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# Interaction patterns and combined toxic effects of acetamiprid in combination with seven pesticides on honey bee (*Apis mellifera* L.)

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ABSTRACT

The neonicotinoid insecticide acetamiprid (ACT) and seven pesticides [abamectin (ABA), emamectin benzoate (EMB), dicrotophos (DIC), bifenthrin (BIF), cypermethrin (CYP), *lambda*-cyhalothrin (LCY) and tetraconazole (TET)] are widely applied agrochemicals worldwide. Since most previous studies on these pesticides are performed merely based on toxicity tests with individual active ingredients, only finite knowledge is available on the mixture toxicities of these formulated compounds to crop pollinators. In this study, we examined their toxicities of binary, ternary, quaternary, quinquenary, senary, septenary and octonary mixtures to honey bee (*Apis mellifera* L.) with feeding toxicity test. Results showed that EMB and ABA had the highest toxicities to *A. mellifera* with $LC_{50}$ values of 0.033 (0.028–0.038) and 0.047 (0.039–0.056) μg a. i. $mL^{-1}$ after exposure for 7 days, respectively, followed by DIC with an $LC_{50}$ value of 1.22 (1.01–1.41) μg a. i. $mL^{-1}$. In contrast, relatively low toxicities were found from pyrethroid insecticides, ACT, and TET with their $LC_{50}$ values ranged from 44.76 (38.75–50.89) to 251.7 (198.4–297.3) μg a. i. $mL^{-1}$. Most of pesticide mixtures containing ACT and TET elicited synergistic interactions to honey bees. Besides, four pesticide mixtures of ACT + BIF, ACT + BIF + CYP, ACT + BIF + LCY and ACT + CYP + DIC + EMB also displayed synergistic effects. Among 98 tested binary to octonary mixtures of ACT in combination with seven pesticides, 44.90% of combinations exhibited synergistic effects on honey bees. Considering ACT was permitted to use on flowering crops, more attention should be paid to its application in the fields due to the synergistic effects of ACT in combination with other pesticides on *A. mellifera* under laboratory conditions.

## 1. Introduction

Honey bee (*Apis mellifera* L.) are invaluable to commercial agriculture, plant propagation and human society (Garantonakis et al., 2016). Besides, they ensure the pollination of many wild flowers, thus greatly contribute to plant biodiversity (Martins et al., 2015; Hung et al., 2018). However, there has been increasing concern about the losses of honey bee colonies in several parts of the world over the past decade (McMenamin and Genersch, 2015; Beyer et al., 2018). Such declines threaten the economic viability of the beekeeping industry and have serious implications to pollination services for both cultivated and wild plants (Clermont et al., 2015). Many factors have been linked to colony declines, such as parasitic mites, pathogens, loss of foraging habitat, and widespread pesticide application (Cutler et al., 2014; Goulson et al., 2015; Sánchez-Bayo et al., 2016; Abbo et al., 2017; O'Neal et al., 2018). Although the causes of honey bee decline may be complex and subjected to disagreement, the large-scale application of pesticides has been indicated as a potential contributing factor (Diao et al., 2018; Tosi et al., 2018).

By targeting nicotinic acetylcholine receptor (nAChR) in the insect nervous system, neonicotinoid insecticides cause over-stimulations to nerve cells resulting in paralysis and death (Casida, 2018). Being less toxic to mammals and possessing systemic activity, neonicotinoids have become agronomically important insecticides worldwide since the early 1990s. Currently, they are extensively applied as seed treatment on bee-visiting crops, such as oilseed rape and ornamental garden plants or as foliar sprays on fruits, such as apples and pears (Manjon et al., 2018; Wang et al., 2018a). By systemic spreading through the xylem in the treated plants, neonicotinoids contaminate pollens and nectars, that thereafter are collected by foragers and transferred to the hive to feed youngsters (Stewart et al., 2014; Silvina et al., 2017). Some studies have indicated that neonicotinoid residues are detected in many water

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resources, such as surface water and guttation water, which can be deemed as another oral exposure to *A. mellifera* (Thompson, 2010; Wirte et al., 2018). Additionally, neonicotinoids persist in the soil, from where they can translocate to wild flowers in the field margins, thus prolonging the exposure well beyond crop flowering, and even into the following year (Botías et al., 2015; Krupke and Long, 2015). Since honey bees can expose to neonicotinoids through multiple routes, it is necessary to explicate the potential toxic effects of neonicotinoids on crop pollinators (Halm et al., 2006; Hladik et al., 2018).

Honey bees are exposed to pesticides in different situations. For example, they may be poisoned during pesticide application or via contact with residue on treated plants, as well as by ingestion of pesticide-contaminated food (Stewart et al., 2014; Zhu et al., 2015). Foliar sprays may be still applied on long blooming plants in later season, which may incur significantly acute poisoning of honey bee workers (Thompson et al., 2014; Tosi et al., 2018). Consequently, colonies may not have enough time to recover and to prepare for overwintering (Silvina et al., 2017). While contaminated storage food like nectar is used instantly, pollen needs to be fermented first to become digestible for bees, and can thus be used post the foraging season, particularly during winter (van der Sluijs et al., 2013; Wood and Goulson, 2017). Oral exposure is estimated to be the highest risk for foragers, winter bees and larvae (Fairbrother et al., 2014). The systemic properties of neonicotinoids facilitate its translocation to nectar, pollen and guttation droplets, and it can reach honey bees mainly through feeding (Blacquière et al., 2012; Godfray et al., 2014; Johnson, 2015). Therefore, feeding toxicity test was used to determine the mixture toxicities in this study.

Neonicotinoids are moderately to highly toxic to honey bees depending on the particular active ingredients (Johnson, 2015; Christen et al., 2016). Because the nitro-substituted neonicotinoids, including imidacloprid, thiamethoxam, and clothianidin, have high intrinsic toxicity to honey bees, their risks to *A. mellifera* have been intensively examined around the world (Schmuck and Lewis, 2016; Li et al., 2017; Hernando et al., 2018; Jiang et al., 2018). In contrast, the cyano-substituted neonicotinoid insecticide acetamiprid (ACT) has less toxic to honey bees compared with the nitro-substituted neonicotinoids (Iwasa et al., 2004; Feyereisen, 2018). Thus, the more bee-friendly characteristics of ACT have led to the less strict criteria of its usage, e.g. it is permitted to use on flowering crops during daylight when honey bees are actively foraging (Godfray et al., 2014). Numerous studies have reported about the toxicity of single pesticides, while the combined toxic effects and possible interactions among different pesticides were neglected by laboratory studies (Pilling et al., 1995; Iwasa et al., 2004; Thompson et al., 2014; Sgolastra et al., 2017). In fact, ACT and other types of pesticides often co-exist as mixtures of complex compounds in realistic environments, which may induce extra effects on honey bees compared with ACT alone (Iwasa et al., 2004; David et al., 2016; Feyereisen, 2018). To the best of our knowledge, only few studies have assessed the mixture toxicity risk of ACT in combination with other commonly used pesticides to honey bees. Therefore, such co-existence of ACT and other types of pesticides deserves further investigation. In this study, we aimed to evaluate the interaction patterns and combined toxic effects of ACT in combination with other currently used pesticides in order to mitigate the side-effects of neonicotinoids to crop pollinators.

## 2. Materials and methods

### 2.1. Colony rearing of honey bees

Honey bees (25–30 colonies) were originally obtained from commercial beekeepers located in the pine forest and pasture areas of southeastern Mississippi near Magee and Perkinston. Honey bee colonies were maintained in an isolated apiary in the Mississippi Wildlife Management Area near Stoneville, Mississippi (USA). Approximately 15% new colonies (package bees with Italian queen) were purchased each year from beekeepers near Little Rock, Arkansas and added to the bee yard. To obtain enough bees for conducting a few experiments, eight frames with > 50% coverage of healthy brood were pulled out from 5–7 colonies and transferred to a container which contains ventages covered with 8 mesh screen and a lid to prevent bees from escaping. The container was held in a laboratory incubator set at 33 ± 0.5 °C, 65 ± 3% relative humidity, and no light. Twenty newly emerged honey bee workers from mixed frames (colonies) were randomly transferred daily into a cage (diameter × height: 9.3 × 10 cm) and fed with a vial of 50% sucrose solution and a vial of d-$H_2O$ placed upside down at the top of each cage. Caged bees with the same age were maintained in incubators under the same conditions described above for 4 days before being used for treatments.

### 2.2. Pesticides

Commercial formulations, instead of active ingredients, were used to include potential additive and/or synergistic toxicity to bees from formulating reagents (Zhu et al., 2014; Mullin et al., 2015). In addition, we aimed to simulate field conditions and assess potential interactive mixture toxicity to honey bees from formulations used by farmers. Eight formulated commercial pesticides from five classes of chemicals were examined in the current study, including one neonicotinoid insecticide acetamiprid (ACT, Intruder 70 WP, 70% active ingredient (A.I.), Gowan, Yuma, AZ, USA), two avermectin insecticides abamectin (ABA, Agri-Mek 0.15EC, 2% A.I., Syngenta) and emamectin benzoate (EMB, Denim 0.16 EC, 2.15% A.I., Syngenta), one organophosphate insecticide dicrotophos (DIC, Bidrin 8 EC, 82% A.I., AMVAC Chemical Co.), three pyrethroid insecticides bifenthrin (BIF, Brigade 2 EC, 25.1% A.I., Agrisolutions), cypermethrin (CYP, Holster, 30.6% A.I., Agrisolutions Loveland) and *lambda*-cyhalothrin (LCY, Karate Z 2.08 CS, 22.8% A.I., Syngenta) and one triazole fungicide tetraconazole (TET, Domark 230 ME, 20.5% A.I., Valent). The tested pesticides with different modes of action (MOA) have been extensively applied in agriculture globally.

### 2.3. Toxicity test method

The acute toxicity test of emerged workers of *A. mellifera* was performed according to Yao et al. (2018) with slight modifications. A preliminary experiment was conducted to indicate the concentration range that produced the 100% mortality rate at 7 days post exposure to the pesticides. A certain amount of formulated pesticides were directly spiked into 50% sucrose solution to make final concentration. The tested concentration range of each pesticide was provided in Table S1 as the supplemental materials. To obtain $LC_{50}$, at least six concentrations in a geometric series and a 50% sucrose solution-only as control were used for each pesticide. Three cages, each containing 20 adult workers (4 days old), were used for each concentration. Each caged bees were fed with 50% sucrose solution containing pesticide (or only 50% sucrose solution as a control) and d-$H_2O$. Considering the slow toxic action of ABA, EMB and DIC, the exposure lasted for 7 days to determine acute and subacute toxicities of pesticides to *A. mellifera*. The external environment during exposure, including temperature, humidity and light cycle, were maintained at the same conditions described above. Honey bees were regarded as dead when a complete immobility was noticed after a gentle prod with a fine bristle. The cumulative mortality of honey bees was recorded 2 days, 4 days and 7 days post-exposure, respectively. At the end of each test, the control mortality should less than 10%. If control mortality was over 10%, the experiment would be repeated.

### 2.4. Combined toxicity test

In order to evaluate the combined toxic effects, the binary, ternary, quaternary, quinquenary, senary, septenary and octonary mixtures of

ACT and other pesticides were prepared at a equitoxic constant mixture ratio of 1:1 (50% of the 4 d-$LC_{50}$ of each pesticide), 1:1:1 (33.3% of the 4 d-$LC_{50}$ of each pesticide), 1:1:1:1 (25% of the 4 d-$LC_{50}$ of each pesticide), 1:1:1:1:1 (20% of the 4 d-$LC_{50}$ of each pesticide), 1:1:1:1:1:1 (16.7% of the 4 d-$LC_{50}$ of each pesticide), 1:1:1:1:1:1:1 (14.3% of the 96 h-$LC_{50}$ of each pesticide), 1:1:1:1:1:1:1:1 (12.5% of the 4 d-$LC_{50}$ of each pesticide), respectively, based on the determined individual $LC_{50}$ values. The proportion of equitoxic mixture were used to avoid that the toxicity of an individual pesticide varies greatly, one pesticide would have a dominant toxicity and mask the toxic effect of the other pesticide, thus changing the joint action characteristics of the mixed pesticides. Complete concentration-response correlations were experimentally established by varying the total concentration of each mixture and maintaining all the above-mentioned ratios constant. Each treatment was performed in triplicate for each tested concentration.

### 2.5. Statistical analysis

SAS probit analysis (SAS/STAT 9.2 User's Guide, Cary, NC) was employed to calculate $LC_{50}$ values of tested pesticides to *A. mellifera*. Significant level of mean separation ($P < 0.05$) determined was based on the lack of overlap between the 95% confidence interval of two $LC_{50}$ values. Marking's (Marking, 1985) additive index (AI) method is a useful tool to determine mixture toxicities of chemicals, which has received extensive attention in recent years (Cang et al., 2017; Wang et al., 2017). Therefore, the AI was adopted to assess combined toxicity as follows:

$$S = (Am/Ai) + (Bm/Bi)$$

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

The AI value was determined from the sum of $S$ based on the appropriate formulas as follows:

$$AI = (1/S) - 1 \text{ for } S < 1.0 \text{ and } AI = 1 - S \text{ for } S \geq 1.0.$$

Combined toxicities were classified as antagonistic effect ($AI \leq -0.2$), additive ($-0.2 < AI \leq 0.25$) or synergistic effect ($AI > 0.25$) accordingly. The greater the AI value, the greater the pesticide synergy (Wang et al., 2018b).

## 3. Results

### 3.1. Single pesticide toxicity

The toxicities of eight pesticides to *A. mellifera* were summarized in Table 1. Various pesticides displayed a wide range of feeding toxicities. Besides, different pesticides within the same chemical class incurred different toxicities to the pollinators. After 2-day exposure, $LC_{50}$ values of the pesticides to 4-d old honey bee workers ranged from 0.13 (0.10–0.17) to 875.6 (772.6–1014) μg a. i. $mL^{-1}$. The order of toxicity for the eight pesticides was ranked as follows: EMB, ABA > DIC > CYP > TET, ACT ≥ LCY > BIF. Among the tested pesticides, the feeding toxicities of EMB and ABA were the highest with the $LC_{50}$ values of 0.13 (0.10–0.17) and 0.17 (0.14–0.22) μg a. i. $mL^{-1}$, respectively. In contrast, BIF had the lowest toxicity with an $LC_{50}$ value of 875.6 (772.6–1014) μg a. i. $mL^{-1}$. Therefore, EMB and ABA were 6735 and 5150 times more toxic than BIF to *A. mellifera* according to their $LC_{50}$ values, respectively.

After feeding exposure for 4 days, the $LC_{50}$ values of the 8 pesticides tested to honey bees ranged from 0.048 (0.042–0.056) to 342.1 (295.8–390.1) μg a. i. $mL^{-1}$. The order of toxicity (high-low) for the eight pesticides was ranked as follows: EMB > ABA > DIC > CYP > LCY, ACT ≥ TET > BIF. Amongst the investigated pesticides, EMB and ABA still showed the greatest toxicities with $LC_{50}$ values of 0.048 (0.042–0.056) and 0.092 (0.078–0.11) μg a. i. $mL^{-1}$, respectively. Meanwhile, BIF still displayed the lowest toxicity with an $LC_{50}$ value of 342.1 (295.8–390.1) μg a. i. $mL^{-1}$. Therefore, the toxicities of EMB and ABA were 7127 and 3718 times higher than that of BIF, respectively.

After the feeding exposure for 7 days, the $LC_{50}$ values of the selected pesticides to bees ranged from 0.033 (0.028–0.038) to 251.7 (198.4–297.3) μg a. i. $mL^{-1}$. Based on $LC_{50}$ values, the toxicities of the 8 pesticides to *A. mellifera* were ranked as follows: EMB > ABA > DIC > CYP > LCY > ACT, TET, BIF. Among the assessed pesticides, EMB and ABA still exhibited the highest toxicities with $LC_{50}$ values of 0.033 (0.028–0.038) and 0.047 (0.039–0.056) μg a. i. $mL^{-1}$, respectively. In contrast, BIF still exhibited the lowest toxicity with an $LC_{50}$ value of 251.7 (198.4–297.3) μg a. i. $mL^{-1}$. Consequently, the toxicities of EMB and ABA were 7627 and 5355 times more toxic than BIF, respectively.

The toxicities of all the evaluated pesticides (with the exception of TET) to honey bees after 4-day exposure were significantly higher compared with their respective toxicities after 2-day exposure. Furthermore, the toxicities of ABA, LCY, DIC and EMB to *A. mellifera* after 7-day exposure were significantly higher compared with their respective toxicities after 4-day exposure. Taking together, the toxicities of all the tested pesticides after 7-day exposure were significantly higher compared with their respective toxicities after 2-day exposure, indicating that their toxicities were positively correlated with exposure period. Among the tested pesticides, the toxicities of LCY, DIC, BIF and ABA to *A. mellifera* after 7-day exposure were 6.06, 4.87, 3.48 and 3.62 times higher compared with those after 2-day exposure, respectively. Overall, avermectin insecticides EMB and ABA exhibited the highest toxicities, followed by organophosphate insecticide DIC, while the triazole fungicide TET showed a relatively low toxicity to *A. mellifera*.

### 3.2. Toxicities of pesticide mixtures

In order to explore synergistic toxicity of ACT in combination with

**Table 1**
Acute toxicity of eight pesticides to honey bee workers at different duration, expressed as median lethal concentration ($LC_{50}$: μg a.i. $mL^{-1}$).

| Pesticides | 2 d interval | | 4 d interval | | 7 d interval | |
|---|---|---|---|---|---|---|
| | Slope (SE) | $LC_{50}$ (95% CI) μg a.i. $mL^{-1}$ | Slope (SE) | $LC_{50}$ (95% CI) μg a.i. $mL^{-1}$ | Slope (SE) | $LC_{50}$ (95% CI) μg a.i. $mL^{-1}$ |
| ACT | 3.82 (0.36) | 365.5 (316.3–414.2) | 3.74 (0.36) | 251.9 (205.4–294.3) | 4.22 (0.45) | 187.6 (141.9–227.9) |
| ABA | 1.79 (0.17) | 0.17 (0.14–0.22) | 2.16 (0.18) | 0.092 (0.078–0.11) | 4.15 (0.43) | 0.047 (0.039–0.056) |
| BIF | 3.41 (0.27) | 875.6 (772.6–1014) | 3.45 (0.28) | 342.1 (295.8–390.1) | 4.87 (0.51) | 251.7 (198.4–297.3) |
| CYP | 2.17 (0.19) | 79.58 (67.65–95.67) | 2.49 (0.21) | 56.19 (48.53–65.06) | 3.29 (0.31) | 44.76 (38.75–50.89) |
| *LCY* | 2.04 (0.21) | 502.4 (387.8–719.1) | 1.84 (0.15) | 174.4 (146.8–209.5) | 2.75 (0.23) | 82.94 (68.89–96.67) |
| TET | 3.30 (0.29) | 312.7 (271.2–356.6) | 3.64 (0.34) | 258.7 (221.2–295.3) | 3.77 (0.36) | 216.8 (180.7–250.2) |
| DIC | 1.49 (0.15) | 5.94 (4.63–8.26) | 2.94 (0.24) | 2.06 (1.81–2.35) | 3.70 (0.35) | 1.22 (1.01–1.41) |
| EMB | 2.06 (0.18) | 0.13 (0.10–0.17) | 2.48 (0.19) | 0.048 (0.042–0.056) | 2.82 (0.23) | 0.033 (0.028–0.038) |

*$LC_{50}$* lethal concentration that kill 50% honey bee workers, *CI* confidence interval, *ACT* Acetamiprid, *ABA* Abamectin, *BIF* Bifenthrin, *CYP* Cypermethrin, *LCY lambda*-Cyhalothrin, *TET* Tetraconazole, *DIC* Dicrotophos, *EMB* Emamectin benzoate.

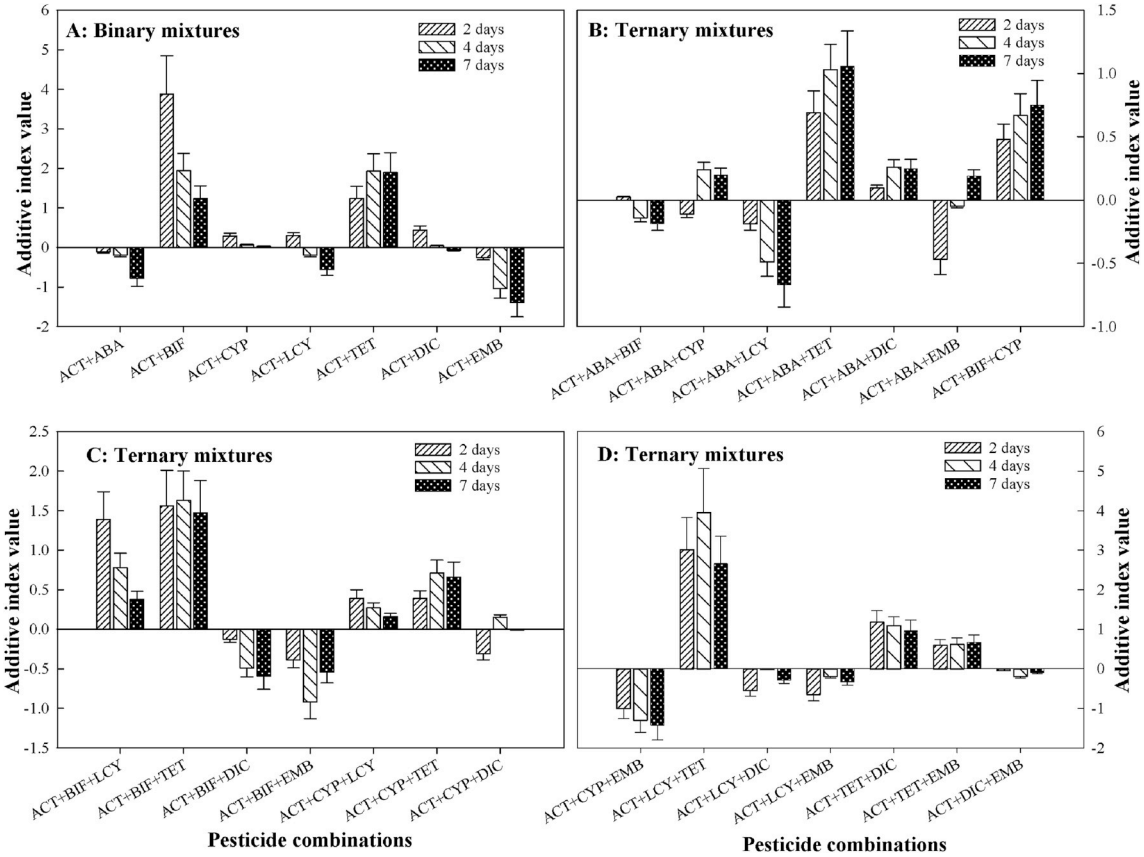


**Fig. 1.** Combined toxicities of binary and ternary mixtures of acetamiprid (ACT) in combination with seven pesticides to honey bees. *ACT* Acetamiprid, *ABA* Abamectin, *BIF* Bifenthrin, *CYP* Cypermethrin, *LCY lambda*-Cyhalothrin, *TET* Tetraconazole, *DIC* Dicrotophos, *EMB* Emamectin benzoate.

other pesticides to *A. mellifera*, $LC_{50}$ values of the pesticides applied individually and in different combinations were comparatively analyzed for their interactions after feeding exposure for 2 days, 4 days and 7 days. The obtained AI value was used to evaluate the combined toxic effect of each pesticide combination on honey bees (Table S2).

### 3.2.1. Combined toxicities of binary and ternary mixtures

The two binary mixtures of ACT + BIF and ACT + TET elicited synergistic effects on honey bee mortality, and their AI values ranged from 1.24 to 3.88 after exposure for 2 days, from 1.93 to 1.94 after exposure for 4 days and from 1.24 to 1.90 after exposure for 7 days. On the contrary, the AI values of ACT + EMB after 2-day, 4-day and 7-day exposures were −0.25, −1.04 and −1.39, respectively, suggesting antagonistic effects. However, all the other binary mixtures showed multivariate effects after exposure for different durations (Fig. 1A).

Eight ternary mixtures of ACT + ABA + TET, ACT + BIF + CYP, ACT + BIF + LCY, ACT + BIF + TET, ACT + CYP + TET, ACT + LCY + TET, ACT + TET + DIC and ACT + TET + EMB showed synergistic responses with AI values ranging from 0.39 to 3.01 after 2-day exposure, from 0.62 to 3.95 after 4-day exposure and from 0.38 to 2.66 after 7-day exposure. In contrast, two ternary mixtures of ACT + BIF + EMB and ACT + CYP + EMB had antagonistic effects on the pollinators with AI values ranging from −1.01 to −0.39 after 2-day exposure, from −1.30 to −0.92 after 4-day exposure and from −1.43 to −0.54 after 7-day exposure. However, three ternary mixtures of ACT + ABA + BIF, ACT + ABA + CYP and ACT + DIC + EMB displayed additive responses, and their AI values ranged from −0.045 to 0.024 after exposure for 2 days, from −0.19 to 0.24 after exposure for 4 days and from −0.19 to 0.20 after exposure for 7 days. The other eight ternary mixtures showed dual effects of combined toxicity, including additive-antagonistic or additive-synergistic responses toward *A. mellifera* (Fig. 1B–D).

### 3.2.2. Combined toxicities of quaternary mixtures

All the quaternary mixtures containing ACT and TET (with the exception of ACT + ABA + TET + DIC) and one quaternary mixture of ACT + CYP + DIC + EMB elicited synergistic effects on honey bees with AI values ranging from 0.26 to 4.43 after exposure for 2 days, from 0.41 to 3.64 after exposure for 4 days and from 0.37 to 2.52 after exposure for 7 days. In contrast, two quaternary mixtures of ACT + ABA + CYP + DIC and ACT + ABA + LCY + EMB displayed antagonistic responses to the pollinators with AI values ranging from −0.31 to −0.29 after exposure for 2 days, from −1.35 to −0.46 after exposure for 4 days and from 0.37 to 2.52 after exposure for 7 days. However, two quaternary mixtures of ACT + ABA + LCY + DIC and ACT + CYP + LCY + DIC had additive effects, and their AI values ranged from 0.087 to 0.13 after exposure for 2 days, from −0.095 to 0.15 after exposure for 4 days and from −0.11 to −0.054 after exposure for 7 days. The other 16 quaternary mixtures exhibited dual (additive-antagonism and additive-synergism) or triple (antagonism-additive-synergism) effects of combined toxicity after exposure for different durations (Fig. 2A–G).

### 3.2.3. Combined toxicities of quinquenary to octonary mixtures

All the quinquenary mixtures (with the exception of ACT + CYP + LCY + TET + DIC) containing ACT and TET exhibited synergistic responses to *A. mellifera* with AI values ranging from 0.33 to 1.76 after exposure for 2 days, from 0.94 to 2.39 after exposure for 4 days and from 0.85 to 2.0 after exposure for 7 days. In contrast, four quinquenary mixtures of ACT + ABA + BIF + CYP + EMB,

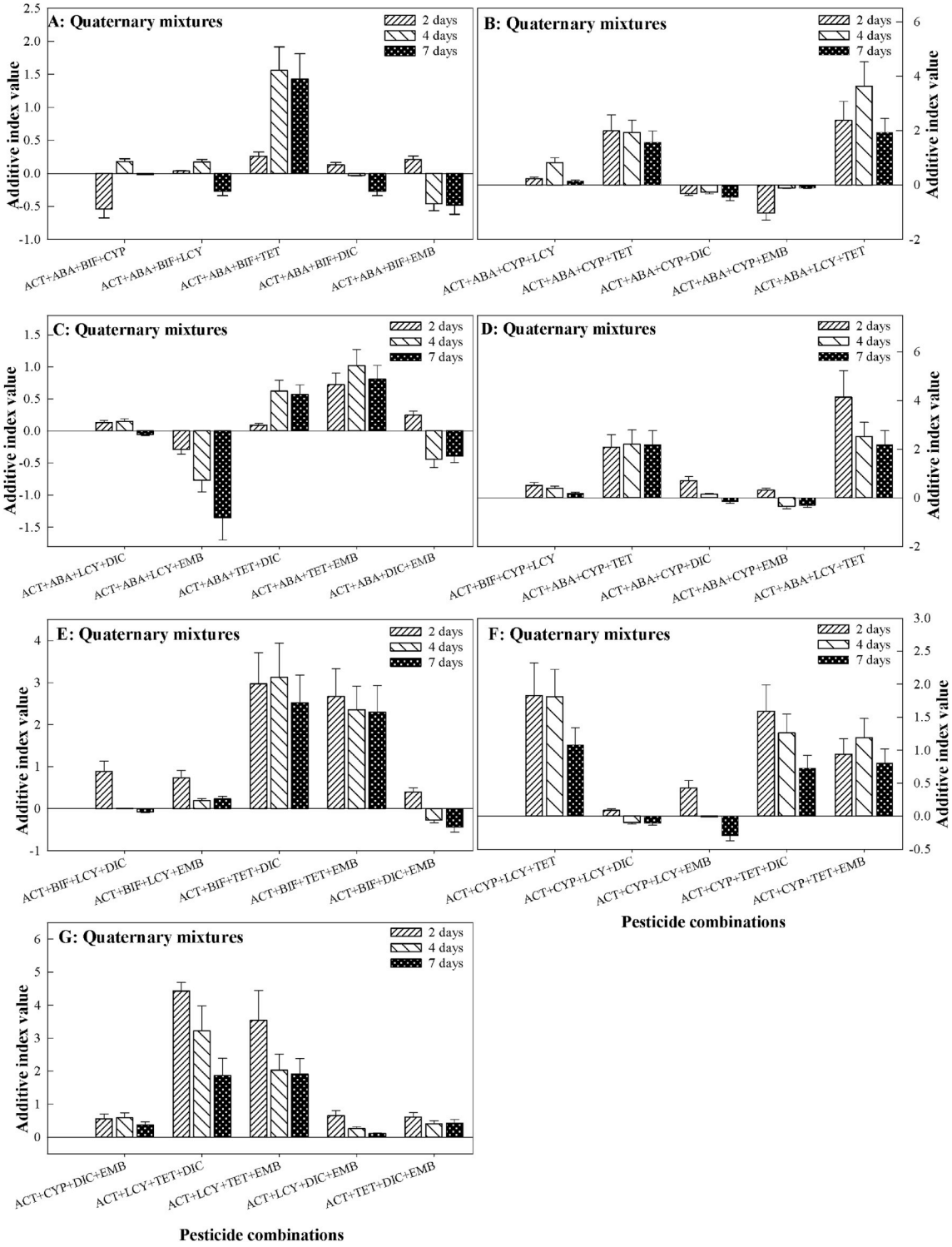


**Fig. 2.** Combined toxicities of quaternary mixtures of acetamiprid (ACT) in combination with seven pesticides to honey bees. *ACT* Acetamiprid, *ABA* Abamectin, *BIF* Bifenthrin, *CYP* Cypermethrin, *LCY lambda*-Cyhalothrin, *TET* Tetraconazole, *DIC* Dicrotophos, *EMB* Emamectin benzoate.

ACT + ABA + BIF + LCY + DIC, ACT + ABA + BIF + LCY + EMB and ACT + CYP + LCY + DIC + EMB had antagonistic effects on honey bees with AI values ranging from −1.42 to −0.51 after exposure for 2 days, from −1.99 to −0.28 after exposure for 4 days and from −2.45 to −0.79 after exposure for 7 days. The other nine quinquenary mixtures exhibited dual or triple effects of combined toxicity (Fig. 3A–D).

All the senary mixtures containing ACT and TET exhibited synergistic responses with AI values ranging from 1.84 to 3.31 after exposure for 2 days, from 0.93 to 1.94 after exposure for 4 days and from 0.77 to 1.54 after exposure for 7 days. The AI values of ACT + BIF + CYP + LCY + DIC + EMB after exposure for 2 days, 4 days and 7 days were 0.21, −0.15 and −0.14, respectively, suggesting additive effects on *A. mellifera*. However, the other three senary mixtures had dual (additive-antagonism and additive-synergism) effects of combined toxicity (Fig. 4A–B).

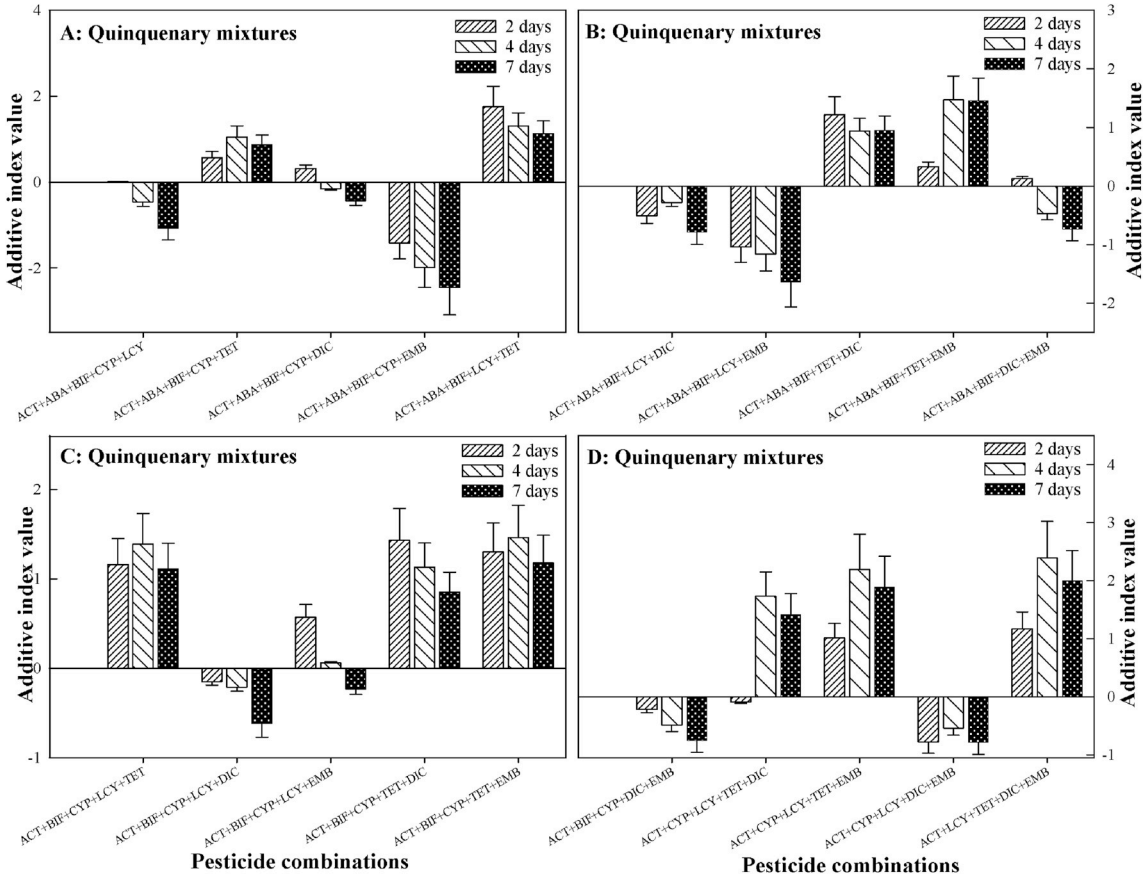


**Fig. 3.** Combined toxicities of quinquenary mixtures of acetamiprid (ACT) in combination with seven pesticides to honey bees. *ACT* Acetamiprid, *ABA* Abamectin, *BIF* Bifenthrin, *CYP* Cypermethrin, *LCY lambda*-Cyhalothrin, *TET* Tetraconazole, *DIC* Dicrotophos, *EMB* Emamectin benzoate.

All the septenary mixtures containing ACT and TET had synergistic effects on honey bees with AI values ranging from 2.28 to 3.63 after exposure for 2 days, from 0.4 to 1.99 after exposure for 4 days and from 0.8 to 1.25 after exposure for 7 days. For the septenary mixture of ACT + ABA + BIF + CYP + LCY + DIC + EMB, the calculated AI values were −0.018, −0.47 and −0.59 after exposure for 2 days, 4 days and 7 days, indicating additive and antagonistic effects, respectively. The octonary mixture of ACT + ABA + BIF + CYP + LCY + TET + DIC + EMB showed AI values of 1.68, 1.01 and 0.87 after exposure for 2 days, 4 days and 7 days, respectively, suggesting synergistic responses (Fig. 4C).

### 3.3. Statistics of interaction patterns

#### 3.3.1. Interaction patterns of binary and ternary mixtures

A total of 28 binary and ternary mixtures of ACT in combination with seven pesticides were evaluated in this study. All the binary and ternary mixtures containing ACT and TET elicited synergistic effects on honey bees. Overall, 35.71% of binary and ternary mixtures exhibited synergistic effects on *A. mellifera*. Besides, 14.29% of combinations showed additive-synergistic effects. In contrast, only 10.71% of pesticide mixtures displayed antagonism to the pollinators. In addition, 25% and 10.71% of combinations had additive-antagonistic and additive effects, respectively (Fig. 5A).

#### 3.3.2. Interaction patterns of quaternary mixtures

A total of 35 quaternary mixtures of ACT in combination with seven pesticides were tested in the current study. We showed that 14 out of 15 quaternary mixtures containing ACT and TET exerted synergistic effects on honey bees. Moreover, 42.86% of quaternary mixtures displayed synergistic effects. In addition, 20% and 8.57% of combinations exhibited additive-synergistic and antagonistic-additive-synergistic effects to *A. mellifera*, respectively. In contrast, only 5.71% and 17.14% of combinations had antagonistic and additive-antagonistic responses, respectively. Furthermore, 5.71% of combinations showed additive effects on the pollinators (Fig. 5B).

#### 3.3.3. Interaction patterns of quinquenary to octonary mixtures

A total of 35 quinquenary to octonary mixtures of ACT in combination with and other pesticides were determined in this study. We showed that 19 out of 20 pesticide mixtures containing ACT and TET had synergistic effects on the pollinators. Moreover, 54.29% of quinquenary to octonary mixtures exerted synergistic effects, all of which contained ACT and TET. Besides, 8.57% and 2.86% of combinations showed additive-synergistic and antagonistic-additive-synergistic effects on honey bees, respectively. In contrast, 11.43% and 20% of combinations elicited antagonistic and additive-antagonistic responses, respectively. Only 2.86% of combinations had additive effects on the pollinators (Fig. 5C).

Overall, 98 binary to octonary mixtures of ACT in combinations with other seven pesticides were assessed in the current study. We showed that 44.90% of combinations showed synergistic effects on honey bees. Besides, 14.29% and 5.10% of combinations exerted additive-synergistic and antagonistic-additive-synergistic responses, respectively. In contrast, 9.18% and 20.41% of combinations elicited antagonistic and additive-antagonistic effects on the pollinators, respectively. Only, 6.12% of combinations had additive effects. Moreover, 40 out of 42 pesticide mixtures containing ACT and TET exhibited synergistic effects on *A. mellifera*. This means that 95.24% of pesticide mixtures containing ACT and TET exerted synergistic responses (Fig. 5D).

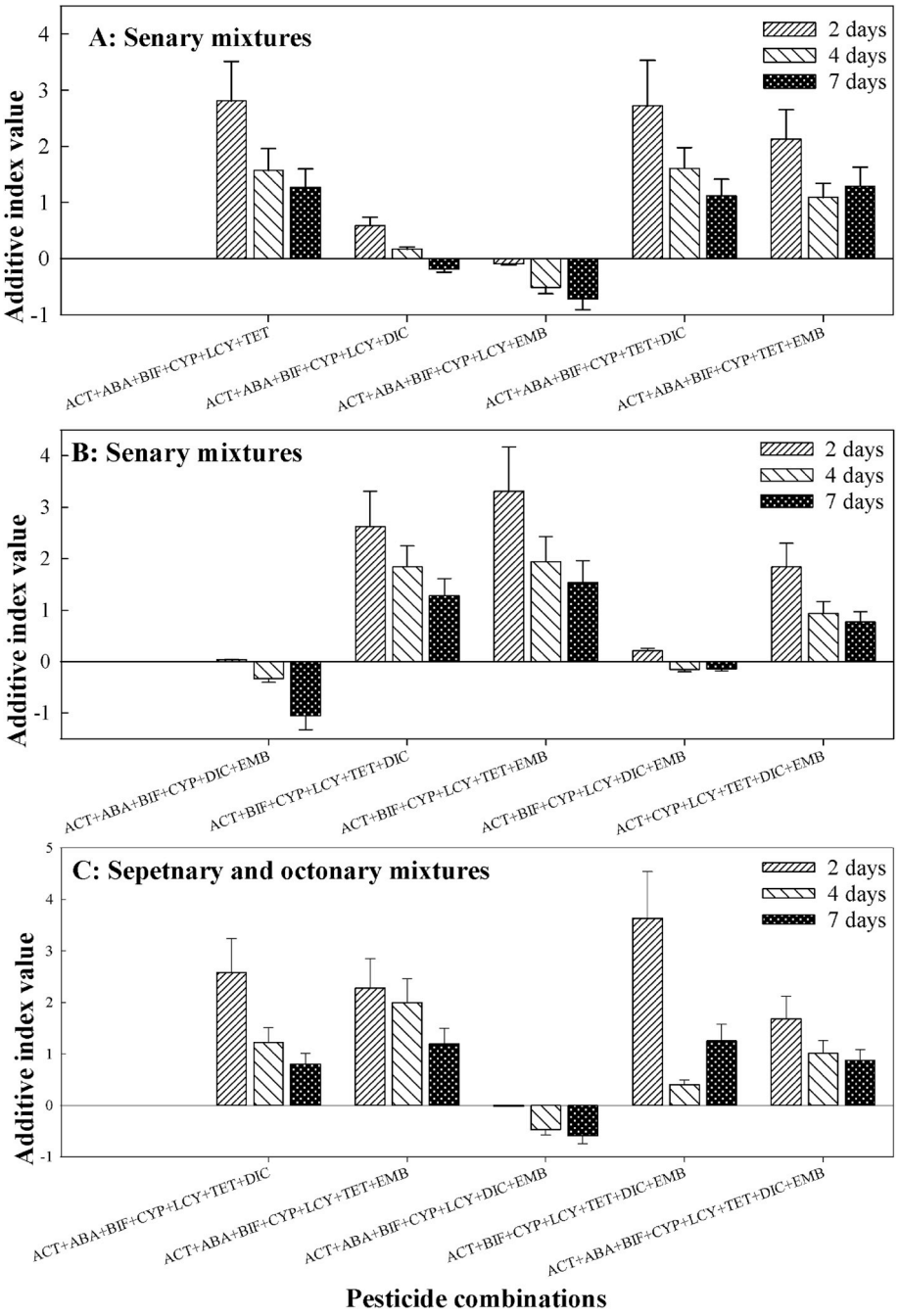


**Fig. 4.** Combined toxicities of senary mixtures of acetamiprid (ACT) in combination with seven pesticides to honey bees. *ACT* Acetamiprid, *ABA* Abamectin, *BIF* Bifenthrin, *CYP* Cypermethrin, *LCY lambda*-Cyhalothrin, *TET* Tetraconazole, *DIC* Dicrotophos, *EMB* Emamectin benzoate.

## 4. Discussion

Results from single toxicity tests showed that honey bees were most sensitive to EMB and ABA, followed by DIC, and they were least sensitive to pyrethroids, ACT and TET. Previous studies have revealed that the 24-h $LC_{50}$ values of EMB and ABA via oral feeding are 2.19 (0.27–4.69) and 0.66 (0.10–2.98) μg a. i. $mL^{-1}$, respectively, confirming their high toxicity to honey bees (Abdu-Allah and Pittendrigh, 2018). Some studies have shown that DIC also exhibits a strong toxic effect on *A. mellifera* with contact toxicity test (Zhu et al., 2015). The high toxicities of EMB, ABA and DIC may be attributed to less pronounced ability to metabolize the compounds in *A. mellifera* (Berenbaum and Johnson, 2015). In contrast, the lower feeding toxicities of pyrethroids, ACT, and TET may be caused by high enzymatic metabolic activity, which leading to only a low amount of poison to the site of pesticide action (Gong and Diao, 2017). Considering the high efficacy of EMB and ABA toward target pests, pesticide managers should cautiously assess their usage in pest control to prevent severe damages to crop pollinators.

Honey bees are usually exposed to a mixture of pesticides instead of individual compounds in natural environments (Gill et al., 2012; David et al., 2016). This is particularly true for honey bees foraging on a crop treated with different pesticides, either applied sequentially or in a tank mixture, when honey bees forage on various plants contaminated with different pesticides (Böhme et al., 2017; Sgolastra et al., 2017). A survey of honey bees, their hive wax and pollen in the United States has demonstrated that most of samples are contaminated with at least one pesticide, and a total of 121 different agrochemicals are detected in

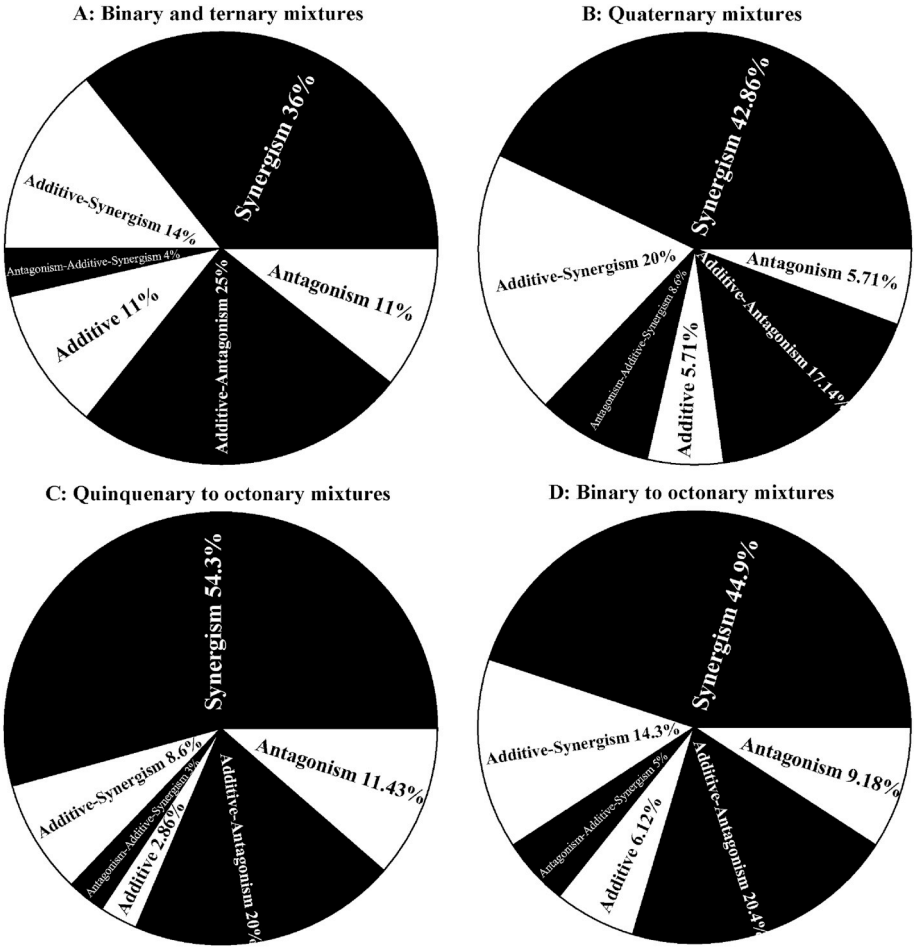


**Fig. 5.** Interaction patterns of acetamiprid (ACT) in combination with seven pesticides to honey bees.

samples (Halm et al., 2006; David et al., 2016). However, most of studies on toxic effects of pesticides on honey bees have focused on effects of individual chemicals (Badiou-Bénéteau et al., 2012; Zhu et al., 2015). Although the concentrations of individual pesticides may be low in natural ecosystems, interactions among pesticide mixtures result in significant toxicity to the pollinators (Pilling et al., 1995; Naggar et al., 2015; David et al., 2016; Böhme et al., 2017). Therefore, it is essential to examine the potential interaction patterns of pesticide mixtures with different MOA to *A. mellifera*.

Synergistic interactions of mixed pesticides can cause serious affects on honey bee colonies (Gill et al., 2012; Stanley et al., 2015). The understanding of interaction patterns among pesticides is very important for the restriction of using defined mixture with negative effects and also for the prediction of potential toxicity of newly developed compounds in agriculture and apiculture (Gill et al., 2012; Gong and Diao, 2017). Our data showed that most of the pesticide mixtures containing both ACT and TET had synergistic toxicities to *A. mellifera*. The toxicity of mixture, neonicotinoid insecticide and triazole fungicide, have been well documented, because neonicotinoids were frequently co-applied with fungicides that may inhibit P450-mediated detoxification in bees (David et al., 2016; Raimets et al., 2018). The ergosterol biosynthesis inhibitor (EBI) fungicides inhibit the cytochrome P450-mediated detoxification of neonicotinoids, resulting in an increase in ACT toxicity to honey bees (Iwasa et al., 2004; Johnson, 2015). Therefore, synergistic toxicities are often observed in the mixtures of neonicotinoids when combined with triazole fungicides (Thompson et al., 2014; Sgolastra et al., 2017). Since most chemicals are assumed to have additive toxicity, the increased toxicity can cause adverse effects on honey bee colonies, threatening the normal functioning of ecosystems (Rizzati et al., 2016).

ACT and BIF are often formulated as tank-mixture in order to allow a broader spectrum of pest control and to reduce pesticide resistance (Hernández et al., 2017; Belden and Brain, 2018). Synergistic effects were found from one binary mixture of ACT + BIF, two ternary mixtures of ACT + BIF + CYP and ACT + BIF + LCY on *A. mellifera*. They would pose greater-than-expected threat to crop pollinators (Rizzati et al., 2016). Interactions between compounds can influence several processes in organisms, including bioavailability, uptake, metabolization, excretion, etc. One possible explanation for the synergy in the simultaneous presence of ACT and BIF could be probably related with changes in the metabolic enzyme activities. One similar example was observed with the organophosphate coumaphos that enhanced the toxicity when mixed with the pyrethroid tau-fluvalinate (Johnson et al., 2009). Here the observed synergism may result from competition between ACT and BIF for access to detoxicative P450s. Therefore, in order to alleviate the adverse effects of ACT and BIF on *A. mellifera*, the use of mixture of ACT + BIF should be avoided and an alternative mixture should be sought to minimize the risk.

Previous studies about combined toxicity of pesticides to honey bees have mainly focused on the interactions between two pesticides (Thompson et al., 2014; Sgolastra et al., 2017). The current study simulated the exposure of honey bees to a variety of different pesticides in the actual environment (David et al., 2016), and assessed the interactive toxicities of ACT in combination with seven pesticides to *A. mellifera*. Due to the observed synergistic effects of ACT when in combination with other pesticides on honey bees, its usage should be

cautiously evaluated in integrated pest management for avoiding serious harm to benefit organisms under field conditions.

## 5. Conclusions

EMB and ABA were the most toxic compounds among the eight determined pesticides. Synergistic effects on honey bees were detected from most of pesticide mixtures containing ACT and TET. Moreover, four pesticide mixtures of ACT + BIF, ACT + BIF + CYP, ACT + BIF + LCY and ACT + CYP + DIC + EMB also exhibited synergistic effects. In addition, 44.90% of binary to octonary mixtures of ACT in combination with seven pesticides displayed synergistic effects on *A. mellifera*. These findings mimicked the environmental condition in the field condition and the combinations of ACT + TET can influence the interaction patterns of the pesticides with the honey bees. The results emphasized that the co-occurrence of several pesticides in the ecosystems might lead to increased toxicity, leading to serious side-effects on the crop pollinators compared with their individual toxicities. Although ACT had low toxicity to *A. mellifera*, this neonicotinoid insecticide should be carefully applied under field conditions due to its synergistic effects in combination with other pesticides on crop pollinators.

## Author contributions

Yanhua Wang, Yu Cheng Zhu and Wenhong Li conceived and designed experiments; Yanhua Wang, Yu Cheng Zhu and Wenhong Li performed experiments; Yanhua Wang, Yu Cheng Zhu and Wenhong Li analyzed data; Yu Cheng Zhu contributed reagents/material/analysis tools; Yanhua Wang, Yu Cheng Zhu and Wenhong Li wrote the paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.110100.

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