Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Evidence of diet supplementation with vitamin C protecting honeybees from Imidacloprid induced peroxidative damage: a study with *Apis cerana indica*

Syama P. S., Sreeranjit Kumar C. V.

P. G. and Research Department of Zoology, Govt. Victoria College, Palakkad, Kerala, India

Article History

Edited by
Evandro Nascimento Silva, UEFS, Brazil
Received 01 February 2022
Initial acceptance 23 May 2022
Final acceptance 17 June 2022
Publication date 07 September 2022

Keywords
Neonicotinoids, Ascorbic acid, Antioxidant enzymes, Peritrophic membrane, Honey bees.

Corresponding author
Syama P. S.
P. G. and Research Department of Zoology, Govt. Victoria College Palakkad, Kerala.
E-Mail: syamaktp@gmail.com

Abstract

Neonicotinoids are one of the major stresses contributing to the decline in the population of honeybees. Worker bees are prone to various stress factors during foraging and are susceptible to Imidacloprid due to the reduction in the number of genes encoding for the major enzyme families responsible for the detoxification of toxins. The present study worked on the hypothesis that the dietary supplementation of Ascorbic acid (VIT C) could reduce the peroxidative damage in the worker bees of Apis cerana indica exposed to sub-lethal concentration of imidacloprid (IMD). Furthermore, we also evaluated the role and efficacy of VIT C supplementation on the cytoarchitecture of midgut tissues on exposure to IMD. Colonies of honeybees were maintained by providing sugar syrup to the control group and sugar syrup supplemented with 0.2% VIT C for the experimental group for six months. Worker bees from both groups were randomly collected and exposed to 0.001 mg/mL IMD. To study the peroxidative damage, the activities of various enzymes were analyzed. The activities of antioxidant enzymes including Catalase, Superoxide Dismutase, Glutathione S Transferase, and Glutathione Peroxidase in the hemolymph and midgut tissues of worker bees were significantly decreased due to exposure to IMD as a single agent. However, their activities showed a significant elevation under diet supplementation with VIT C. Histological examination revealed midgut tissue damage and the rupture of peritrophic membrane among the workers exposed to IMD as compared with the control group. The damage to the midgut was alleviated and the peritrophic membrane was found to be intact in the worker bees supplemented with VIT C. Our results indicated that the dietary supplementation of VIT C has the potential to maintain the redox status and thereby can offer protective potential against the peroxidative damages induced by the sub-lethal concentration of IMD.

Introduction

Multiple interacting factors including exposure to pesticides, attack of parasites and pathogens and habitat loss have been suggested as chronic sub lethal stress associated with the decline in the population of honeybees (Bryden et al., 2013). A group of neurotoxic insecticides, the neonicotinoids, has been singled out from all the stress factors due to its wide use in crop protection (Godfray et al., 2014). Neonicotinoids are synthetic analogs of nicotine which mimics the action of acetylcholine, the main excitatory neurotransmitter in the brain of honeybees (Casida et al., 2013). Neonicotinoids, which comprise imidacloprid, acetamiprid, clothianidin, thiomethoxam, thiacloprid, dinotefuran and nitenpyram were extensively used in seed treatments, soil applications and as foliar sprays to control crop pests (Blacquiere et al., 2012). They target nicotinic- Acetylcholine receptors (**nAChR**) and disrupt the functioning of the central nervous system

by overstimulation (Matsuda et al., 2001). This group of pesticides may cause behavioral problems in honeybees such as defective or delayed communication, navigation, homing and foraging (Henry et al., 2012) and adversely affect their immunity (Di Prisco et al., 2013). As these are systemic insecticides, their traces are found in nectar and pollen, bee products like bee bread, honey and beeswax (Blacquiere et al., 2012).

Imidacloprid (IMD) is widely used against insect pests because of its diverse application methods and lower toxicity to non-target organisms (Medrzycki et al., 2003). IMD and its metabolites were detected in pollen, honey samples, and from different honeybee body parts such as hemolymph, midgut, thorax and rectum (Suchail et al., 2004). Sub lethal effects manifested in bees exposed to IMD include inhibition in associative learning (Decourtye et al., 2004), abnormal foraging behavior (Yang et al., 2008), reduction in mobility and loss of communicative ability (Medrzycki et al., 2003). Considering the effect on metabolism, IMD impairs brain metabolism (Decourtye et al., 2004) and reduces mitochondrial activity (Nicodemo et al., 2014).

The midgut of honeybees is highly sensitive to poisonous substances (Bielenin & Ibek, 1980) and malnutrition (Szymas & Przybyl, 2007) due to the absence of chitinous lining. The midgut lumen has a protective covering called peritrophic membrane (PM) that surrounds the food (Snodgrass, 2018). In the lumen, PM acts as a semipermeable membrane and protects the midgut epithelium from mechanical damage, attack of pathogens (Lehane et al., 1997) and from toxic substances (Bielenin & Ibek, 1980). Metabolic resistance is one of the principal mechanisms used by insects to escape the adverse effects of natural and synthetic toxins (Esther et al., 2015). Organisms are endowed with a wide variety of endogenous antioxidative enzymes that protect cells from peroxidative damage, such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GSR) and Glutathione S-transferase (GST). As the honeybees have a lower number of genes coding for antioxidant proteins in their genome, the ability of honeybees to defend against ROS is strongly limited as compared to other insects (Corona et al*.,* 2006). The antioxidant enzymes in honeybees are efficient in detoxification of ROS and SOD acts as the first line defense against oxygen free radicals (Corona et al., 2006). H_2O_2 is eliminated by the action of CAT (Aebi, 1984) and GPx (Mannervik, 1985). GST conjugates the toxic compounds to glutathione to facilitate their removal from the tissues (Hinton et al., 1995; Jakoby, 1985; Lee, 1991).

Vitamin C is an important water-soluble antioxidant which can neutralize ROS and reduce oxidative stress (Verma et al., 2007). It can work from both inside and outside the cells to combat free radical damage and may act as a source of electrons to free radicals such as hydroxyl and superoxide radicals in order to quench their reactivity (Bendich et al., 1990; Bindhumol et al., 2003). Earlier studies showed that the dietary supplementation of VIT C positively influences the antioxidant defense and in turn reduces the winter mortality rate (Farjan et al., 2012; Andi & Ahmadi, 2014). The present study was designed to examine the protective effect of dietary supplementation of Ascorbic acid on the antioxidant defense system and histological alterations in the midgut of honeybee workers under IMD exposure.

Materials and methods

1. Experimental Protocol

1.1. Field colonies used in the experiments

The honeybee colonies of *A. cerana indica* used in the experiment were maintained in a domestic garden (Palakkad District, latitude-10.89, longitude-76.4). Experimental colonies were provided with 0.2 % VIT C in 250 ml sugar syrup (1:1 sugar and water) while the control colonies were provided with 250 ml sugar syrup alone. They were fed once in a week, throughout the experiment over a period of six months. The colonies were periodically examined for the viability of the colony.

1.2. Laboratory Experiment

Worker bees were randomly collected from the brood of control colonies and were divided into two groups. Out of this one group was maintained as control by providing sugar syrup alone (**Control)** and the other group was administered with a sub lethal concentration of 0.001 mg/mL imidacloprid along with sugar syrup (**IMD**). Similarly, the vitamin C supplemented bees were also grouped into two. Out of this one group was maintained as control with vitamin C supplementation (**VIT C Control**) and the other group was administered with imidacloprid at a sub lethal concentration of 0.001 mg/mL **(VIT C + IMD**) and all the groups were left undisturbed for an hour after treatment (the sub lethal concentration of IMD was achieved by evaluating LD_{50} as per the guidelines mentioned in OECD, 1998).

2. Enzyme assays

Ten worker bees were randomly selected from each treatment, and they were anesthetized by keeping them at 4 ºC for five minutes. The hemolymph was collected using microcapillary tubes after incising it dorsally between the 5th and 6th abdominal segment by using a sterile needle. The midgut was dissected out according to the protocol described by Carreck et al. (2013). They were pooled and homogenized in saline solution, centrifuged at 10,000 rpm for 20 minutes at 4 ºC, then the supernatants were decanted and used for enzyme assays. The enzymatic activity of Catalase (Luck, 1974), Peroxidase (Reddy et al., 1995), Superoxide dismutase (Paoletti et al., 1986), Glutathione S-transferases (Habig et al., 1974), Glutathione peroxidase (Rotruck et al., 1973), Glutathione Reductase (David & Richard, 1983) of the hemolymph and the midgut tissues were determined. The experiment was repeated three times.

EXPERIMENTAL DESIGN

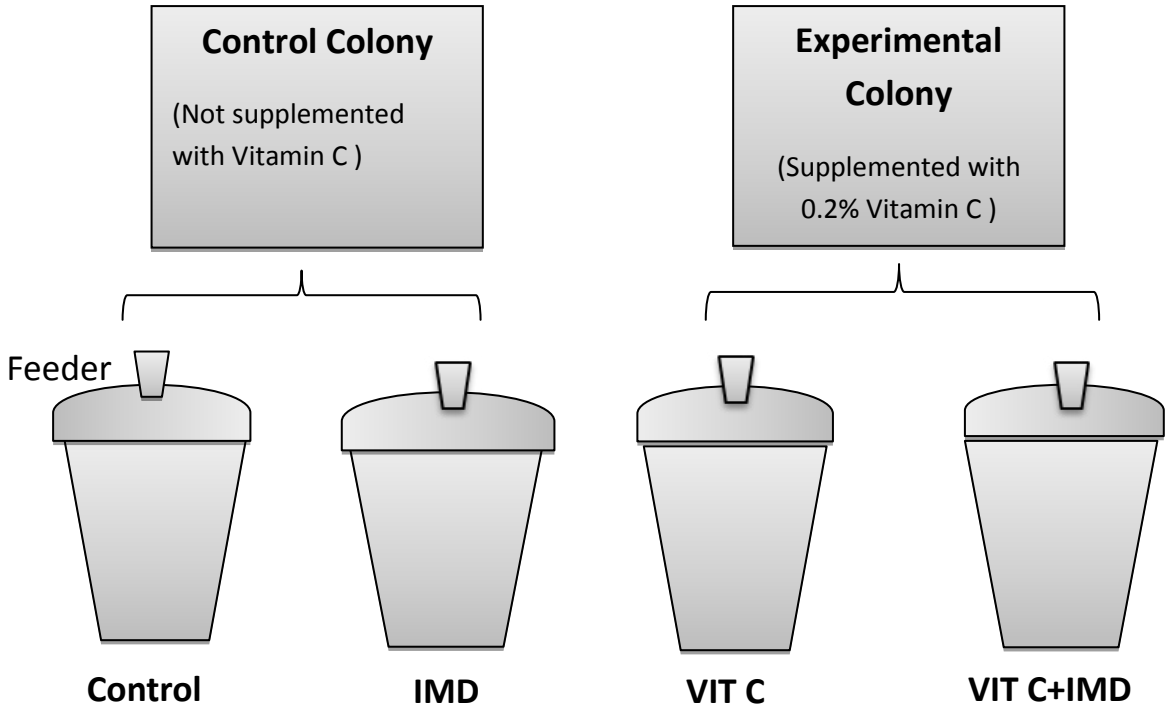

Fig 1. **Control**: Workers from control colony, **IMD**: Workers from Control colony exposed to **IMD** at 0.001mg/mL, **VIT C**: Workers from Experimental colony, **VIT C + IMD**: Workers from Experimental colony exposed to IMD at 0.001mg/mL.

3. Histological analysis

The midgut of worker bees from each treatment were dissected out and fixed in Bouin's fixative for the histological studies. Serial sections were dewaxed and dehydrated using different grades of alcohol and were double stained with Hematoxylin and Eosin. The histological analysis was done and photographs were taken by using a **LEICA DM 750** light microscope. Scaling and labelling were done by using Image J software.

4. Statistical analysis

A probit analysis was done for the LD_{50} calculation. Significant differences among treatments were identified by One Way ANOVA and pairwise analysis was carried out using DMRT (Duncan Multiple Range Test) at 5% ($p < 0.05$) level of significance with statistical software 'R' Version 4.1.1.

Results

1.Enzymes of Antioxidant System

1.1. Catalase activity

The exposure of honeybees to IMD significantly inhibited catalase activity in the hemolymph and midgut, since in this treatment the CAT activity was 4.7 ± 2.5 and 57.9 ± 13.1, respectively. There was a clear decrease in the CAT activity in comparison to the control (12.06 ± 3.38 and 101.1 ± 11.8, for hemolymph and midgut, respectively). When the diet was enriched only with VIT C, the CAT activity (34.9 ± 5.9 for hemolymph and 146.2 ± 0 for midgut) presented the highest and statistically significant effect. Finally, the supplementation of VIT C in the diet of honeybees exposed to IMD contributed to suppress the negative effects the insecticide, reestablishing the CAT activity to levels higher than in the control for the hemolymph and equal to the control for the midgut (Fig 2).

1.2. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was assessed in both hemolymph and midgut (Fig 3). No significant change ($p \geq 0.05$) was observed in the activity of SOD in the hemolymph of worker bees, when they were exposed to IMD (2.0 ± 0.005) compared to control (2.0 ± 0). In the midgut tissues, the IMD treatment resulted in a significant ($p \leq 0.05$) decrease of enzyme activity (2.01 ± 0.008). A significant increase in the SOD activity in the hemolymph and midgut of worker bees was noticed in VIT C supplemented treatment (2.06 ± 0.008 for hemolymph and 2.04 ± 0.02 for midgut) against the control (2.0 ± 0 for hemolymph and 2.03 ± 0.01 for midgut). When worker bees were exposed to IMD but the diet was supplemented with VIT C, the SOD activity was found to

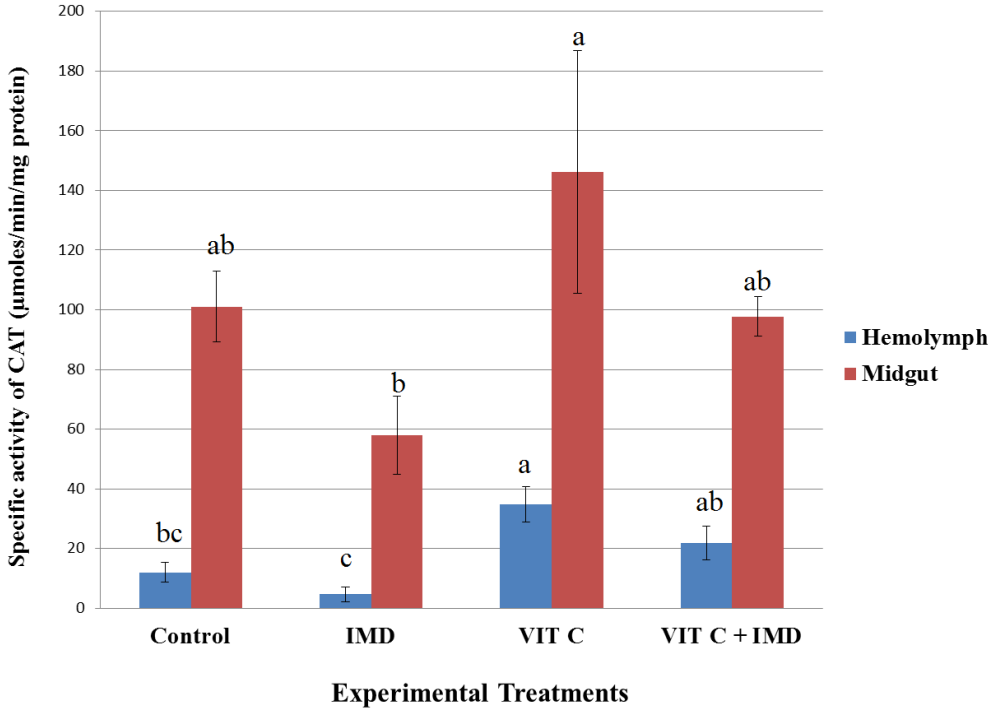

Fig 2. Specific activity of CAT in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

be increased significantly in the hemolymph (2.03 ± 0.01) when compared to control, but no significant difference was observed in the midgut in comparison to the control.

1.3. Peroxidase activity

Peroxidase (POD) activity was assessed both in the hemolymph and midgut tissues of worker bees (Fig 4). No significant difference ($p \geq 0.05$) was recorded in the activity of POD in the hemolymph of worker bees from treated groups and control. An increase of activity was recorded in IMD fed bees (2846 ± 1223), but no significant difference ($p \geq 0.05$) was found in other treatments. The IMD treatment resulted in a significant elevation ($p \leq 0.05$) of enzymatic activity in the midgut of worker bees (11660 ± 1725) as compared to the control treatment (2837 ± 18.02). A significant decrease of POD activity was observed in the midgut of worker bees from VIT C treatment (3055 ± 212.3) and VIT C + IMD treatment (5670 ± 515) when compared to IMD treatment (11660 ± 1725).

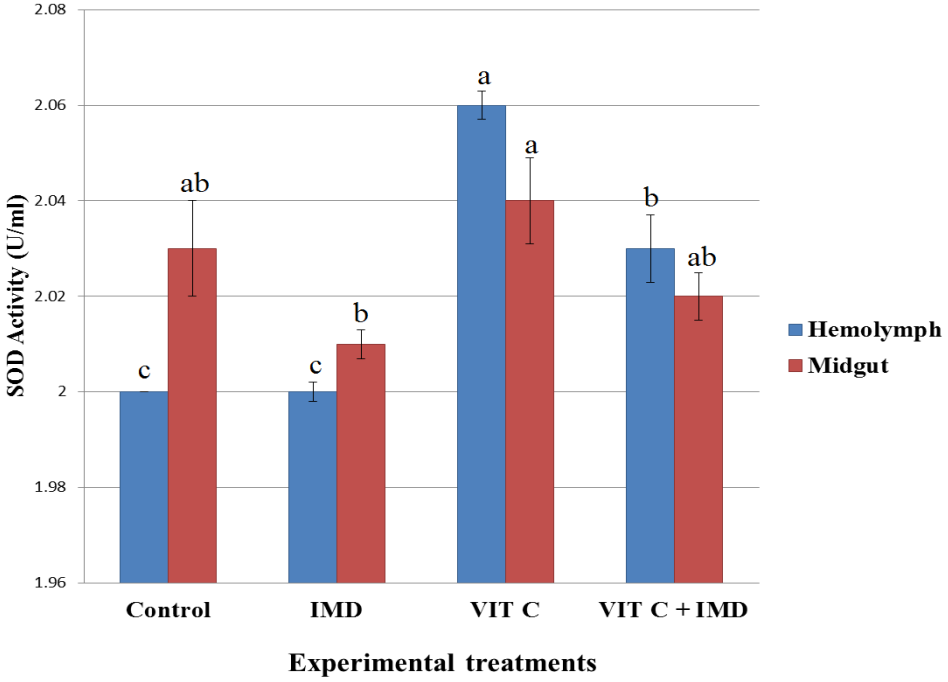

Fig 3. Specific activity of SOD in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

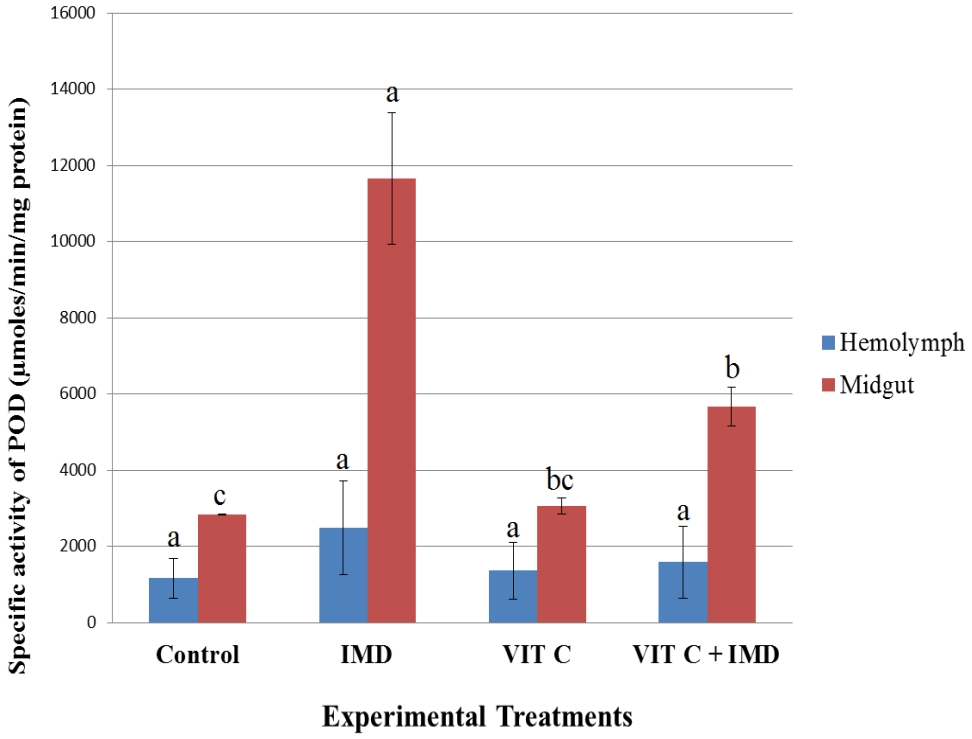

Fig 4. Specific activity of POD in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

1.4. Glutathione S-transferases activity

Statistical analysis performed on the data showed that worker bees in the IMD treatment had a significantly ($p \leq 0.05$) lower GST activity both in the hemolymph (2.24 ± 0.6) and midgut (0.7 ± 0.3) compared to the control (6.4 5± 1.0 and 1.01 ± 0.3). The worker bees from the VIT C treatment (11.4 ± 2.1 and 2.0 ± 0.3) and VIT C + IMD treatment (7.44 ± 0.9 and 2.6 ± 0.3) had a significantly ($p \leq 0.05$) higher GST activity both in hemolymph and midgut as compared with the control (Fig 5).

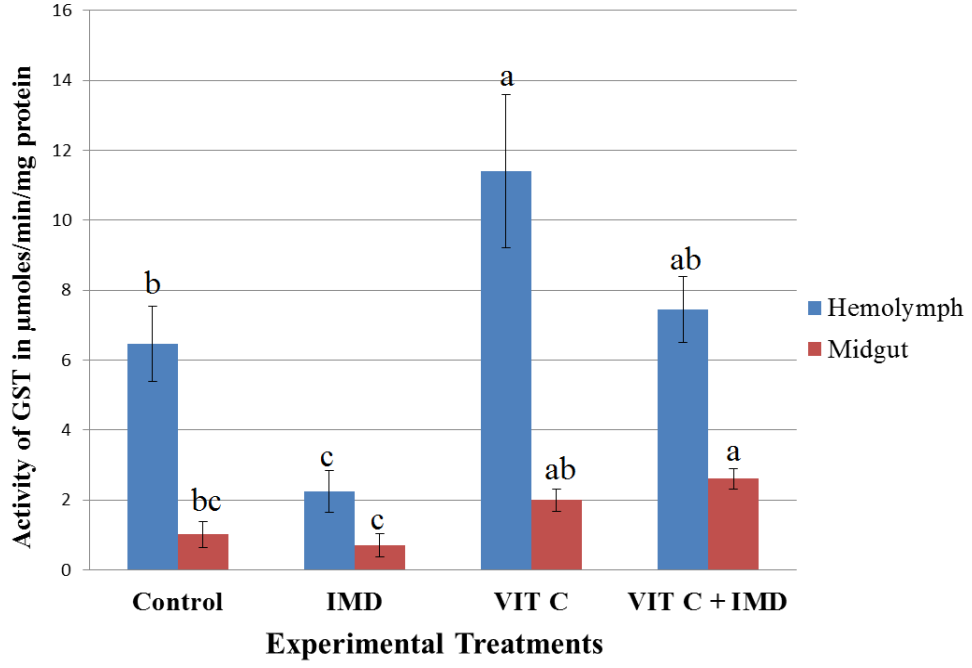

Fig 5. Specific activity of GST in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

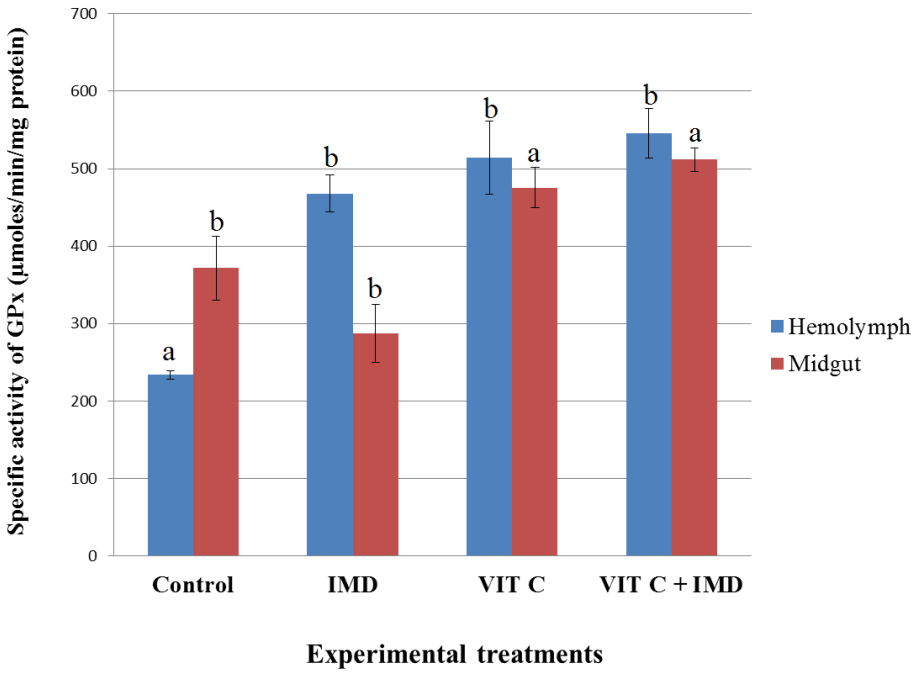

Fig 6. Specific activity of GPx in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

1.5. Glutathione peroxidase activity

The activity of the enzyme Glutathione peroxidase was measured in the hemolymph and midgut of worker bees (Fig 6). The activity of this enzyme in the hemolymph was significantly higher with the treatments with exposure to IMD and/or supplementation with VIT C, in comparison with the control. Regarding midgut tissues, the exposure of honeybees to IMD did not affect the enzyme activity. However, the supplementation of the diet with VIT C alone or combined with the exposure of honeybees to IMD significantly increased the enzyme activity.

1.6. Glutathione Reductase activity

GSR activity was measured in the hemolymph and midgut of worker bees (Fig 7). The statistical analysis performed on all data showed an effect of VIT C in increasing enzyme activity in the hemolymph (0.76 ± 0.09) of worker

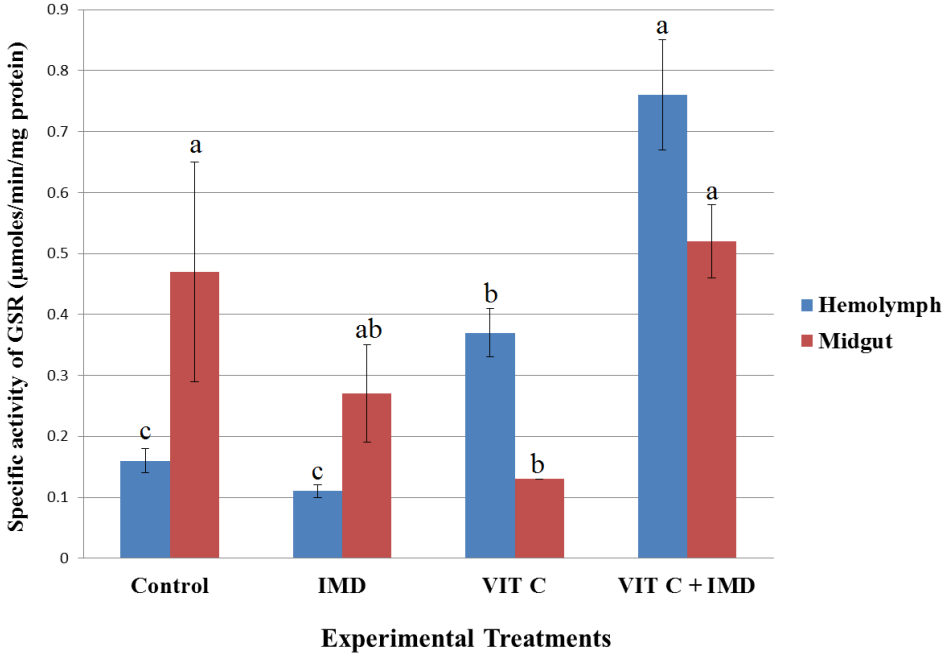

Fig 7. Specific activity of GSR in the hemolymph and mid gut tissues of worker bees (treatment means obtained with n = 6). Significant differences with p-value ≤ 0.05 are marked with different letters.

bees in comparison to the control (0.16 ± 0.02). The activity of the enzyme was found to be reduced when the worker bees were exposed to IMD.

2. Histological analysis

The histological analysis of the midgut of honeybees from the Control (Figure 8-A) showed a typical morphology of epithelium which contains a thin gelatinous layer, multiple layers of peritrophic membrane (PM) and a wide lumen between PM and epithelium. In some regions, the midgut epithelium was higher and, in some places, it was not well defined. At the base of the epithelium, the nuclei are surrounded with lighter areas (hallo's) with vacuolated cell cytoplasm. Pollen grains were surrounded by numerous layers of peritrophic membranes. Tearing of the PM from the gut epithelium was clearly visible in some regions. Intestinal lumen is wider in the control group.

In the Vitamin C supplemented group (Figure 8-B), the analysis of the midgut showed a well-defined epithelium with a thick gelatinous layer. The nuclei were covered with hallo's and the cell cytoplasm was less vacuolated. The epithelium was higher in some places and the epithelial folds were not well defined. Pollen grains present in the midgut region were covered with multiple layers of peritrophic membranes. Tearing of the peritrophic membrane was seen at some regions. Intestinal lumen was slightly wide.

Cyto-architectural analysis of the midgut of bees in the IMD treatment (Figure 8-C) showed adverse effects, where the peritrophic membrane was totally ruptured and the midgut contents were dispersed in the lumen. The gut epithelium was degenerated and without the gelatinous matrix, and the cells had large vacuoles.

The severity of the damage to the histological architecture was comparatively less on VIT C +IMD treatment (Figure 8-D). The thickness of the epithelial lining was found to be the same as in VIT C treatment, but large vacuoles were present in the epithelium. The peritrophic membrane was found intact, degeneration of epithelial cells and loss of gelatinous matrix were not observed.

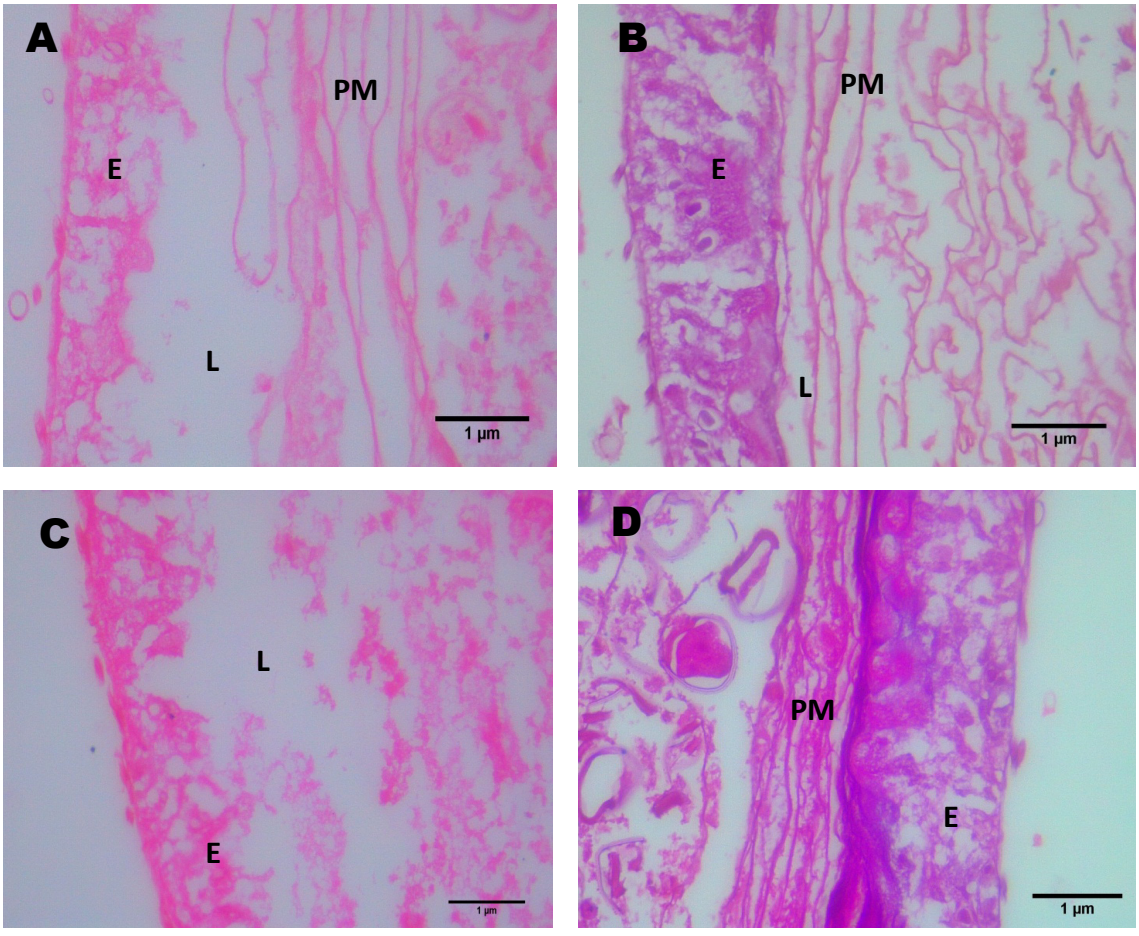

Fig 8. Histology of midgut cells of worker honeybee (***A. cerana indica***) stained with H & E. **A-D** includes, **A**. midgut cells of honeybee workers received sugar syrup presenting thin gelatinous layer of epithelium with wide lumen and intact peritrophic membrane. **B**. midgut cells of honeybee workers supplemented with Vitamin **C** showing well defined epithelium, slightly wide lumen and multiple layers of peritrophic membrane. **C**. midgut cells of honeybee workers exposed to **IMD** showing degenerated epithelial cells and completely ruptured peritrophic membrane. **D**. midgut cells of ascorbic acid supplemented worker bees exposed to imidacloprid presenting thick epithelium and multi layered peritrophic membrane. **E** Epithelium; **PM** Peritrophic Membrane; **L** Lumen.

Discussion

Oxidative stress refers to an imbalance in the redox status of the body due to the overproduction of free radicals beyond the capability of the antioxidant defense system to neutralize it. The uncontrolled scenario of free radical production may pose a serious threat to the survival of the body tissues due to the damage of the vital cellular components such as the membranes, lipids, nucleic acids, and proteins (Hodgson & Smart, 2001). Aerobic organisms are endowed with protective mechanisms comprising enzymatic and non-enzymatic antioxidants that are habitually efficient in blocking the detrimental effects of free radicals, including ROS.

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are the key components to maintain the redox balance since they scavenge the excess of free radicals and can be the first line of defense against various types of such oxidative radicals. SOD and CAT are the enzymes which act as subsequent constituents in the antioxidant response system of the cells. CAT converts superoxide ($O2^-$) into H_2O_2 and then SOD converts the so formed H_2O_2 into H_2O and O_2. Various enzymatic free radical scavengers including catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), peroxidase, and superoxide dismutase (SOD) are reported to be present in vast quantities in bees (Weirich et al., 2002; Strachecka et al., 2014; Strachecka et al., 2017). The elevation in the levels of antioxidant enzymes may therefore be an important indication of an organism's endeavor to counter an induced oxidative stress.

The present study brings forth new insights to the capability of dietary supplementation of Vitamin C in diminishing the peroxidative damages generated by IMD. The specific levels of antioxidant enzymes in the honeybee *A. cerana indica* were studied to understand the tolerance and detoxification strategies against IMD toxicity. The present observations indicate that the exposure of honeybees to IMD had a significant effect on the activities of antioxidant enzymes. The activation of antioxidant enzymes observed in the present study is supposed to be a defensive mechanism to scavenge the excess ROS to alleviate the adverse effects induced by the pesticide.

The primary function of catalase enzymes is the conversion of hydrogen peroxide (H_2O_2) into H_2 and O_2. H_2O_2 is one of the most stable reactive oxygen species and plays a key role in the pathologies of numerous diseases, including Alzheimer's disease. Catalase is an antioxidant enzyme found in many cell types and is constituted by several sulf-hydryl groups (mainly of the amino acid, cysteine) in its internal structure. Neutralization of free radicals by this antioxidant is through the expenditure of its sulf-hydryl groups (Deisseroth & Dounce, 1970; Chance et al., 1979; Egaas et al., 1999; Milton, 2004; Enayati et al., 2005; Kirkman & Gaetani, 2007). In the present investigation we observed a significant reduction in the activity of catalase enzyme in both the midgut and hemolymph regions of worker bees exposed to IMD.

Down regulation of catalase activity is coupled with augmented defenselessness to oxidative stress. Uncontrolled free radical production may be due to the saturation of the available sulf-hydryl groups of catalase enzyme. This imbalance in the redox balance may in turn attack the catalase enzyme and can cause denaturation of its structure. The overall result is the damage to the cell membranes by H_2O_2 induced oxidative damages (Goth et al., 2004; Ho et al., 2004). Vitamin C as an adjuvant was found to elevate the levels of catalase enzyme significantly in both the midgut and hemolymph regions of worker bees. The protective potential of this vitamin is supposed to be due to its capability to reduce the free radical induced damages by either reducing the levels of free radicals or by stimulating the activity of catalase enzyme.

Superoxide dismutases (SODs) refer to a group of metalloenzymes that are present in all kingdoms of life. SODs serve as the front-line defense mechanism against tissue injuries due to reactive oxygen species (ROS). These proteins catalyze the dismutation of superoxide anion free radical (O_2) into molecular oxygen and hydrogen peroxide (H_2O_2) and thereby decreasing O_2 levels which otherwise can damage the cells at excessive concentrations (Younus, 2018). Our study showed a reduction in the activity of SOD in the hemolymph and midgut tissues of honeybees exposed to IMD. The reduced activity of the scavenging enzymes observed in the study reflects the excess of ROS generation due to the exposure to IMD. Our findings are in line with Kapoor et al., (2010). On the other hand, Vitamin C supplementation was found to enhance the activity of SOD in the corresponding group in the present study. The elevated level of SOD is supposed to be due to the boosting of the dismutation process by IMD which in turn can facilitate the elimination of ROS.

The major purpose of the peroxidase enzyme is the breakdown of H_2O_2 to nontoxic components (Thangudu & Su, 2021). A significant elevation in POD levels was observed in honeybees exclusively exposed to IMD. This elevation in peroxidase levels is an indication of the enhanced synthesis of peroxidase enzyme to counter the increased volume of free radicals in the tissues. Vitamin C as an adjuvant was found to cause a significant reduction in peroxidase enzyme levels and this probably indicates the capability of this vitamin in alleviating the stress generated by oxidative stress.

Glutathione peroxidase refers to an enzyme family endowed with peroxidase activity which is assigned with the biological role of protecting the organisms from oxidative damage. GPx defends free radical attacks at the expense of GSH. El-Gendy et al. (2010) reported that the main function of GPx is to reduce lipid hydroperoxides to their consequent alcohols and to reduce free hydrogen peroxide, thereby regulating the redox balance in the body. In the present study, we observed a significant reduction in GPx levels in the midgut of worker bees exposed to IMD. This is an indication of the probable damage of midgut cell membranes due to the enhanced production of hydroperoxides and other free radicals because of the failure of GPx in neutralizing them.

Our observations are in line with the findings of Kapoor et al. (2010) who also reported a reduction in GPx activity on IMD exposure.

The enhanced level of GPx in the hemolymph in IMD treated bees indicates the probable defense mechanism against the enhanced production of hydroperoxides, which in turn can reduce the damage of cell membranes. The different mechanisms of action of GPx in the midgut and the hemolymph may be due to the difference in basal concentrations of this key free radical scavenging enzyme in the two regions. Vitamin C supplementation was found to boost the activity of GPx in both the midgut and hemolymph in worker bees. This is an indication of the capability of Vitamin C in enhancing GPx production in various tissues, irrespective of the basal concentrations. This clearly indicates that the administration of Vitamin C in the diet of beehives subject to foraging in areas or agricultural landscapes with IMD application can alleviate the toxic aspects of this insecticide in various tissues. This line of investigation can be of great importance to the practice of apiculture and the prevention of colony decline or even collapse. Our study presents interesting results in support of diet supplementation as a strategy to counteract the deleterious effects of IMD in the metabolic defenses of honeybees against toxic agrochemical compounds.

Glutathione S-transferases (GSTs) comprise a family of Phase II detoxification enzymes that are assigned with the function of safeguarding the different cellular macromolecules from attack by reactive electrophiles. GSTs regulate the levels of an enormous range of electrophiles by conjugating them with glutathione (GSH). Glutathione conjugation is the primary step in the mercapturic acid pathway that facilitates the eradication of toxic compounds (Townsend & Tew, 2003). Observations from our study indicate the significant reduction of GST levels in the hemolymph and midgut tissues of worker bees exposed to IMD. This may be due to the enhanced utilization of GSH in neutralizing the larger volumes of electrophilic toxic compounds produced due to IMD administration. On the other hand, Vitamin C as an adjuvant was found to present a different scenario with a significant enhancement of GST levels in the respective groups. The enhancement of GST activity may be due to the alleviation of the levels of oxidative radicals by Vitamin C, which in turn resulted in the reduced usage of GSH. This elevated concentration of GSH can offer protection against cellular stress by scavenging the excess free radicals.

Glutathione Reductase (GSR) is a key enzyme that is involved in the redox metabolic cycle of GSH along with GPx, which in turn catalyzes the oxidation of GSH (reduced form) to GSSG (oxidized form). Once GSSG is formed it is reduced by GSR, which utilizes NADPH as the reducing factor (Jefferies et al., 2003). A significant reduction in GSR level was observed in the hemolymph and midgut tissues exposed to IMD alone. Since the concentration of GSR in cells is much lower than that of GPx, any variation in the redox balance due to enhanced oxidative stress can cause a significant reduction in these enzyme levels. It can be noted that Vitamin C supplementation was effective in enhancing the activities of these antioxidant enzymes in the hemolymph and midgut tissues. GSR also follows the same pattern of activity. Such a positive up regulation of antioxidant status along with the down regulation of oxidative stress may be the reason for maintaining the proper redox status in the hemolymph and midgut tissues.

The digestive system is the element of contact with pathogens and poisonous substances and is responsible for the detoxification of harmful substances (Higes et al., 2013). Histological analysis of the midgut epithelium may reveal the toxicity of ingested xenobiotics (Han et al., 2012). The midgut epithelial cells of bees fed with sugar syrup elicited normal appearance, the peritrophic membrane (PM) appeared visible and intact with normal thickness (Figure 8-A). The midgut cells of honeybees supplemented with Vitamin C elicited well defined epithelium, slightly wide lumen and multiple layers of the peritrophic membranes, indicating normal tissue morphology (Figure 8-B). Microscopic examination of the midgut cells exposed to IMD reveals degenerated epithelial cells and completely ruptured peritrophic membrane, indicating damage to the tissue membranes. The damage may be due to the irregular redox balance in tissues due to the enhanced production of free radicals on exposure to IMD (Figure 8-C) (Balieira et al., 2018; Yucel & Kayis, 2019). Peritrophic membrane plays an important role in protecting epithelial tissues from the adverse effects of chemical and microbial components in food (Terra, 1988). Any alterations in the midgut epithelial cells and PM can be considered as an indicator of environmental stress (Pawert et al., 1996).

Histological observations of the midgut epithelium of Vitamin C treated bees exposed to IMD showed a multi-layered peritrophic membrane. This indicates the capability of Ascorbic acid supplementation in protecting the tissues from IMD induced toxic effects by the proper maintenance of the cellular redox balance (Figure 8-D). Multiple layers of peritrophic membrane in bees are associated with better utilization of nutrients (Szymas et al., 2012; Crailsheim, 1988). The modifications in the cyto-architecture of gut epithelium give supplementary evidence for the protective role of VIT C against IMD toxicity.

Conclusion

The present study revealed the potential of imidacloprid in adversely affecting the redox balance in the midgut tissues and hemolymph of honeybees. The redox imbalance thus generated has resulted in the accumulation of free radicals in the midgut tissues, which in turn resulted in the building up of oxidative stress. The scavenging of electrons from the cell membranes by the free radicals has resulted in the altered morphology of tissues as evidenced from the histological

analysis. On the contrary, Vitamin C supplementation as an adjuvant was found to properly regulate the antioxidant defense system in the tissues as evidenced from the activities of scavenging enzymes. The maintenance of normal morphology of the tissues by Vitamin C serves as strong visual evidence for such regulation. Hence, we suggest that Vitamin C can be used as an effective natural supplement in relieving the toxic aspects of pesticide induced oxidative stress.

Acknowledgements

We are grateful to the University Grants Commission (UGC) of India for funding this study.

Authors' Contributions

SPS: Conceptualization, methodology, formal analysis, investigation, writing and editing.
SCV: Conceptualization, methodology, supervision, writing and editing.

References

Aebi H. (1984). Catalase in vitro. Methods in Enzymology. 105: 121-126. doi: 10.1016/S0076-6879(84)05016-3

Andi, M.A. & Ahmadi, A. (2014). Influence of vitamin C in sugar syrup on brood area, colony population, body weight and protein in honeybees. International Journal of Biosciences, 4: 32-36. doi:10.12692/ijb/4.6.32-36.

Balieira, K.V.B., Mazzo, M., Bizerra, P.F.V., Guimarães, A.R.D.J.S., Nicodemo, D. & Mingatto, F.E. (2018). Imidacloprid-induced oxidative stress in honey bees and the antioxidant action of caffeine. Apidologie, 49: 562-572. doi: 10.1007/s13592-018-0583-1.

Bendich, A., (1990). Antioxidant Micronutrients and Immune Responses, Micronutrients and Immune Functions. New York Academy of Sciences, New York. p. 175.

Bielenin, I. & Ibek, Z. (1980). Influence of sodium fluoride on the midgut epithelium of the honey bee, *Apis mellifera* L. (Apidae, Hymenoptera). Zeszyty Naukowe Akademii Rolniczej w Krakowie. Zootechnika, 20159: 49-69.

Bindhumol, V., Chitra, K.C. & Mathur, P.P., (2003). Bisphenol A induces reactive oxygen species generation in the liver of male rats. Toxicology, 188: 117-124. doi: 10.1016/S0300-483X(03)00056-8

Blacquiere, T., Smagghe, G., Van Gestel, C.A. & Mommaerts, V. (2012). Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology, 21: 973-992. doi: 10.1007/s10646-012-0863-x.

Bryden, J., Gill, R.J., Mitton, R.A., Raine, N.E. & Jansen, V.A. (2013). Chronic sublethal stress causes bee colony failure. Ecology Letters, 16: 1463-1469. doi:10.1111/ele.12188

Carreck, N.L., Andree, M., Brent, C.S., Cox-Foster, D., Dade, H.A., Ellis, J.D. & Van Englesdorp, D. (2013). Standard methods for *Apis mellifera* anatomy and dissection. Journal of Apicultural Research, 52: 1-40. doi: 10.3896/IBRA.1.52.4.03.

Casida J.E. & Durkin K.A. (2013). Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. Annual Review of Entomology. 58: 99-117, doi: 10.1146/annurev-ento-120811-153645

Chance, B., Sies, H. & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. Physiological Reviews, 59: 527-605. doi: 10.1152/physrev.1979.59.3.527

Corona, M. & Robinson, G.E. (2006). Genes of the antioxidant system of the honeybee: annotation and phylogeny. Insect Molecular Biology, 15: 687-701.doi: 10.1111/j.1365-2583.2006.00695.x.

Crailsheim, K. (1988). Transport of leucine in the alimentary canal of the honeybee (*Apis mellifera* L.) and its dependence on season. Journal of Insect Physiology, 34: 1093-1100. doi: 10.1016/0022-1910(88)90210-7.

David, M & Richard, J.S. (1983). In: Methods of enzymatic analysis. Bergmeyer, J and Grab M. (Eds), Verlag Chemie Wenhein Deer Field, Beach Floride, Pp 358.

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M. & Pham-Delègue, M.H. (2004). Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). Pesticide Biochemistry and Physiology, 78: 83-92. doi: 10.1016/j.pestbp.2003.10.001.

Deisseroth, A. & Dounce, A.L. (1970). Catalase: Physical and chemical properties, mechanism of catalysis, and physiological role. Physiological Reviews, 50: 319-375. doi: 10.1152/physrev.1970.50.3.319.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F. & Pennacchio, F. (2013). Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proceedings of the National Academy of Sciences, 110: 18466-18471. doi: 10.1073/pnas.1314923110.

Egaas, E., Sandvik, M., Fjeld, E., Kallqvist, T., Goksoyr, A. & Svensen, A. (1999). Some effects of the fungicide propiconazole on cytochrome P450 and glutathione S-transferase in brown trout (*Salmotrutta).* Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 122: 337-344. doi: 10.1016/S0742-8413(98)10133-0.

El-Gendy, K.S., Aly, N.M., Mahmoud, F.H., Kenawy, A. & El-Sebae, A.K.H. (2010). The role of vitamin C as antioxidant in protection of oxidative stress induced by imidacloprid. Food and Chemical Toxicology, 48: 215-221. doi:10.1016/j.fct.2009.10.003.

Enayati, A.A., Ranson, H. & Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. Insect

Molecular Biology, 14: 3-8. doi: 10.1111/j.1365-2583.2004.00529.x.

Esther, E., Smit, S., Beukes, M., Apostolides, Z., Pirk, C.W., & Nicolson, S.W. (2015). Detoxification mechanisms of honey bees (*Apis mellifera*) resulting in tolerance of dietary nicotine. Scientific Reports, 5: 1-11. doi: 10.1038/srep11779.

Farjan, M., Dmitryjuk, M., Lipinski, Z., Biernat-Lopienska, E. & Żołtowska, K. (2012). Supplementation of the honeybee diet with vitamin C: The effect on the antioxidative system of *Apis Mellifera* carnica brood at different stages. Journal of Apicultural Research, 51: 263-270. doi: 10.3896/IBRA.1.51.3.07.

Godfray, H.C.J., Blacquiere, T., Field, L. M., Hails, R.S., Petrokofsky, G., Potts, S.G. & McLean, A.R. (2014). A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proceedings of the Royal Society B: Biological Sciences, 281: 20140558. doi: 10.1098/rspb.2014.0558.

Goth, L., Rass, P. & Pay, A. (2004). Catalase enzyme mutations and their association with diseases. Molecular Diagnosis, 8: 141-149. doi:10.1007/BF03260057.

Habig, W.H. Pabst, M.J. & Jokoby, W.B. (1974). Glutathione transferase: A first enzymatic step in mercapturic acid III formation. Journal of Biological Chemistry, 249: 7130-7139. doi: 10.1016/S0021-9258(19)42083-8.

Han, P., Niu, C.Y., Biondi, A., Desneux, N., (2012). Does transgenic Cry1Ac + CpTI cotton pollen affect hypopharyngeal gland development and midgut proteolytic enzyme activity in the honey bee *Apis mellifera* L. (Hymenoptera, Apidae) Ecotoxicology, 21: 2214-2221. doi: 10.1007/s10646-012-0976-2.

Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.F., Aupinel, P. & Decourtye, A. (2012). A common pesticide decreases foraging success and survival in honey bees. Science, 336: 348-350. doi: 10.1126/science.1215039.

Higes, M., Meana, A., Bartolome, C., Botías, C. & Martín Hernandez, R. (2013). *Nosema ceranae* (Microsporidia), a controversial 21st century honeybee pathogen. Environmental Microbiology Reports, 5: 17-29. doi: 10.1111/1758-2229.12024.

Hinton, B.T., Palladino, M.A., Rudolph, D. & Labus, J.C. (1995). The epididymis as protector of maturing spermatozoa. Reproduction, Fertility and Development, 7: 731-745.

Ho, Y.S., Xiong, Y., Ma, W., Spector, A. & Ho, D.S. (2004). Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. Journal of Biological Chemistry, 279: 32804-32812. doi: 10.1074/jbc.M404800200.

Hodgson, E., Smart, R.C. (2001). Introduction to Biochemical Toxicology, in: Wiley-Inter Science (3 Eds.), Hoboken, pp. 309-323.

Jakoby, W.B. (1985). Glutathione transferases: An Overview. Methods in Enzymology, 113: 495-499. doi: 10.1016/S0076-6879(85)13064-8.

Jefferies, H., Coster, J., Khalil, A., Bot, J., McCauley, R.D. & Hall, J.C. (2003). Glutathione. ANZ Journal of Surgery, 73: 517-522. doi: 10.1046/j.1445-1433.2003.02682.x.

Kapoor, U., Srivastava, M.K., Bhardwaj, S. & Srivastava, L.P. (2010). Effect of imidacloprid on antioxidant enzymes and lipid peroxidation in female rats to derive its No Observed Effect Level (NOEL). The Journal of Toxicological Sciences, 35: 577-581. doi: 10.2131/jts.35.577.

Kirkman, H.N. & Gaetani, G.F. (2007). Mammalian catalase: a venerable enzyme with new mysteries. Trends in Biochemical Sciences, 32: 44-50. doi: 10.1016/j.tibs.2006.11.003.

Lee, K. (1991). Glutathione S-transferase activities in phytophagous insects: induction and inhibition by plant phototoxins and phenols. Insect Biochemistry, 21: 353-361. doi: 10.1016/0020-1790(91)90001-U.

Lehane, M.J. (1997). Peritrophic matrix structure and function. Annual Review of Entomology, 42: 525-550. doi: 10.1146/annurev.ento.42.1.525.

Luck, H. (1963). Methods of enzymatic analysis. Enzyme Assays in-vivo. New York, London: Academic Press.

Mannervik, B. (1985). Glutathione peroxidase. In Methods in Enzymology, 113: 490-495. Academic Press. doi: 10.1016/S0076-6879(85)13063-6.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M. & Sattelle, D.B. (2001). Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends in Pharmacological Sciences, 22: 573-580. doi: 10.1016/S0165-6147(00)01820-4.

Medrzycki, P., Giffard, H., Aupinel, P., Belzunces, L.P., Chauzat, M.P., Claßen, C. & Vidau, C. (2013). Standard methods for toxicology research in *Apis mellifera*. Journal of Apicultural Research, 52: 1-60. doi: 10.3896/IBRA.1.52.4.14.

Medrzycki, P., Montanari, R., Bortolotti, L., Sabatini, A.G., Maini, S. & Porrini, C. (2003). Effects of imidacloprid administered in sub-lethal doses on honey bee behaviour. Laboratory tests. Bulletin of Insectology, 56: 59-62.

Milton, N.G. (2004). Role of hydrogen peroxide in the aetiology of Alzheimer's disease. Drugs and Aging, 21: 81-100.

Nicodemo, D., Maioli, M.A., Medeiros, H.C., Guelfi, M., Balieira, K.V., De Jong, D. & Mingatto, F.E. (2014). Fipronil and imidacloprid reduce honeybee mitochondrial activity. Environmental Toxicology and Chemistry, 33: 2070-2075. doi:10.2165/00002512-200421020-00002.

Paoletti, F., Aldinucci, D., Mocali, A. & Caparrini, A. (1986). A sensitive spectrophotometric method for the determination

of superoxide dismutase activity in tissue extracts. Analytical Biochemistry, 154: 536-541. doi: 10.1016/0003-2697(86)90026-6.

Pawert, M., Triebskorn, R., Graff, S., Berkus, M., Schulz, J. & Köhler, H.R. (1996). Cellular alterations in collembolan midgut cells as a marker of heavy metal exposure: ultrastructure and intracellular metal distribution. Science of The Total Environment, 181: 187-200. doi: 10.1016/0048-9697(95)05009-4.

Reddy, K.P., Subhani, S.M., Khan, P.A., & Kumar, K.B. (1985). Effect of light and benzyladenine on dark-treated growing rice (*Oryza sativa*) leaves II. Changes in peroxidase activity. Plant and Cell Physiology, 26: 987-994. doi: 10.1093/oxfordjournals.pcp.a077018.

Rotruck, T.T., Ganther, H.E., Swanson, A.B., Hafeman, D.G. & Hoeckstra, W.G. (1973). Selenium: Biochemical role as a component of glutathione peroxidase. Science, 179: 588-590. doi: 10.1126/science.179.4073.588.

Snodgrass, R.E. (2018). Anatomy of the honey bee. Cornell University Press.

Strachecka, A., Chobotow, J., Paleolog, J., Los, A., Schulz, M., Teper, D. & Grzybek, M. (2017). Insights into the biochemical defence and methylation of the solitary bee *Osmia rufa* L: A foundation for examining eusociality development. Plos One, 12: e0176539. doi: 10.1371/journal.pone.0176539.

Strachecka, A., Krauze M., Olszewski, K., Borsuk, G., Paleolog, J., Merska M., Chobotow, J., Bajda, M. & Grzywnowicz, K. (2014). Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). Biochemistry, 79: 1192-1201. doi: 10.1134/S0006297914110066.

Suchail, S., Debrauwer, L. & Belzunces, L.P. (2004). Metabolism of imidacloprid in *Apis mellifera.* Pest Management Science: formerly Pesticide Science, 60: 291-296. doi: 10.1002/ps.772.

Szymas, B. & Przybyl, A. (2007). Midgut histological picture of the honey bee (*Apis mellifera* L.) following consumption of substitute feeds supplemented with feed additives. Nauka Przyroda Technologie, 1: 48.

Szymas, B., Langowska, A. & Kazimierczak-Baryczko, M. (2012). Histological structure of the midgut of honeybees (*Apis mellifera* L.) fed pollen substitutes fortified with probiotics. Journal of Apicultural Science, 56: 5-12. doi: 10.2478/v10289-012-0001-2.

Terra, W.R., Espinoza-Fuentes, F.P., Ribeiro, A.F. & Ferreira, C. (1988). The larval midgut of the housefly (*Musca domestica*): ultrastructure, fluid fluxes and ion secretion in relation to the organization of digestion. Journal of Insect Physiology, 34: 463-472. doi: 10.1016/0022-1910(88)90187-4.

Thangudu, S. & Su, C.H., (2021). Peroxidase mimetic nanozymes in cancer phototherapy: Progress and perspectives. Biomolecules, 11: 1015. doi: 10.3390/biom11071015.

Townsend, D.M. & Tew, K.D., (2003). The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene, 22: 7369-7375. doi:10.1038/sj.onc.1206940.

Verma, R.S., Mehta, A. & Srivastava, N. (2007). In vivo chlorpyrifos induced oxidative stress: attenuation by antioxidant vitamins. Pesticide Biochemistry and Physiology, 88: 191-196. doi: 10.1016/j.pestbp.2006.11.002.

Weirich, G.F., Collins, A.M. & Williams, V.P. (2002). Antioxidant enzymes in the honeybee, *Apis mellifera*. Apidologie, 33: 3-14 doi: 10.1051/apido:200100.

Yang, E.C., Chuang, Y.C., Chen, Y.L. & Chang, L.H. (2008). Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). Journal of Economic Entomology, 101: 1743-1748. doi: 10.1603/0022-0493-101.6.1743.

Younus, H. (2018). Therapeutic potentials of superoxide dismutase. International Journal of Health Sciences, 12: 88.

Yucel, M.S. & Kayis, T. (2019). Imidacloprid induced alterations in oxidative stress, biochemical, genotoxic, and immunotoxic biomarkers in non-mammalian model organism *Galleria mellonella* L. (Lepidoptera: Pyralidae). Journal of Environmental Science and Health, Part B, 54: 27-34. doi: 10.1080/03601234.2018.1530545.