Ecotoxicology and Environmental Safety 220 (2021) 112379

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Sublethal effects of Isoclast™ Active (50% sulfoxaflor water dispersible granules) on larval and adult worker honey bees (*Apis mellifera* L.)

Jiahuan Li [a,b,1], Liuwei Zhao [b,1], Suzhen Qi [b,*], Wenting Zhao [c], Xiaofeng Xue [b], Liming Wu [b], Shaokang Huang [a,d,**]

[a] College of Animal Sciences (College of Bee Science), Fujian Agriculture and Forestry University, Fuzhou 350002, China
[b] Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing 100093, China
[c] Key Laboratory of Urban Agriculture In North China , Ministry of Agriculture and Rural Affairs, Beijing University of Agriculture, Beijing 102206, China
[d] Fujian Honey Bee Biology Observation Station, Ministry of Agriculture and Rural Affairs, Fuzhou 350002, China

ARTICLE INFO

Edited by Dr. Yong Liang

Keywords:
Sulfoxaflor
Honeybee
Larval toxicity
Adult toxicity
Oxidative stress

ABSTRACT

Sulfoxaflor is a novel sulfoximine insecticide which is widely used to control crop pests. Risk assessments have reported its high toxicity to pollinators. However, sulfoxaflor is not persistent in the environment and few studies have addressed its negative effects on larval and newly emerged honeybees at environmentally relevant concentrations. In the present study, the sublethal effects of a sulfoxaflor commercial product, Isoclast™ Active, were evaluated in the laboratory using larvae and newly emerged worker honeybees. The results of 96-h acute toxicity showed that Isoclast is moderately toxic to adult bees, and it could induce significant death and growth failure of larvae after continuous dietary intake. In addition, Isoclast induced significant changes in antioxidative (SOD, CAT), lipid peroxidation (POD, LPO, MDA), detoxification (GST, GR, GSH) and signal transduction-related (AChE, ACh) enzymes or products both in larvae and adult honey bees under residue levels. Here we firstly reported the lethal and sublethal effects of commercial sulfoxaflor to honeybees' larvae and young workers. All these findings revealed the potential risks of sulfoxaflor residue in environment to honey bees, and may also to other pollinators. This is a laboratory mimic studies, and further studies are still needed to investigate the risks and in-depth mechanisms of sulfoxaflor to bees in field.

1. Introduction

Sulfoxaflor (CAS: 946578-00-3), the first marketed sulfoximine-based insecticide, was registered in 2010 and has been successfully used to protect multiple crops against many pests (Rossaro et al., 2018). Although sulfoxaflor and neonicotinoids both target insect acetylcholine receptors (nAChRs), they bind to different sites on these receptors (Watson et al., 2011; Babcock et al., 2011). Thanks to its high potency and low cross-resistance with other insecticides, sulfoxaflor is considered an alternative to, and even better than, some neonicotinoids (Babcock et al., 2011). The risks of sulfoxaflor, when used as a pesticide, to humans, the environment and non-target organisms were assessed systematically by the European Food Safety Authority in 2014, and reassessed in 2019 for its potential risks to pollinators (https://www.greenfacts.org/en/sulfaxoflor-pesticide-bees/l-2/index.htm#0). The conclusion of these assessments was that sulfoxaflor is moderately toxic to mammals and birds and slightly toxic to most aquatic species, but that it poses high risks to honeybees and bumblebees when the bees come into contact with spray droplets shortly after application, especially during the flowering stage (Al Naggar and Paxton, 2021; Chakrabarti et al., 2020).

Some studies have reported the high toxicity of sulfoxaflor to bees. Sulfoxaflor is registered as having a contact acute LD_{50} of 0.379 μg/bee and an oral acute LD_{50} of 0.146 μg/bee for honeybees (*Apis* spp.) in the PPDB (Pesticide Properties DataBase, http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/1669.htm). For bumblebees (*Bombus* spp.), the acute contact LD_{50} is 7.55 μg/bee and the acute oral LD_{50} is 0.027 μg/bee. In addition to high acute toxicity, sublethal effects of sulfoxaflor on bees were also observed. Siviter et al. performed a series of studies on the effects of sulfoxaflor on bees and found that a post-spray field exposure

* Correspondence to: No.1 Xiangshan Beigou, Haidian District, Beijing 100093, China.
** Correspondence to: College of Bee Science, Fujian Agriculture and Forestry University, Fujian 350002 China.
E-mail addresses: suzhen_qi@126.com (S. Qi), skhuang@fafu.edu.cn (S. Huang).
[1] Co-first authors.

https://doi.org/10.1016/j.ecoenv.2021.112379
Received 6 December 2020; Received in revised form 28 April 2021; Accepted 24 May 2021
Available online 28 May 2021

of 5ppb reduced bumblebee colony reproductive success, leading to fewer workers and fewer reproductive offspring (Siviter et al., 2018). Under another microcolony condition, the same concentration of sulfoxaflor reduced the number of eggs and larvae, but no effect was found on ovarian development (Siviter et al., 2020). Interestingly, under acute exposure, Siviter et al. Siviter et al. (2019) did not find a significant impact of sulfoxaflor on olfactory conditioning performance in either bumblebees (*B. terrestris*) or honeybees (*A. mellifera*).

Sulfoxaflor is not persistent in many matrices. For example, the half-life of sulfoxaflor was found to range from 1.36 to 5.10 days in soil and 6.13–9.37 days in cotton leaves, and the final residue in both soil and cotton was lower than 0.05 mg/kg 7 days after the last application of 0.6–0.9 g/30 m^2 of 50% water dispersible granules (WDGs) (Qin et al., 2014). In Xinjiang Uygur Autonomous Region of China, the application of sulfoxaflor at 450 g active ingredient (a.i.) ha^{-1} through drip irrigation at different times before or during flowering resulted in a residue amounts of sulfoxaflor (~17 μg/kg) and when applied before flowering at 700 g a.i. ha^{-1}, the residue level was $\leq$ 14.2 μg kg^{-1} in pollen and $<$ 0.68 μg kg^{-1} in nectar (Jiang et al., 2020).

However, pesticide exposure and residue in environment can have detrimental impacts on bees. For example, Chakrabarti et al. Chakrabarti et al. (2020) found that application of Sivanto (flupyradifurone) and Transform (sulfoxaflor) at recommended field rates significantly increased the average consumption of water and sugar syrup by bees. They also found higher amounts of total reactive oxygen species (ROS) and total reactive nitrogen species (RNS), as well as caspase-3 activity, which indicated that sulfoxaflor can increase oxidative stress and induce apoptosis in honeybees. Despite these valuable studies, the physiological effects of sulfoxaflor, especially in its marketed formulation, on honeybees have not been explored systemically. To fill this gap, we exposed larvae and newly emerged honeybee adults to sublethal concentrations of a sulfoxaflor formulation Isoclast™ Active, 50% WDG, and evaluated the effects on survival, development, and physiological fitness. The main objective of this study was to assess the effects and possible mechanisms of Isoclast™ Active on bees at different development stages. Sulfoxaflor and other sulfoximine-based insecticides are of increasing global importance. The data from this study can provide a reference for risk assessment of this class of insecticides.

2. Materials and methods

2.1. Chemicals and honeybees

Isoclast™ active, 50% sulfoxaflor WDG (Dow AgroSciences, USA), was purchased from a local pesticide shop in Beijing, China. Pure water was used as a solvent to prepare stock and test solutions. Standard sulfoxaflor with 97% purity was purchased from A Chemtek Inc. (MA, USA) and used for calculating the stability of sulfoxaflor in sucrose solution (Supporting Information). All other chemicals used for food preparation and enzyme activity analysis were of the highest commercially available purity and were purchased from the Beijing Chemical Reagent Company (Beijing, China).

The honeybees (*Apis mellifera L.*) were maintained at the Xiangshan apiaries in the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences (IAR, CAAS), Beijing, China. All the colonies were maintained according to standard beekeeping practices and no drugs were applied at least 1 month before tests (Qi et al., 2020b).

2.2. Toxicity tests of adult bees

According to the results of stability calculation (Supporting Information), Isoclast in sucrose solution was not stable beyond 24 h, so all the test solutions were freshly prepared and renewed every day to maintain consistent exposure concentrations. Newly emerged adult bees ($<$ 24 h) were collected and used for the following acute and chronic experiments.

Acute toxicity calculation: this test was performed according to the OECD guideline 213 (OECD, 1998) which was also used in our earlier studies (OECD, 1998). Based on the limit range test, five concentrations were used to determine the LC_{50} of Isoclast: 1.2, 1.4, 1.6, 1.8, and 2.0 mg/L. Firstly, Isoclast was dissolved in water and then diluted to the appropriate concentrations by 50% sucrose solution (wt/vol). As only water was used as solvent, 50% sucrose solution without sulfoxaflor was used as a blank control to calculate the natural death rate. Twenty newly emerged adult bees were collected and then placed into one plastic box as one replicate. After starvation for 2 h, 1.5 mL of each test solution was provided to bees in each cage. The test lasted 96 h and the solutions were changed every day. Each treatment had three replicates. The culture conditions were 30 ± 1 °C and relative humidity (RH) of 50% under darkness. The 96-h half-lethal effective concentration (LC_{50}) was calculated using probit analysis.

Chronic toxicity calculation: based on the 96-h LC_{50} value, the upper limit of the sublethal concentration was set to 1.0 mg/L. Comprehensively considering the residue levels of sulfoxaflor in pollen and nectar (Jiang et al., 2020), two lower concentrations of 0.01 and 0.1 mg/L were also set for the 14-day chronic toxicity test. Every 20 newly emerged bees collected from brood combs were transferred into one plastic transparent container and cultured in an incubator under 24 h of darkness, 30 ± 1 °C, and 50% RH. Bees in each container were fed continuously with 2 mL of control or treated sucrose solution for 14 days. The survival and food consumption of bees were calculated every 24 h. Each treatment consisted of three replicates. After 14 days of exposure, surviving bees in each container were collected and stored at − 80 °C before use (Zhu et al., 2020; Qi et al., 2020a).

2.3. Toxicity tests of honeybee larvae

Honeybee larvae were reared in vitro according to OECD guideline 239, which is also used in our earlier study with some modification (Qi et al., 2020b). According to the preliminary tests and the residue levels of sulfoxaflor in bee products, five concentrations of Isoclast at 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mg/L were used to determine its toxicity to honeybee larvae.

One-day-old ($<$ 24 h) larvae were transferred from combs to 48-well tissue culture plates with 20 μL artificial food A (50% royal jelly, 6% D-glucose, 6% D-fructose, 1% yeast extract, and 37% deionized water) in each well. No food was provided on day 2. On day 3, 20 μL diet B (50% royal jelly, 7.5% D-glucose, 7.5% D-fructose, 1.5% yeast extract, and 33.5% deionized water) without (control) or with (treatment groups) Isoclast was provided to each larva. On days 4, 5, and 6, 30, 40, and 50 μL, respectively, of diet C (50% royal jelly, 9% D-glucose, 9% D-fructose, 2% yeast extract, and 30% deionized water) sulfoxaflor solution was offered to each larva. Stop feeding on day 7 and on day 8, larvae were moved to new plates for pupation and eclosion (Dai et al., 2019). The conditions were 35 °C and darkness during the entire exposure, and RH was 95% for the larval stage, 75% for pupae (Days 8–15), and RH 50% for adult emergence (Days 15–21). Each treatment consisted of three replicates with 16 larvae per replicate. Survival of larvae and pupae was monitored every day, and dead bees were removed at the same time. Mortality of larvae and rates of pupation and eclosion were calculated on days 8, 15, and 22 as described in OECD guidance 237 (OECD, 2016). A replicate of this experiment was performed, and larvae samples were collected on day 8 for enzyme analysis. Samples were stored at − 80 °C until assayed.

2.4. Determination of enzyme activity

Samples of larvae and the abdomens of 14-day-old adults in a pre-cooled 0.9% sodium chloride solution were homogenized on ice followed by centrifugation at 1902*g* for 10 min. After protein concentration calculation (Enhanced BCA Protein Assay Kit- P0009, Biyuntian, China), the supernatant was used to measure the enzyme activities of superoxide

dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), peroxidase (POD), and acetylcholinesterase (AChE) and the contents of glutathione (GSH), lipid peroxidation products (LPO), and malondialdehyde (MDA) using commercial assay kits purchased from Nanjing Jiancheng Bioengineering Institute, China. All of the enzyme activities were expressed in enzyme units (U) per protein content for comparison, and one unit was defined as the amount of enzyme that degraded one unit of the substrate supplied with the kits.

2.5. Statistical analysis

All of the data are presented as mean ± SD (standard deviation). The 96-h LC_{50} of Isoclast was calculated by probit regression using SPSS 21.0 (IBM, Chicago, USA). Survival curves for larval and adult bees were plotted using the built-in methods of Graphpad Prism 7.0 (San Diego, CA, USA). The statistical differences between treatments and controls were determined using a one-way analysis of variance (ANOVA), followed by a post-hoc Dunnett's test at $p = 0.05$ and $p = 0.01$ using Graphpad Prism 7.0 (San Diego, CA, USA).

3. Results

3.1. Stresses induced by sulfoxaflor in adult bees

The 96-h acute LC_{50} of Isoclast to adult bees was 1.72 (95% CI: 1.63–1.83) mg/L, and the linear regression formulation was $Y = -1.691 + 7.194$ lgX with a correlation coefficient of $R^2 = 0.986$. (Refer to STable 1 for more LCx information). With increasing exposure time, sulfoxaflor concentrations between 0.01 and 1.0 mg/L induced significant survival stress. Survival of bees in the control and 0.01 mg/L groups was higher than that in 0.10 and 1.0 mg/L of sulfoxaflor treatment groups (Fig. 1A). At the 14th day, mortality was 13.33% ± 9.43% in the control group and 6.67% ± 2.36% in 0.01 mg/L group, and significantly increased to 31.67% ± 15.35% and 75% ± 18.71% in the 0.10 and 1.0 mg/L groups, respectively. Though the difference between each survival curve was significant, the averaged food consumption per bee was similar between the groups at each day of exposure, except the 1.0 mg/L group, which had an obvious reduction in consumption. The total cumulative food consumption of each bee after 14 days of exposure was 866.91 ± 32.46 mg/bee in the control group and only 613.27 ± 61.52 mg/bee in the 1.0 mg/L group (Fig. 1B).

In addition to the lethal effects, significant differences in antioxidative and detoxification reactions were also observed by measuring the changes in the activities of enzymes and the levels of their products (Fig. 2). In abdomen tissues, the activity of the enzyme SOD in the three Isoclast treatment groups ranged from 6.50 to 6.93 U/mg protein, which was significantly lower than the activity, 7.43 ± 0.37 U/mg protein, in the control group. There was a greater decrease in CAT activity upon Isoclast treatment, and the 1 mg/L group had the lowest activity, 45.08 ± 2.18 U/g protein, which was only 46% of the control level (98.0 ± 3.68 U/g protein).

No significant difference in GSH content was observed. However, the enzymes GST and GR were significantly activated by all concentrations of Isoclast, except for GST at 1.0 mg/L. GST activity in control was 32.82 ± 2.53 U/mg protein, and increased to slightly higher levels, 36.53 ± 0.68 and 36.97 ± 0.39 U/mg protein, in bees treated with 0.01 and 0.1 mg/L Isoclast, respectively. However, GST activity decreased to 23.95 ± 0.67 U/mg protein in the 1 mg/L group, corresponding to a 27% reduction compared with the control. GR activity increased 2.49, 1.53, and 1.18 fold compared with the control activity (6.22 ± 0.20 U/g protein) in the 0.01, 0.10, and 1.0 mg/L groups, respectively.

The activity of POD (Fig. 2F–H), was significantly reduced to 3.49 ± 0.31 and 3.90 ± 0.06 U/mg protein after exposure to 0.1 and 1 mg/L Isoclast, respectively. No difference in POD activity was observed between the 0.01 mg/L and control (5.15 ± 0.38 U/mg protein) groups. The changes in the products of lipid peroxidation, MDA and LPO, were different. No significant effect of Isoclast on MDA was observed, while the content of LPO decreased in an obvious dose-dependent manner. The contents of LPO were 1.26 ± 0.16, 0.89 ± 0.11, and 0.58 ± 0.14 μmol/g protein in the 0.01, 0.10, and 1.0 mg/L groups, respectively, which were all significantly lower than the content of 1.48 ± 0.14 μmol/g protein observed in the control.

3.2. Lethal effects of Isoclast on larval bees

After continuously consuming Isoclast-containing food, a mortality rate of 12.5% was calculated in control group, which meets the requirement of OECD 239 guideline and earlier studies (OECD, 2016; Tavares et al., 2017).

Seen from Fig. 3, survival of larvae decreased along with increasing exposure time and increasing Isoclast concentration. Of the larvae fed the highest concentration of Isoclast (0.1 mg/L), 45.83% ± 2.95% died by exposure day 8%, and 29.17% ± 2.95% of larvae fed the lowest concentration (10^{-5} mg/L) died by the same time point. Along with these stresses given by Isoclast at larval stage, honeybees also went through a difficult in the following pupation and emergence stages. Death during pupation is much less than that during the larval stages. For example, the mortality of pupae was only 16.7% in the 10^{-4} group and ranged from 25% to 27% in other four treatment groups (Fig. 3B). Due to the continuous death, the final emergence rate was only 27.08% ± 2.95% in the 10^{-1} mg/L group, 37.5% ± 5.10% in the 10^{-3} mg/L group, and 43.75% ± 5.10% in the 10^{-5} mg/L group; all of these rates were significantly lower than that of the control group (70.8%).

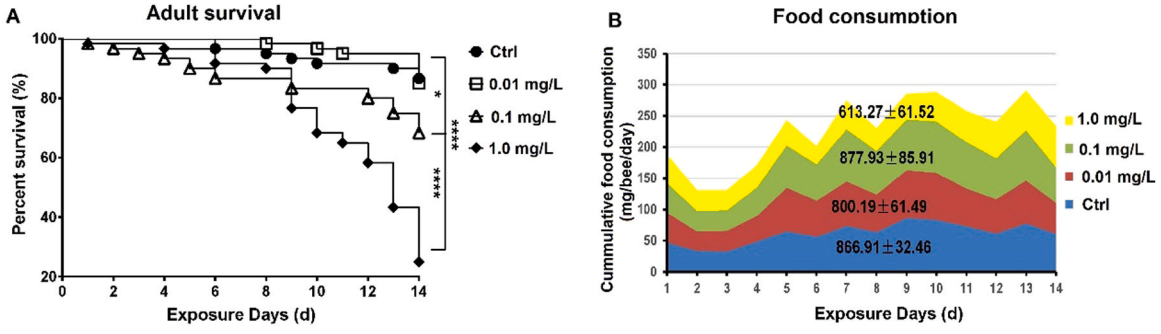

Fig. 1. Effects of sulfoxaflor on mortality (A) and food consumption (B) of new emerged adult worker bees during 14 days exposure. The statistical analysis was performed by Graph pad Prism using the built-in Log-rank (Mantel-Cox) test and Logrank test for trend. The results of these two tests showed that the difference between survival curves were significant, with a p value < 0.0001. Difference between each curve were compared use t-test and significant differences were marked as $^{*}p < 0.01$, $^{****}p < 0.0001$.

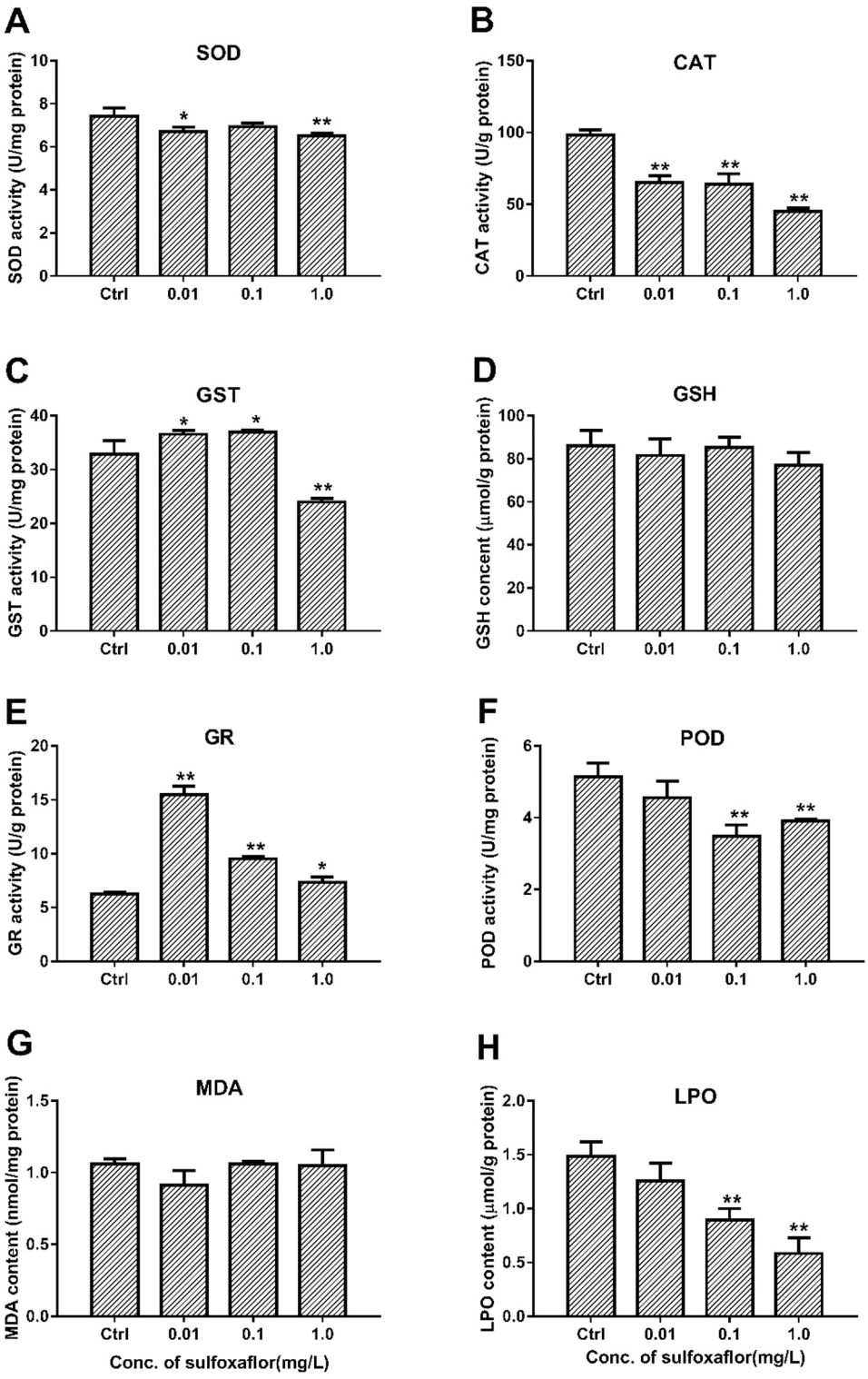

Fig. 2. Effects of sulfoxaflor on target abdomen enzymes in adult honey bees after 14 days exposure. New emerged adult bees were exposed to sulfoxaflor sucrose solution for 14 days and the abdomen tissues were homogenized for enzyme activity or content analysis. The calculated indexes were oxidative stress-related enzymes, (A) Sod and (B) Cat; metabolic detoxification-related enzymes, (C) GST, (D) GSH, (E) GR, and Lipid peroxidation-related enzymes, (F) POD, (G) MDA and (H) LPO. Significant differences relative to the Ctrl (control) group, $*p < 0.05$, $**p < 0.01$. Error bars represent SD fold changes.

3.3. Effects of sulfoxaflor on enzymes in bee larvae

Sulfoxaflor induced significant changes in enzyme activity in bee larvae. The activities of enzymes GST and GR, were significantly higher in all sulfoxaflor treatment groups compared with the control group, but showed different trends (Fig. 4A1,A2). GST activity generally increased along with increasing Isoclast concentration. The highest activity was 58.53 ± 1.97 U/mg protein in the 0.1 mg/L group compared with an activity of 35.14 ± 2.60 U/mg protein in the control. In contrast, GR activity increased to the highest level (11.54 ± 0.60 U/g) in the 10^{-5} mg/L group, which was 1.38 fold higher than that in control (4.84 ± 0.71 U/g protein), and then decreased with further increases in Isoclast concentration. However, GR activity remained higher than that in the control group. The contents of GSH in the treatment groups were not significantly different from those in the control (Fig. 4A3).

The activity of the enzyme POD was not greatly affected by Isoclast, except for a significant decrease in the 10^{-4} mg/L group (1.87 ± 0.08 U/mg protein) compared with the control (2.72 ± 0.19 U/

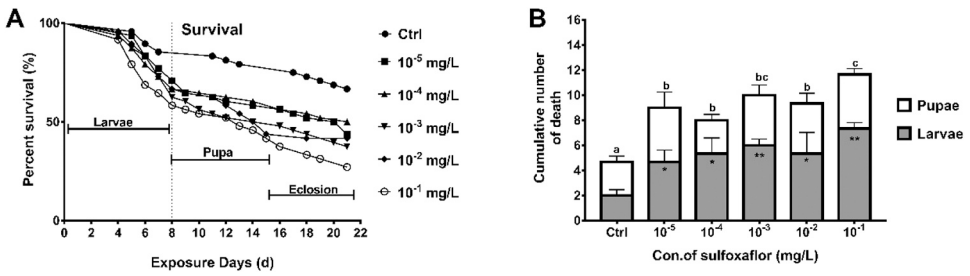

Fig. 3. Survival stresses induced by sulfoxaflor through larval bee development stages. Artificial food with (10^{-5}~10^{-1} mg/L) or without (Ctrl) sulfoxaflor were provided from 1st to 6th day old larvae, and death was calculated every day. (A) Showed the survival curve through whole period, (B) showed the cumulative number of death during larval or pupal stage. Significant differences relative to the Ctrl (control) group, $^{*}p < 0.05$, $^{**}p < 0.01$. For survival curves (A), the statistical analysis was performed by Graph pad Prism using the built-in Log-rank (Mantel-Cox) test and Logrank test for trend. The results of these two tests showed that the difference between survival curves were significant, with a p value of 0.0037 and 0.0001, respectively. Different letters in (B) column means the differences between control and treatments in the total cumulative death number of larvae and pupae were significant $p < 0.05$.

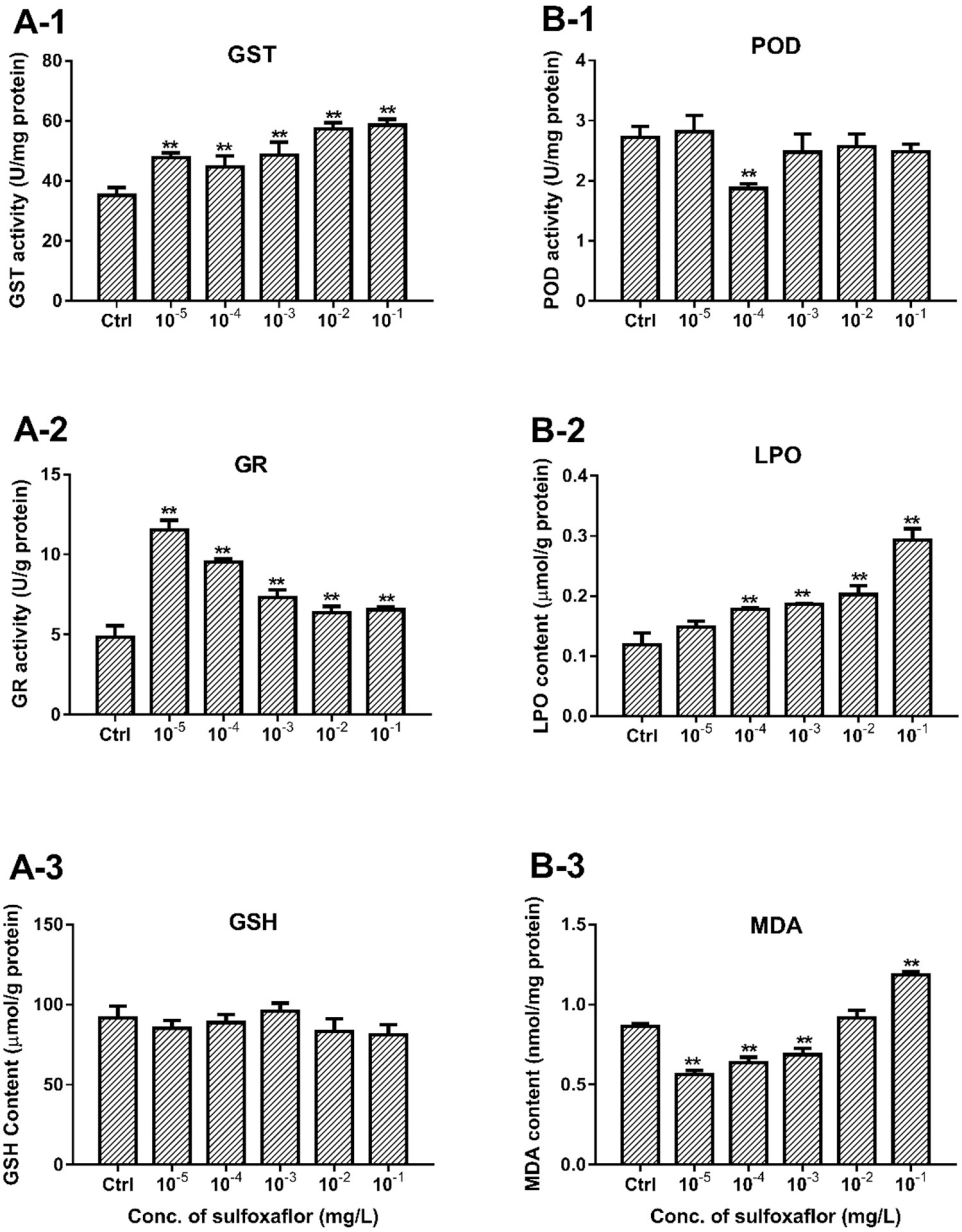

Fig. 4. Effects of sulfoxaflor on target enzymes in larvae honey bees after exposure. The calculated indexes were metabolic detoxification-related enzymes: (A-1) GST, (A-2) GR, (A-3) GSH, and Lipid peroxidation-related enzymes: (B-1) POD, (B-2) LPO and (B-3) MDA. Significant differences relative to the Ctrl (control) group, $^{*}p < 0.05$, $^{**}p < 0.01$. Error bars represent SD fold changes.

mg protein). The products of lipid peroxidation, LPO and MDA, were influenced significantly by Isoclast. The LPO content clearly increased in response to increasing concentrations of sulfoxaflor. The LPO content was 0.12 ± 0.02 μmol/g protein in the control group, and steadily increased to 0.29 ± 0.02 μmol/g protein in the 10^{-5} mg/L group. Compared with the control (0.86 ± 0.02 nmol/mg protein), the content of MDA decreased in response to 10^{-5}, 10^{-4}, and 10^{-3} mg/L (0.56 ± 0.02, 0.64 ± 0.03, and 0.69 ± 0.04 nmol/mg protein, respectively) and increased in the 10^{-2} mg/L (0.92 ± 0.05 nmol/mg protein) and 10^{-1} mg/L (1.18 ± 0.02 nmol/mg protein) Isoclast treatment groups.

Measurement of SOD and CAT activities revealed that sulfoxaflor induced oxidative stress. SOD was activated by Isoclast in the 10^{-3}, 10^{-2} and 10^{-1} mg/L groups, and CAT was inhibited only in the 10^{-1} mg/L group (Fig. 5). SOD activity was 8.79 ± 0.33 U/mg protein in the control group and 10.95 ± 0.19 U/mg protein in the 10^{-1} mg/L group, while CAT activity was 234.72 ± 15.82 U/g protein in the control and 186.41 ± 9.18 U/g protein in the 10^{-1} mg/L Isoclast treatment group.

We also measured the activity of AChE and content of acetylcholine (Ach). AChE activity was significantly higher in all the Isoclast treatment groups compared with the control (Fig. 5); the highest activity was 0.15 ± 0.01 U/mg protein in the 10^{-3} mg/L treatment group, which was 2.85 fold higher than the control level (0.04 ± 0.01 U/mg protein). Consistent with this, the contents of Ach were also significantly higher in all treatment groups compared with the control. The groups with higher AChE activity generally had a relatively low Ach content.

4. Discussion

Given the effects observed in this study, sulfoxaflor was toxic to both honeybee larvae and adults. Under long-term exposure, even at environment relevant concentrations, sulfoxaflor could influence the survival and food consumption of worker bees, and interfere the development of bee larvae by resulting significant oxidative stresses in both adults and larvae.

4.1. Lethal risks of sulfoxaflor to honeybees depends on exposure conditions

Synthetic pesticides were widely used in field, and considering their persistency and intense application, pesticides residue in water, pollen and nectar may lead to long periods of exposure to bees and colonies (Sanchez-Bayo and Goka, 2014). Thus, the accurate time-to-effect scaling is an important tool for estimating the effect of pesticide exposure on honeybees (Simon-Delso et al., 2018). During the 14 days exposure of sulfoxaflor to adults in the present study, mortality was low during the first 7 days in all treatments but significantly increased to 31.7% and 75% in 0.1 mg/L and 1.0 mg/L respectively at the end of tests. These high mortalities at 1.0 mg/L can attribute to the high concentration designed, which was close to the 96-h LC_{50}. The time-reinforced effect (TRT) might be another reason for the increased death in sulfoxaflor treatments. TRT is a common mechanism of pesticides to organisms. Simon-Delso et al. performed a 33 days exposure of boscalid to bees rather standard 10-day test, and the cumulative toxicity was not observed until the 17–18 days when a tolerant inflexion of bees to toxicant appeared (Simon-Delso et al., 2018). Similar time-cumulated effects were also found for neonicotinoids imidacloprid and fipronil (Holder et al., 2018; Rondeau et al., 2014). Given the wide application and high risks of sulfoxaflor to bees as well as the time-cumulative effects of pesticides over time in environmental situation, more studies combined standard studies and chronic toxicity tests mimic real conditions are necessary for risk assessments of pesticides to bees.

Bee larvae are sensitive to sulfoxaflor. In the present study, significant death was found from the lowest dosage of 10^{-5} mg/L to the highest 10^{-1} mg/L through the whole development stage. Notably, the more constants honeybees accumulated at larvae stage, the more death

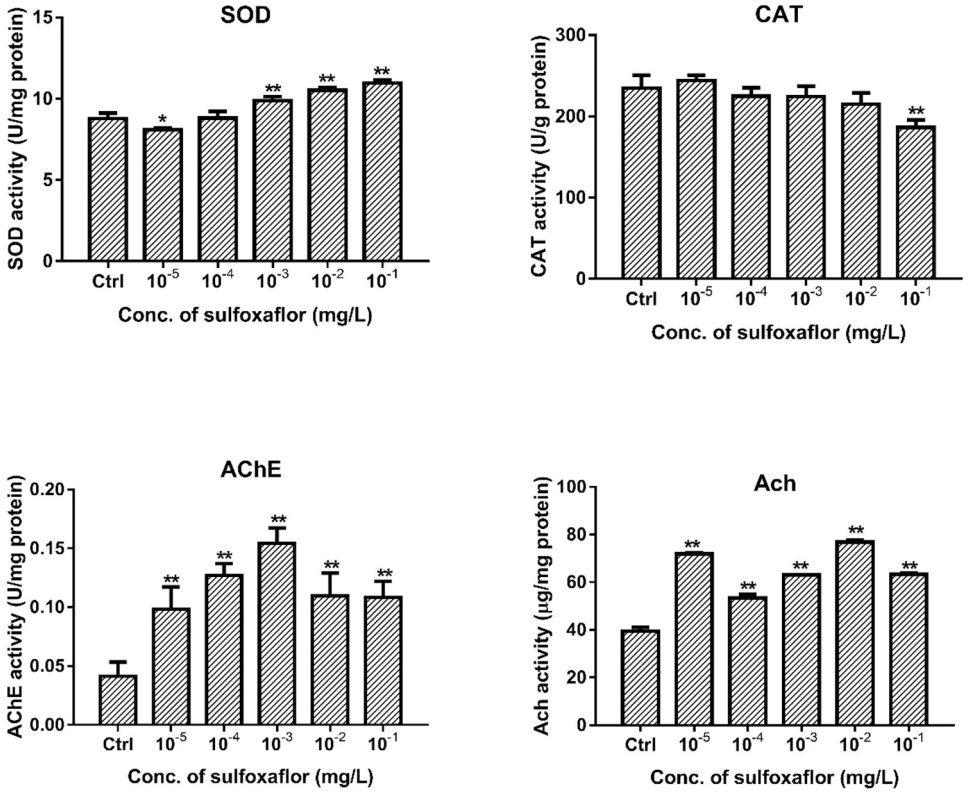

Fig. 5. Effects of sulfoxaflor on antioxidative enzymes Sod and Cat, and AChE, Ach in bee larvae after exposure. Significant differences relative to the Ctrl (control) group, *$p < 0.05$, **$p < 0.01$. Error bars represent SD fold changes.

can be calculated in the following pupa and emergence stages. This dose and time related relationship has been reported for many pesticides, like imidacloprid, and our earlier studies of flumehtrin (Qi et al., 2020b, 2020a). Lückmann et al. evaluated the toxicity of 138 active substances or formulated products to honeybee larvae and found that the toxicity of herbicides were higher than fungicides and insecticides are the most toxic, which are about 6–8 times toxic than fungicides (Lückmann et al., 2019). Larvae are the basis for colony's growth and development, because most of them grow into worker bees which are responsible for all the routine works in beehives, and the adequate adults will in return positively guarantee the food storage and baby caring. Some models have been developed to explore the effects of different factors on colony dynamics and relative compensate mechanisms (Torres et al., 2015; Khoury et al., 2013, 2011). Honey bees are highly social insect and known as super-organisms, thus any influence on the balance of each age castes (eggs, larvae, pupae and adults) and division of labor (nursing and foraging bees) could impact the growth and development of colonies, finally lead to colony failure (Khoury et al., 2011).

Since from 2006, the colony collapse disorder (CCD) has been reported over the whole world and diagnostic of this syndrome are often vacant hives containing dead brood and few or no adult bees (Khoury et al., 2013; Ellis et al., 2010). Among a variety of factors, pesticides could present risks to bees at both individual and colony levels, but the sensitivity of bees to chemicals can vary depending on age, nutritional status, diseases, exposure conditions, synergistic effects with other compounds and or factors (Wade et al., 2019). Dai et al. (2017) tested the 72-h acute toxicity of amitraz, chlorpyrifos, coumaphos, fluvalinate, and imidacloprid to honeybee larvae reared in vitro, and the calculated LD_{50}s of these five pesticides were 14.83, 0.46, 2.70, 0.83, and 4.17 μg/larvae, respectively. The registered oral LD_{50}s of chlorpyrifos and imidacloprid for *Apis* spp. in Pesticide Properties DataBase (PPDB) are 0.25 and 0.0037 μg/bee, respectively, which indicates that adults have a higher sensitivity to these pesticides than larvae. Here, a total of 160 μL of food containing sulfaxaflor was provided to each larva during exposure, the concentrations of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} mg/L theoretically corresponded to a total dose of 1.60×10^{-3}, 1.60×10^{-2}, 0.16, 1.60, and 16 ng sulfoxaflor per larva, respectively. These doses are much lower than those used for adult bees, as LC_{50} or LD_{50} was not calculated for larvae, so there's no direct comparison between the sensitivity of larval and adult bees to sulfoxaflor. However, if easily considering the lowest observed effect concentrations (LOEC), we speculate that honeybee adults are more tolerant to sulfoxaflor than larvae. The different sensitivities between larvae and adults might be due to the presence of insect fat bodies, which play important role in metabolism (Dai et al., 2017; Staron et al., 2017). In the same study of Lückmann et al. Lückmann et al. (2019), results indicated that toxicity did not increase during the pupation period between D8 and D22 in most cases and the larval growing period between D3 and D8 represents the most sensitive period of the pre-imaginal development. Comprehensively considering the high sensitivity and mortality of bee larvae to sulfoxaflor through whole develop history, further studies could focus on assessing the risk of chemicals to honey bee larvae at the individual or colony level, but the exposure duration has not to be fixed for evaluating chronic toxicity.

4.2. Sulfoxaflor induced physiological stresses in bees

Oxidative metabolism is a universal process in all aerobic organisms, and imbalances in ROS can cause oxidation of proteins, RNA, and DNA, and peroxidation of membrane lipids, finally resulting in damage, aging, and death (Weirich et al., 2002; Leza et al., 2019). The significant changes in the activities of the antioxidative enzymes SOD and CAT and the lipid peroxidation enzyme POD and the contents of MDA and LPO indicated that sulfoxaflor induced significant oxidative stress and damage both in honeybee larvae and adults.

The honey bees' larvae and adults have different responding mechanisms to stresses induced by sulfoxaflor. For example, SOD activity was increased after sulfoxaflor exposure in larvae but slightly decreased in adults. The content of MDA in larvae was lower than control at low concentrations but higher in the high-concentration groups; however, the levels of MDA in adult didn't change significantly. This phenomenon can be explained by the different sensitivities of larvae and adults to chemicals and by the different distribution of enzymes in tissues (Weirich et al., 2002). Hsu (Hsu and Hsieh, 2014) and Sagona et al. (2020) investigated the changes in antioxidant enzyme activities during aging in honeybees and found that SOD activity was significantly higher in young workers than in larvae, pupae, and old workers, while CAT and glutathione peroxidase activities were lower in young workers than in old workers. In addition, the activities of SOD, CAT, and GST also differ between tissues in bees. GSTs are important for the phase II detoxification of electrophilic xenobiotics in bees and also participate in phase I detoxification by binding and sequestering toxins (Berenbaum and Johnson, 2015). In this study, the activities of GST and GR changed both in larvae and adult bees, but the GSH content did not. These findings indicate that GSTs participated in the detoxification of sulfoxaflor in bees and help hosts resist xenobiotics. Similar effects were also found in earthworms (Fang et al., 2018), an important non-target organism in soil. Considering the universal importance of these indicators monitored here, they can not only be widely used in the toxicity studies of sulfoxaflor and other pesticides, but also efficient biomarkers for environment risks assessment.

Sulfoxaflor is the only Group 4C neonicotinoid insecticide and shares the same target of nAChRs with other neonicotinoids. Ach is the main excitatory neurotransmitter in honeybee brain, thus any interference in the Ach balance would impact the central nervous system and finally result in abnormal behavior output and colony failure (Grunewald and Siefert, 2019; Cabirol and Haase, 2019). A lot of studies and reviews have introduced the important function of Ach and its catalyzing enzyme AChE in bees and they also have been widely used as general biomarker of neural toxins in the fields of environmental and occupational medicine (Lionetto et al., 2013). In the present study, the content of Ach was significantly higher in sulfoxaflor-treated larvae than that in control, and the AChE activity changed in parallel. Some studies have shown that field-realistic doses of sulfoxaflor affect egg-laying rates and reproductive success of bumble bees and its lethal risks to honeybees could increase when combined exposure with neonicotinoids (Azpiazu et al., 2021). Honeybees undergo complete metamorphosis and the larvae experience huge changes during this stage, and the results found here indicated that sulfoxaflor has a highly toxic to honey bee larvae.

Except the important role of Ach and AChE in central nervous system, the non-neuronal Ach and AChE also exist in insects and are crucial during all developmental stages (Grunewald and Siefert, 2019; Wessler et al., 2016). In the same study, Grunewald and Siefert (Grunewald and Siefert, 2019) detailedly reviewed the function of Ach and AChE in the development of honeybees and assumed that neonicotinoids cause behavior impairment on adult bees by neuron effects, and disturb the development of larvae and adult by non-neuronally actions. Compared to vertebrates, the non-neuronal cholinergic system of insects is largely understudied, and whether sulfoxaflor alter the function of glands is still unknown. In the present study, AChE and Ach are sensitive biomarkers for the sulfoxaflor-induced effects in honeybee larvae. As the honeybees are highly social insects, and the development success of each caste is strongly associated with other castes, so AChE and Ach can also be used as biomarkers for environmental risk assessment in field at colony level.

5. Conclusion

Sulfoxaflor is a promising novel neonicotinoid compound for present and future use to control insects that have high resistance to current insecticides, especially those have high toxicity to mammals. Taken the results of the present study together with those notable previous reports of sulfoxaflor risks to non-target organisms, the potential harm of

sulfoxaflor to honeybees at realistic doses needs more attention. In particular, more work is needed to understand the life stage-dependent sensitivity to sulfoxaflor (high purity or different formulations) at the individual and colony level. The present study not only fills the gap in sulfoxaflor risk assessments but also provides a resource for future research on the effects of pollution in bees.

CRediT authorship contribution statement

Suzhen Qi and Shaokang Huang conceived and designed the experiments; Jiahuan Li, Liuwei Zhao and Suzhen Qi performed the experiments and data analysis; Wenting Zhao and Xiaofeng Xue contributed to the language check and polish; Liming Wu is responsible for supervision and resources allocation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No. 31702194 and No. 21707162); and also kindly supported by Project of Key Laboratory of Urban Agriculture In North China in 2020 (Grant No. kf2020013). We are grateful to Wei Du and Cuiling Wu from Agilent Beijing for their assistance with HPLC/MS/MS.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112379.

References

Al Naggar, Y., Paxton, R.J., 2021. The novel insecticides flupyradifurone and sulfoxaflor do not act synergistically with viral pathogens in reducing honey bee (Apis mellifera) survival but sulfoxaflor modulates host immunocompetence. Microb. Biotechnol. 14 (1), 227–240.

Azpiazu, C., Bosch, J., Bortolotti, L., Medrzycki, P., Teper, D., Molowny-Horas, R., Sgolastra, F., 2021. Toxicity of the insecticide sulfoxaflor alone and in combination with the fungicide fluxapyroxad in three bee species. Sci. Rep. 11 (1), 6821.

Babcock, J.M., Gerwick, C.B., Huang, J.X., Loso, M.R., Nakamura, G., Nolting, S.P., Rogers, R.B., Sparks, T.C., Thomas, J., Watson, G.B., Zhu, Y., 2011. Biological characterization of sulfoxaflor, a novel insecticide. Pest Manag. Sci. 67 (3), 328–334.

Berenbaum, M.R., Johnson, R.M., 2015. Xenobiotic detoxification pathways in honey bees. Curr. Opin. Insect Sci. 10, 51–58.

Cabirol, A., Haase, A., 2019. The neurophysiological bases of the impact of neonicotinoid pesticides on the behaviour of honeybees. Insects 10 (10).

Chakrabarti, P., Carlson, E.A., Lucas, H.M., Melathopoulos, A.P., Sagili, R.R., 2020. Field rates of SivantoTM (flupyradifurone) and Transform® (sulfoxaflor) increase oxidative stress and induce apoptosis in honey bees (Apis mellifera L.). PLoS One 15 (5), 0233033.

Dai, P., Jack, C.J., Mortensen, A.N., Ellis, J.D., 2017. Acute toxicity of five pesticides to Apis mellifera larvae reared in vitro. Pest Manag. Sci. 73 (11), 2282–2286.

Dai, P., Wang, M., Geng, L., Yan, Z., Yang, Y., Guo, L., Ma, S., Diao, Q., 2019. The effect of Bt Cry9Ee toxin on honey bee brood and adults reared in vitro, Apis mellifera (Hymenoptera: Apidae). Ecotoxicol. Environ. Saf. 181, 381–387.

Ellis, J.D., Evans, J.D., Pettis, J., 2010. Colony losses, managed colony population decline, and Colony Collapse Disorder in the United States. J. Apic. Res. 49 (1), 134–136.

Fang, S., Zhang, Y., You, X., Sun, P., Qiu, J., Kong, F., 2018. Lethal toxicity and sublethal metabolic interference effects of sulfoxaflor on the earthworm (Eisenia fetida). J. Agric. Food Chem. 66 (45), 11902–11908.

Grunewald, B., Siefert, P., 2019. Acetylcholine and its receptors in honeybees: involvement in development and impairments by neonicotinoids. Insects 10 (12).

Holder, P.J., Jones, A., Tyler, C.R., Cresswell, J.E., 2018. Fipronil pesticide as a suspect in historical mass mortalities of honey bees. Proc. Natl. Acad. Sci. USA 115 (51), 13033–13038.

Hsu, C.Y., Hsieh, Y.S., 2014. Oxidative stress decreases in the trophocytes and fat cells of worker honeybees during aging. Biogerontology 15 (2), 129–137.

Jiang, H., Chen, J.J., Zhao, C., Tian, Y.Q., Zhang, Z.X., Xu, H.H., 2020. Sulfoxaflor residues in pollen and nectar of cotton applied through drip irrigation and their potential exposure to Apis mellifera L. Insects 11 (2).

Khoury, D.S., Barron, A.B., Myerscough, M.R., 2013. Modelling food and population dynamics in honey bee colonies. PLoS One 8 (5), 59084.

Khoury, D.S., Myerscough, M.R., Barron, A.B., 2011. A quantitative model of honey bee colony population dynamics. PLoS One 6 (4), 18491.

Leza, M., Herrera, C., Marques, A., Roca, P., Sastre-Serra, J., Pons, D.G., 2019. The impact of the invasive species Vespa velutina on honeybees: a new approach based on oxidative stress. Sci. Total Environ. 689, 709–715.

Lionetto, M.G., Caricato, R., Calisi, A., Giordano, M.E., Schettino, T., 2013. Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. BioMed Res. Int. 2013, 1–8.

Lückmann, J., Becker, R., Miles, M., Alix, A., Dinter, A., Kroder, S., Pilling, E., Ruddle, N., Schneider, C., Sharples, A., Oger, L., 2019. Evaluation of honey bee larvae data: sensitivity to PPPs and impact analysis of EFSA Bee GD, in: Hazards of Pesticides to Bees - 14th International Symposium of the ICP-PR Bee Protection Group, Bern, Switzerland, vol. 465, pp. 82–89.

OECD, 1998. OECD Guidelines for the Testing of Chemicals-Honeybees, Acute Oral Toxicity Test. In Wageningen, Netherlands.

OECD, 2016. Guidance Document on Honey Bee (Apis mellifera) Larval Toxicity Test, Repeated Exposure. No. 239.

Qin, X., Xu, Y.M., Yang, S., Zhao, L.J., Liu, Y.T., 2014. Residue determination and degradation of sulfoxaflor in cotton and soil. J. Agric. Resour. Environ. 31 (4), 381–387.

Qi, S.Z., Niu, X.Y., Wang, D.H., Wang, C., Zhu, L.Z., Xue, X.F., Zhang, Z.Y., Wu, L.M., 2020a. Flumethrin at sublethal concentrations induces stresses in adult honey bees (Apis mellifera L.). Sci. Total Environ. 700, 134500.

Qi, S., Zhu, L., Wang, D., Wang, C., Chen, X., Xue, X., Wu, L., 2020b. Flumethrin at honey-relevant levels induces physiological stresses to honey bee larvae (Apis mellifera L.) in vitro. Ecotoxicol. Environ. Saf. 190, 110101.

Rondeau, G., Sanchez-Bayo, F., Tennekes, H.A., Decourtye, A., Ramirez-Romero, R., Desneux, N., 2014. Delayed and time-cumulative toxicity of imidacloprid in bees, ants and termites. Sci. Rep. 4.

Rossaro, B., Convertini, S., Bacci, L., 2018. A review of sulfoxaflor, a derivative of biological acting substances as a class of insecticides with a broad range of action against many insect pests. J. Èntomol. Acarol. Res. 50 (3).

Sagona, S., Betti, L., Casini, L., Palego, L., Giannaccini, G., Felicioli, A., 2020. Antioxidant enzymes activity during age polyethism in Apis mellifera L., 1758. J. Apic. Res. 1–11.

Sanchez-Bayo, F., Goka, K., 2014. Pesticide residues and bees–a risk assessment. PLoS One 9 (4), 94482.

Simon-Delso, N., Martin, G.S., Bruneau, E., Hautier, L., 2018. Time-to-death approach to reveal chronic and cumulative toxicity of a fungicide for honeybees not revealed with the standard ten-day test. Sci. Rep. 8, 7241.

Siviter, H., Brown, M.J.F., Leadbeater, E., 2018. Sulfoxaflor exposure reduces bumblebee reproductive success. Nature 561 (7721), 109–112.

Siviter, H., Horner, J., Brown, M.J.F., Leadbeater, E., 2020. Sulfoxaflor exposure reduces egg laying in bumblebees Bombus terrestris. J. Appl. Ecol. 57 (1), 160–169.

Siviter, H., Scott, A., Pasquier, G., Pull, C.D., Brown, M.J.F., Leadbeater, E., 2019. No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. Peerj 7, 7208.

Staron, M., Sabo, R., Sobekova, A., Sabova, L., Legath, J., Lohajova, L., Javorsky, P., 2017. Formetanate toxicity and changes in antioxidant enzyme system of Apis mellifera larvae. Environ. Sci. Pollut. Res. Int. 24 (16), 14060–14070.

Tavares, D.A., Dussaubat, C., Kretzschmar, A., Carvalho, S.M., Silva-Zacarin, E.C.M., Malaspina, O., Berail, G., Brunet, J.L., Belzunces, L.P., 2017. Exposure of larvae to thiamethoxam affects the survival and physiology of the honey bee at post-embryonic stages. Environ. Pollut. 229, 386–393.

Torres, D.J., Ricoy, U.M., Roybal, S., 2015. Modeling honey bee populations. PLoS One 10 (7), 0130966.

Wade, A., Lin, C.H., Kurkul, C., Regan, E.R., Johnson, R.M., 2019. Combined toxicity of insecticides and fungicides applied to california almond orchards to honey bee larvae and adults. Insects 10 (1).

Watson, G.B., Loso, M.R., Babcock, J.M., Hasler, J.M., Letherer, T.J., Young, C.D., Zhu, Y., Casida, J.E., Sparks, T.C., 2011. Novel nicotinic action of the sulfoximine insecticide sulfoxaflor. Insect Biochem. Mol. Biol. 41 (7), 432–439.

Weirich, G.F., Collins, A.M., Williams, V.P., 2002. Antioxidant enzymes in the honey bee, Apis mellifera. Apidologie 33 (1), 3–14.

Wessler, I., Gartner, H.A., Michel-Schmidt, R., Brochhausen, C., Schmitz, L., Anspach, L., Grunewald, B., Kirkpatrick, C.J., 2016. Honeybees produce millimolar concentrations of non-neuronal acetylcholine for breeding: possible adverse effects of neonicotinoids. PLoS One 11 (6), 0156886.

Zhu, L., Qi, S., Xue, X., Niu, X., Wu, L., 2020. Nitenpyram disturbs gut microbiota and influences metabolic homeostasis and immunity in honey bee (Apis mellifera L.). Environ. Pollut. 258, 113671.