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Binary and ternary toxicological interactions of clothianidin and eight commonly used pesticides on honey bees (*Apis mellifera*)

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ABSTRACT

Although many toxicological evaluations have been conducted for honey bees (*Apis mellifera*), most of these studies have only focused on the effects of individual chemicals. However, honey bees are usually exposed to pesticide mixtures under field conditions. In this study, we examined the effects of individual pesticides and mixtures of clothianidin (CLO) with eight other pesticides [carbaryl (CAR), thiodicarb (THI), chlorpyrifos (CHL), *beta*-cyfluthrin (BCY), *gamma*-cyhalothrin (GCY), tetraconazole (TET), spinosad (SPI) and indoxacarb (IND)] on honey bees using a feeding method. Toxicity tests of a 4-day exposure to individual pesticides revealed that CLO had the highest toxicity to *A. mellifera,* with an LC_{50} value of 0.24 μg a.i. mL^{-1}, followed by IND and CHL with LC_{50} values of 3.40 and 3.56 μg a.i. mL^{-1}, respectively. SPI and CAR had relatively low toxicities, with LC_{50} values of 7.19 and 8.42 μg a.i. mL^{-1}, respectively. In contrast, TET exhibited the least toxicity, with an LC_{50} value of 258.7 μg a.i. mL^{-1}. Most binary mixtures of CLO with other pesticides exerted additive and antagonistic effects. However, all the ternary mixtures containing CLO and TET (except for CLO+TET+THD) elicited synergistic responses to bees. Either increased numbers of components in the mixture or/and a unique mode of action appeared to be responsible for the higher toxicity of mixtures. Our findings emphasized the need for risk assessment of pesticide mixtures rather than the individual chemicals. Our data also provided information that might help growers avoid increased toxicity and unnecessary injury to pollinators.

1. Introduction

The use of pesticides remains one of the main pest management strategies in modern agriculture (Jactel et al., 2019). However, the unintentional misuse of pesticides in farming areas can pose certain risks to the environment and non-target organisms, such as bees (Thompson et al., 2019; Kadlikova et al., 2021). Honey bees (*Apis mellifera*) play a major role in the maintenance of plant biodiversity and food security through agricultural productivity (Klein et al., 2018). In the last several decades, an alarming level of loss of honey bee colonies has been reported in the United States, Canada and parts of Europe, and the number of colonies is decreasing even while global demand for crop pollination is growing (Bryden et al., 2013; Harwood and Dolezal, 2020). Honey bees are the predominantly managed pollinator worldwide, so it is important to understand and mitigate the causes of current declines in honey bee populations (Lourenço et al., 2019). It has been frequently reported that the use of pesticides is a factor reducing bee health, especially neonicotinoid insecticides, which are widely applied, highly toxic, and persistent in soil and water (Zhu et al., 2015; Diao et al., 2018; Crenna et al., 2020). The systemic property of neonicotinoids leads to chemical diffusion through the xylem in growing plants, causing the compound to be found in pollen, nectar, and water of guttation, sources that are used by honey bees for food and energy (Schmuck and Lewis, 2016). Therefore, special attention should be paid to the toxic effects of neonicotinoids to mitigate their harm to honey bees (Tison et al., 2019; Annoscia et al., 2020).

The data available on toxicities of pesticide mixtures are relatively limited compared with what has been reported for those of individual pesticides (Jiang et al., 2018; Thompson et al., 2019). However, in agricultural environments, honey bee populations are generally not

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exposed to individual pesticides because farmers often use pesticide mixtures (Liu et al., 2017; Carnesecchi et al., 2019). One study has found that on average there are residues of 6.5 pesticides in North American honey bee colonies (Mullin et al., 2010). In addition, honey bees appear to be deficient in detoxifying enzyme systems, making them specifically susceptible to pesticides (Gong and Diao, 2017). It is known that pesticide mixtures can lead to changes in toxicity, and thus the results from individual-pesticide experiments do not reflect field conditions where multiple pesticides or pesticide mixtures are present (Prado et al., 2019). Studies show that the synergistic effect of different pesticide mixtures in honey bees has large impacts on honey bee colonies and may significantly contribute to honey bee decline (Han et al., 2019). Due to the ubiquitous nature of exposure to multiple pesticides and the paucity of data for toxicity of mixtures, it is urgently necessary to consider the effects of pesticide mixtures on *A. mellifera* (Wang et al., 2020).

Clothianidin (CLO) is one of the most commonly used neonicotinoids worldwide, both as a systemic pesticide in seed coating, and directly sprayed on crops (Zhu et al., 2017a; Schmolke et al., 2019). Although CLO and imidacloprid are in the same pesticide group (the "N-nitroguanidines"), CLO acts as a super agonist of nicotinic acetylcholine receptor (nAChR) while imidacloprid is only a partial agonist of nAChR (Schmuck and Lewis, 2016; Matsuda et al., 2020). Due to the extensive use of CLO, its residues have frequently been detected in nectar and pollen samples (Sánchez-Hernández et al., 2016). Recent evidence has also shown that CLO and other classes of pesticides are often present in the same floral resources, posing a potential threat to crop pollinators (Sgolastra et al., 2018). The carbamates carbaryl (CAR) and thiodicarb (THI), the organophosphate chlorpyrifos (CHL), the pyrethroids *beta*-cyfluthrin (BCY) and *gamma*-cyhalothrin (GCY), the triazole fungicide tetraconazole (TET), the spinosyn insecticide spinosad (SPI), and the oxadiazine insecticide indoxacarb (IND) are widely used in the management of key agricultural pests and diseases, and they represent a variety of chemical classes recommended by extension specialists (Insect Control Guide Committee, 2020). Mixtures of CLO and the above-mentioned different insecticides are very often used in agricultural applications, which are co-applied to control striped flea beetle [*Phyllotreta striolata* (F.)], greenbug [*Schizaphis graminum* (Rondani)], the greenhouse whitefly [*Trialeurodes vaporariorum* (Westwood)], white grubs [*Alissonotum impressicolle* (Arrow)], and so on (Belden and Brain, 2018; Wade et al., 2019). Therefore, there is a growing concern about their interactive toxicity to honey bees (Yao et al., 2018). Here, we examined whether there was synergistic toxicity in mixtures of CLO and eight other commonly used pesticides for honey bees. Our findings could provide a scientific basis for the realistic assessment of the potential negative effects of CLO and its mixtures on honey bees.

2. Methods

2.1. Honey bee cultures

The honey bee (*A. mellifera*) queenright colonies used in our experiments were originally purchased from beekeepers located in pine forest and pasture areas of Mississippi near the cities of Magee and Perkinston. Bee colonies were installed in an isolated bee yard in the Mississippi Wildlife Management Area (North Stoneville, MS, USA). Frames with > 50% coverage of sealed brood were transferred into a laboratory incubator set at 33 ± 0.5 ℃, relative humidity of 65 ± 3%, and no light. A group of 20 newly emerged worker bees were transferred into a testing cage (diameter × height: 9.3 × 10 cm) and provided one scintillation vial of 50% sucrose solution and one scintillation vial of d-H_2O at the top of the cage. Caged bees were maintained in incubators under the same conditions described above for 4 days before for further experiments.

2.2. Pesticides

Nine pesticides from seven chemical classes were selected in this study, including CLO (Belay 50 WDG, Valent), CAR (Sevin XLR Plus, Bayer CropScience) and THI (Larvin 3.2F, Bayer), CHL (Lorsban 4E, Dow AgroSciences), BCY (Baythroid XL 1 EC, Bayer) and GCY (Declare, Cheminova), TET (Domark 230 ME, Valent), SPI (Tracer 4 SC, Dow AgroSciences) and IND (Steward EC, DuPont). Commercial formulations of these pesticides rather than their active ingredient were used since we aimed to simulate field situations and evaluate the potential interactive toxicities of chemical mixtures to honey bees from formulations commonly applied under field conditions.

2.3. Toxicity test method

Bioassays were performed by feeding worker bees sucrose solutions containing the desired concentrations of particular pesticides, as per Yao et al. (2018), with minor modifications. Preliminary studies were conducted to determine the range of concentrations that caused 0–100% honey bee mortality. To establish the concentration-mortality relationship, honey bees were exposed to six different concentrations of each pesticide with a geometrical ratio. Sucrose solution alone was used as the control. Each tested concentration was carried out in triplicate. One cage of 20 bees was used as a replicate. The exposure lasted for 7 days. During the test period, the bees were maintained in the dark (except for the period during observations) in an incubator at 33 ± 0.5 °C with a relative humidity of 65 ± 3%. Honey bees incapable of righting themselves were recorded as dead. The mortality of honey bees was recorded after exposure for 2, 4, and 7 days, respectively.

2.4. Mixture toxicity test

To reveal potential interactive effects of pesticide mixtures on honey bees, CLO was binarily and ternarily mixed with the other eight pesticides. The toxicities of chemical mixtures were assessed using honey bee workers exposed to sucrose solution containing CLO and other chemicals. We directly compared the toxicity between the single compounds and their combinations in concurrent testing. To assess the mixed effects of pesticide combinations, honey bees were challenged by serial dilutions of each chemical with a fixed equitoxic constant mixture ratio (the same toxicity effect from each chemical) according to individual 4-day LC_{50} values. Pesticide combinations were tested to six dilutions with a geometrical ratio. To establish the complete concentration-response correlations, the contents of each chemical were different, while the above-mentioned ratios in the combinations remained unchanged. The experiment for each tested level was performed three times (cages), and there were 20 honey bee workers (4 days old) in each cage. The other procedures for assaying mixtures were similar to those used for the individual pesticide assays.

2.5. Statistical analysis

SAS probit analysis (SAS/STAT 9.2 User's Guide, Cary, NC) was used to calculate LC_{50} values of assessed pesticides for honey bees. Toxicities were considered significantly different if the 95% confidence intervals (CIs) of two LC_{50} values did not overlap.

Marking's (1985) additive index (AI) of co-effect was adopted to evaluate mixture toxicity as follows:

$$S=(A\mathrm{m}/A\mathrm{i})+(B\mathrm{m}/B\mathrm{i})$$

where S presents the sum of the toxic effect of pesticides A and B; Am indicates the LC_{50} of pesticide A in the mixture; Ai is the LC_{50} of individual pesticide A; Bm indicates the LC_{50} of pesticide B in the mixture; and Bi is the LC_{50} of individual pesticide B.

The AI value was calculated from the sum of S based on the corresponding formulas as follows:

$$\mathrm{AI}=(1/S)-1 \text{ for } S<1{\cdot}0 \text{ and } \mathrm{AI}=1-S \text{ for } S\geq1{\cdot}0{\cdot}$$

Mixture toxicities were scored as antagonistic effect (AI ≤ −0.2), additive (−0.2 < AI ≤ 0.25) or synergistic effect (AI > 0.25) following the criteria defined by Su et al. (2016). The greater the AI value, the stronger the pesticide synergy.

3. Results

3.1. Single pesticide toxicity

A high degree of variation in toxicity was found among various classes of pesticides and pesticides within the same class (Table 1). After 2 days of exposure, the oral toxicity (LC_{50}) of the nine selected pesticides to *A. mellifera* ranged from 0.38 to 312.7 μg a.i. mL^{-1}. The toxicity of the nine pesticides could be ranked in descending order as follows: CLO > CHL, IND ≥ SPI, CAR ≥ BCY > GCY > THD > TET. CLO, CHL and IND were 822.9, 63.2 and 54.9 times more toxic than TET to *A. mellifera* according to their LC_{50} values, respectively. After 4 days of exposure, the LC_{50} values of the examined pesticides to honeybees ranged from 0.24 to 258.7 μg a.i. mL^{-1}. The order of toxicity (high-low) for the nine pesticides was ranked as follows: CLO > IND, CHL > SPI, CAR > BCY > GCY > THD > TET. CLO, IND and CHL were 1087, 76.1 and 72.7 times more toxic than TET, respectively.

After 7 days of exposure, the LC_{50} values of the assessed pesticides to the pollinators ranged from 0.20 to 216.8 μg a.i. mL^{-1}. The descending order of toxicity of the nine pesticides after exposure for 7 days was the same as that after 4 days of exposure. CLO, IND and CHL were 1084, 80.6 and 74.5 times more toxic than TET, respectively.

The toxicities of CLO, CHL, CAR and THD to *A. mellifera* after 4 days of exposure were significantly higher compared with their respective toxicities after 2 days of exposure. The toxicities of all the evaluated pesticides to honey bees after 7 days of exposure were similar to their respective toxicities after 4 days of exposure, but were significantly higher than their respective toxicities after the 2 days of exposure, by 2.12-, 1.96-, 1.72- and 1.7-fold for IND, CLO, CAR and CHL, respectively. In general, longer exposure was positively, but not linearly, correlated with the toxicity of the chemicals tested. Overall, the neonicotinoid insecticide CLO displayed the highest toxicity among the nine pesticides tested, followed by oxadiazine insecticide IND and organophosphate insecticide CHL. Spinosyn insecticide SPI and carbamate insecticide CAR showed relatively low toxicities, and triazole fungicide TET showed the lowest toxicity to *A. mellifera*.

3.2. Toxicity of pesticide mixtures

3.2.1. Toxicities of binary mixtures

The AI values for the binary mixture of CLO+TET after 2, 4, or 7 days of exposure were − 0.035, 0.19 and 0.37, respectively, indicating that the mixture had additive and synergistic effects on honey bees. Two binary mixtures of CLO+IND and CLO+THD exhibited additive effects from 2 days to 7 days after exposure. However, four binary mixtures, CLO+CHL, CLO+BCY, CLO+GCY and CLO+CAR, exhibited additive and antagonistic effects from 2 days to 7 days after exposure. In contrast, the binary mixture of CLO+SPI had antagonistic effects on the pollinators from 2 days to 7 days after exposure (Table 2).

3.2.2. Toxicities of ternary mixtures

All the ternary mixtures containing CLO and TET (except for CLO+TET+THD) displayed synergistic responses to honey bees from 2 days to 7 days after exposure. However, two ternary mixtures, CLO+-SPI+CAR and CLO+BCY+GCY, had additive effects from 2 days to 7 days after exposure. In contrast, six ternary mixtures, CLO+CHL+SPI, CLO+CHL+BCY, CLO+CHL+GCY, CLO+CHL+CAR, CLO+SPI+IND and CLO+BCY+THD, exhibited antagonistic effects on *A. mellifera* from 2 days to 7 days after exposure. The other 13 ternary mixtures showed additive and antagonistic effects with their AI values ranging from − 0.51 to − 0.04 after 2 days of exposure, from − 0.84 to − 0.16 after 4 days of exposure, and from − 0.44–0.088 after 7 days of exposure (Tables 3–6).

3.3. Interaction patterns of pesticide mixtures

Among the binary mixtures of CLO in combination with each of the other eight pesticides, 12.5% of the binary mixtures elicited additive-synergistic effects (showing additive effects at shorter exposure and synergism at longer exposure) on *A. mellifera*. In addition, 25.0% of combinations showed additive effects, and 12.5% of the pesticide mixtures exhibited antagonism. The rest (50.0%) of the combinations showed antagonistic-additive effects on honey bees (Fig. 1A).

A total of 28 ternary mixtures of CLO in combination with eight pesticides were investigated in the present study. Approximately 21.43% of ternary mixtures exerted synergistic effects. In addition, 3.57% and 7.14% of combinations exhibited additive-synergistic and additive effects on *A. mellifera*, respectively. In contrast, 46.43% and

Table 1
Summary of parameter estimates for the acute toxicity of the nine pesticides to honey bee (*Apis mellifera*).

Pesticides	2 d interval)			4 d interval			7 d interval		
	Slope (SE)	LC_{50} (95% FL) μg a.i. mL^{-1}	Predicted LD_{50} (ng a.i. bee^{-1})	Slope (SE)	LC_{50} (95% FL) μg a.i. mL^{-1}	Predicted LD_{50} (ng a.i. bee^{-1})	Slope (SE)	LC_{50} (95% FL) μg a.i. mL^{-1}	Predicted LD_{50} (ng a.i. bee^{-1})
CLO	3.09 (0.28)	0.38 (0.33–0.44)	19.76	3.02 (0.26)	0.24 (0.20–0.27)	24.96	3.35 (0.31)	0.20 (0.17–0.23)	36.4
CHL	2.11 (0.17)	4.95 (4.21–5.83)	257.4	2.57 (0.21)	3.56 (3.03–4.11)	370.24	3.43 (0.32)	2.91 (2.44–3.36)	529.62
TET	3.30 (0.29)	312.7 (271.2–356.6)	16,260.4	3.64 (0.34)	258.7 (221.2–295.2)	26,904.8	3.77 (0.36)	216.8 (180.7–250.2)	39,457.6
SPI	3.01 (0.26)	8.87 (6.83–12.02)	461.24	2.98 (0.25)	7.19 (6.24–8.26)	747.76	3.37 (0.30)	6.03 (5.22–6.87)	1097.46
BCY	2.17 (0.19)	14.27 (12.05–17.13)	742.04	2.24 (0.19)	11.41 (9.67–13.48)	1186.64	2.58 (0.22)	10.05 (8.60–11.69)	1829.1
GCY	1.93 (0.18)	45.99 (37.33–59.69)	2391.48	2.56 (0.22)	33.03 (28.26–39.46)	3435.12	2.52 (0.22)	29.46 (25.27–34.86)	5361.72
CAR	3.81 (0.37)	11.19 (9.86–13.01)	581.88	3.09 (0.29	8.42 (7.33–9.69)	875.68	3.46 (0.32)	6.49 (5.60–7.40)	1181.18
IND	2.11 (0.26)	5.69 (4.03–9.74)	295.88	2.10 (0.21)	3.40 (2.69–4.69)	353.6	1.93 (0.18)	2.69 (2.17–3.53)	489.58
THD	2.49 (0.22)	72.91 (61.96–88.19)	3791.32	2.79 (0.23)	49.85 (43.16–57.79)	5184.4	2.85 (0.24)	44.76 (38.71–51.65)	8146.32

FL fiducial limit, CLO Clothianidin, CHL Chlorpyrifos, TET Tetraconazole, SPI Spinosad, BCY Beta-cyfluthrin, GCY Gamma-cyhalothrin, CAR Carbaryl, INO Indoxacarb, THD Thiodicarb
Predicted LD_{50} was obtained based on LC_{50}, treatment duration (days), and average feeding volume (μl/day/bee [Zhu et al., 2017b]).

Table 2
Binary toxicological effects of clothianidin and other pesticides on honey bee *(Apis mellifera)*.

Exposure time (day)	Pesticide A	*Am* LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide B	*Bm* LC_{50} (95% FL) μg a.i. mL^{-1}	AI
2	CLO	0.21 (014–0.25)	+	CHL	3.06 (2.63–3.71)	-0.17
4	CLO	0.17 (0.12–0.29)	+	CHL	2.58 (1.92–4.32)	-0.43
7	CLO	0.14 (0.10–0.24)	+	CHL	2.18 (1.59–3.56)	-0.45
2	CLO	0.17 (0.15–0.20)	+	TET	183.9 (163.0–213.9)	-0.035
4	CLO	0.10 (0.089–0.11)	+	TET	109.4 (95.87–123.2)	0.19
7	CLO	0.073 (0.061–0.084)	+	TET	79.27 (66.43–90.44)	0.37
2	CLO	0.29 (0.18–2.09)	+	SPI	8.80 (5.51–62.87)	-0.75
4	CLO	0.25 (0.16–1.01)	+	SPI	7.52 (4.97–30.13)	-1.08
7	CLO	0.21 (0.14–0.40)	+	SPI	6.21 (4.46–12.07)	-1.09
2	CLO	0.25 (0.21–0.33)	+	BCY	12.32 (10.30–15.91)	-0.52
4	CLO	0.14 (0.12–0.16)	+	BCY	6.69 (5.85–7.74)	-0.17
7	CLO	0.091 (0.077–0.10)	+	BCY	4.31 (3.67–4.94)	0.13
2	CLO	0.22 (0.17–0.28)	+	GCY	29.93 (24.69–38.31)	-0.23
4	CLO	0.13 (0.11–0.16)	+	GCY	19.03 (16.49–22.18)	-0.12
7	CLO	0.11 (0.10–0.13)	+	GCY	15.95 (13.94–18.21)	-0.089
2	CLO	0.17 (0.15–0.20)	+	CAR	6.09 (5.40–7.03)	-0.001
4	CLO	0.14 (0.12–0.16)	+	CAR	5.04 (4.53–5.66)	-0.18
7	CLO	0.12 (0.10–0.16)	+	CAR	4.44 (3.52–5.69)	-0.28
2	CLO	0.18 (0.15–0.22)	+	IND	2.60 (2.24–3.12)	0.075
4	CLO	0.13 (0.11–0.15)	+	IND	1.89 (1.66–2.16)	-0.098
7	CLO	0.10 (0.094–0.12)	+	IND	1.51 (1.33–1.69)	-0.059
2	CLO	0.16 (0.14–0.19)	+	THD	33.75 (29.75–38.97)	0.13
4	CLO	0.12 (0.10–0.14)	+	THD	25.39 (22.63–28.51)	-0.008
7	CLO	0.099 (0.088–0.11)	+	THD	20.62 (18.31–22.97)	0.047

FL fiducial limit, AI additive index, CLO Clothianidin, CHL Chlorpyrifos, TET Tetraconazole, SPI Spinosad, BCY Beta-cyfluthrin, GCY Gamma-cyhalothrin, CAR Carbaryl, INO Indoxacarb, THD Thiodicarb.

Table 3
Ternary toxicological effects of clothianidin + chlorpyrifos and other pesticides on honey bee *(Apis mellifera)*.

Exposure time (day)	Pesticide A	*Am* LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide B	*Bm* LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide C	*Cm* LC_{50} (95% FL) μg a.i. mL^{-1}	AI
2	CLO	0.084 (0.071–0.10)	+	CHL	1.25 (1.06–1.55)	+	TET	90.96 (77.41–112.9)	0.31
4	CLO	0.062 (0.054–0.072)	+	CHL	0.91 (0.80–1.07)	+	TET	66.64 (58.55–77.68)	0.29
7	CLO	0.051 (0.045–0.057)	+	CHL	0.75 (0.66–0.84)	+	TET	54.53 (48.61–61.74)	0.31
2	CLO	0.22 (0.18–0.31)	+	CHL	3.32 (2.67–4.54)	+	SPI	6.71 (5.40–9.21)	-1.00
4	CLO	0.16 (0.13–0.20)	+	CHL	2.40 (2.04–2.97)	+	SPI	4.85 (4.12–6.01)	-1.01
7	CLO	0.094 (0.082–0.11)	+	CHL	1.39 (1.22–1.58)	+	SPI	2.81 (2.47–3.19)	-0.42
2	CLO	0.17 (0.14–0.21)	+	CHL	2.52 (2.15–3.14)	+	BCY	8.04 (6.84–9.99)	-0.52
4	CLO	0.12 (0.10–0.14)	+	CHL	1.79 (1.58–2.08)	+	BCY	5.71 (5.03–6.61)	-0.50
7	CLO	0.097 (0.086–0.11)	+	CHL	1.44 (1.27–1.65)	+	BCY	4.61 (4.07–5.25)	-0.44
2	CLO	0.20 (0.16–0.27)	+	CHL	2.99 (2.47–3.94)	+	GCY	27.81 (22.90–36.61)	-0.74
4	CLO	0.13 (0.11–0.16)	+	CHL	1.99 (1.72–2.37)	+	GCY	18.49 (16.02–21.99)	-0.66
7	CLO	0.092 (0.081–0.11)	+	CHL	1.37 (1.20–1.56)	+	GCY	12.67 (11.14–14.42)	-0.36
2	CLO	0.14 (0.11–0.17)	+	CHL	2.04 (1.72–2.49)	+	CAR	4.81 (4.07–5.87)	-0.22
4	CLO	0.098 (0.085–0.11)	+	CHL	1.46 (1.26–1.69)	+	CAR	3.45 (2.99–4.01)	-0.23
7	CLO	0.075 (0.065–0.085)	+	CHL	1.11 (0.97–1.26)	+	CAR	2.63 (2.28–2.97)	-0.86
2	CLO	0.14 (0.12–0.17)	+	CHL	2.18 (1.88–2.63)	+	IND	2.08 (1.79–2.51)	-0.17
4	CLO	0.099 (0.087–0.11)	+	CHL	1.47 (1.30–1.68)	+	IND	1.41 (1.24–1.61)	-0.24
7	CLO	0.074 (0.065–0.084)	+	CHL	1.11 (0.97–1.24)	+	IND	1.05 (0.92–1.18)	-0.14
2	CLO	0.14 (0.12–0.18)	+	CHL	2.15 (1.84–2.61)	+	THD	30.17 (25.88–36.49)	-0.22
4	CLO	0.11 (0.093–0.12)	+	CHL	1.56 (1.38–1.81)	+	THD	21.96 (19.35–25.22)	-0.34
7	CLO	0.074 (0.064–0.084)	+	CHL	1.09 (0.95–1.24)	+	THD	15.39 (13.43–17.38)	-0.09

FL fiducial limit, AI additive index, CLO Clothianidin, CHL Chlorpyrifos, TET Tetraconazole, SPI Spinosad, BCY Beta-cyfluthrin, GCY Gamma-cyhalothrin, CAR Carbaryl, INO Indoxacarb, THD Thiodicarb

21.43% of combinations had additive-antagonistic and antagonistic responses, respectively (Fig. 1B).

4. Discussion

Acute toxicity tests are usually the first step in evaluating the potential risk of pesticides to honey bees (Spruill et al., 2019). Previous studies have shown that the 2-day LC_{50} value of CLO via feeding exposure is 0.077 (0.05–0.11) μg a.i. mL^{-1}, indicating that it is an extremely toxic pesticide to honey bees (Laurino et al., 2011). Some studies have reported that CHL is highly toxic to *A. mellifera,* with a 2-day and 10-day feeding toxicity (LC_{50}) of 2.29 (0.63–1.29) and 0.58 (0.49–0.71) μg a.i. mL^{-1}, respectively (Zhao et al., 2008; Cheng et al., 2016). IND also exhibits high toxicity, with a 2-day LC_{50} value of 3.54 (3.28–3.82) μg a.i. mL^{-1} in a previous study (Yu et al., 2009), which is similar to our findings in this study. The high toxicity of CLO, IND, and CHL may be attributed to the lower ability of honey bees to metabolize these compounds (Berenbaum and Johnson, 2015; Milone et al., 2020). In contrast, the

Table 4
Ternary toxicological effects of clothianidin + tetraconazole and other pesticides on honey bee *(Apis mellifera)*.

Exposure time (day)	Pesticide A	*Am* LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide B	*Bm*LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide C	*Cm* LC_{50} (95% FL) μg a.i. mL^{-1}	AI
2	CLO	0.081 (0.068–0.099)	+	TET	86.95 (74.06–107.2)	+	SPI	2.42 (2.06–2.98)	0.31
4	CLO	0.063 (0.055–0.074)	+	TET	67.82 (59.54–79.22)	+	SPI	1.88 (1.65–2.21)	0.27
7	CLO	0.051 (0.041–0.067)	+	TET	55.22 (44.12–72.36)	+	SPI	1.54 (1.23–2.01)	0.31
2	CLO	0.043 (0.037–0.049)	+	TET	46.09 (40.24–52.61)	+	BCY	2.03 (1.77–2.32)	1.50
4	CLO	0.033 (0.028–0.037)	+	TET	35.34 (30.37–40.14)	+	BCY	1.55 (1.34–1.77)	1.44
7	CLO	0.028 (0.023–0.031)	+	TET	30.25 (25.43–34.46)	+	BCY	1.33 (1.12–1.52)	1.42
2	CLO	0.043 (0.038–0.049)	+	TET	46.71 (41.18–52.83)	+	GCY	5.97 (5.26–6.75)	1.55
4	CLO	0.037 (0.032–0.041)	+	TET	39.84 (34.96–44.66)	+	GCY	5.09 (4.46–5.71)	1.16
7	CLO	0.033 (0.028–0.037)	+	TET	35.85 (31.12–40.14)	+	GCY	4.58 (3.97–5.13)	1.06
2	CLO	0.068 (0.059–0.081)	+	TET	73.54 (64.01–87.24)	+	CAR	2.38 (2.07–2.83)	0.58
4	CLO	0.056 (0.049–0.065)	+	TET	60.93 (53.74–70.26)	+	CAR	1.97 (1.74–2.28)	0.43
7	CLO	0.045 (0.034–0.058)	+	TET	48.34 (37.08–62.94)	+	CAR	1.56 (1.20–2.04)	0.45
2	CLO	0.069 (0.061–0.082)	+	TET	75.26 (66.02–89.02)	+	IND	0.98 (0.86–1.17)	0.68
4	CLO	0.057 (0.051–0.065)	+	TET	61.66 (55.60–70.17)	+	IND	0.81 (0.73–0.92)	0.40
7	CLO	0.051 (0.033–0.00)	+	TET	55.48 (35.61–120.1)	+	IND	0.72 (0.47–1.57)	0.29
2	CLO	0.090 (0.076–0.11)	+	TET	97.38 (82.12–123.1)	+	THD	18.77 (15.83–23.73)	0.24
4	CLO	0.067 (0.059–0.079)	+	TET	72.69 (63.84–85.53)	+	THD	14.01 (12.31–16.49)	0.19
7	CLO	0.053 (0.041–0.075)	+	TET	57.68 (44.59–81.19)	+	THD	11.12 (8.59–15.65)	0.28

FL fiducial limit, AI additive index, CLO Clothianidin, CHL Chlorpyrifos, TET Tetraconazole, SPI Spinosad, BCY Beta-cyfluthrin, GCY Gamma-cyhalothrin, CAR Carbaryl, INO Indoxacarb, THD Thiodicarb

Table 5
Ternary toxicological effects of clothianidin +spinosad and other pesticides on honey bee *(Apis mellifera)*.

Exposure time (day)	Pesticide A	*Am* LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide B	*Bm*LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide C	*Cm* LC_{50} (95% FL) μg a.i. mL^{-1}	AI
2	CLO	0.16 (0.13–0.20)	+	SPI	4.91 (4.17–6.05)	+	BCY	7.72 (6.57–9.53)	-0.51
4	CLO	0.093 (0.082–0.11)	+	SPI	2.80 (2.47–3.19)	+	BCY	4.41 (3.88–5.02)	-0.16
7	CLO	0.073 (0.063–0.082)	+	SPI	2.18 (1.89–2.46	+	BCY	3.43 (2.98–3.88)	-0.071
2	CLO	0.16 (0.13–0.19)	+	SPI	4.86 (4.17–5.96)	+	GCY	22.31 (19.13–27.35)	-0.45
4	CLO	0.11 (0.10–0.14)	+	SPI	3.54 (3.13–4.10)	+	GCY	16.25 (14.35–18.79)	-0.44
7	CLO	0.071 (0.061–0.081)	+	SPI	2.12 (1.81–2.44)	+	GCY	9.73 (8.34–11.16)	-0.034
2	CLO	0.10 (0.087–0.12)	+	SPI	3.03 (2.64–3.49)	+	CAR	3.53 (3.07–4.08)	0.089
4	CLO	0.077 (0.066–0.089)	+	SPI	2.33 (2.01–2.67)	+	CAR	2.72 (2.34–3.12)	0.036
7	CLO	0.056 (0.046–0.065)	+	SPI	1.67 (1.38–1.95)	+	CAR	1.95 (1.61–2.27)	0.17
2	CLO	0.18 (0.15–0.24)	+	SPI	5.57 (4.66–7.14)	+	IND	2.63 (2.20–3.37)	-0.56
4	CLO	0.12 (0.10–0.14)	+	SPI	3.71 (3.24–4.34)	+	IND	1.75 (1.52–2.05)	-0.53
7	CLO	0.10 (0.092–0.12)	+	SPI	3.13 (2.76–3.58)	+	IND	1.47 (1.30–1.69)	-0.57
2	CLO	0.14 (0.12–0.18)	+	SPI	4.46 (3.83–5.40)	+	THD	30.92 (26.57–37.42)	-0.29
4	CLO	0.099 (0.086–0.12)	+	SPI	2.98 (2.60–3.45)	+	THD	20.68 (18.01–23.91)	-0.24
7	CLO	0.079 (0.069–0.090)	+	SPI	2.38 (2.07–2.71)	+	THD	16.49 (14.35–18.78)	-0.16

FL fiducial limit, AI additive index, CLO Clothianidin, CHL Chlorpyrifos, TET Tetraconazole, SPI Spinosad, BCY Beta-cyfluthrin, GCY Gamma-cyhalothrin, CAR Carbaryl, INO Indoxacarb, THD Thiodicarb

lower feeding toxicity of THD and TET may be caused by their low water solubility or higher enzymatic metabolic activity against these compounds by honey bees, or both, allowing only low concentrations to reach the pesticide site of action (Johnson, 2015; Gong and Diao, 2017). Considering the high efficacy of CLO, IND, and CHL against various target pests, special attention should be paid to their use in integrated pest management (IPM) programs to prevent serious harm to crop pollinators.

Although fungicides are considered to be relatively safe for honey bees compared with insecticides, some fungicides can increase insecticide toxicity (Raimets et al., 2018; Han et al., 2019; Almasri et al., 2020). Most binary mixtures of CLO in combination with eight pesticides exhibited additive and antagonistic effects, while all the ternary mixtures containing CLO and TET (except for CLO+TET+THD) had synergistic responses to *A. mellifera*. In other words, the synergistic interactions of CLO and TET could exceed the influence of the additive and antagonistic effects of other pesticide mixtures. This phenomenon has also been noted in a previous study, in which the synergistic interaction between atrazine and methyl-parathion can mask the influence of other interactions, resulting in synergistic toxicity of a ternary combination of atrazine, methyl-parathion and methoxychlor to the aquatic midge (*Chironomus tentans*) (Pape-Lindstrom and Lydy, 1997). Therefore, pesticide mixtures containing CLO and TET in combination with additional pesticides might lead to aggravated risks to crop pollinators.

The interactive toxicity of neonicotinoid insecticides and triazole fungicides have been comparatively well established because commercial mixtures and farmers' tank-mixes of neonicotinoids in combination with the ergosterol-biosynthesis inhibitor (EBI) type fungicides are frequently used to reduce operational or production costs (or both) in agricultural production (Belden and Brain, 2018; Almasri et al., 2020). The EBI fungicide TET inhibits the cytochrome P450-mediated detoxification of neonicotinoid insecticide CLO, resulting in an increase in CLO toxicity to honey bees (Hernández et al., 2017; Haas and Nauen, 2021). It is urgent necessary to pay more attention to the toxicity of neonicotinoid mixtures with EBI fungicides in honeybees because the

Table 6
Ternary toxicological effects of clothianidin + *Beta*-cyfluthrin (*Gamma*-cyhalothrin / Carbaryl / Indoxacarb) and other pesticides on honey bee (*Apis mellifera*).

Exposure time (day)	Pesticide A	*Am* LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide B	*Bm* LC_{50} (95% FL) μg a.i. mL^{-1}		Pesticide C	*Cm* LC_{50} (95% FL) μg a.i. mL^{-1}	AI
2	CLO	0.13 (0.11–0.16)	+	BCY	6.28 (5.33–7.61)	+	GCY	18.18 (15.45–22.05)	-0.18
4	CLO	0.075 (0.065–0.085)	+	BCY	3.57 (3.10–4.06)	+	GCY	10.34 (8.98–11.76)	0.066
7	CLO	0.062 (0.053–0.071)	+	BCY	2.97 (2.55–3.35)	+	GCY	8.60 (7.39–9.71)	0.11
2	CLO	0.12 (0.10–0.15)	+	BCY	5.95 (5.20–6.98)	+	CAR	4.39 (3.83–5.15)	-0.13
4	CLO	0.099 (0.087–0.11)	+	BCY	4.72 (4.17–5.37)	+	CAR	3.48 (3.08–3.96)	-0.24
7	CLO	0.061 (0.051–0.069)	+	BCY	2.88 (2.43–3.32)	+	CAR	2.12 (1.79–2.45)	0.088
2	CLO	0.20 (0.16–0.27)	+	BCY	9.52 (7.63–12.92)	+	IND	2.84 (2.27–3.85)	-0.68
4	CLO	0.10 (0.091–0.12)	+	BCY	4.92 (4.34–5.63)	+	IND	1.47 (1.29–1.67)	-0.28
7	CLO	0.084 (0.074–0.095)	+	BCY	4.02 (3.56–4.53)	+	IND	1.19 (1.06–1.35)	-0.26
2	CLO	0.14 (0.12–0.18)	+	BCY	7.12 (6.01–8.78)	+	THD	31.13 (26.31–38.39)	-0.29
4	CLO	0.10 (0.090–0.12)	+	BCY	4.91 (4.32–5.64)	+	THD	21.48 (18.89–24.66)	-0.84
7	CLO	0.079 (0.069–0.090)	+	BCY	3.78 (3.31–4.28)	+	THD	16.54 (14.48–18.73)	-0.14
2	CLO	0.12 (0.11–0.15)	+	GCY	17.61 (15.29–20.82)	+	CAR	4.48 (3.89–5.30)	-0.11
4	CLO	0.11 (0.094–0.12)	+	GCY	14.86 (13.01–17.19)	+	CAR	3.78 (3.31–4.37)	-0.36
7	CLO	0.081 (0.071–0.091)	+	GCY	11.07 (9.77–12.45	+	CAR	2.82 (2.48–3.17)	-0.21
2	CLO	0.17 (0.14–0.22)	+	GCY	23.52 (19.66–29.76)	+	IND	2.42 (2.02–3.07)	-0.38
4	CLO	0.11 (0.092–0.12)	+	GCY	14.59 (12.74–16.92)	+	IND	1.50 (1.31–1.74)	-0.34
7	CLO	0.072 (0.049–0.098)	+	GCY	9.97 (6.79–13.57)	+	IND	1.02 (0.84–1.29)	-0.068
2	CLO	0.12 (0.11–0.15)	+	GCY	17.86 (15.46–21.21)	+	THD	26.99 (23.36–32.06)	-0.074
4	CLO	0.10 (0.091–0.12)	+	GCY	14.31 (12.54–16.51)	+	THD	21.63 (18.95–24.93)	-0.28
7	CLO	0.085 (0.075–0.096)	+	GCY	11.72 (10.33–13.24)	+	THD	17.71 (15.61–20.01)	-0.22
2	CLO	0.12 (0.11–0.15)	+	CAR	4.47 (3.88–5.31)	+	IND	1.81 (1.57–2.15)	-0.04
4	CLO	0.099 (0.087–0.11)	+	CAR	3.49 (3.07–4.01)	+	IND	1.41 (1.24–1.62)	-0.24
7	CLO	0.063 (0.053–0.071)	+	CAR	2.19 (1.88–2.50)	+	IND	0.89 (0.76–1.01)	0.019
2	CLO	0.12 (0.11–0.15)	+	CAR	4.43 (3.94–5.13)	+	THD	26.29 (23.39–30.49)	-0.079
4	CLO	0.11 (0.10–0.13)	+	CAR	3.95 (3.55–4.49)	+	THD	23.46 (21.08–26.69)	-0.39
7	CLO	0.096 (0.072–0.13)	+	CAR	3.35 (2.53–4.57)	+	THD	19.94 (15.05–27.16)	-0.44
2	CLO	0.13 (0.11–0.16)	+	IND	1.89 (1.63–2.25)	+	THD	27.68 (23.96–32.93)	-0.053
4	CLO	0.11 (0.098–0.13)	+	IND	1.59 (1.39–1.84)	+	THD	23.30 (20.46–26.95)	-0.39
7	CLO	0.097 (0.086–0.11)	+	IND	1.37 (1.21–1.57)	+	THD	20.23 (17.86–23.06)	-0.44

FL fiducial limit, AI additive index, CLO Clothianidin, CHL Chlorpyrifos, TET Tetraconazole, SPI Spinosad, BCY Beta-cyfluthrin, GCY Gamma-cyhalothrin, CAR Carbaryl, INO Indoxacarb, THD Thiodicarb.

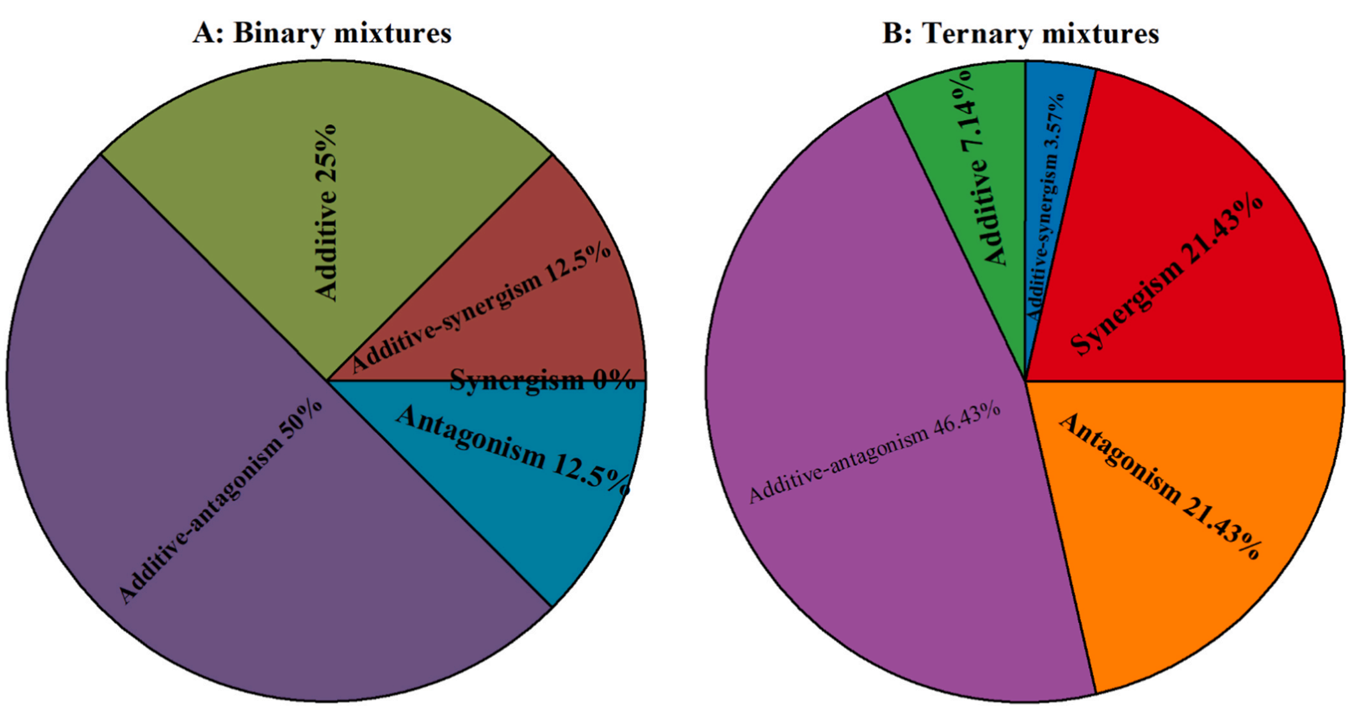

Fig. 1. Interaction patterns in binary mixtures (A) and ternary mixtures (B) of clothianidin with eight other pesticides in honey bees.

assessment of the risk of pesticides against honeybees has been focused only on individual pesticides (Sgolastra et al., 2017; Han et al., 2019).

Several pesticide combinations (i.e. CLO+TET, CLO+CAR and CLO+CHL+THD) exhibited different joint toxic effects with the prolongation of exposure periods. The joint toxic effects include aspects of toxicokinetics and toxicodynamics, and these mechanisms may differ at different exposure periods of pesticide combinations (Hernández et al., 2017). To explore the nature of these joint effects, biochemical- and molecular-level understanding of pesticide interactions is required for developing a more effective detoxification strategy (Gong and Diao, 2017; Zhu et al., 2020). Our results exemplified the notion that the effects of pesticide combinations are very hard to predict solely based on information of the single compounds, which most probably is the result of biological complexity and redundancy in response pathways.

Given the large number of pesticides to which honey bees are potentially exposed, and the practical impossibility of testing every possible combination, it might be the most sensible approach to focus on the most frequently used ones when performing assays of bees' exposure (Carnesecchi et al., 2019). These multi-exposure scenarios are especially important to consider in cases when the synergy of two or more pesticides exists and is detectable in honey bees (Sgolastra et al., 2017; Almasri et al., 2020). However, the complexity of toxicological interactions can lead to unpredictable effects of pesticide mixtures (Hernández et al., 2017). Improvements are required to make them more realistic while keeping the cost of regulatory tests affordable, posing a considerable regulatory challenge (Wade et al., 2019). The identification of core features of pesticide mixtures at a molecular level, such as gene expression profiles, could be helpful to assess or predict the occurrence of interactive effects giving rise to unpredicted responses (Tomé et al., 2020).

The concentrations of individual pesticides in the mixture fluctuate greatly, leading to difficulties to determine the toxicity of every chemical (Carnesecchi et al., 2019). Plenty of approaches have been developed to predict toxicities of chemical combinations to environmental organisms, such as the concentration addition (CA) model, independent action (IA) model, quantitative structure-activity relationship (QSAR) approach, and AI method (Liu et al., 2017; Carnesecchi et al., 2020; Wang et al., 2020). Both the CA and IA models presume that no interactions exist among mixture components. Nevertheless, such a situation rarely happens in mixtures in natural environments (Escher et al., 2020). Moreover, it must be assumed that the modes of action (MOA) of mixture components can be precisely measured (Sigurnjak et al., 2020). It is not suitable to use the QSAR model to assess the pesticide classes or chemicals with distinct chemical structures (Xu et al., 2021). However, the AI model can be used to measure the potential toxicities of mixtures (but only at one effect level) (Wang et al., 2020). Therefore, we adopted the AI model to determine the mixture toxicity of pesticides in our current work.

In the present investigation, we only assessed the combinations of chemicals with equitoxic ratios. However, the contents of pesticides greatly vary in natural environments (Calatayud-Vernich et al., 2018). Thus, such multiple combinations should be examined at a series of proportions when conducting the toxicity assessment in the further survey (Carnesecchi et al., 2019). It is quite important to explore the mixture toxicities of pesticides since various chemicals may concurrently remain in the environment (Almasri et al., 2020). Besides, the regulatory total maximum daily loads (TMDLs) have been issued only for single chemicals (Tosi et al., 2018). Therefore, the latent additive/-synergistic risks from chemical combinations are largely unknown (Araújo et al., 2021). The results from our current study offered valuable insights into the complex impacts of pesticide combinations on pollinating insects and promoted a comprehensive understanding of the interaction patterns of various chemicals (Wang et al., 2020). Collectively, we should pay more attention to the impacts of pesticide combinations on crop pollinators in future risk assessments.

The information obtained based on single pesticides may not be suitable for mixture outcomes, since pollinators, such as bees, are commonly exposed to pesticide combinations under field conditions (Rizzati et al., 2016; Wade et al., 2019). It is highly necessary to further assess the pesticide mixtures. A rapid, simple, and cost-effective toxicity approach would be greatly beneficial in monitoring ecosystems (Carnesecchi et al., 2019). To better understand the ecotoxicology of pesticide combinations, additional research is required to adopt this and similar approaches (e.g., honey bee field study) on actual and simulated environments under different conditions (Agatz et al., 2019; Kuivila et al., 2021). Our results greatly contributed to environmental risk assessment, and we, for the first time, confirmed that honey bees were differently susceptible when exposed to a wide range of pesticides simultaneously under field conditions. Synergism is likely to occur among compounds in natural ecosystems and should be assessed (Almasri et al., 2020). This is particularly true for triazole fungicides, which are routinely sprayed when plants are blooming under the assumption that these compounds are safe for bees (Almasri et al., 2021). Synergistic interactions might increase the propensity of honey bee colonies to fail. Second, chronic and sublethal toxicity tests of pesticide mixtures should be conducted systematically, especially for systemic pesticides with a long-term action (Almeida et al., 2021). Finally, the toxic effects of pesticide mixtures on larvae and queens should also be examined because the success of honey bee colonies depends on the health of the larvae and queens (Milone et al., 2020; Botías et al., 2021).

5. Conclusions

CLO was the most toxic one among the nine tested pesticides. Most of the binary mixtures of CLO in combination with other pesticides exhibited additive and antagonistic interactions to *A. mellifera*. All the ternary mixtures containing CLO and TET (except for CLO+TET+THD) displayed synergistic effects on honey bees. This study demonstrated that pesticide mixtures could provoke greater toxicity to honey bees than tested individually. Prospective environment risk assessments based on individual exposure might hence not adequately protect crop pollinators. More attention should be paid to the synergistic effects of pesticide mixtures because they can pose a serious threat to natural ecosystems.

CRediT authorship contribution statement

Yanhua Wang: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing. **Yu-Cheng Zhu**: Conceptualization, Methodology, Software, Formal analysis, Investigation, Resources, Data Curation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition, Corresponding. **Wenhong Li**: Investigation, Data Curation. **Jianxiu Yao**: Investigation, Data Curation, Writing – Review & Editing. **Gadi V.P. Reddy**: Writing – Review & Editing, Administration, Funding acquisition. **Lu Lv**: Data Curation, Writing – Review & Editing.

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.

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References

Agatz, A., Kuhl, R., Miles, M., Schad, T., Preuss, T.G., 2019. An evaluation of the beehave model using honey bee field study data: insights and recommendations. Environ. Toxicol. Chem. 38 (11), 2535–2545.

Almasri, H., Tavares, D.A., Diogon, M., Pioz, M., Alamil, M., Sené, D., Tchamitchian, S., Cousin, M., Brunet, J.L., Belzunces, L.P., 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.

Almasri, H., Tavares, D.A., Pioz, M., Sené, D., Tchamitchian, S., Cousin, M., Brunet, J.L., Belzunces, L.P., 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.

Almeida, C.H.S., Haddi, K., Toledo, P.F.S., Rezende, S.M., Santana, W.C., Guedes, R.N.C., Newland, P.L., Oliveira, E.E., 2021. Sublethal agrochemical exposures can alter honey bees' and Neotropical stingless bees' color preferences, respiration rates, and locomotory responses. Sci. Total Environ. 779, 146432.

Annoscia, D., Di Prisco, G., Becchimanzi, A., Caprio, E., Frizzera, D., Linguadoca, A., Nazzi, F., Pennacchio, F., 2020. Neonicotinoid clothianidin reduces honey bee immune response and contributes to Varroa mite proliferation. Nat. Commun. 11 (1), 5887.

Araújo, R.D.S., Bernardes, R.C., Martins, G.F., 2021. A mixture containing the herbicides Mesotrione and Atrazine imposes toxicological risks on workers of *Partamona helleri*. Sci. Total Environ. 763, 142980.

Belden, J.B., Brain, R.A., 2018. Incorporating the joint toxicity of co-applied pesticides into the ecological risk assessment process. Integr. Environ. Assess. Manag. 14 (1), 79–91.

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

Botías, C., Jones, J.C., Pamminger, T., Bartomeus, I., Hughes, W.O.H., Goulson, D., 2021. Multiple stressors interact to impair the performance of bumblebee *Bombus terrestris* colonies. J. Anim. Ecol. 90 (2), 415–431.

Bryden, J., Gill, R.J., Mitton, R.A., Raine, N.E., Jansen, V.A., 2013. Chronic sublethal stress causes bee colony failure. Ecol. Lett. 16 (12), 1463–1469.

Calatayud-Vernich, P., Calatayud, F., Simó, E., Picó, Y., 2018. Pesticide residues in honey bees, pollen and beeswax: assessing beehive exposure. Environ. Pollut. 241, 106–114.

Carnesecchi, E., Svendsen, C., Lasagni, S., Grech, A., Quignot, N., Amzal, B., Toma, C., Tosi, S., Rortais, A., Cortinas-Abrahantes, J., Capri, E., Kramer, N., Benfenati, E., Spurgeon, D., Guillot, G., Dorne, J.L.C.M., 2019. Investigating combined toxicity of binary mixtures in bees: meta-analysis of laboratory tests, modelling, mechanistic basis and implications for risk assessment. Environ. Int. 133 (Pt B), 105256.

Carnesecchi, E., Toma, C., Roncaglioni, A., Kramer, N., Benfenati, E., Dorne, J.L.C.M., 2020. Integrating QSAR models predicting acute contact toxicity and mode of action profiling in honey bees (*A. mellifera*): data curation using open source databases, performance testing and validation. Sci. Total Environ. 735, 139243.

Cheng, Y., Tan, L.C., Bu, Y.Q., Ge, C.N., Zhou, J.Y., Shan, Z.J., 2016. Chronic oral toxicity of chlorpyrifos and imidacloprid to adult honey bees (*Apis mellifera* L.). Asian J. Ecotoxicol. 11 (2), 715–719.

Crenna, E., Jolliet, O., Collina, E., Sala, S., Fantke, P., 2020. Characterizing honey bee exposure and effects from pesticides for chemical prioritization and life cycle assessment. Environ. Int. 138, 105642.

Diao, Q., Li, B., Zhao, H., Wu, Y., Guo, R., Dai, P., Chen, D., Wang, Q., Hou, C., 2018. Enhancement of chronic bee paralysis virus levels in honeybees acute exposed to imidacloprid: a Chinese case study. Sci. Total Environ. 630, 487–494.

Escher, B., Braun, G., Zarfl, C., 2020. Exploring the concepts of concentration addition and independent action using a linear low-effect mixture model. Environ. Toxicol. Chem. 39 (12), 2552–2559.

Gong, Y., Diao, Q., 2017. Current knowledge of detoxification mechanisms of xenobiotic in honey bees. Ecotoxicology 26 (1), 1–12.

Haas, J., Nauen, R., 2021. Pesticide risk assessment at the molecular level using honey bee cytochrome P450 enzymes: a complementary approach. Environ. Int. 147, 106372.

Han, W., Yang, Y., Gao, J., Zhao, D., Ren, C., Wang, S., Zhao, S., Zhong, Y., 2019. Chronic toxicity and biochemical response of *Apis cerana* cerana (Hymenoptera: Apidae) exposed to acetamiprid and propiconazole alone or combined. Ecotoxicology 28 (4), 399–411.

Harwood, G.P., Dolezal, A.G., 2020. Pesticide-Virus interactions in honey bees: challenges and opportunities for understanding drivers of bee declines. Viruses 12 (5), 566.

Hernández, A.F., Gil, F., Lacasaña, M., 2017. Toxicological interactions of pesticide mixtures: an update. Arch. Toxicol. 91 (10), 3211–3223.

Insect Control Guide Committee, 2020. Mississippi State University Extension Publication 2471. Starkville, MS, US.

Jactel, H., Verheggen, F., Thiéry, D., Escobar-Gutiérrez, A.J., Gachet, E., Desneux, N., Neonicotinoids Working Group, 2019. Alternatives to neonicotinoids. Environ. Int. 129, 423–429.

Jiang, J., Ma, D., Zou, N., Yu, X., Zhang, Z., Liu, F., Mu, W., 2018. Concentrations of imidacloprid and thiamethoxam in pollen, nectar and leaves from seed-dressed cotton crops and their potential risk to honeybees (*Apis mellifera* L.). Chemosphere 201, 159–167.

Johnson, R.M., 2015. Honey bee toxicology. Annu. Rev. Entomol. 60, 415–434.

Kadlikova, K., Vaclavikova, M., Halesova, T., Kamler, M., Markovic, M., Erban, T., 2021. The investigation of honey bee pesticide poisoning incidents in Czechia. Chemosphere 263, 128056.

Klein, A.M., Boreux, V., Fornoff, F., Mupepele, A.C., Pufal, G., 2018. Relevance of wild and managed bees for human well-being. Curr. Opin. Insect Sci. 26, 82–88.

Kuivila, K.M., Judd, H., Hladik, M.L., Strange, J.P., 2021. Field-level exposure of bumble bees to fungicides applied to a commercial cherry orchard. J. Econ. Entomol. 114 (3), 1065–1071.

Laurino, D., Porporato, M., Patetta, A., Manino, A., 2011. Toxicity of neonicotinoid insecticides to honey bees: laboratory tests. Bull. Insectol. 64, 107–113.

Liu, Y., Liu, S., Zhang, H., Gu, Y., Li, X., He, M., Tan, H., 2017. Application of the combination index (CI)-isobologram equation to research the toxicological interactions of clothianidin, thiamethoxam, and dinotefuran in honeybee, *Apis mellifera*. Chemosphere 184, 806–811.

Lourenço, A.P., Martins, J.R., Torres, F.A.S., Mackert, A., Aguiar, L.R., Hartfelder, K., Bitondi, M.M.G., Simões, Z.L.P., 2019. Immunosenescence in honey bees (*Apis mellifera* L.) is caused by intrinsic senescence and behavioral physiology. Exp. Gerontol. 119, 174–183.

Marking, L.L., 1985. Toxicity of chemical mixtures. In: Rand, G., Petroceli, S. (Eds.), Fundamentals of Aquatic Toxicology. Hemisphere Publishing Corporation, Washington DC, pp. 164–176.

Matsuda, K., Ihara, M., Sattelle, D.B., 2020. Neonicotinoid insecticides: molecular targets, resistance, and toxicity. Annu. Rev. Pharmacol. Toxicol. 60, 241–255.

Milone, J.P., Rinkevich, F.D., McAfee, A., Foster, L.J., Tarpy, D.R., 2020. Differences in larval pesticide tolerance and esterase activity across honey bee (*Apis mellifera*) stocks. Ecotoxicol. Environ. Saf. 206, 111213.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., Vanengelsdorp, D., Pettis, J.S., 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS One 5 (3), 9754.

Pape-Lindstrom, P.A., Lydy, M.J., 1997. Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. Environ. Toxicol. Chem. 16 (11), 2415–2420.

Prado, A., Pioz, M., Vidau, C., Requier, F., Jury, M., Crauser, D., Brunet, J.L., Le Conte, Y., Alaux, C., 2019. Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees. Sci. Total Environ. 650 (Pt 1), 1250–1260.

Raimets, R., Karise, R., Mänd, M., Kaart, T., Ponting, S., Song, J., Cresswell, J.E., 2018. Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.). Pest Manag. Sci. 74 (3), 541–546.

Rizzati, V., Briand, O., Guillou, H., Gamet-Payrastre, L., 2016. Effects of pesticide mixtures in human and animal models: an update of the recent literature. Chem. Biol. Interact. 254, 231–246.

Sánchez-Hernández, L., Hernández-Domínguez, D., Martín, M.T., Nozal, M.J., Higes, M., Bernal, Y.J.L., 2016. Residues of neonicotinoids and their metabolites in honey and pollen from sunflower and maize seed dressing crops. J. Chromatogr. A 1428, 220–227.

Schmolke, A., Abi-Akar, F., Hinarejos, S., 2019. Honey bee colony-level exposure and effects in realistic landscapes: an application of beehive simulating clothianidin residues in corn pollen. Environ. Toxicol. Chem. 38 (2), 423–435.

Schmuck, R., Lewis, G., 2016. Review of field and monitoring studies investigating the role of nitro-substituted neonicotinoid insecticides in the reported losses of honey bee colonies (*Apis mellifera*). Ecotoxicology 25 (9), 1617–1629.

Sgolastra, F., Blasioli, S., Renzi, T., Tosi, S., Medrzycki, P., Molowny-Horas, R., Porrini, C., Braschi, I., 2018. Lethal effects of Cr (III) alone and in combination with propiconazole and clothianidin in honey bees. Chemosphere 191, 365–372.

Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M.T., Tosi, S., Bogo, G., Teper, D., Porrini, C., Molowny-Horas, R., Bosch, J., 2017. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. Pest Manag. Sci. 73 (6), 1236–1243.

Sigurnjak, M., Ukić, Š., Cvetnić, M., Markić, M., Novak, S.M., Rasulev, B., Kušić, H., Lončarić, B.A., Rogošić, M., Bolanča, T., 2020. Combined toxicities of binary mixtures of alachlor, chlorfenvinphos, diuron and isoproturon. Chemosphere 240, 124973.

Spruill, S.E., O'Neill, B.F., Hinarejos, S., Cabrera, A.R., 2019. A comparison of acute toxicity endpoints for adult honey bees with technical grade active ingredients and typical end-use products as test substance. J. Econ. Entomol. 113 (2), 1015–1017.

Su, L.S., Yang, G.L., Wu, S.G., Pi, T.X., Wang, Q., 2016. The single and joint toxicity of tiazophos and cyhalothrin to earthworm. Asian J. Ecotoxicol. 11, 294–301.

Thompson, H., Overmyer, J., Feken, M., Ruddle, N., Vaughan, S., Scorgie, E., Bocksch, S., Hill, M., 2019. Thiamethoxam: long-term effects following honey bee colony-level exposure and implications for risk assessment. Sci. Total Environ. 654, 60–71.

Tison, L., Rößner, A., Gerschewski, S., Menzel, R., 2019. The neonicotinoid clothianidin impairs memory processing in honey bees. Ecotoxicol. Environ. Saf. 180, 139–145.

Tomé, H.V.V., Schmehl, D.R., Wedde, A.E., Godoy, R.S.M., Ravaiano, S.V., Guedes, R.N.C., Martins, G.F., Ellis, J.D., 2020. Frequently encountered pesticides can cause multiple disorders in developing worker honey bees. Environ. Pollut. 256, 113420.

Tosi, S., Costa, C., Vesco, U., Quaglia, G., Guido, G., 2018. A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. Sci. Total Environ. 615, 208–218.

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 honeybee larvae and adults. Insects 10 (1), 20.

Wang, Y., Zhu, Y.C., Li, W., 2020. Interaction patterns and combined toxic effects of acetamiprid in combination with seven pesticides on honey bee (*Apis mellifera* L.). Ecotoxicol. Environ. Saf. 190, 110100.

Xu, X., Zhao, P., Wang, Z., Zhang, X., Wu, Z., Li, W., Tang, Y., Liu, G., 2021. In silico prediction of chemical acute contact toxicity on honey bees via machine learning methods. Toxicol. In Vitro 72, 105089.

Yao, J., Zhu, Y.C., Adamczyk, J., 2018. Responses of honey bees to lethal and sublethal doses of formulated clothianidin alone and mixtures. J. Econ. Entomol. 111 (4), 1517–1525.

Yu, R.X., Zhao, X.P., Wu, C.X., Wu, S.G., Cang, T., Chen, L.P., Wang, Q., 2009. Evaluation of indoxacarb to environmental organisms. Agrochemicals 48 (1), 47–49.

Zhao, X.P., Cang, T., Chen, C., Wu, C.X., Chen, L.P., Wu, S.G., Yu, R.X., Wang, Q., 2008. Evaluation of effects of some pesticides on the toxicity and safety to bee. Zhejiang Agric. Sci. 6, 748–750.

Zhu, Y.C., Adamczyk, J., Rinderer, T., Yao, J., Danka, R., Luttrell, R., Gore, J., 2015. Spray toxicity and risk potential of 42 commonly used formulations of row crop pesticides to adult honey bees (Hymenoptera: Aphidae). J. Econ. Entomol. 108 (6), 2640–2647.

Zhu, Y.C., Caren, J., Reddy, G.V.P., 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.

Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017a. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). PLoS One 12 (5), e0176837.

Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017b. 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.