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ORIGINAL RESEARCH ARTICLE

Effects of clothianidin on antioxidant enzyme activities and malondialdehyde level in honey bee drone semen

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Neonicotinoids can cause a variety of adverse sublethal effects in workers and queens honey bees. However, despite their key role in reproduction, drones have not received much attention on how neonicotinoids can affect their fertility. The aim of this study was to assess the influence of clothianidin exposure of drones at sexual maturity stage, on antioxidant enzyme activities, malondialdehyde (MDA) level and on protein content of semen. Our results show for the first time that clothianidin elicits significant increases in superoxide dismutase, of glutathione peroxidase, of catalase and of MDA level. Protein content in semen of drones exposed to clothianidin was significantly decreased. This study suggested that drones exposed to clothianidin at the stage of sexual maturity could induce oxidative stress in spermatozoa of drones which could affect the semen quality and therefore the queen fecundity.

Keywords: clothianidin; drones; semen; antioxidant enzymes; malondialdehyde; protein content

Introduction

Honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), have been the subject of many studies in recent years (Potts et al., 2010) due to the highly complex and significant role bees play in the ecosystem and the progressive decline in their number (Neumann & Carreck, 2010). It is thought that the impact of multiple pathogens and pesticides weaken the immunity of bees and affect their physiology and development (Frazier, Mullin, Frazier, & Ashcraft, 2008). Neonicotinoids have been the most widely used pesticides. They act as agonists to the nicotinic acetylcholine receptors (nAChRs) of insects with much higher affinity than to those of mammals. With the recent expanding use of neonicotinoids in farming, forestry, and the building industry, honey bee colony collapse disorder (CCD) has inflicted great damage on crop production in many parts of the world (Hirano et al., 2015). Neonicotinoids have thus attracted much attention as a strongly suspected agent of CCD. They were proved to disturb the foraging and homing behaviors of honey bees, weakening their colonies (Gill, Ramos-Rodriguez, & Raine, 2012; Henry et al., 2012). They are also known to cause significant oxidative stress across a wide range of animal taxa including insects (Qiao, Seidler, & Slotkin, 2005).

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds an organism's natural antioxidative defense mechanism and such imbalance may result from an overabundance in ROS-producing stressors in the environment (Velki, Kodrík, Večeřa, Hackenberger, & Socha, 2011) such as ultraviolet radiation, bacterial infections, antibiotics, and pesticides. Therefore, insects have developed a suite of antioxidant enzymes to overcome oxidative stress and to neutralize the toxicity of ROS. The value of an antioxidant enzyme system depends upon its location relative to where the oxygen radicals are generated. Semen is proved to possess a large amount of antioxidants, thereby protecting gonadal cells and mature spermatozoa from oxidative damage, especially after leaving the testicles (Huang et al., 2018). A substantial amount of researches has been done on antioxidant enzyme systems in human and insects (Buyukguzel, 2009) such as honey bees, specially workers that are susceptible to oxidative stress because of their lifestyle (Korayem, Khodairy, Abdel-Aal, & El-Sonbaty, 2012). However, there is no report exploring the oxidative stress to which drone spermatozoa are subjected to and the relative antioxidant enzyme activities when drones feed on contaminated food.

The focus of this research was to study the effect of a sublethal exposure of clothianidin on the activity of some antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP)), on malondialdehyde (MDA) level and protein content of drone's semen.

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Materials and methods

Toxicity test

Queenless and droneless nuclei composed of 5000 honey bees, one brood frame, and four empty frames with no food storage were placed in an outdoor tunnel as described by Ben Abdelkader et al. (2014). Three hundred emerging drones were introduced in each droneless nucleus and enabled for free movement in the colony. Treated and controls nuclei were placed separately in different but adjacent insect proof tunnel compartments (8×8 m) in order to control the available food. In each compartment, two nuclei were introduced. A feeder placed at equal distance from the nuclei consisted of a yellow cup containing sugar syrup with a final concentration of 50% sucrose, 0.1% DMSO (v/v), and 0.1 μg/L clothianidin for treated group. An exposure of bees to such clothianidin concentration (0.1 μg/L) is relevant considering the levels of nectar contamination by clothianidin observed in fields (EFSA, 2013; Pilling, Campbell, Coulson, Ruddle, & Tornier, 2013; Rolke, Persigehl, Peters, Sterk, & Blenau, 2016; Solomon & Stephenson, 2017; Xu et al., 2016). Controls received sugar syrup containing 50% sucrose and 0.1% DMSO. Honey bees were fed or exposed to clothianidin from 9:00 to 12:00 a.m. each day for a period of 20 days. Outside this period, honey bees received water and pollen *ad libitum*.

Semen collection and biochemical analyses

Drones were collected after 20 days and semen was gathered by a manual eversion of the endophallus. Fresh semen from each sample was transferred into a microcentrifuge tube, diluted twofold in Kiev solution and centrifuged at 4 °C for 20 min at 16,000*g*. The supernatants (diluted seminal plasma) were kept for the SOD assay. The pellets were washed twice with 100 μL of Kiev solution, resuspended in the same Kiev volume and centrifuged at 4 °C for 20 min at 16,000*g*. Supernatants were discarded and pellets containing spermatozoa were lysed with a lysis buffer (10% initial semen volume) [10 mM NaCl, 1% (w/v) Triton X-100 and 40 mM sodium phosphate pH 7.4] containing 2 μg/mL antipain, leupeptin and pepstatin A, 25 units/mL aprotinin and 0.1 mg/mL soybean trypsin inhibitor as protease inhibitors (Belzunces, Theveniau, Masson, & Bounias, 1990) to perform GP assays. The cellular debris were removed by centrifugation for 15 min at 15,000*g* before measuring CAT activity.

Biochemical analyses were performed using a TECAN Infinite® F500 plate reader. All enzyme assays were performed in triplicate at 25 °C.

Superoxide dismutase

SOD activity was indirectly measured in seminal fluid (SF) using the xanthine/xanthine oxidase system to generate O_2^- and nitro blue tetrazolium (NBT). SOD competes with xanthine oxidase and limits the generation of reduced NBT, which was followed at 560 nm. The reaction medium contained 0.1 mM EDTA, 0.1 mM xanthine, 0.025 mM NBT, 0.00833 U/mL xanthine oxidase, and 50 mM phosphate/carbonate pH 7.8.

Glutathione peroxidase

The GP catalyzes the destruction of peroxides, such as H_2O_2, by oxidizing GSH and generating H_2O and GSSG. GP activity was monitored in a reaction mixture containing lysed spermatozoa using tert-butyl hydroperoxide (TBHP) as the substrate. The generated oxidized glutathione (GSSG) was reduced by glutathione reductase (GR) in the presence of NADPH to generate GSH and NADP. The conversion of NADPH in NADP + was followed at 340 nm. The reaction medium contained 1 mM EDTA, 0.2 mM TBHP, 0.85 mM GSSG, 0.16 mM NADPH, 0.25 U/mL GR, and 50 mM Na/K phosphate pH 7.4.

Catalase

The CAT catalyzes the decomposition of H_2O_2 into oxygen (O_2) and water (H_2O) to protect cells against oxidative stress. The decomposition of H_2O_2 by CAT was followed in spermatozoa at 240 nm. The reaction medium contained 30 mM H_2O_2 and 100 mM sodium phosphate pH 7.0 (Beers & Sizer, 1952).

Malondialdehyde level

Thio barbituric acid reactive substances (TBARS) are naturally present in biological specimens and include lipid hydroperoxides and aldehydes which increase in concentration as a response to oxidative stress (Yagi, 1998). The malondialdehyde-thio barbituric acid (MDA-TBA) adducts formed by the reaction of MDA and TBA under higher temperature and acidic conditions are measured colorimetrically according to the recommendations of the manufacturer. This test was performed in PBS containing 0.5% Triton-X100 per mL. TBARS assay values are usually reported in MDA equivalent which is a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides. This test was performed with an assay kit from Cayman Chemical's (BertinPharma, France).

Protein content

Proteins were assayed according to the method of Lowry, Rosebrough, Farr, and Randall (1951). This test was performed with Bio-Rad DC Protein Assay Reagents Package Kit. Every well in the microplate contained 2.5 μL of diluted semen, 25 μL of DC™ Protein Assay Reagent A, and 200 μL DC™ Protein Assay

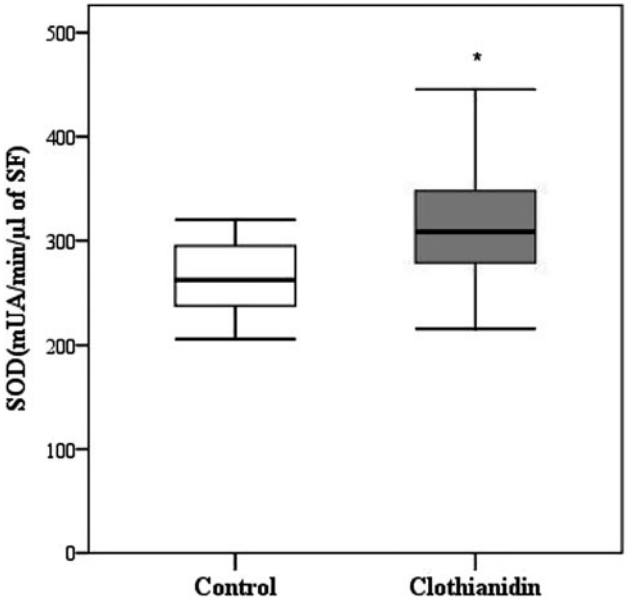

Figure 1. Assessment of superoxyde dismutase activity (SOD) (milli units of absorbance.min^{-1}.µL^{-1} of seminal fluid) in seminal fluid of drone honey bees (*Apis mellifera*) chronically exposed to clothianidin for 20 days after emergence compared with controls. All boxplots show the inter-quartile range (box), the median (black line within box), and data range (horizontal black lines from box). A significant difference between treatment groups is indicated by $^{*}P < 0.05$.

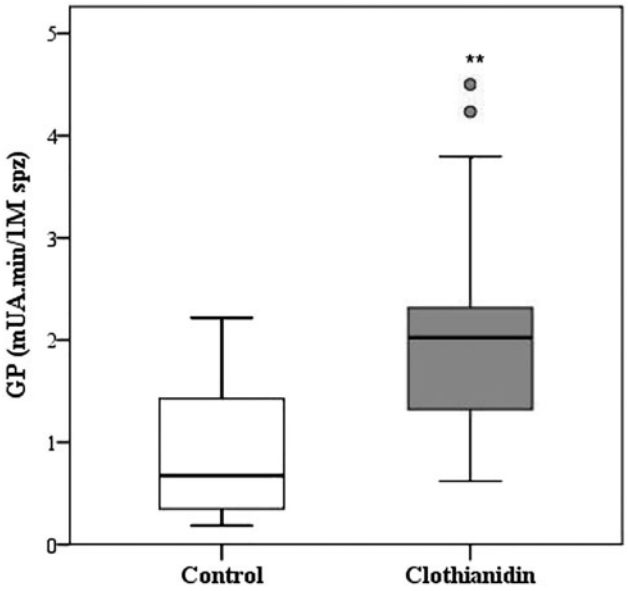

Figure 2. Assessment of gluthatione peroxidase activity (GPx) (milli units of absorbance.min^{-1}.million^{-1} of spermatozoa) in spermatozoa of drone honey bees (*Apis mellifera*) chronically exposed to clothianidin for 20 days after emergence compared with controls. All boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from box), and outliers (black dots). A significant difference between treatment groups is indicated by $^{**}P < 0.01$.

Reagent B. The microplate was kept in darkness for 20 min. Absorbance was read at 750 nm.

Statistical analysis

The statistical analysis was carried out using a generalized linear mixed model to assess the statistical significance of the differences between the exposed group and control. All data are expressed as mean ± standard error (SE). The level of significance was taken as $P < 0.05$. All statistical analyses were performed using an SPSS V. 16.

Results

Physiological markers related to antioxidant defense (SOD, CAT, and GP) were tested in drone semen. The SOD activity was determined in SF whereas the CAT and GP activities were determined in spermatozoa. After sublethal chronic exposure to clothianidin, a significant increase was observed in SOD activity (314 ± 56.7 mUA.min^{-1}.µL^{-1} of SF, $P < 0.05$, Figure 1). GPx (Figure 2) and CAT (Figure 3) activities were also significantly higher in spermatozoa of drones exposed to clothianidin. They were determined to be 2.11 ± 1.2mU.min^{-1}.10^{-6}of spz ($P < 0.01$) and 226.69 ± 91 mU.min^{-1}.10^{-6} of spz ($P < 0.05$) respectively.

To address our hypothesis that chronic clothianidin exposure resulted in increased oxidative stress, we measured MDA concentration, a marker of lipid peroxidation. In fact, our results showed a statistically

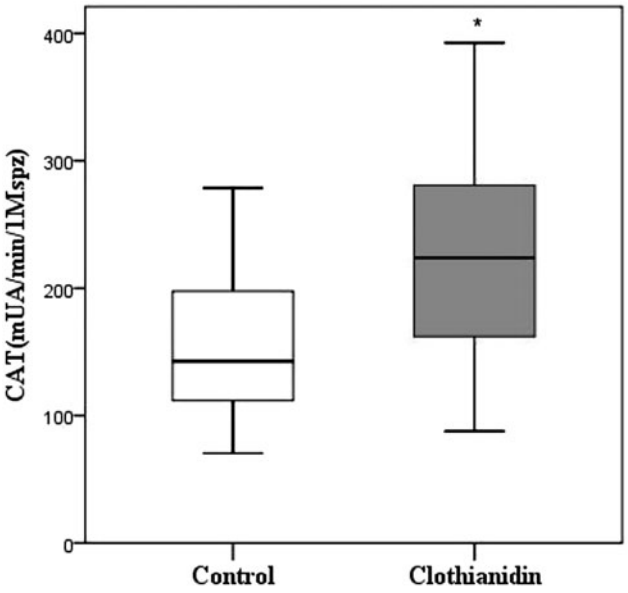

Figure 3. Assessment of catalase activity (CAT) (milli units of absorbance.min^{-1}.million^{-1} of spermatozoa) in spermatozoa of drone honey bees (*Apis mellifera*) chronically exposed to clothianidin for 20 days after emergence compared with controls. All boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from box), and outliers (black dots). A significant difference between treatment groups is indicated by $^{*}P < 0.05$.

significant increase in the MDA level in semen of drones exposed to clothianidin (0.24 ± 0.09 µM, $P < 0.05$, Figure 4).

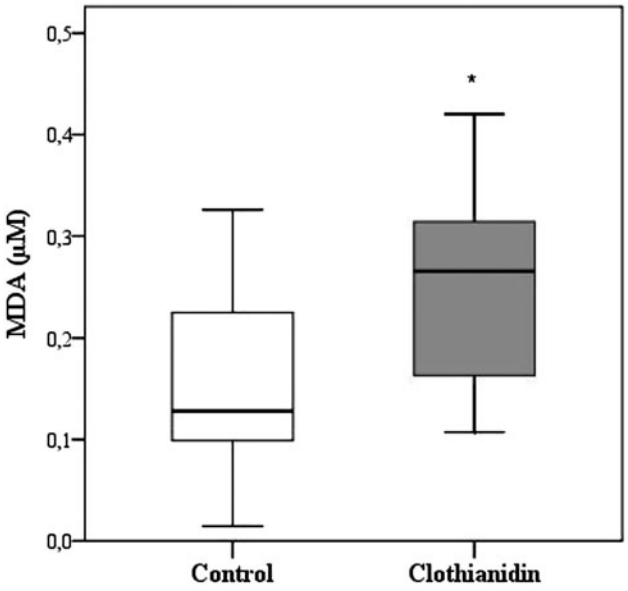

Figure 4. Assessment of malondialdehyde level (MDA) (µM) in spermatozoa of drone honey bees (*Apis mellifera*) chronically exposed to clothianidin for 20 days after emergence compared with controls. All boxplots show the inter-quartile range (box), the median (black line within box), and data range (horizontal black lines from box). A significant difference between treatment groups is indicated by $^{*}P < 0.05$.

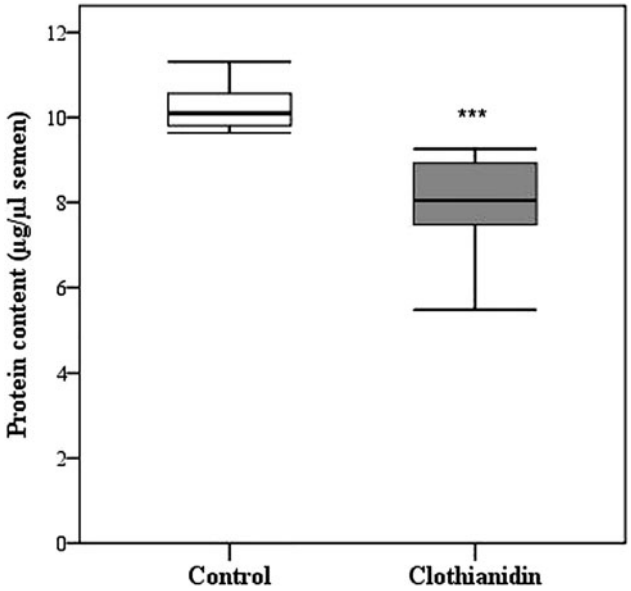

Figure 5. Assessment of protein content (µg. μL^{-1} of semen) in semen of drone honey bees (*Apis mellifera*) chronically exposed to clothianidin for 20 days after emergence compared with controls. All boxplots show the inter-quartile range (box), the median (black line within box), and data range (horizontal black lines from box). A significant difference between treatment groups is indicated by $^{***}P < 0.001$.

Seminal antioxidant activity is also supplemented by non-enzymatic antioxidants in semen present in the form of vitamin C, vitamin E, beta carotenes, carotenoids, flavonoids, and metal binding proteins. Protein content in the present study was affected by clothianidin exposure. It was significantly reduced in semen of exposed group ($P < 0.001$, Figure 5).

Discussion

Honey bee colonies are often exposed to neonicotinoids through contaminated nectar and pollen stored and subsequently shared among nestmates including drones (DeGrandi-Hoffman, Chen, & Simonds, 2013). Neonicotinoids are known to have strong oxidizing properties that affect the reproductive and central nervous systems (Duzguner & Erdogan, 2012) by producing ROS, leading to oxidative stress and alterations in radical scavenging enzymes in insects (Buyukguzel, 2006; Felton & Summers, 1995). However, organisms have mechanisms to counteract the effect of free radicals generated by cell metabolism. The most important antioxidant enzymes are SOD, CAT, GPx, and GST. SOD is one of the first enzymatic line of antioxidant defenses. It scavengers both intracellular and extracellular superoxide radical and prevents the lipid peroxidation of the plasma membrane (Atig et al., 2012). GP could also protect the sperm against oxidative damages. It plays an important role in sperm maturation from the early events up to the onset of fertilization (Eskiocak et al., 2005). The presence of CAT has been demonstrated in the semen of drones (Weirich, Collins, & Williams, 2002). It has a protective role in the aging process and in the control of oxidative stress in cells, mainly resulting from H_2O_2 (Bucak et al., 2007). Our study clearly demonstrates that clothianidin can have significant sublethal effects on honey bee drones. These toxic effects of clothianidin may be induced by oxidative stress, as clothianidin appeared to increase the activity of antioxidant enzymes SOD in seminal plasma and GPx, and CAT activities in spermatozoa. In honey bee antioxidants enzymes have been proposed to reduce oxidative risk during sperm storage (Weirich et al., 2002). Therefore, higher SOD activity in semen of exposed drones indicates that SOD makes a significant contribution to the protection of drone spermatozoa against peroxidative damage. The increase in GP activity in spermatozoa could be a result of an up-regulation triggered by a chronic oxidative stress (Espinoza et al., 2008). In fact, it has been showed that the exposure to sublethal concentrations of cyclodiene insecticides increased the GP activity in CHO-K1 cells (Bayoumi et al., 2001). The highest CAT activities could provide protection against H_2O_2 generated internally or entering the cells by diffusion (Weirich et al., 2002). Antioxidant enzymes constitute a mutually supportive team of defense against ROS. Thus, SOD, CAT, and GP should be conjugated in order to prevent the action of hydrogen peroxide (Atig et al., 2012). Environmental contaminants, as for this study the exposure to clothianidin, could affect the balance between the extent of free radical production and antioxidant capacity of the semen and provoke an excessive production of ROS

that is effectively scavenged by endogenous antioxidant defense system (Jamieson, 1989) of the spermatozoa and surrounding seminal plasma. Therefore, the semen quality and sperm fertilizing ability could be affected by a disruption of this balance (Partyka, Łukaszewicz, & Niżański, 2012).

The occurrence of this stress is demonstrated also by the increase in MDA level spermatozoa of drones exposed to clothianidin which may cause changes in the sperm and diminish fertility (Hsieh, Chang, & Lin, 2006). Pesticides such as dimethoate induce significant oxidative damages in spermatozoa as evidenced by increased MDA levels (Ben Abdallah, Fetoui, Zribi, Fakfakh, & Ammar-Keskes, 2012). The increase in MDA concentration is probably due to the clothianidin-induced excessive production of ROS and consequently elevated lipid peroxidation. According to Buyukguzel (2006), MDA is an indicator of lipid peroxidation and will also react with DNA, protein, and other biomolecules leading to oxidative damage.

Oxidative stress is one of the major causes of defective sperm function and not only disrupts the integrity of sperm DNA but also limits the fertilizing potential of these cells as a result of collateral damage to proteins in the sperm plasma membrane (Aitken, Smith, Jobling, Baker, & De Iuliis, 2014). In fact, several SF enzymes detected in the SF proteomes of honey bees (Baer, Heazlewood, Taylor, Eubel, & Millar, 2009) are predicted to protect sperm from microbial attacks or by reducing oxidative stress in the sperm. However, in our study protein content of drone exposed to clothianidin was lower than that of the control group. Protein concentration in semen can be influenced by external factors such as seasonal changes (Muino-Blanco, Perez-Pe, & Cebrian-Perez, 2008). Tyszkiewiczl (2002) found also that protein concentration in seminal plasma of boar decrease with the age of boar. Therefore, the decreased protein content could be a response of an oxidative stress caused by clothianidin exposure.

Conclusion

In conclusion, this study is the first report investigating the effect of exposure to clothianidin, via contaminated food brought to the hive by foragers, on the antioxidant status of honey bee drone semen. The increase in antioxidant enzymes, MDA concentration, and the decrease in protein content in semen report that spermatozoa of drones exposed to clothianidin could be under an oxidative stress. These results reveal for the first time that the exposure of drones to neonicotinoid insecticides during the sexual maturity could induce an oxidative stress to the drone spermatozoa and could have serious consequences for the life cycle of an *A. mellifera* colony by transmitting poor quality semen. The results introduce a new perspective on the studies on drone fertility impairment that could lead to the decrease in queen performances.

Disclosure statement

No potential conflict of interest was reported by the authors.

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