Chronic in-hive exposure to a field-relevant concentration of Closer™ SC (24% sulfoxaflor) insecticide altered immunological and physiological markers of honey bee foragers (Apis mellifera)

Fatma S Ahmed, Walid S Helmy, Hatem Sharaf El-Din, Yahya Al Naggar

Apidologie (2023) 54:4

https://doi.org/10.1007/s13592-022-00987-6

Original Article

Chronic in-hive exposure to a field-relevant concentration of Closer™ SC (24% sulfoxaflor) insecticide altered immunological and physiological markers of honey bee foragers (*Apis mellifera*)

Fatma S. Ahmed[1], Walid S. Helmy[1], Hatem Sharaf El-Din[1], and Yahya Al Naggar[2]

[1] Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University, Giza 12613, Egypt
[2] Zoology Department, Faculty of Science, Tanta University, Tanta 31527, Egypt

Received 19 September 2022 – Revised 15 December 2022 – Accepted 16 December 2022

Abstract – Sulfoxaflor (SULF), a well-known alternative for the banned neonicotinoids, is not environmentally persistent, yet numerous studies using field-realistic levels have demonstrated its detrimental impact on honey bee colonies. Despite this, even just a limited number of studies have addressed its residue-level effects on the physiological and immunological biomarkers of foraging honey bees in semi-field conditions. In the present study, we determined the LT_{50} of sulfoxaflor (Closer™ 24% SC) on forager bees in the laboratory at concentrations that were 12 to 300,000-fold lower than the field-recommended concentration. Following that, we exposed bee colonies in a semi-field in-hive experiment for 21 days to a concentration that is 600,000-fold lower than the field-recommended concentration to assess potential effects on the physiological state of honey bee foragers. To do that, a total of six colonies were treated with Closer™ (0.3 µg a.i./L) or control treatment, and the activities of acetylcholinesterase (AChE), glucose oxidase (GOX), and carboxylesterase (CaEs) in head tissues and invertase (IV), alkaline phosphatase (ALP), and glutathione-S-transferase (GST) in the midgut tissues of forager bees were assessed at 5-, 10-, 15-, and 20-day post-exposure. The LT_{50} results for the least and greatest concentrations of Closer™ were 0.37 and 74.3 h, respectively. Furthermore, all measured physiological and immunological biomarkers were negatively impacted in the chronic exposure to 0.3 µg a.i./L concentration of Closer™. This data demonstrated the importance of reviewing the risk assessment of various sulfoxaflor formulations on bees and rephrasing the legislation that governs how beekeepers deal with sulfoxaflor-sprayed fields.

sulfoxaflor / semi-field experiment / forager bees / AChE / glucose oxidase / pollinators

1. INTRODUCTION

Sulfoxaflor (SULF), the first commercially available sulfoximine, is a systemic insecticide that has been used successfully to control a wide range of pests on multiple crops (Bacci et al. 2018). Like neonicotinoids, it targets insect nicotinic acetylcholine receptors (nAChRs), but at different sites (Watson et al. 2011). There has been no evidence of cross-resistance in sap-feeding insect pests that are likely resistant to commercial neonicotinoid insecticides due to metabolic detoxification mechanisms (Watson et al. 2021). As a result, many countries have begun to use sulfoximine-based insecticides in all outdoor crops to fill the hole left by the prohibition of neonicotinoids (Brown et al. 2016), and SULF (Transform®) has been registered in more than 81 countries worldwide for a wide variety

Corresponding author: Y. Al Naggar, Yehia.elnagar@science.tanta.edu.eg
Manuscript editor: Cedric Alaux

Published online: 03 January 2023

of crops including wheat, oilseed rape, cotton, or tomato in all inhabited continents.

As a result of the expansion of the use of SULF, several studies have evaluated its effects on bees. Some studies have reported that SULF has a contact acute LD_{50} of 0.379 µg/bee and an oral acute LD_{50} of 0.146 µg/bee toxicity for honey bees (*Apis* spp.) (Pesticide Properties DataBase (PPDB), http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/1669.htm). Although some studies suggest it is a safe chemical with no negative effects on either honey bee survival (Al Naggar and Paxton 2021) or bumblebee olfactory conditioning or working memory (Siviter et al. 2019), other studies have revealed sublethal effects of SULF on honey bee survival (Cheng et al. 2018) as well as bumblebee egg-laying and reproductive success (Siviter et al. 2018, 2020). Furthermore, honey bees exposed to a field-relevant concentration of SULF, either alone or in combination with the fungicide azoxystrobin, developed gut microbiota dysbiosis, which was linked to an increase in the relative abundance of opportunistic bacterial pathogens (Al Naggar et al. 2022). Despite their importance, these studies used the active ingredient of SULF. The addition of adjuvants to the active ingredient of an insecticide may result in a difference in its toxic effects (Mesnage and Antoniou 2017). Furthermore, formulations have been shown to be more toxic to bees than active substances (Mullin et al. 2015; Caliani et al. 2021).

Several commercial forms of SULF had been evaluated for their toxic effect on honey bees and bumble bees under colony and semi-field conditions. For example, the impact of the maximum rates for spray applications (0.4 L ha^{-1}) of Closer™ (formulated product of SULF, 120 g L^{-1} sulfoxaflor, i.e., 48 g a.i. ha^{-1}) on honey bee and bumble bee colony development and foraging activity was assessed (Tamburini et al. 2021a, b). Although the authors found no significant effects of Closer™ on honey bee colony development or foraging activity, they found effects on bumble bee colony growth and size, as well as foraging activity. Additionally, in our recent work, we found that chronic exposure of honey bee colonies to a sublethal and field-relevant concentration (0.3 ppb) of another formulation of SULF (Closer® SC, 240 g L^{-1} sulfoxaflor) negatively impacted honey bee colony development as well as the weight and foraging activity of forager bees (El-Din et al. 2022). These adverse effects of Closer™ on both bumblebees and honey bees could be attributed to physiological effects, particularly on the immune system and metabolism as shown in previous research (Badiou-Bénéteau et al. 2012; Carvalho et al. 2013; Al Naggar et al. 2015; Roat et al. 2017; Al Naggar and Baer 2019). However, this has not yet been investigated and must be confirmed or refuted.

The median lethal time (LT_{50}) is the time it takes for 50% of organisms exposed to a toxicant to die and is commonly used in toxicology studies to quantify the amount of a stressor necessary to kill an organism. LT_{50} can be used in conjunction with LD_{50} or LC_{50} for even more precise quantification (Zhao and Newman 2004). As a result, the goal of this study was to estimate the time required for sublethal and field-relevant Closer™ concentrations to kill 50% of honey bee foragers in the laboratory, as well as to investigate the potential adverse effects of chronic exposure to Closer™ under semi-field and in-hive conditions on immunological and physiological markers of foragers. We, therefore, measured levels of immunological and metabolic enzymes (glucose oxidase (Gox) (Alaux et al. 2010), alkaline phosphatase (ALP) (Chen et al. 2011), and invertase (IV)), detoxification enzymes (carboxylesterase (CaEs), glutathione-S-transferase (GST)), and acetylcholinesterase (AChE) in the head and midgut of honey bee foragers. These six biomarkers have been found to be useful in evaluating pesticide impacts on honey bees in various biological compartments (Alaux et al. 2010; Chen et al. 2011; Zhu et al. 2017; Yao et al. 2018; Almasri et al. 2020, 2021). We expected that the activity of these enzymes may alter over time as a result of exposure to the pesticide.

2. MATERIALS AND METHODS

2.1. Honey bees

Colonies of *Apis mellifera carnica* led by mated sisters' queens were maintained in the apiary yard of the agricultural experimental station, Faculty of Agriculture, Cairo University, Egypt, from June to August 2021. Colonies had no visible honey bee diseases, enough bee bread and honey stores, and a large number of nurses honey bee workers, and each colony comprised of eight bee-covered combs, including two to three combs of the brood.

2.2. Pesticide

Closer™ (240 g L^{-1} SULF, i.e., 40 g a.i. ha^{-1}, registration number L9694) was purchased from Corteva, Inc. (Corteva™ Agriscience). The formulation was kept at room temperature in its original packaging. Stock solutions were freshly prepared with tap water and diluted immediately at room temperature.

2.3. Determination of median lethal time (LT_{50}) of Closer™

The median lethal time LT_{50} (time until death of 50% of organisms exposed to a toxicant) of sublethal concentrations of Closer™ were conducted by oral administration through poisoned sugar syrup under controlled laboratory conditions. The tested concentrations were 15,000, 12,000, 6000, 2000, 600, 60, 6, and 0.6 µg a.i./L which represent 12-, 15-, 30-, 90-, 300-, 3000-, 30,000-, and 300,000-fold lower than the field recommended concentration on cotton (≈ 150 cm^3 a.i/200 L, according to Closer™ 24% SC SDS https://www.corteva.co.za/products-and-solutions/crop-protection/closer-240-sc.html), respectively.

Closer™ sublethal concentrations were chosen based on a study conducted by the US Environmental Protection Agency (2016). This study used a 0.089 lb a.i./acre application rate on cotton plants (equivalent to 40 cm^3 active compound/200 L) applied twice at 5-day intervals. That application rate, which is 3.75-fold lower than the currently recommended concentration, results in 6.09–38.15 µg a.i./L residues of SULF in nectar and 107.76–1647.10 µg a.i./L in pollen (U.S. EPA 2016).

From the hive entrance of three chosen colonies, we collected honey bee foragers (older than 3 weeks) who were returning with pollen grains on their legs. We chose forager bees over in-hive bees (OECD Test No. 213 *Test No. 213: Honeybees, Acute Oral Toxicity Test*, 1998) because they are more likely to be directly exposed to contaminated nectar. Furthermore, Barascou et al. (2022) showed that foragers are more sensitive to pesticides than nurse bees. The tested concentrations were prepared in 500 g per L sucrose (50% w per v). Each concentration was tested with three replicates, each replicate had twenty foragers, and they were allowed to feed on sugar syrup containing the tested concentrations or sugar syrup free of the pesticide (control). The cages used for oral toxicity bioassay were made of wood and had a rectangle shape (40 cm height × 30 cm diameter). The front face of these cages was designed as an openable door made of metal wire with wooden edges. Glass tubes 50 mm × 10 mm in size with an open-end narrowed to 2 mm in diameter and containing 200 µL of 50% sugar syrup at the appropriate concentration were used as feeders. The bees were starved for 2 h before the experiment, and moribund bees were replaced with healthy ones. Once the sugar syrup had been consumed (within 1–2 h), the feeders were removed and replaced with one containing untreated sugar syrup. The cages were kept in the dark at a room temperature of 25 ± 2 °C and relative humidity of 60 ± 10% (OECD 1998). For determining the oral median lethal time (LT_{50}), the mortalities were assessed at successive intervals: 0.2, 0.3, 0.5, 0.8, 1, 2, 3, 4, 6, 12, 24, 48, and 72 h post-treatment. Then LT_{50} was calculated using the LdP LineR program using the log-probit model Ehabsoft (http://www.ehabsoft.com/ldpline).

2.4. Chronic toxicity of Closer™

To investigate the potential adverse effects of chronic exposure for 20 days to a sublethal and field-relevant concentration of Closer™ on the physiological state of forager bees, honey bee colonies ($n = 3$) were exposed to 0.3 µg a.i./L (represented 600,000-fold lower than field recommended concentration) of Closer™ that was incorporated into 1 L of sugar solution 50% (w/v) per colony. Three colonies were used as a control group and received the same preparations as the experimental group but without the pesticide. The colonies that were chosen had at least 10,000–15,000 bees and 5–6 brood frames. The 1-L feeding volume was chosen to be consumed within 24 h, allowing for the introduction of fresh preparations every 2 days for 21 days. Every 5 days, twenty forager bees were collected from the hive entrance from each replicate colony of both treated and non-treated colonies, decapitated, the heads and midgut separated, and then stored at − 80 °C for enzyme activity assays to investigate the effect of pesticide exposure on enzyme activities over time.

2.5. Enzyme assays

The following chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA): *o*-dianisidine, horseradish peroxidase, 4-nitrophenyl acetate (4-NPA), 4-nitrophenol, acetylthiocholine iodide (ATChI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), ρ-hydroxybenzhydrazide (PAHBAH), *para*-nitrophenylphosphatase (pNPP), L-glutathione reduced (GSH), and 1-chloro-2,4 dinitrobenzene (CDNB).

Bee foragers were decapitated, and the heads were used to determine GOx, AChE, and CaE activities, while the midgut tissues were used to determine the activities of invertase, GST, and ALP enzymes. Three pooled samples of five foragers randomly selected from each replicate colony were homogenized using Surom™ homogenizer in three periods that did not exceed 30 s each. The extraction medium contained 40 mM sodium phosphate buffer (SPB) pH 7.4, 0.3% (w/v) Triton X-100 and protease inhibitor (Badiou-Bénéteau et al. 2012) to produce 10% (w/v) tissue extracts. The homogenates were then centrifuged at 12,000 rpm (4 °C) for 15 min, and the supernatants were collected for the enzyme activity assays listed below. The protein concentration of the homogenates was determined using the Bradford reaction with bovine serum albumin as a standard (Bradford 1976) and expressed as mg protein/mL homogenate. The protein concentration of each sample was used to standardize the enzyme activities to a per-milligram protein basis.

2.6. Acetylcholinesterase (AChE)

The head extract was used to determine the activity of AChE (EC 3.1.1.7) using acetylthiocholine (ATC) as a substrate, as described by Ellman et al. (1961) with modifications (Zhu et al. 2017). In a 200 µL reaction mixture, 50 µL supernatant of foragers head extract was mixed with 0.25 mM ATC and 0.4 mM DTNB in 150 µL of 0.1 M phosphate buffer pH 7.5. The change in absorbance (A) was measured kinetically at 405 nm using FLUOstar® Omega multi-mode microplate reader (BMG Labtech Co., Germany). The molar extinction coefficient of the colored product, DTNB (1.36×10^4 M^{-1} cm^{-1}) was used. Specific activities were expressed as nmole ATC hydrolyzed per min per mg protein.

2.7. Glucose oxidase (GOx)

The oxidation of *o*-dianisidine by H_2O_2 at 430 nm was conducted to determine the activity of GOx (EC 1.1.3.4) in the supernatant of foragers head extract according to the method of Alaux et al. (2010). The reaction medium contained 100 µL of sample supernatant, 200 µL of 2.5-unit peroxidase, 100 µL of 100 mM glucose, 100 µL of 0.3 mM o-dianisidine, and 500 µL of 125 mM monopotassium phosphate, pH 7.0. The quantification of GOx was performed using the H_2O_2 standard curve and expressed as muA per minute per milligram protein.

2.8. Carboxylesterases (CaEs)

The activity of CaEs (EC 3.1.1.1) was assessed using 4-nitrophenyl acetate (4-NPA) as a substrate according to the method of Chanda et al., (1997). The test media (2 mL total) was made up of 1,925 µL of 20 mM Tris–HCl buffer (pH 8.0) with 1 mM ethylenediaminetetraacetic acid (EDTA), 25 µL of 5 mM 4-NPA, and 50 µL of supernatant of foragers head extract. After 5 min of incubation at 25 °C, the formation of yellow 4-nitrophenol was measured at 405 nm with a Thermo Scientific Evolution 100 UV–Visible Spectrophotometer and plotted against a standard curve of 4-nitrophenol. The specific activity was expressed as µmoles of hydrolyzed 4-NPA per mg protein per minute.

2.9. Invertase (IV)

Using sucrose as a substrate and ρ-hydroxybenzhydrazide (PAHBAH) as a coloring reagent, the activity of the invertase enzyme (EC 3.2.1.26) was determined in supernatant of foragers head extract according to the method of Lever (1972) which was modified by Khandekar et al. (2014). A 1 mL reaction mixture containing 100 µL of supernatant and 900 µL of 1% w/v sucrose solution (in 100 mM acetate buffer, pH 4.5) was incubated at 55 °C for 20 min. Then 50 µL of the reaction mixture was withdrawn and added to 1.45 mL of freshly prepared 0.5% w/v PAHBAH solution (in 0.5 M freshly prepared NaOH). The mixture was then boiled at 90 °C for 5 min followed by cooling to room temperature. A blank sample was incubated without enzyme solution. The absorbance was measured at 410 nm by Thermo Scientific Evolution 100 UV–Visible Spectrophotometer. The specific activity of the invertase enzyme was determined by hydrolyzing 1.0 µmole of sucrose to glucose and fructose per minute per milligram protein under assay conditions.

2.10. Alkaline phosphatase (ALP)

The activity of ALP (EC 3.1.3.1) was assessed using *para*-nitrophenylphosphatase (pNPP) as a chromogenic substrate, as described by Bounias et al. (1996). The reaction medium contained 20 µL of supernatant of foragers midgut extract, 50 µL of 20 µM $MgCl_2$, 80 µL of 2 mM p-NPP, and 100 µL of 400 mM Tris–HCl, pH 8.5. The FLUOstar® Omega multi-mode microplate reader was used to monitor ALP at 410 nm. ALP-specific activities were calculated using a pure 4-nitrophenol calibration curve and expressed as µmol pNPP formed per minute per milligram protein.

2.11. Glutathione-S-transferase (GST)

Midgut GST (EC 2.5.1.18) activities were assayed using 1-chloro-2,4 dinitrobenzene (CDNB) as a substrate, based on the method of Habig et al. (1974) with adjustments in CDNB and glutathione (GSH) concentrations. A 10 µL of supernatant of foragers midgut extract was incubated for 10 min at 25 °C with 950 µL of phosphate buffer (100 mM, PH 7.4) and 30 µL of 50 mM glutathione (GSH) in an incubator. The reaction was initiated by the addition of 10 µL of 75 mM CDNB in ethanol. The change in absorbance was recorded at 340 nm with the Thermo Scientific Evolution 100 UV–Visible Spectrophotometer, using its kinetic mode, at 60-s intervals during a 5-min reaction period. The amount of conjugated CDNB formed was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig et al. 1974). The GST-specific activities were expressed as nmole CDNB-GSH conjugate per minute per milligram protein.

For all enzyme assays, three replicates from both treated and non-treated colonies were used, and they were corrected for non-enzymatic conjugation that occurred in a sample where the enzyme solution was replaced by the buffer. All enzymatic activities were also normalized to the protein concentration in the heads and midguts of treated and control groups.

2.12. Data analysis

Statistical analyses were performed using R studio version 4.1.2 (R Core Team (2022),

while data visualization was carried out by use of GraphPad Prism version 8.00 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com). To compare the change in enzyme activities between treated and non-treated colonies at different time points, we used ANOVA (Type II) tests in a generalized linear mixed model (GLMM). Pesticide exposure and time of assessment were used as independent, fixed factors (predictors), while the colony was included as a random factor. To test for significant interactive effects of exposure to pesticide exposure and time of assessment of investigated enzymes, we inspected the pesticide × time interaction terms in all models. For all analyses, the level of type I error was set as $p < 0.05$.

3. RESULTS

3.1. LT_{50} of sublethal concentrations of Closer™

The mortality % of foragers exposed to different concentrations of Closer™ was recorded at successive intervals (up to 72 h after treatment). Table I shows the LT_{50} and LT_{90} values at different times (h) for the eight tested concentrations. As expected, the LT_{50} values increased as Closer™ concentrations decreased. The least and greatest LT_{50} recorded were 0.37 and 74.3 h for the least (6 µg a.i. /L) and greatest concentrations (15,000 µg a.i. /L) of Closer™, respectively. The least and greatest concentrations are 300,000 and 12-fold lower than the field-recommended concentration. After 72 h of treatment, the mortality rate in the control group was 3.3%.

3.2. Enzyme activities

When we compared the activity of AChE, GOx, and CaEs at different time points in the head tissues of foragers exposed to a sublethal concentration of Closer™ (0.3 µg a.i./L) for 21 days, we found significant pesticide × time interaction effects for only AChE (GLMM, $p < 0.05$) indicating that the activity of this enzyme changed (increased) over time as a result of pesticide treatment (Table II).

Activities of GOx, and CaEs changed significantly in treated colonies compared to non-treated colonies (control). The activity of CaEs was always lower in forager bees collected from treated colonies compared to those collected from non-treated colonies (Figure 1, Table II).

Table I The median lethal time (LT_{50} and LT_{90}) (hours) of honey bee foragers ($n = 60$ bee/treatment) after exposure to Closer™ (active ingredient sulfoxaflor) at sub-field recommended concentrations

Conc. (µg a.i./L)	-fold lower than field recommended conc.[a]	LT_{50}(h)[b]	95% CL[c](lower–upper)	LT_{90}(h)[b]	95% CL (lower–upper)	Slope (mean ± SE)	$\chi 2$
15,000	12	0.37	0.32–0.43	1.04	0.85–1.38	2.89 ± 0.32	4.41
12,000	15	0.86	0.75–0.98	2.88	2.30–3.87	2.44 ± 0.21	9.37
6000	30	1.23	1.07–1.39	3.06	2.63–3.72	3.24 ± 0.28	4.21
2000	90	1.81	1.57–2.08	6.71	5.31–9.23	2.25 ± 0.20	1.18
600	300	5.99	5.27–6.85	18.2	14.7–24.3	2.66 ± 0.24	6.09
60	3000	14.7	11.8–18.1	95.2	66.4–161	1.58 ± 0.17	3.85
6	30,000	31.3	25.9–38.6	146	101–255	1.91 ± 0.22	1.64
0.6	300,000	74.3	56.0–115	423	227–1301	1.69 ± 0.26	6.93

[a]The field recommended concentration on cotton (≈150 cm^3/200 L, according to Closer™ 240 SC SDS)

[b]LT_{50} and LT_{90} are expressed in h

[c]Confidence intervals at a 95% probability level

We also found significant pesticide × time interaction effects for only GST (GLMM, $p < 0.05$) when we compared the activity of GST, invertase, and ALP enzymes at different time points in the midgut tissues of foragers exposed to a sublethal concentration of Closer™ (0.3 µg a.i./L), indicating that the activity of this enzyme changed over time as a result of pesticide treatment (Table II).

The activities of invertase and ALP changed significantly in treated colonies compared to non-treated colonies (control). The activity of ALP was always higher in forager bees collected from treated colonies compared to those collected from non-treated colonies (Figure 2, Table II).

4. DISCUSSION

Pesticides can have lethal effects on bees (Henry et al. 2012; Tosi and Nieh 2019), as well as sublethal effects such as cognitive impairments (Chakrabarti et al. 2019), flight and homing disruptions (Henry et al. 2012), and detrimental physiological alterations (Pettis et al. 2012; Chakrabarti et al. 2020). Consequently, the probable links between honey bee survival and pesticide residues have received a great deal of attention, and previous research indicated that pesticide residues may have a deleterious impact on honey bee population survival (Di Prisco et al. 2013; Sanchez-Bayo and Goka 2014; Zhu et al. 2017).

Closer™ (SULF) has been shown to be toxic to insects that pollinate plants (Bacci et al. 2018). As a result, this insecticide is used under strict regulations to limit risk of toxicity for these useful insects. One of these regulations is to avoid exposing bees to excessive concentrations of this insecticide. Despite that, bees may be exposed to residues of SULF while foraging on blooming plants adjacent to the treated fields, and this exposure may occur more than once.

In the current study, we exposed foragers to Closer™ concentrations that ranged from 12 to 300,000-fold lower than the field-recommended concentration (150 cm^3 active compound/200 L) under laboratory conditions. The results revealed that exposing forager bees to these concentrations resulted in 50% mortality in less than 48 h post-treatment, while exposure to concentration of 300,000-fold less than the recommended concentration resulted in 50% mortality in around 3 days. In fact, the mortality data obtained in the current study is consistent with the Closer™ fact sheet, which states that this chemical is harmful to bees and advises farmers to allow at least 3 h between application and bee foraging period. Despite this, the concentrations tested are quite low and several orders of magnitude lower than what bees may be exposed to after 3 h of foraging. According to the U.S. EPA (2016), when an application rate of 40 cm^3 active compound/200 L was applied, they detected a 6.09–38.15 µg a.i./L of SULF in nectar and 107.76–1647.10 µg a.i./L in pollen. These levels are significantly

Table II Results from GLMM analyses testing effects of exposure to a sublethal concentration (0.3 µg a.i./L) of Closer® (active ingredient sulfoxaflor) for 21 days on the activity of some enzymes in bee heads (*AChE* acetylcholinesterase, *GOx* glucose oxidase, and *CaEs* 4-nitrophenyl acetate carboxylase) and midgut tissues (*GST* glutathione-S-transferase, *IV* invertase (IV), and *ALP* alkaline phosphatase) at different time points of exposure

	(Type III Wald chi-square tests) (*p*-value)						
Source	**Df**	**AChE**	**GO_X**	**CaEs**	**GST**	**Invertase**	**ALP**
Pesticide	**1**	**< 0.001**	**0.02**	**< 0.001**	**< 0.001**	**0.04**	**< 0.001**
Time	**1**	**< 0.001**	0.05	0.54	0.06	0.71	**< 0.001**
Pesticide × time	**1**	**< 0.001**	0.25	0.77	**0.01**	0.07	0.19

Values in bold indicate statistically significant differences between treatments

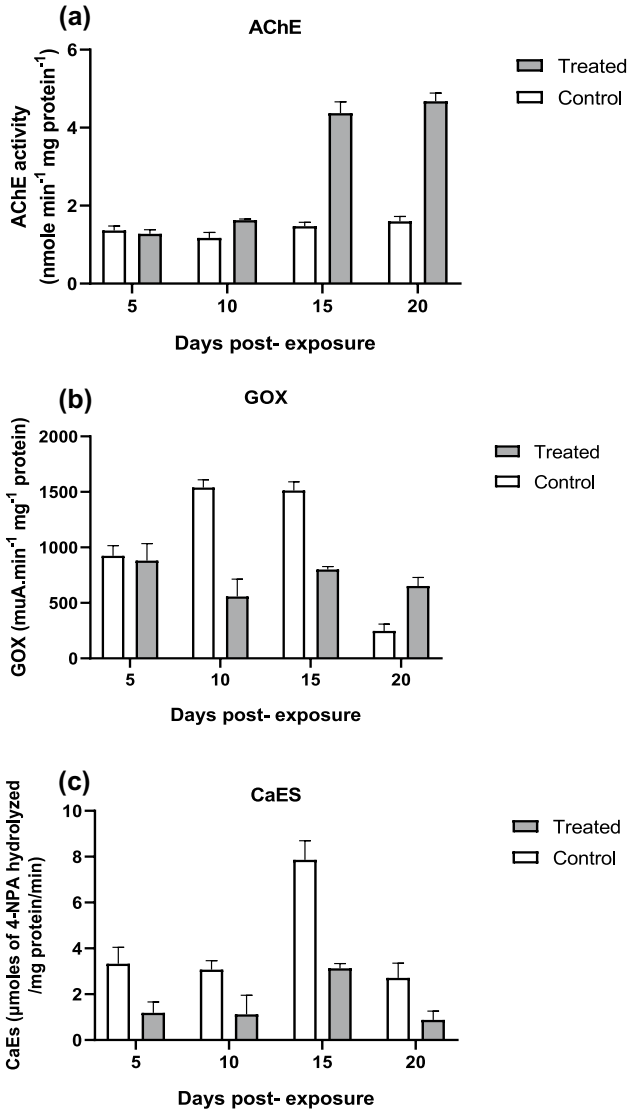

Figure 1. Effects of exposure of foraging bees to a sublethal concentration (0.3 µg a.i./L) of Closer® (active ingredient sulfoxaflor) for 21 days under semi-field in-hive conditions on the activity of **a** acetylcholinesterase (AChE), **b** glucose oxidase (GOx), and **c** 4-nitrophenyl acetate carboxylase (CaEs)) in bee heads at different time points of exposure. The statistical details are addressed in Table II, bars depict (mean ± SEM).

higher than the tested levels in our study, indicating a substantial risk to forager bees.

In our recent study, we showed that a 21-day chronic exposure to a very low concentration of Closer™ (SULF) had a detrimental effect on honey bee colony growth and foraging activity (El-Din et al. 2022). We assumed that the negative impact of chronic exposure to Closer™ (0.3 µg a.i./L) on foraging activity and weight of foragers could be attributed to physiological implications mainly in the immune system, detoxification, and metabolism of honey bee foragers as found in previous research (Christen et al., 2021; Al Naggar et al. 2015; Roat et al.

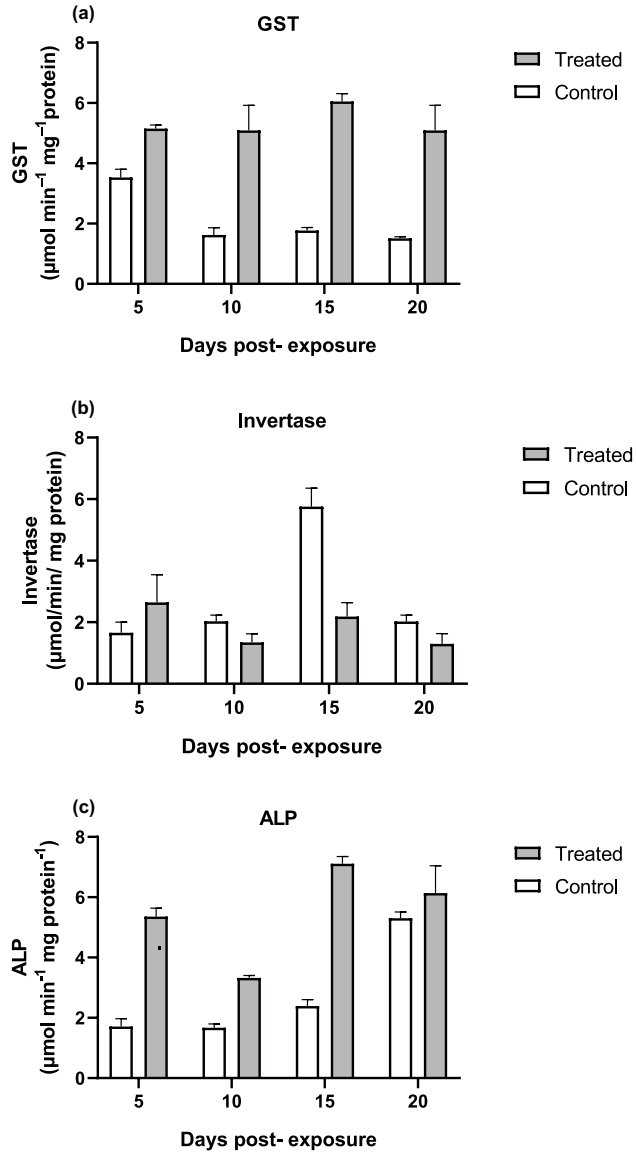

Figure 2. Effects of exposure of foraging bees to a sublethal concentration (0.3 µg a.i./L) of Closer® (active ingredient sulfoxaflor) for 21 days under semi-field in-hive conditions on the activity of **a** glutathione-S-transferase (GST), **b** invertase (IV), and **c** alkaline phosphatase (ALP) enzymes in bee midgut tissues at different time points of exposure. The statistical details are addressed in Table II, bars depict (mean ± SEM).

2017; Al Naggar and Baer 2019; Wu et al. 2023). To validate our assumption, we assessed the potential adverse effect of chronic exposure to Closer™ (0.3 µg a.i./L) on the activities of some enzymes involved in immunity (Gox and ALP, ALP also involved in metabolism (Carvalho et al. 2013), detoxification (GST and CaEs), honey production (IV), and neurotransmission (AChE) in foragers over time under in-hive conditions.

The immunological defense in honey bees is well-developed and includes hygienic behavior, which is an antiseptic behavior consisting of

the ability to recognize and remove unhealthy broods from the hive (Wilson-Rich et al. 2008). Any impairments in this immune system would undoubtedly affect the whole hive's vitality. Intriguingly, GOX-specific activity was significantly altered by chronic Closer™ exposure in the current study. The GOx enzyme, a social immunity parameter (Alaux et al. 2010), sterilizes larval food and honey (Bucekova et al. 2014), hence contributing to colony-food sterilization and thus disease prevention. Consequently, if the colony was unable to maintain levels of GOX activity, a decrease in antiseptics in the colony would impact not just adult nestmates but also brood survival, weakening the colony in the long run (Alaux et al. 2010). Our findings showed that the colony was unable to counteract the negative effects of Closer™ chronic exposure on the GOx activity, suggesting that the GOx may be a useful biomarker for assessing the previously observed disruptive effects of sulfoxaflor on colony growth and development.

Another immunological and metabolic biomarker in our study is alkaline phosphatase (ALP). We found a significant increase in ALP activity in response to Closer™ chronic exposure over the time. Similarly, an increase in the ALP activity in forager bees, however, in response to thiamethoxam (Badiou-Bénéteau et al. 2012) or spinosad (Carvalho et al. 2013) was reported. Fipronil and AMISTAR® XTRA (a commercial fungicide composed of azoxystrobin and cyproconazole) on the contrary significantly reduced the ALP activity in bees (Carvalho et al. 2013; Caliani et al. 2021). The alkaline phosphatase family of enzymes oversees cell signaling, the transport of metabolites, and is involved in digestion processes (Bounias et al. 1985). Insecticides, fungicides (Bounias et al. 1985), or acaricides (Bounias et al. 1996) have been shown to modulate ALP in the honey bee, even though it is not involved in pesticide detoxification. As a result, the elevation in ALP activity in our work could be interpreted as a defect in the immunological state of forager bees.

GST and CaEs are detoxification enzymes that eliminate a wide range of toxic compounds (Badawy et al. 2015). Despite this, these enzymes have not been thoroughly explored in honey bees (Zhu et al. 2017), and limited data suggested that GST and CaEs are less relevant in the honey bee detoxification system (Iwasa et al. 2004). On the contrary, several studies recorded a modulation in GST and/or CaEs activities of bees after pesticides exposure (Badiou-Bénéteau et al. 2012; Carvalho et al. 2013; Badawy et al. 2015; Almasri et al. 2020, 2021).

In our study, we found a significant induction in GST-specific activity over the 21 days of chronic exposure to Closer™. In addition to its role in detoxification, GST is thought to be a bioindicator of oxidative stress induction. This enzyme converts biomolecule peroxidation products formed during oxidative stress into less toxic hydroxyl derivatives (Yan et al. 2013). Thus, the increased GST activity is most likely due to increased lipid peroxidation (Taric et al. 2020). Badawy et al. (2015) also reported a 186% increase in GST activity following the treatment with acetamiprid, pymetrozine, and pyridalyl at lower doses. In addition, Nielsen et al. (2000), Papadopoulos et al. (2004), Carvalho et al. (2013), Roat et al. (2017), and Caliani et al. (2021) reported increased GST-specific activity in bees exposed to deltamethrin, flumethrin, spinosad, fipronil and AMISTAR®, respectively. The increase in GST-specific activity strongly implies the induction of oxidative stress in the midgut tissues of foragers due to exposure to SULF.

Carboxylesterase is involved in the biochemical mechanism of pesticide resistance in certain pest species and plays an important role in the detoxification of numerous endogenous and exogenous agrochemicals through hydrolysis (Wheelock et al. 2005). CaEs-specific activity was increased in the heads of honey bee foragers when acetamiprid, pymetrozine, and pyridalyl pesticides were applied at different rates and was inhibited by dinotefuran, indicating that carboxylesterase activity is pesticide dependent (Badawy et al. 2015). In the current study, we observed a significant decrease in CaEs-specific activity over the chronic exposure period to Closer™, implying a reduction in honey bee foragers detoxification capacity and that foragers are

very sensitive to Closer™, which could explain the negative effects reported on foraging activity and bee weight in our previous research (El-Din et al. 2022).

We further evaluated the effect of SULF on invertase, which is the enzyme responsible for honey production by hydrolyzing nectar sucrose to form fructose and glucose (Sahin et al. 2020). This enzyme also consider a vital parameter for estimating honey quality and freshness (Julika et al. 2020). We detected a significant decrease of invertase activity beginning on the tenth-day post-exposure compared to the control, indicating a detrimental effect on invertase, which might influence honey production. This assumption is consistent with the findings of Sandrock et al. (2014), who reported a 29% decline in honey production in colonies exposed to thiamethoxam and clothianidin on a long-term basis and could also be attributed to the decrease in foraging activity revealed in our previous study (El-Din et al. 2022). On contrary of our results, Zhu et al. (2017) found that a 3000 µg a.i./L of Transform 5G® (another formulation form of SULF) had no negative effects on invertase activity.

Many studies and reviews have highlighted the importance of acetylcholine and its catalyzing enzyme AChE in bees, and they have also been widely used as general biomarkers of neural toxins in environmental and occupational medicine (Lionetto et al. 2013). AChE inhibits impulse transmission by rapidly hydrolyzing the neurotransmitter acetylcholine in cholinergic receptors (Colović et al. 2013). In the current study, we found in the head of foragers collected from Closer™treated colonies an increase over time of AChE-specific activity. This is consistent with the findings of previous research that demonstrated higher AChE activity in advice™ (imidacloprid) and Transform™ (SULF)-treated bees at the residual level (Zhu et al. 2017) as well as in the brain tissues of bees exposed to coumaphos and aldicarb (Williamson et al. 2013). In contrast, AChE activity was inhibited in the head and thorax of honey bee foragers exposed to different rates of acetamiprid, pymetrozine, dinotefuran, and pyridalyl pesticides (Badawy et al. 2015).

The increase in AChE-specific activity that occurs in foragers after chronic exposure to Closer™ could be attributed to a defense response, thereby protecting overall bee health (Qi et al. 2020). AChE is also involved in memory and learning processing (Guez et al. 2010). As a result, changes in AChE activity may impair honey bee learning and memory performance, as recently reported (Cartereau et al. 2022), potentially reducing foraging performance as observed in our previous research (El-Din et al. 2022).

We employed forager bees rather than any other hive member in either the acute (LT_{50}) or chronic (enzyme assays) exposure experiments in the current study. This is due to the fact that forager bees conduct all outside work like nectar, pollen, water, and propolis collection (Bucekova et al. 2014). While performing this duty, forager bees may be accidentally exposed to pesticide residues (Zhu et al. 2020). Moreover, they might transfer the contaminated nectar and pollen to the hive leading to the accumulation of numerous pesticide residues in the beehive matrices (Almasri et al. 2021).

5. CONCLUSION

The cumulative intake of Closer™ at a very low level modulates immunological parameters, causing oxidative stress and neurotoxicity, which may affect forager body mass and activity, and therefore colony growth. These findings lend credence to the belief that Closer™ poses significant risks to pollination services, necessitating the passage of critical legislation prohibiting the use of such products or limiting exposure of honey bee colonies.

AUTHOR CONTRIBUTION

FA: study concept and design, acquisition of data, and writing of the manuscript. WH: data curation, and methodology. FA and WH: performed enzyme assays and funded all chemical reagents. HE: handling of honey bees and data collection. YA: study concept and design, data analysis, visualization, supervision, writing-reviewing and editing.

DATA AVAILABILITY

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

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval No approval of the Research Ethics Committee was required to achieve the goals of this study, as the experimental work involved an unregulated invertebrate species (*Apis mellifera*).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

REFERENCES

Al Naggar Y, Baer B (2019) Consequences of a short time exposure to a sublethal dose of Flupyradifurone (Sivanto) pesticide early in life on survival and immunity in the honeybee (*Apis mellifera*). Sci Rep 9(1):19753. https://doi.org/10.1038/s41598-019-56224-1

Al Naggar Y, Paxton RJ (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. https://doi.org/10.1111/1751-7915.13673

Al Naggar Y, Singavarapu B, Paxton RJ, Wubet T (2022) Bees under interactive stressors: the novel insecticides flupyradifurone and sulfoxaflor along with the fungicide azoxystrobin disrupt the gut microbiota of honey bees and increase opportunistic bacterial pathogens. Sci Total Environ 849:157941. https://doi.org/10.1016/j.scitotenv.2022.157941

Al Naggar Y, Wiseman S, Sun J, Cutler GC, Aboul-Soud M, Naiem E, Mona M, Seif A, Giesy JP (2015) Effects of environmentally-relevant mixtures of four common organophosphorus insecticides on the honey bee (*Apis mellifera* L.). J Insect Physiol 82:85–91. https://doi.org/10.1016/j.jinsphys.2015.09.004

Alaux C, Ducloz F, Crauser D, Le Conte Y (2010) Diet effects on honeybee immunocompetence. Biol Let 6(4):562–565. https://doi.org/10.1098/rsbl.2009.0986

Almasri H, Tavares DA, Diogon M, Pioz M, Alamil M, Sené D, Tchamitchian S, Cousin M, Brunet J-L, Belzunces LP (2021) Physiological effects of the interaction between Nosema ceranae and sequential and overlapping exposure to glyphosate and difenoconazole in the honey bee *Apis mellifera*. Ecotoxicol Environ Saf 217:112258. https://doi.org/10.1016/j.ecoenv.2021.112258

Almasri H, Tavares DA, Pioz M, Sené D, Tchamitchian S, Cousin M, Brunet J-L, Belzunces LP (2020) Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter *Apis mellifera* honey bees. Ecotoxicol Environ Saf 203:111013. https://doi.org/10.1016/j.ecoenv.2020.111013

Bacci L, Stefano C, Rossaro B (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 Entomol Acarol Res 50. https://doi.org/10.4081/jear.2018.7836

Badawy MEI, Nasr HM, Rabea EI (2015) Toxicity and biochemical changes in the honey bee *Apis mellifera* exposed to four insecticides under laboratory conditions. Apidologie 46(2):177–193. https://doi.org/10.1007/s13592-014-0315-0

Badiou-Beneteau A, Carvalho SM, Brunet J-472 L, Carvalho GA, Bulete A, Giroud B, Belzunces LP (2012) Development of biomarkers of exposure to xenobiotics in the honey bee Apis mellifera: application to the systemic insecticide thiamethoxam. Ecotoxicol Environ Saf 82:22-31 https://doi.org/10.1016/j.ecoenv.2012.05.005

Barascou L, Sene D, Le Conte Y, Alaux C (2022) Pesticide risk assessment: honeybee workers are not all equal regarding the risk posed by exposure to pesticides. Environ Sci Pollut Res Int 29(60):90328–90337. https://doi.org/10.1007/s11356-022-21969-2

Bounias M, Dujin N, Popesković DS (1985) Sublethal effects of a synthetic pyrethroid, deltamethrin, on the glycemia, the lipemia, and the gut alkaline phosphatases of honeybees. Pestic Biochem Physiol 24(2):149–160. https://doi.org/10.1016/0048-3575(85)90124-5

Bounias M, Kruk I, Nectoux M, Popeskovic D (1996) Toxicology of cupric salts on honeybees. V. Gluconate and sulfate action on gut alkaline and acid phosphatases. Ecotoxicol Environ Saf 35(1), 67–76. https://doi.org/10.1006/eesa.1996.0082

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1006/abio.1976.9999

Brown MJ, Dicks LV, Paxton RJ, Baldock KC, Barron AB, Chauzat MP, Stout JC (2016) A horizon scan of future threats and opportunities for pollinators and pollination. Peer J, 4, e2249

Bucekova M, Valachova I, Kohutova L, Prochazka E, Klaudiny J, Majtan J (2014) Honeybee glucose oxidase—its expression in honeybee workers and comparative analyses of its content and H2O2-mediated antibacterial activity in natural honeys. Naturwissenschaften 101(8):661–670. https://doi.org/10.1007/s00114-014-1205-z

Caliani I, Campani T, Conti B, Cosci F, Bedini S, D'Agostino A, Ammendola A, Di Noi A, Gori A, Casini S (2021) Multi-biomarker approach and IBR index to evaluate the effects of different contaminants on the ecotoxicological status of Apis mellifera. Ecotoxicol Environ Saf 208:111486. https://doi.org/10.1016/j.ecoenv.2020.111486

Cartereau A, Pineau X, Lebreton J, Mathé-Allainmat M, Taillebois E, Thany SH (2022) Impairments in learning and memory performances associated with nicotinic receptor expression in the honeybee Apis mellifera after exposure to a sublethal dose of sulfoxaflor. PLoS ONE 17(8):e0272514. https://doi.org/10.1371/journal.pone.0272514

Carvalho SM, Belzunces LP, Carvalho GA, Brunet J-L, Badiou-Beneteau A (2013) Enzymatic biomarkers as tools to assess environmental quality: a case study of exposure of the honeybee Apis mellifera to insecticides. Environ Toxicol Chem 32(9):2117–2124. https://doi.org/10.1002/etc.2288

Chakrabarti P, Carlson EA, Lucas HM, Melathopoulos AP, Sagili RR (2020) Field rates of Sivanto™ (flupyradifurone) and Transform® (sulfoxaflor) increase oxidative stress and induce apoptosis in honey bees (*Apis mellifera* L.). PloS One 15(5), e0233033. https://doi.org/10.1371/journal.pone.0233033

Chakrabarti P, Sarkar S, Basu P (2019) Pesticide induced visual abnormalities in Asian honey bees (Apis cerana L.) in intensive agricultural landscapes. Chemosphere 230:51–58. https://doi.org/10.1016/j.chemosphere.2019.05.050

Chanda SM, Mortensen SR, Moser VC, Padilla S (1997) Tissue-specific effects of chlorpyrifos on carboxylesterase and cholinesterase activity in adult rats: an in vitro and in vivo comparison. Fundamental and Applied Toxicology : Official Journal of the Society of Toxicology 38(2):148–157

Chen KT, Malo MS, Beasley-Topliffe LK, Poelstra K, Millan JL, Mostafa G, Alam SN, Ramasamy S, Warren HS, Hohmann EL, Hodin RA (2011) A role for intestinal alkaline phosphatase in the maintenance of local gut immunity. Dig Dis Sci 56(4):1020–1027. https://doi.org/10.1007/s10620-010-1396-x

Cheng Y, Bu Y, Tan L, Wu W, Li J, Zhou J, Zhai A, Shan Z (2018) A semi-field study to evaluate effects of sulfoxaflor on honey bee *(Apis mellifera)*. 9

Christen V, Grossar D, Charrière JD, Eyer M, Jeker L (2021) Correlation between increased homing flight duration and altered gene expression in the brain of honey bee foragers after acute oral exposure to thiacloprid and thiamethoxam. Front Insect Sci 19

Colović MB, Krstić DZ, Lazarević-Pašti TD, Bondžić AM, Vasić VM (2013) Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol 11(3):315–335. https://doi.org/10.2174/1570159X11311030006

Di Prisco G, Cavaliere V, Annoscia D, Varricchio P, Caprio E, Nazzi F, Gargiulo G, Pennacchio F (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proc Natl Acad Sci 110(46):18466–18471. https://doi.org/10.1073/pnas.1314923110

El-Din HS, Helmy WS, Al Naggar Y, Ahmed FS (2022) Chronic exposure to a field-realistic concentration of Closer® SC (24% sulfoxaflor) insecticide impacted the growth and foraging activity of honey bee colonies. Apidologie 53(2):22. https://doi.org/10.1007/s13592-022-00937-2

Ellman GL, Courtney KD, Andres VJ, Feather-stone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Guez D, Zhu H, Zhang SW, Srinivasan MV (2010) Enhanced cholinergic transmission promotes recall in honeybees. J Insect Physiol 56(9):1341–1348. https://doi.org/10.1016/j.jinsphys.2010.04.022

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249(22):7130–7139

Henry M, Béguin M, Requier F, Rollin O, Odoux J-F, Aupinel P, Aptel J, Tchamitchian S, Decourtye A (2012) A common pesticide decreases foraging success and survival in honey bees. Science 336(6079):348–350. https://doi.org/10.1126/science.1215039

Iwasa T, Motoyama N, Ambrose JT, Roe RM (2004) Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee. Apis Mellifera Crop Protection 23(5):371–378. https://doi.org/10.1016/j.cropro.2003.08.018

Julika WN, Ajit A, Ismail N, Aqilah N, Naila A, Sulaiman AZ (2020) Sugar profile and enzymatic analysis of stingless bee honey collected from local market in Malaysia. IOP Conference Series: Materials Science and Engineering 736(6):062001. https://doi.org/10.1088/1757-899x/736/6/062001

Khandekar DC, Palai T, Agarwal A, Bhattacharya PK (2014) Kinetics of sucrose conversion to fructo-oligosaccharides using enzyme (invertase) under free condition. Bioprocess Biosyst Eng 37(12):2529–2537. https://doi.org/10.1007/s00449-014-1230-5

Lever M (1972) A new reaction for colorimetric determination of carbohydrates. Anal Biochem 47(1):273–279. https://doi.org/10.1016/0003-2697(72)90301-6

Lionetto MG, Caricato R, Calisi A, Giordano ME, Schettino T (2013) Acetylcholinesterase as a

biomarker in environmental and occupational medicine: new insights and future perspectives. Biomed Res Int 2013:321213. https://doi.org/10.1155/2013/321213

Mesnage R, Antoniou MN (2017) Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. Front Public Health 5:361. https://doi.org/10.3389/fpubh.2017.00361

Mullin CA, Chen J, Fine JD, Frazier MT, Frazier JL (2015) The formulation makes the honey bee poison. Pestic Biochem Physiol 120:27–35. https://doi.org/10.1016/j.pestbp.2014.12.026

Nielsen SA, Brødsgaard CJ, Hansen H (2000) Effects on detoxification enzymes in different life stages of honey bees (*Apis mellifera* L., Hymenoptera: Apidae) treated with a synthetic pyrethroid (Flumethrin). Altern Lab Anim, ATLA 28(3):437–443

OECD. (1998). Test No. 213: Honeybees, Acute Oral Toxicity Test. https://www.oecd-ilibrary.org/content/publication/9789264070165-en

Papadopoulos AI, Polemitou I, Laifi P, Yiangou A, Tananaki C (2004) Glutathione S-transferase in the developmental stages of the insect *Apis mellifera* macedonica. Comp Biochem Physiol c: Toxicol Pharmacol 139(1):87–92. https://doi.org/10.1016/j.cca.2004.09.009

Pettis JS, vanEngelsdorp D, Johnson J, Dively G (2012) Pesticide exposure in honey bees results in increased levels of the gut pathogen Nosema. Naturwissenschaften 99(2):153–158. https://doi.org/10.1007/s00114-011-0881-1

Qi S, Niu X, Wang D, hui Wang C, Zhu L, Xue X, Zhang Z, Wu L (2020) Flumethrin at sublethal concentrations induces stresses in adult honey bees (*Apis mellifera* L.). Sci Total Environ 700:134500. https://doi.org/10.1016/j.scitotenv.2019.134500

R Core Team (2022) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Roat TC, Carvalho SM, Palma MS, Malaspina O (2017) Biochemical response of the Africanized honeybee exposed to fipronil. Environ Toxicol Chem 36(6):1652–1660. https://doi.org/10.1002/etc.3699

Sahin H, Kolayli S, Beykaya M (2020) Investigation of variations of invertase and glucose oxidase degrees against heating and timing options in raw honeys. J Chem 2020:5398062. https://doi.org/10.1155/2020/5398062

Sanchez-Bayo F, Goka K (2014) Pesticide residues and bees—a risk assessment. PLoS ONE 9(4):e94482. https://doi.org/10.1371/journal.pone.0094482

Sandrock C, Tanadini M, Tanadini LG, Fauser-Misslin A, Potts SG, Neumann P (2014) Impact of chronic neonicotinoid exposure on honeybee colony performance and queen supersedure. PLoS ONE 9(8):e103592. https://doi.org/10.1371/journal.pone.0103592

Siviter H, Brown MJF, Leadbeater E (2018) Sulfoxaflor exposure reduces bumblebee reproductive success. Nature 561(7721):109–112. https://doi.org/10.1038/s41586-018-0430-6

Siviter H, Horner J, Brown MJF, Leadbeater E (2020) Sulfoxaflor exposure reduces egg laying in bumblebees Bombus terrestris. J Appl Ecol 57(1):160–169. https://doi.org/10.1111/1365-2664.13519

Siviter H, Scott A, Pasquier G, Pull CD, Brown MJF, Leadbeater E (2019) No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. PeerJ 7:e7208. https://doi.org/10.7717/peerj.7208

Tamburini G, Wintermantel D, Allan MJ, Dean RR, Knauer A, Albrecht M, Klein A-M (2021a) Sulfoxaflor insecticide and azoxystrobin fungicide have no major impact on honeybees in a realistic-exposure semi-field experiment. Sci Total Environ 778:146084. https://doi.org/10.1016/j.scitotenv.2021.146084

Tamburini G, Pereira-Peixoto MH, Borth J, Lotz S, Wintermantel D, Allan MJ, Klein AM (2021b) Fungicide and insecticide exposure adversely impacts bumblebees and pollination services under semi-field conditions. Environ Int 157:106813

Taric E, Glavinic U, Vejnovic B, Stanojkovic A, Aleksic N, Dimitrijevic V, Stanimirovic Z (2020) Oxidative stress, endoparasite prevalence and social immunity in bee colonies kept traditionally vs. those kept for commercial purposes. Insects 11(5). https://doi.org/10.3390/insects11050266

Test No (1998) 213: Honeybees, Acute Oral Toxicity. TEST. https://doi.org/10.1787/9789264070165-EN

Tosi S, Nieh JC (2019) Lethal and sublethal synergistic effects of a new systemic pesticide, flupyradifurone (Sivanto®), on honeybees. Proc Royal Soc B 286(1900). https://doi.org/10.1098/RSPB.2019.0433

U.S. EPA (2016) Addendum to the Environmental Fate and Ecological Risk Assessment for Sulfoxaflor Registration. (p. 27). United states environmental protection agency. https://www.regulations.gov/document/EPAHQ-OPP-2010-0889-0409

Watson GB, Loso MR, Babcock JM, Hasler JM, Letherer TJ, Young CD, Zhu Y, Casida JE, Sparks TC (2011) Novel nicotinic action of the sulfoximine insecticide sulfoxaflor. Insect Biochem Mol Biol 41(7):432–439. https://doi.org/10.1016/j.ibmb.2011.01.009

Watson GB, Siebert MW, Wang NX, Loso MR, Sparks TC (2021) Sulfoxaflor—A sulfoximine insecticide: review and analysis of mode of action, resistance and cross-resistance. Pestic Biochem Physiol 178:104924. https://doi.org/10.1016/j.pestbp.2021.104924

Wheelock CE, Shan G, Ottea J (2005) Overview of carboxylesterases and their role in the metabolism of insecticides. J Pestic Sci 30(2):75–83. https://doi.org/10.1584/jpestics.30.75

Williamson SM, Moffat C, Gomersall MAE, Saranzewa N, Connolly CN, Wright GA (2013) Exposure to acetylcholinesterase inhibitors alters the physiology and motor function of honeybees. Front Physiol 4:13. https://doi.org/10.3389/fphys.2013.00013

Wilson-Rich N, Dres ST, Starks PT (2008) The ontogeny of immunity: development of innate immune strength in the honey bee (*Apis mellifera*). J Insect Physiol 54(10–11):1392–1399. https://doi.org/10.1016/j.jinsphys.2008.07.016

Wu X, Li Z, Yang H, He X, Yan W, Zeng Z (2023) The adverse impact on lifespan, immunity, and forage behavior of worker bees (Apis mellifera Linnaeus 1758) after exposure to flumethrin. Sci Total Environ 858:160146. https://doi.org/10.1016/j.scitotenv.2022.160146

Yan H, Jia H, Gao H, Guo X, Xu B (2013) Identification, genomic organization, and oxidative stress response of a sigma class glutathione S-transferase gene (AccGSTS1) in the honey bee. Apis Cerana Cell Stress & Chaperones 18(4):415–426. https://doi.org/10.1007/s12192-012-0394-7

Yao J, Zhu YC, Adamczyk J, Luttrell R (2018) Influences of acephate and mixtures with other commonly used pesticides on honey bee (*Apis mellifera*) survival and detoxification enzyme activities. Comp Biochem Physiol c: Toxicol Pharmacol 209:9–17. https://doi.org/10.1016/j.cbpc.2018.03.005

Zhao Y, Newman MC (2004) Shortcomings of the laboratory-derived median lethal concentration for predicting mortality in field populations: exposure duration and latent mortality. Environ Toxicol Chem 23(9):2147–2153. https://doi.org/10.1897/03-557

Zhu YC, Caren J, Reddy GVP, Li W, Yao J (2020) Effect of age on insecticide susceptibility and enzymatic activities of three detoxification enzymes and one invertase in honey bee workers (*Apis mellifera*). Comp Biochem Physiol c: Toxicol Pharmacol 238:108844. https://doi.org/10.1016/j.cbpc.2020.108844

Zhu YC, Yao J, Adamczyk J, Luttrell R (2017) Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (*Apis mellifera*). PLoS ONE 12(6):e0178421. https://doi.org/10.1371/journal.pone.0178421