 ng/mL ($LD_{50}/5$)) for 48 h and control group (C). The oral acute toxicity (LD_{50}) for thiacloprid is 17.94 µg/bee and for clothianidin 3.68 ng/bee. Results are expressed as mean ± standard deviation of three biological replicas. Asterisk (*) indicates a statistically significant difference between control and individual treatments, according to Tukey test for $p < 0.05$.

proPOx and POx. Activity of SOD and CAT, as first-line antioxidative enzymes, increasing only after exposure of honey bees to clothianidin, but GST activity decreasing in all thiacloprid treated groups and after the highest concentration of clothianidin exposure of honey bees.

In our study both NEOCs, thiacloprid and clothianidin, increased activity of AChE. These results are in accordance with the study of Boily et al. (2013), which proposed that the increased activity of AChE is a potential biomarker of exposure of bees to neonicotinoids. In comparison with exposure for 10 days in chronic study of Boily et al. (2013), increased AChE activity after 48 h in our study suggests that AChE is also a sensitive biomarker of early NEOCs exposure. AChE is an enzyme involved in termination of nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine (Čolović et al. 2013). Boily et al. (2013) explained the increased activity of AChE by NEOCs occupying the binding site of acetylcholine, consequently inducing an accumulation of this molecule in the synapses, and stimulating the activity of AChE in a typical substrate-enzyme cellular response. On the other hand, similar study of Badawy et al. (2015) reported decreasing activity of AChE after 24-h oral exposure of honey bees to NEOCs, which suggested that the effect of NEOCs to AChE activity is probably time-dependent and may have a biphasic response. NEOCs or their metabolites can either entirely inhibit or just reduce AChE activity by unspecific protein binding, which was detected as a decrease in AChE activity in the honey bee after acute oral treatment for 24 h (Badawy et al. 2015). Compounded with the accumulation of acetylcholine in the synapses (Boily et al. 2013), there could be an increase in the expression and synthesis of AChE in the honey bee, which may elevate AChE activity after prolonged treatments.

Many studies predicted that oxidative stress is one of the possible mechanisms of pesticide toxicity (Badiou-Bénéteau et al. 2012; Balieira et al. 2018; Olgun et al. 2020). The results in our study clearly indicated induction of oxidative stress in honey bees after an exposure to sublethal doses of thiacloprid and clothianidin. We found that

MDA levels were significantly higher in honey bees after exposures to the highest tested doses (C3 and T3) of both NEOCs in comparison with control, and that CAT and SOD activities were increased only for clothianidin exposure. Malondialdehyde (MDA) is the end product of lipid peroxidation indicating disturbance of cell membrane as a consequence of oxidative stress (Ayala et al. 2014; Balieira et al. 2018). Our results are in accordance with Balieira et al. (2018) that reported increased MDA level about 2.5-fold after exposure of honey bee to imidacloprid, as well as increased SOD, CAT, and GPx activities. Additionally, Abdelkader et al. (2019) reported twofold MDA level increase in drone semen after their exposure to sublethal concentration of clothianidin, also followed by increased SOD, CAT and GPx activities.

Exposure of honey bees to all tested sublethal doses of thiacloprid significantly lowered GST activity, but for the clothianidin, only the highest dose (C3) significantly reduced GST activity. Also, the corrected mortality for abovementioned doses reached high values (above 5%), suggesting that decreased GST activity could be a sensitive biomarker of the adverse effects of NEOCs. GST is one of the major detoxification enzymes in honey bee, but due to glutathione peroxidase (GPx) activities, GST is also considered as part of ADS. There are many studies which reported increased GST activity as a consequence to NEOCs' exposure (Vidau et al. 2011; Badiou-Bénéteau et al. 2012; Dussaubat et al. 2016). However, Yao et al. (2018) reported no effects on GST activity in honey bee after exposure to clothianidin sublethal dose of 2.6 ng/mL. Our results are in agreement with this study because in our study lower concentrations of clothianidin (C1 and C2) did not have effect on GST activity, but only the highest one (C3). It is very interesting that a reduction of GST activity was accompanied by an increasing level of its cofactor, GSH. Many studies reported a decrease in GSH level in animal tissue after exposure to insecticides (Bebe and Panemangalore 2005; El-Gendy et al. 2010). GSH is an antioxidant and a component of non-enzymatic ADS, but also a reducing equivalent for action of GPx. Possibly,

reported increase of GPx activity (Balieira et al. 2018; Abdelkader et al. 2019) and/or decrease in GST activity induced the elevation of GSH level to protect sensitive protein thiol groups (-SH). In our study, the tested sublethal doses of thiacloprid had no effect on protein thiol groups, but lower doses of clothianidin increased protein thiols in honey bees. It could be that some protein thiols have an important role to protect honey bee to sublethal doses of clothianidin such as thioredoxin and glutaredoxin as important part of insect's thiol-redox homeostasis (Yao et al. 2014). In addition, the two tested lower doses of clothianidin in our study had no effect on MDA level, GST activity, and GSH level in honey bees; also mortality is less than 5% in these experimental groups, which could be in relation to before mentioned increasing of protective protein's thiol groups.

The exposure of honey bees to sublethal doses of thiacloprid and clothianidin reduced the activities of proPOx and POx in hemolymph of honey bee. All tested doses of clothianidin significantly decrease activity of both enzymes, but thiacloprid only for the two higher doses (T2 and T3). These results correspond to toxicity of examined NEOCs. Decreasing activities of both enzymes are in accordance with the results obtained in the study Brandt et al. (2016) that sublethal doses of NEOCs, including thiacloprid and clothianidin, affected hemocytes count, encapsulation reaction, and antimicrobial activity in honey bee. Also, the study Di Prisco et al. (2013) reported that clothianidin negatively modulates NF-κB immune signaling and adversely affects antiviral immune defenses in honey bee. It could be that the activity of POx and proPOx are under control of the signaling pathway regulated by this transcription factor. In the study of Li et al. (2017), no effect of sublethal dose of clothianidin on proPOx protein expression was observed. Reasons for this disagreement with our result could be due to the author's measurement of proPOx by ELISA assay which detects only proteins, but no enzyme activities. Also, for assay they used brain and thorax tissues. As proPOx and POx are plasma proteins in insect hemolymph, testing activities of these enzymes in hemolymph in our study is a more representative procedure. Furthermore, there are different mechanisms for control of enzyme activity, and protein expression and activity are not necessarily in direct correlation.

Results obtained in our study clearly indicate that reduced activity of enzymes involved in detoxification (GST) and immune response (proPOx and POx), as well as increased level of lipid peroxidation, could be responsible for vulnerability of honey bee to parasite and virus infection induced by their exposure to sublethal doses of NEOCs and at least partly responsible for the losses of managed honey bee colonies in broader context. Also, neural enzyme (AChE) is modulated by exposure to both NEOCs, leading to neurotransmission disturbance. It is necessary to emphasize that thiacloprid, which is classified as a less toxic NEOC than clothianidin, still has a significant adverse effect on analyzed enzymes. In order to protect the honey bees, complete restrictions of its use in the future should be considered, especially for bee foraging plants. One of the possible solutions could be the transition from a conventional chemical pesticide to use of alternative biopesticides for biological control of pests. Also, regarding the process for assessing risks of pesticides to honey bees based on determination of lethal dose, it is important to mention that an absence of mortality does not always imply a functional integrity of individual bees. In this study, we raised concerns about the NEOCs-mediated effects on enzymes responsible for regulating important physiological processes that may in turn affect the health and survival of individual bees and possibly lead to the colony collapse. Therefore, assessment of recognized honey bee biomarkers at sublethal pesticide doses should be mandatory in the review process of existing pesticides or registration of new formulations.

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AUTHOR CONTRIBUTION

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DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval   Not applicable.

Consent to participate   Not applicable.

Consent for publication   Not applicable.

Conflict of interest   The authors declare no competing interests.

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