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Chronic toxicity and biochemical response of *Apis cerana cerana* (Hymenoptera: Apidae) exposed to acetamiprid and propiconazole alone or combined

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Abstract
Acetamiprid and ergosterol-inhibiting fungicide (EBI) are frequently applied to many flowering plants, while honey bees are pollinating agents or pollinators of the flowers. Hence honey bees are often exposed to these pesticides. But until now, the effects of theses combinations at field-realistic doses on honey bee health have been poorly investigated. In this study, we explore the synergistic mortality and some physiological effects in surviving honey bees after chronic oral exposure to acetamiprid and/or propiconazole in the laboratory. The results indicated that chronic combined exposure to acetamiprid and propiconazole produced a significant synergistic effect on mortality both for newly emerged bees (50% mortality in 7.2 days) and forager bees (50% mortality in 4.8 days). Honey bee weight of newly emerged bees was decreased after feeding food with a field concentration of acetamiprid and propiconazole, alone or combined for 10 days. Combination of acetamiprid and propiconazole also modulated the activities of P450s, GST and CAT in newly emerged bees and forager bees than either alone, but neither pesticide affected the activity of AChE. These results show that chronic combined exposure to pesticides of relatively low toxicity may caused severely physiological disruptions that could be potentially damaging for the honey bees.

Keywords *Apis cerana cerana* · Synergistic mortality · Detoxification enzymes · Physiological effects

Introduction

Apis cerana cerana is an important indigenous species, it is distributed widely in various geographic zones throughout China and brings huge economic benefits to agriculture (Klein et al. 2007; Li et al. 2016). *A. c. cerana* play an indispensable role in maintaining ecological balance and biological diversity (Liu et al. 2017). In recent years, the western honey bee populations have plummeted and this phenomenon has elicited global concern (Lee et al. 2015; Potts et al. 2010; vanEngelsdorp and Meixner 2010). Similarly, *A. c. cerana* are currently also suffering from the significant population (Chen et al. 2017; Xu et al. 2009) and natural distribution area (Gemeda et al. 2017) decreases. The causes of the phenomenon was related with multiple environmental stressors (Goulson et al. 2015), but insecticides have been considered to play a significant role (Krupke et al. 2012; Pisa et al. 2015).

In modern agriculture, the co-application of pesticides is a common phenomenon, such as insecticides and fungicides are frequently either co-applied in a tank mix or sequentially applied (Robinson et al. 2017), and also used in seed coatings, which contain almost always a mixture of a neonicotinoid and EBI fungicide. Some companies have a patent on such mixtures because they have proven to be more effective (i.e. toxic) to control pests. Hence foraging bees are often simultaneously exposed directly to a variety of pesticides and hive bees can be exposed through pollen

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or nectar contaminated with different pesticides returned to the hive by foragers (David et al. 2016; Dolezal et al. 2016; Johnson et al. 2010). These different pesticides residues at various concentrations have been detected in different matrices of honey bee colonies (David et al. 2015; David et al. 2016; Mullin et al. 2010; Tong et al. 2016; Wu et al. 2011), and their potential hazards to honey bee have been extensively studied. For example, exposure to sublethal levels of pesticides in honey bees can affect behavior (Henry et al. 2012; Matsumoto 2013; Yang et al. 2008), learning (Decourtye et al. 2004a; Decourtye et al. 2004b), colony development (Wu et al. 2011), sperm viability (Chaimanee et al. 2016; Collins and Pettis 2013), and susceptibility to several pathogens (Pettis et al. 2013). However, to date, most studies of toxicity effects of pesticides on bees have tested the effects of single compounds, only several studies have investigated the effects of pesticides associations (Domingues et al. 2017; Gill et al. 2012; Renzi et al. 2016; Sgolastra et al. 2017; Zhu et al. 2017b). Pesticides mixtures can have additive effects through the same or different mode of action or even synergism or antagonism in toxicity. More attention should be paid to specific mixtures because they have been shown synergistic toxicity to bees (Gill et al. 2012; Iwasa et al. 2004; Sanchez-Bayo and Goka 2014). Risk assessment would need to take into consider the magnitude of such interactions.

We assessed the potential synergistic interactions between a neonicotinoid (acetamiprid) and an ergosterol biosynthesis inhibitor (EBI) fungicide (propiconazole) on *A. c. cerana* following oral exposure in the laboratory. Acetamiprid is widely used as second generation chloroneonicotinoids with contact and systemic activity *via* foliar application (Devan et al. 2015). Like all neonicotinoids, acetamiprid is a selective agonist of the nicotinic acetylcholine receptors in the central nervous system of insects (Shimomura et al. 2006). Its acute toxicity is several orders of magnitude lower to honey bees than that of the nitro-substituted neonicotinoids (acute oral LD_{50} for *Apis mellifera*: 15–17 µg/honey bee, acute contact LD_{50} for *A. mellifera*: 8.1–39 µg/honey bee) (Lundin et al. 2015). Because the comparatively more "bee-friendly" properties of acetamiprid, it is permitted to be sprayed on flowering crops (Godfray et al. 2014). Others, like imidacloprid, clothianidin, and thiamethoxam have been permanent banned in agricultural fields and outdoors from 27 April 2018 by the European Union (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:L:2018:132:TOC), because of growing concerns about the risk they may pose on honey bees and other pollinators (Gross 2013), and they have been largely replaced by acetamiprid and thiacloprid in recent years. Honey bees are also exposed to fungicides because they are commonly used during plants flowering period to control fungi born-diseases. Propiconazole is a broad-spectrum systemic triazole fungicide commonly used in agriculture. Hence, in agricultural environments, honey bees are inevitably exposed to acetamiprid and propiconazole in combination. The residues of acetamiprid and propiconazole have been detected in pollen and honey bees body (Johnson et al. 2010; Mullin et al. 2010; Tong et al. 2016; van der Zee et al. 2015). It is reported that exposure to 10 µg/honey bee propiconazole increases the toxicity of acetamiprid 105-fold (Iwasa et al. 2004). Our previous research have shown that the contact and oral toxicities of acetamiprid increased about 10 and 3 folds with the exposure of field-relevant doses of propiconazole on *A. c. cerana* (Han et al. 2017).

There is evidence that chronic exposure to sublethal doses of chemicals can alter physiological functions of organisms. The study of enzymatic modulation induced by pesticides in honey bees is increasingly investigated, revealing specific patterns of response (Badiou-Bénéteau et al. 2012; Carvalho et al. 2013; Roat et al. 2017). Despite the synergism that might occur between neonicotinoids and ergosterol biosynthesis inhibiting fungicides, the physiological mechanisms of interactions remain poorly investigated. Some enzymatic biomarkers are particularly used to assess the physiological effects of environmental stressors. Acetylcholinesterase (AChE) is a neural enzyme that mediates neural transmission by hydrolyzing acetylcholine in cholinergic synapses (Badiou et al. 2007). Cytochrome P450 monooxygenases (P450s) is a detoxification enzyme of phase I, which act on a plethora of endogenous and exogenous substrates directly to reduce their toxicity (Zhang et al. 2016). Glutathione-S-transferase (GST) is a detoxification enzyme involved in the general biotransformation of xenobiotics and endogenous substances of phase II (Hyne and Maher 2003). Plus GST and glutathione (GSH) have a vital role in the repair processes by detoxification of peroxides and oxidized DNA bases, being a key protection mechanism (Forman et al. 2009). Catalase (CAT) is a peroxisomal hydroperoxidase that catalyzes the conversion of hydrogen peroxide into oxygen and water, providing an effective defense system against the toxicity of reactive oxygen species (ROS) (Badiou-Bénéteau et al. 2012).

Our hypothesis here is that chronic exposure to a combination of acetamiprid and propiconazole may have significant effects on honey bee's health. So, in this study, we have investigated the toxic effects of both short-term and long-term exposure to acetamiprid and propiconazole alone or combined at field-relevant doses on newly emerged and forager bees of *A. c. cerana*. This study was focused on mortality measurements and on the physiological disturbances assessed by the modulation of the biomarkers P450s, GST, AChE, CAT. Furthermore, we explored detoxification mechanisms in honey bees through enzymes

activity change induced by insecticides and fungicides with different modes of action.

Materials and methods

Chemicals

Technical-grade chemicals were used for all experiments in this study. Both acetamiprid (purity, 98.2%) and propiconazole (purity, 95%) were obtained from Hainan Bosswell Agrichemical Co., Ltd. NADPHNa4, 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC) were products of Sigma Chemical Co. (St. Louis, MO). Phenylmethylsulfonyl (PMSF), dithiothreitol (DTT), phenylthiourea (PTU), bovine serum albumin (BSA), Aacetylcholinesterase Assay Kits, Glutathione S-transferaseassay Kits, Catalase Assay Kits were obtained from Solarbio Science & Technology Co., Ltd., Beijing. BCA Protein Quantitation Kits were purchased from Biomiga, Inc (San Diego, California). High grade analysis chemicals were purchased from commercial suppliers (Xilong Chemical Co, Shantou).

Honey bees

Honey bees were obtained from queen-right colonies of *A. c. cerana* maintained in the apiary of the Chinese Academy of Tropical Agricultural Sciences, Haikou (N19°32′, E109° 32′). The apiary is located in urban areas, so the honey bees had relatively fewer opportunities to exposure to pesticides than cultivated areas. Experiments were conducted in the Laboratory of Environment and Plant Protection Institute. Colonies and units did not present visible symptom of any known diseases, and hives were not treated with any pesticides.

Test solutions

Base on an acute oral LD_{10} value of 0.183 μg/honey bee for acetamiprid for *A. c. cerana*, determined in a previous study (unpublished), we selected this sublethal dose for our experiments. For propiconazole, a field-realistic exposure would be 1.20 μg/honey bee, derived from the highest residues estimated for this fungicide in Chinese crops [referenced Koch and Weißer (1997): applicated 20 g tracer/ha, residues 35.77 ng/honey bee]. We assumed that 0.183 μg/honey bee acetamiprid and 1.20 μg/honey bee propiconazole were maximum intakes per day of *A. c. cerana* adult. In a pilot study, we found pesticide intakes per honey bee less than the assumed amounts. The actual pesticide consumption was quantified by measuring the daily amount of pesticide-containing sugar solution consumed per honey bee.

Stock solutions (1000 mg/L) of each pesticide was prepared in dimethyl sulfoxide (DMSO) and diluted into sucrose solution (50% w/w sucrose) and stored at −20 °C. The final concentrations of 3.66 ng/μL acetamiprid and 24 ng/μL propiconazole (used to exposure newly emerged bees) or 9.15 ng/μL acetamiprid and 60 ng/μL propiconazole (used to exposure forager bees) were diluted in sucrose solution from stock solutions. The reason for using different concentrations of the two pesticides to the two types of honey bees used is because of forager bees are more susceptible to in-field exposure to pesticide of higher concentrations during their foraging flights compared to in-hive nurse bees. All feeding sucrose solutions contained a final concentration of 0.1% DMSO (v/v) and were freshly prepared daily. In a pilot study, we found no effects of the solvent control (0.1% DMSO) on enzymatic activity change in honey bees.

Experimental design

Newly emerged bees and forager bees were divided into groups and individually treated as later described. Honey bees were kept into cages and these cages were placed in an incubator at controlled conditions (34 ± 2 °C; 60% ± 10% relative humidity; darkness) until the end of the experiment, and all experiments unless otherwise stated were conducted under these conditions. For all experiments, the caged honey bees were fed *ad libitum* on a syrup solution placed on a plastic cap at the bottom of the cage.

Toxicological bioassays in newly emerged bees

Frames of late stage capped brood from the 5 colonies were collected from the apiary and transferred to a dark incubator to monitor the emergence of *A. c. cerana* workers. Newly emerged bees were brushed from frames every 24 h and randomly placed into the wire mesh cages (42 cm × 35 cm × 10 cm), and they were fed *ad libitum* with sucrose solution and pollen (collected from honeycombs) for acclimation about 3 days so that honey bees could be completely developed. In order to investigate the mortality and activities of enzyme biomarkers of both short-term and long-term exposure to acetamiprid and propiconazole alone or combined at field-relevant doses, the newly emerged bees were split in two separate experiments. The two experiments are as follows:

Experiment 1: Newly emerged bees were starved for 2 h, then separated into 4 treatment groups: Control (0.1% DMSO), acetamiprid (3.66 ng/μL), propiconazole (24 ng/μL), and the combination of acetamiprid-propiconazole (3.66 ng/μL + 24 ng/μL). Each experimental group was assayed in six replicates (six iron cages) and each cage (13 cm × 6 cm × 10 cm) contained 20 honey bees from

5 different colonies. All test solutions were provided 1 mL. Test lasted 24 h. Feeders containing the treated solutions were weighed before and after the 1 day exposure to determine the amount consumed by the honey bees. The surviving honey bees from each treatment were collected to determine enzymatic activities.

Experiment II: Four experimental groups of newly emerged bees were set up as in *Experiment I*. Each experimental group was assayed in three replicates (three wooden beehives) and each beehive (23 cm × 18 cm × 20 cm) contained about 150 honey bees from 5 different colonies. In order to mimic the beehive environment as much as possible, a virgin queen was placed in each bee hive. Test lasted 10 days. Honey bees were exposed to the same treatments as experiment I, but only during10 h per day, by feeding with 50% sucrose solution containing the pesticides at the appropriate concentrations. During the remaining 14 h, honey bees were fed with candy and untreated water *ad libitum*. Each day, feeders were replaced and the daily pesticides consumptions were quantified. Throughout the experiment, each beehive was checked every morning and any dead honey bees removed and counted. At the end of the test, the surviving honey bees were collected from each treatment, weighted and stored at −80 °C for analyzing enzymatic activities.

Toxicological bioassays in forager bees

Approximately 2000 newly emerged bees within a period of 8 h were marked on the thorax with maker pen and returned to the colony and captured 18 days later to be used in the following two experiments.

Experiment I: Same as *Experiment I* with newly emerged bees except for the different concentrations used in the four treatments. Four treatment groups set up as follow: control (0.1% DMSO), acetamiprid (9.15 ng/μL), propiconazole (60 ng/μL), and the combination of acetamiprid-propiconazole (9.15 ng/μL + 60 ng/μL).

Experiment II: Same as *experiment II* with newly emerged bees except for using fewer honey bees (100 honey bees) and no queen in each beehive, a shorter exposure (5 days) and different concentrations.

Sample preparation and analysis

The living honey bees from each treatment were anesthetized by cooling at 4 °C for 3 min and sacrificed in ice. AChE was extracted from the head, P450s, GST and CAT were extracted from the midgut. Heads were removed by cutting with a blade and midguts were obtained by pulling the sting from the end of the abdomen. Five heads and five midguts were put in 1.5 mL centrifuge tubes respectively, immediately weighted, flash frozen in liquid N_2, and stored at −80 °C until analysis. Tissues were homogenized at 4 °C using a MY-10 tissue grinder (Germany), then ice-cold extraction medium added and vortexed to make a ten percent (w/v) extract. For P450s [measured as 7-ethoxycoumarin-*O*-deethylase (ECOD) activity)], the extraction medium contained 0.1 M sodium phosphate buffer (pH 7.5), 10% glycerol, 1.0 mM EDTA, 0.1 mM DTT, 1.0 mM PTU and 1.0 mM PMSF. The homogenate was centrifuged at 10,000 g for 20 min at 4 °C and the supernatants were transferred to new tubes to serve as the enzyme sources. For AChE, GST and CAT, the extraction solution comes from their individual kits. Tissue extracts were centrifuged at 4 °C for 10 min at 8000 g and the resulting supernatants were subjected to analysis. For each treatment group, three extracts were achieved for each enzyme and assayed in triplicate. Protein concentrations were estimated using the BCA Protein Quantitation Kit (Biomiga).

7-ethoxycoumarin-O-deethylase (ECOD) assay

The measurement of ECOD activity was done as described by Li et al. (2012). Reaction mixtures contained Tris-HCl buffer (0.25 M, pH 8.0), NADPH (0.25 mM), BSA (0.4 mg/mL), 7-ethoxycoumarin (0.5 mM) and the crude enzyme in a total volume of 1.0 mL. The reaction was started by adding the crude enzyme. The test tubes were incubated for 15 min at 37 °C while shaking at 220 rpm using a thermo-shaker (MSC-100, China). The reaction was stopped by the addition of 0.3 mL of trichloroacetic acid (5%) and the reaction mixtures were centrifuged at 4 °C, 7000 g for 3 min. The supernatant was transferred to new test tube and added 0.7 mL Gly-NaOH (0.6 M, pH 10.4). The production of 7-hydroxycoumarin was quantified by recording the fluorescence (λ_{ex} 368 nm and λ_{em} 456 nm) with a Fluorescence Spectrometer (Lengguang Tech F97Pro, China). The amount of 7-hydroxycoumarin formed by the ECOD activity assay was determined with a standard curve generated from known amounts (0–100 nM) of 7-hydroxycoumarin.

Acetylcholinesterase (AChE) assay

AChE activity was measured using Acetylcholinesterase Assay Kit (Solarbio) according to protocol provided by the manufacturer. The enzyme activity was determined kinetically at 412 nm using a UV spectrophotometer (Shimadzu UV-2600, Japan).

Glutathione-S-Transferase (GST) assay

GST activity was spectrophotometrically assessed by measuring the conjugation of reduced GSH to 1-chloro-2,4-dinitrobenzene (CDNB) using Glutathione-S-transferase

Assay Kit (Solarbio) provided procedure. GST activity was quantified by recording the appearance of conjugated product at 340 nm during 5 min.

Catalase (CAT) assay

CAT activity was measured by Catalase Assay Kit (Solarbio). The reaction was monitored by the decrease in absorbance at 240 nm due to the consumption of H_2O_2.

Data analyses

Daily mortality data was organized in a survival table. Log-rank (Mantel-Cox) with pairwise multi comparison procedures were conducted to compare the survival among the four treatments. Pesticides uptake comparison were analyzed with independent sample T-test. Honey bee weight data and all biochemical assays data were analyzed by one-way analysis of variance (ANOVA) and the means were compared by a Tukey's HSD test. Two-way ANOVA was used to distinguish the interaction effects of exposure time and pesticide treatments. All analyses were performed using the IBM SPSS statistics version 19.0 software program (Statistical Package for Social Science, USA). *P*-values below 0.05 were significantly considered.

Results

Chronic toxicity of acetamiprid and propiconazole, alone or in combination

Survival of newly emerged bees exposed to 3.66 ng/μL acetamiprid or 24 ng/μL propiconazole, alone or in combination, was severely affected in all cases (Fig. 1A). At the end of the experiment (day 10), the mortalities were 18.5% in control with a LT_{50} (time to achieve a lethal concentration for 50% of organisms) of 37.3 d, 48.3% for acetamiprid with a LT_{50} of 15.9 d, 45.3% for propiconazole with a LT_{50} of 11.9 d, and 69.7% for the mixture of the two pesticides with a LT_{50} of 7.2 d.

Survival of forager bees exposed to 9.15 ng/μL acetamiprid or 60 ng/μL propiconazole, alone or in combination, was also severely affected (Fig. 1A). At the end of the experiment (day 5), the mortalities were 19.7% in control, 23.3% for acetamiprid, 30.3% for propiconazole and 55.6% for the mixture. The LT_{50} values were 19.0 d, 16.8 d, 10.3 d, 4.8 d for the control, acetamiprid, propiconazole and the mixture, respectively.

The Log-rank statistic showed significant differences between cumulative survival curves of honey bees exposed to the different treatments [Fig. 1A Log-rank (Mantel-Cox), $\chi^2 = 236.9$, d f = 3, $p < 0.0001$; Fig. 1B Log-rank (Mantel-Cox), $\chi^2 = 77.69$, d f = 3, $p < 0.0001$]. In control groups of forager bees, the higher mortality was occurred in 5th day, the possible reason was that forager bees need to excrete outside the beehive, continued non-excretion lead to increased mortality. In the pairwise comparisons, the acetamiprid and propiconazole combination was more toxic than the control, acetamiprid treated alone and propiconazole treated alone. The survival rate of acetamiprid treated group and propiconazole treated group was not significantly different from each other (Table 1). Both for newly emerged bees and forager bees, the combination produced a clear additive effect.

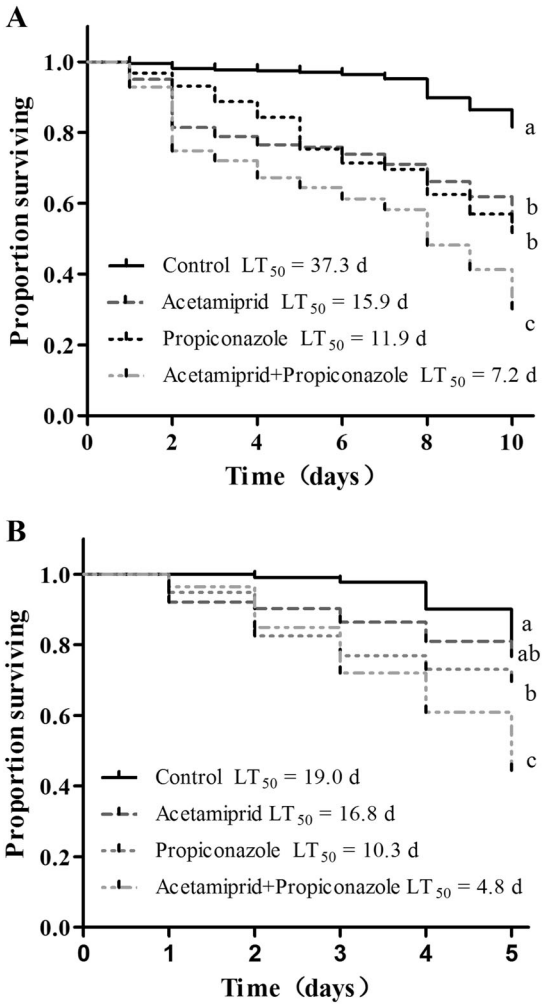

Fig. 1 Survival curves and lethal time (LT_{50}) of newly emerged bees (**A**) and forager bees (**B**) of *Apis cerana cerana* orally continuously exposed to control solution (0.1% DMSO), acetamiprid (3.66 ng/μL or 9.15 ng/μL) propiconazole (24 ng/μL or 60 ng/μL), alone and in combination. Kaplan–Meier with the statistical test Log-Rank. **a** Log-rank (Mantel-Cox) Test: $\chi^2 = 236.9$; d f = 3; $p < 0.0001$. **b** Log-rank (Mantel-Cox) Test: $\chi^2 = 77.69$; d f = 3; $p < 0.0001$. Different letters behind the curves indicate significant differences between treatments

Table 1 Pairwise comparisons of the chronic toxicity of acetamiprid and propiconazole to *Apis cerana cerana*, alone and in combination, 10 d after oral exposure for newly emerged bees and 5 d after oral exposure for forager bees (Log-rank Mantel-Cox test)

Treatments	Newly emerged bees		Forager bees	
	Chi square	*P*-value	Chi square	*P*-value
Control vs. Acetamiprid	101.094	<0.0001	1.792	0.181
Control vs. Propiconazole	93.185	<0.0001	9.980	0.002
Control vs. Combination	275.890	<0.0001	69.298	<0.0001
Acetamiprid vs. Propiconazole	0.672	0.412	3.458	0.063
Acetamiprid vs. Combination	40.705	<0.0001	49.729	<0.0001
Propiconazole vs. Combination	54.437	<0.0001	22.141	<0.0001

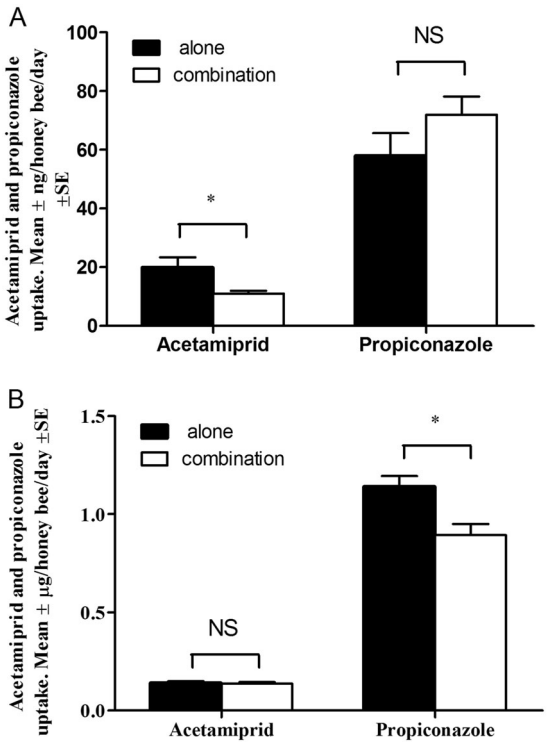

Fig. 2 The mean uptakes of acetamiprid and/or propiconazole per honey bee per day. **A** The uptakes were daily-monitored for 10 days in newly emerged bees. Acetamiprid: independent sample *t*-test, $t_{58} = 2.436$, $p = 0.018$; Propiconazole: Independent sample *t*-test, $t_{58} = -1.415$, $p = 0.162$. **B** The uptakes were daily-monitored for 5 days in forager bees. Acetamiprid: independent sample *t*-test, $t_{28} = 0.233$, $p = 0.818$; Propiconazole: independent sample *t*-test, $t_{28} = 3.161$, $p = 0.004$. NS not significant, $p > 0.05$. Asterisk (*): statistical significance, $p \leq 0.05$

Acetamiprid and propiconazole uptake and honey bee weight

In newly emerged bees (the result of *Experiment II*; Fig. 2A), the mean doses of acetamiprid taken up per honey bee per day averaged 10.98 ± 0.95 ng in the combination treatments. It was significantly lower than that of the alone (19.88 ± 3.53 ng/honey bee/day; *t*-test, $t_{58} = 2.436$, $p = 0.018$). The mean uptakes of propiconazole per honey bee per day (alone: 57.90 ± 7.77 ng; combination: 71.98 ± 6.21 ng) was not significant differences between alone and combination treatments (*t*-test, $t_{58} = -1.415$, $p = 0.162$). In forager bees (the result of *Experiment II*; Fig. 2B), Between single and combination treatments, the mean uptake of acetamiprid pee honey bee per day (alone: 0.139 ± 0.010 µg; combination: 0.136 ± 0.009 µg) had no significant differences (*t*-test, $t_{28} = 0.233$, $p = 0.818$), while the mean uptake of propiconazole pee honey bee per day (alone: 1.141 ± 0.054 µg; combination: 0.893 ± 0.057 µg) had significant differences (*t*-test, $t_{28} = 3.161$, $p = 0.003$). In addition, the actual pesticide consumption per honey bee per day was far less than we assumed.

Honey bee weights were significantly reduced in the treated groups when newly emerged bees were exposed to a field concentration of acetamiprid and propiconazole, alone or combination for 10 days ($F = 25.348$; d f = 3, 103; $p < 0.0001$), but there were no differences between single pesticide and combination treatments (Fig. 3). However, the honey bee weights of forager bees were no differences among the four treatments ($F = 2.171$; d f = 3, 64; $p = 0.100$).

Physiological modulations induced by acetamiprid and propiconazole

The combination of acetamiprid and propiconazole has proven more deadly to honey bees than either alone. Hence, the Physiological reasons were studied by investigating the change of P450s, GST, AChE, CAT activities at different exposure time (1d, 5d or 10d).

In newly emerged bees (Fig. 4A), acetamiprid alone and propiconazole alone did not induce a significant variation of ECOD at either 1 or 10 days, while combination of acetamiprid and propiconazole significantly increased the ECOD activity when compared with the corresponding control at day 1 ($F = 6.584$; d f = 3, 8; $p = 0.015$), but this combination significantly inhibited the ECOD activity when compared with the corresponding control at day 10 ($F = 4.863$; d f = 3, 8; $p = 0.033$). In forager bees (Fig. 4B), acetamiprid treated group and propiconazole treated group

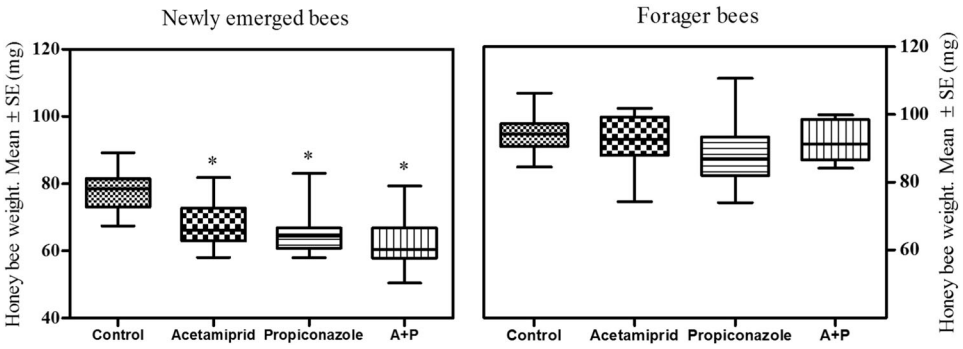

Fig. 3 Effect of acetamiprid and propiconazole alone or in combination on newly emerged bees and forager bees weights of *Apis cerana cerana*. Asterisk (*) indicate a significant difference with the control ($p \leq 0.05$, Tukey's HSD test). A + P: Acetamiprid + Propiconazole

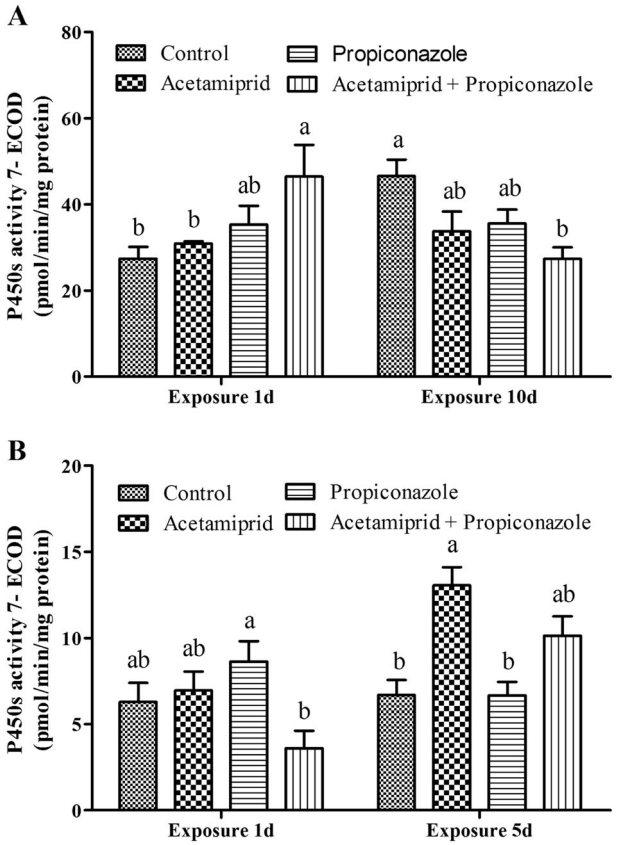

Fig. 4 Effects of acetamiprid and propiconazole alone or combination on the ECOD activities in the midguts of newly emerged bees (**A**) and forager bees (**B**). Data represent the mean ± SE of triplicate assays. Means within bars followed by the same letters are not significantly different ($p > 0.05$, Tukey's HSD)

exhibited the ECOD activity similar to that of control, while combination treated group exhibited an activity significantly lower than propiconazole treated group and similar to that of control and acetamiprid treated group at day 1 ($F = 3.632$; d f = 3, 8; $p = 0.064$). In contrast to 5th day, only acetamiprid alone induced the ECOD activity increase remarkably ($F = 10.031$; d f = 3, 8; $p = 0.004$). Overall, pesticide treatment elicited a modulation of ECOD activity, with a significant interaction with exposure time (Table 2, $p < 0.05$).

Table 2 Two-way ANOVA analysis of the enzyme activities in *Apis cerana cerana* exposed to acetamiprid and propiconazole alone or combined

Enzyme	Bees	Source of variation	d f	*F*	*p*
P450s (ECOD)	NB	Interaction	3,16	7.342	0.0026
		Treatment	3,16	0.5577	0.6505
		Time	1,16	0.08104	0.7796
	FB	Interaction	3,16	8.242	0.0015
		Treatment	3,16	4.634	0.0162
		Time	1,16	14.21	0.0017
GST	NB	Interaction	3,16	4.601	0.0166
		Treatment	3,16	1.931	0.1652
		Time	1,16	20.91	0.0003
	FB	Interaction	3,16	13.22	0.0001
		Treatment	3,16	9.351	0.0008
		Time	1,16	23.42	0.0002
AChE	NB	Interaction	3,16	1.068	0.3905
		Treatment	3,16	0.2173	0.8830
		Time	1,16	29.72	<0.0001
	FB	Interaction	3,16	0.7037	0.5636
		Treatment	3,16	0.6678	0.5840
		Time	1,16	4.885	0.0420
CAT	NB	Interaction	3,16	4.867	0.0136
		Treatment	3,16	18.60	<0.0001
		Time	1,16	7.279	0.0158
	FB	Interaction	3,16	2.210	0.1266
		Treatment	3,16	14.03	<0.0001
		Time	1,16	0.07565	0.7868

Differences were considered significant for $p \leq 0.05$

NB newly emerged bees, *FB* forager bees

In newly emerged bees (Fig. 5A), at day 1, the GST activities were similar between different treatments and the control ($F = 1.721$; d f = 3, 8; $p = 0.240$), and at day 10, the GST activity was reduced in all treatments, although only the acetamiprid alone treatment was statistically significant ($F = 5.370$; d f = 3, 8; $p = 0.026$). In forager bees (Fig. 5B), at day 1, the GST activities were obviously increased in the

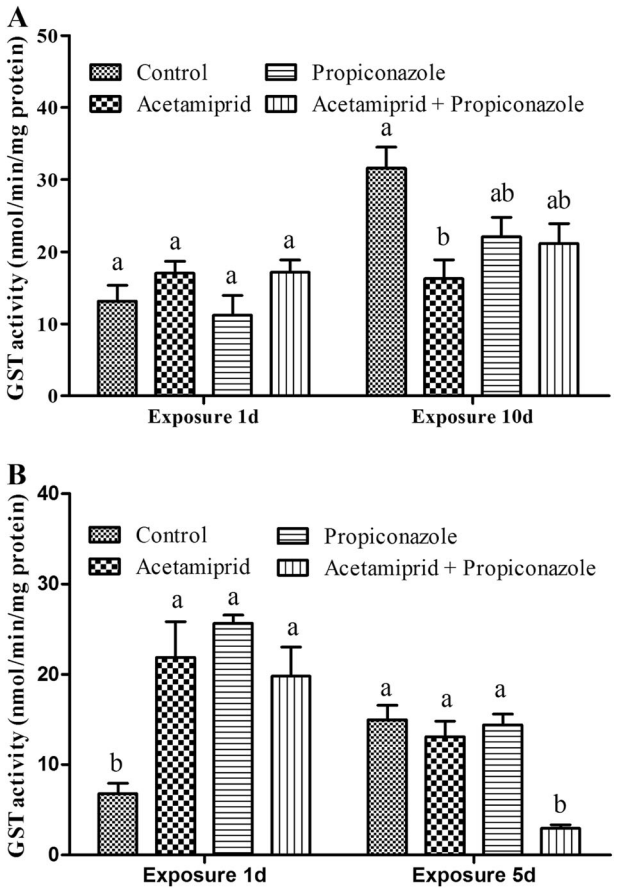

Fig. 5 Effects of acetamiprid and propiconazole alone or combination on the GST activities in the midguts of newly emerged bees (**A**) and forager bees (**B**). Data represent the mean ± SE of triplicate assays. Means within bars followed by the same letters are not significantly different ($p > 0.05$, Tukey's HSD)

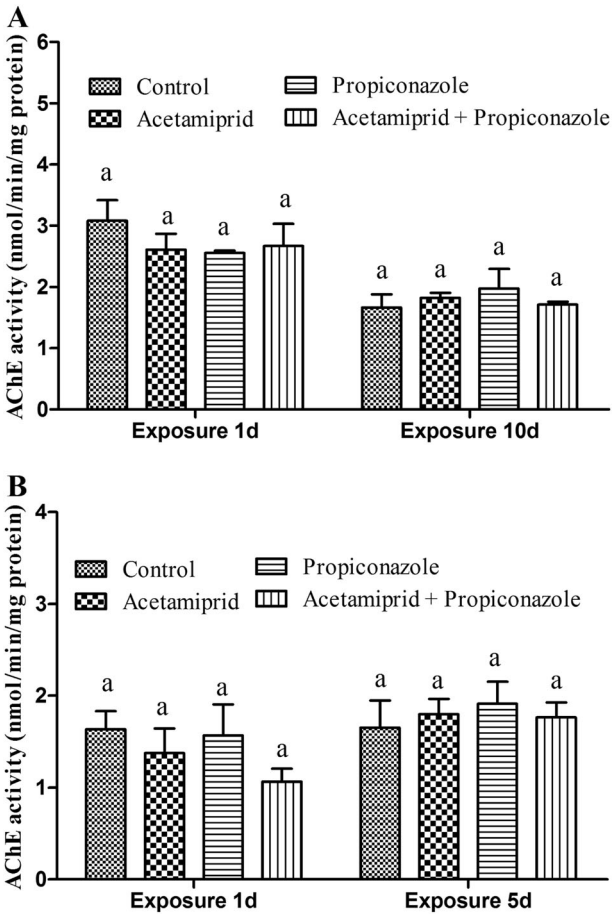

Fig. 6 Effects of acetamiprid and propiconazole alone or combination on the AChE activities in the heads of newly emerged bees (**A**) and forager bees (**B**). Data represent the mean ± SE of triplicate assays. Means within bars followed by the same letters are not significantly different ($p > 0.05$, Tukey's HSD)

treated groups as compared with the control, and there were no significant differences between single and combination treatments ($F = 9.559$; d f = 3, 8; $p = 0.005$). With the extension of exposure time, at day 5, acetamiprid alone and propiconazole alone did not elicite a significant effect on GST activities, while combination of acetamiprid and propiconazole significantly reduced the GST activity ($F = 18.138$; d f = 3, 8; $p = 0.001$). Overall, ANOVA performed on all data showed that the GST activity was significantly modulated by pesticide treatment and exposure time (Table 2, $p < 0.05$).

AChE activity was measured in heads. The interaction between the pesticide treatment and exposure time wasn't statistically significant (Table 2, $p > 0.05$). There were no significant differences for AChE activity between exposed and non exposed honey bees (Fig. 6). That is, the results showed that acetamiprid and propiconazole alone or combination at a sublethal dose didn't affected the activity of AChE in both newly emerged (exposure 1 d: $F = 0.728$; d f = 3, 8; $p = 0.564$; exposure 10 d: $F = 0.476$; d f = 3, 8; $p = 0.708$) and forager bees (exposure 1 d: $F = 1.061$; d f = 3, 8; $p = 0.418$; exposure 5 d: $F = 0.226$; d f = 3, 8; $p = 0.875$) at either sampling date.

CAT overall activity was significantly reduced by pesticide treatments within newly emerged or forager bees (Table 2, $p < 0.0001$). From Fig. 7 it can be observed that CAT activity was significantly suppressed by the mixture of acetamiprid and propiconazole in both newly emerged (exposure 1 d: $F = 8.576$; d f = 3, 8; $p = 0.007$; exposure 10 d: $F = 21.955$; d f = 3, 8; $p < 0.0001$) and forager bees (exposure 1 d: $F = 10.205$; d f = 3, 8; $p = 0.004$; exposure 10 d: $F = 5.437$; d f = 3, 8; $p = 0.025$) at either sampling date. In addition, the CAT activity derived from acetamiprid alone and propiconazole alone treated groups decreased remarkably as the exposure time extended in newly emerged bees.

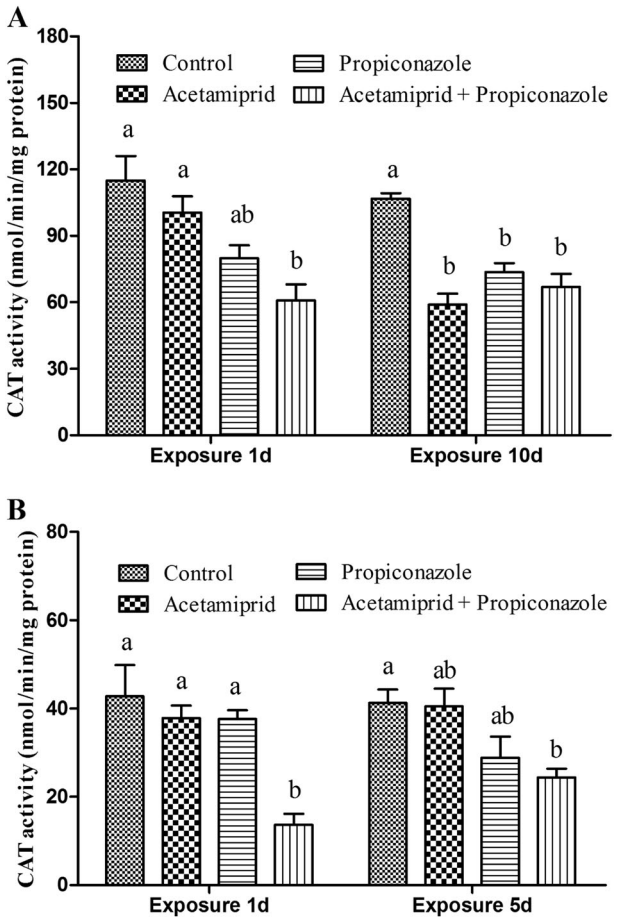

Fig. 7 Effects of acetamiprid and propiconazole alone or combination on the CAT activities in the midguts of newly emerged bees (**A**) and forager bees (**B**). Data represent the mean ± SE of triplicate assays. Means within bars followed by the same letters are not significantly different ($p > 0.05$, Tukey's HSD)

Discussion

In the current agricultural environment, honey bees are inevitably exposed to a complex cocktail of neonicotonoid insecticides and fungicides (David et al. 2016). Here, we evaluated the potential synergistic mortality and physiological changes interaction between a neonicotinoid insecticide acetamiprid and an EBI fungicide propiconazole on *A. c. cerana*. The reasons that our study focusing on acetamiprid and propiconazole were as follows. First, our previous study have shown that sublethal doses of propiconazole (1.2 μg/honey bee) increased the acute toxicity of acetamiprid on *A. c. cerana* (Han et al. 2017). Second, acetamiprid exhibited a much lower toxicity to honey bees (*Apis mellifera*) than the nitro-substituted compounds. However, since *A. c. cerana* is more sensitive to acetamiprid than *A. mellifera* (Yue et al. 2018), acetamiprid and propiconazole are frequently detected in agricultural products and live honey bees owing to its widespread and extensive use (Kiljanek et al. 2017; Robinson et al. 2017; Tong et al. 2016; van der Zee et al. 2015), and it has been recommended for IPM strategies (Jeschke et al. 2011), but sublethal dose of acetamiprid impair activity, memory and sucrose sensitively (El Hassani et al. 2008) and affected gene *vitellogenin* expression of *A. mellifera* (Christen et al. 2017). In addition, propiconazole exposure resulted in oxidative stress in non-target organisms (Tabassum et al. 2016).

This study showed that chronic exposure to acetamiprid and propiconazole alone or combined at sublethal doses severely affected *A. c. cerana's* survival, with 23.3% to 69.7% mortality in a few days. What's need to be defined is whether the doses administered in our experiment are realistic for the honey bees to be exposed to in the field. Acetamiprid has strong contact, stomach and systemic toxicity. Its maximum field recommended dose is 15 g a.i./ha (www.chinapesticide.gov.cn), which is commonly utilized for managing important sucking pests of commercial crops. For example, thrips is an important pest of many plants, and the dose of acetamiprid usually be sprayed to control them by the farmers far exceed the recommended dose. This may lead the honey bees to be exposed to a high dose of acetamiprid. The mean doses of acetamiprid administered in our experiment were 19.88 (alone), 10.98 (combination) ng/honey bee/day for newly emerged bees, and 0.139 (alone), 0.136 (combination) μg/honey bee/day for forager bees respectively. The doses used in our experiment were 160–800 times lower than the published LD_{50} values of acetamiprid [3.208 μg/honey bee for *A. c. cerana* (Han et al. 2017); 15–17 μg/honey bee for *A. mellifera* (Decourtye and Devillers 2010; Lundin et al. 2015)] and therefore can be considered to be field realistic, and yet they produced significant mortality and weight loss amongst honey bees. Propiconazole is a broad-spectrum fungicides, having a long persistence on various crops. The mean doses of propiconazole administered in our experiment were 57.90 (alone), 71.98 (combination) ng/honey bee/day for newly emerged bees, and 1.141 (alone), 0.893 (combination) μg/honey bee/day for forager bees, respectively. These doses corresponds to about 0.01 and 0.2 times the propiconazole oral LD_{50} obtained in a previous study for *A. c. cerana* respectively (Wang et al. 2017), and fall within the range (from 0.0244 to 22.4 μg/honey bee) tested by Thompson et al. (2014) yielding no oral toxic effects in *A. mellifera*. Base on this experimental result, *A. c. cerana* is more sensitive to acetamiprid and propiconazole than *A. mellifera*. The present data also confirmed our hypothesis that chronic oral exposure to the field realistic doses of acetamiprid and propiconazole combination can produce significant synergistic effects on *A. c. cerana*.

Besides the survival data from synergistic toxicity test, we also found that the body weights were substantially

reduced in newly emerged bees fed with pesticides-containing sugar solutions. Possibly, this is a consequence of the increased energetic investment on detoxification and antioxidant.

Furthermore, the biochemical mechanism behind the synergistic was studied. We examined multiple enzyme activities including two detoxification enzymes (P450s and GST), one insecticide target enzyme (AChE), and one antioxidant enzyme (CAT) in surviving honey bees after exposure to acetamiprid and propiconazole, alone or combined. Our data clearly indicated that exposure to sublethal doses of acetamiprid and propiconazole mixture affected *A. c. cerana* physiology.

P450s are a large family of multifunctional enzymes and are found in virtually all living organism, playing a predominant role in phase I of biotransformation mechanisms being involved in the oxidation and detoxification of xenobiotics such as drugs, phytochemicals and synthetic pesticides (Schuler 2011). The activity of P450s have been used as biomarkers of xenobiotic exposure, and P450s activation or inhibition seems to play a key role in the mode of action of a range of synergizing chemicals (Gottardi et al. 2015). In this study, we found ECOD activity changes in new emerged bees larger than in forager bees. The ECOD activity increased significantly after newly emerged bees exposed 1 day to mixture of acetamiprid and propiconazole, whereas ECOD activity has been conspicuously suppressed following 10 days exposure to the mixture. This suggests that for newly emerged bees such effects on ECOD are dose and time dependent. When newly emerged bees short-term exposed to acetamiprid and propiconazole, ECOD activity increased, therefore, a rapid physiological protective response occurred. However, when honey bees were longer exposed to acetamiprid and propiconazole, ECOD activity decreased, the reason for this may be cumulative doses inhibited genes expression of P450. It is well known that EBI fungicides inhibit P450-mediated detoxification (Berenbaum and Johnson 2015). However, our results showed that ECOD activity variation in propiconazole treatments of forager bees (5 d) did not achieve statistical significance.

GST represents an important family of enzymes that catalyze the secondary metabolism of a vast array of compounds oxidized by the cytochrome P450 family, and it appears to contribute to cellular protection against oxidative damage. Therefore, an induction of GST activity has been used as an environmental biomarker (Hyne and Maher 2003). In this study, we found larger GST activity changes in forager bees than in newly emerged bees. GST activities were obviously enhanced after forager bees exposed 1 day to acetamiprid and propiconazole, alone or combination. Five days after the exposure to mixture, a substantial decrease in GST activity was observed. These result indicated that acetamiprid and propiconazole can induce oxidative stress within a short term, but long term exposure the accumulation of ROS could make GST inhibited. A study by Badway et al. (2015) showed that low dose of acetamiprid can significantly increase *A. mellifera* forager GST activity after short term (1d) exposure, but the effects of long term exposure are not available in literature. In addition, acetamiprid significantly inhibited GST activity after 10 days exposure in newly emerged bees.

Therefore, from the ECOD and GST activities variation both in newly emerged and forager bees, we can infer that the response of ECOD and GST, such as induction, inhibition and inactivation, may depending on the physiological differences of young honey bees and foragers, exposure doses and time.

AChE is an enzyme that hydrolyzes the neurotransmitter acetylcholine in cholinergic synapses for the rapid and precise control of nerve transmission. AChE inhibition has been widely used as a biomarker of general exposure to cholinesterase inhibitor pollutants (Badiou-Bénéteau et al. 2012). In this study, acetamiprid and propiconazole at sublethal doses did not inhibit activity of AChE in newly emerged and forager bees either individually or in mixtures. Similarly, no AChE response has been reported in *A. mellifera* following treatment with insecticides such as thiamethoxam (Badiou-Bénéteau et al. 2012), imidacloprid (Alburaki and Steckel 2017; Zhu et al. 2017a; Zhu et al. 2017b). In addition, the combination of acetamiprid and propiconazole was not produce a specific response on AChE.

CAT is a antioxidant enzymes, which is chiefly located in the peroxisomes. It facilitates the removal the hydrogen peroxide and metabolized to molecular oxygen and water (Li et al. 2010a). CAT is consider as the first line of defense against oxygen toxicity (Li et al. 2010b). This enzyme is very active in the honey bee and can be modulated by environmental pollutants (Carvalho et al. 2013). In this study, CAT activity significantly inhibited at any time exposed to mixture of acetamiprid and propiconazole whether in newly emerged or forager bees. Besides, acetamiprid and propiconazole individual processing, CAT activity also significantly inhibited in newly emerged bees at the exposure 10 days. It is reported that oxidative stress will occur if the activities of antioxidant defense systems decrease or ROS production increased (Li et al. 2010b). Hence, our results strong suggest that produced oxidative stress by inhibiting antioxidant enzymes as well as by inducing generation of ROS. Similar result was obtained by Chakroun et al. (2016), who reported that exposure to acetamiprid resulted in a significant decrease the levels of CAT activity in rat liver. Furthermore, Li et al. (2010a) showed that a low concentration of propiconazole can induce CAT in intestine of rainbow trout, but the

accumulation of ROS could make it inhibited strongly. Based on our results, it is clear that *A. c. cerana* co-exposure to acetamiprid and propiconazole, increased the oxidative stress, led to oxidative damage and made the antoxidant enzymes poisoned.

In summary, this study confirmed that chronic combined exposure to relevant sublethal dose of acetamiprid and propiconazole caused synergistic action in *A. c. cerana*, both on mortality and physiology. Our findings showed that acetamiprid and propiconazole are responsible for alterations in detoxification and antioxidant enzymes, which are responsible for regulating physiological processes such as metabolism of xenobiotics and oxidative stress. Therefore, repeated exposure to acetamiprid and propiconazole, the health status of newly emerged and forager bees were affected seriously. Moreover, precise molecular mechanism for these biochemical responses in the *A. c. cerana* are still unclear and also need further investigation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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