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Exposure to acetamiprid influences the development and survival ability of worker bees (*Apis mellifera* L.) from larvae to adults

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ABSTRACT

In most cases, honey bees experience pesticide pollution in a long-term period through direct or indirect exposure, such as the development process from larvae to the pre-harvest stage. At present, little is known about how honey bees respond to pesticide stresses during the continuous development period. This study aims to examine effects of long-term acetamiprid exposure on the development and survival of honey bees, and further present the expression profile in larvae, 1-day-old, and 7-day-old adult worker bees that related to immune, detoxification, acetylcholinesterase (AChE) and memory. Honey bees from 2-day-old larvae to 14-day-old adults except the pupal stage were continuously fed with different acetamiprid solutions (0, 5, and 25 mg/L). We found that acetamiprid over 5 mg/L disturbed the development involving birth weight and emergence rate of newly emerged bees, and reduced the proportion of capped cells of larvae at 25 mg/L; gene expression related to immune and detoxification of worker bees exposed to acetamiprid was roughly activated, returned and then inhibited from larval to emerged and to the late adult stage, respectively. Moreover, lifespans of bees treated with acetamiprid at 25 mg/L were significantly reduced. The present study reflects the potential risk for honey bees continuously exposed to acetamiprid in the development stage.

1. Introduction

Neonicotinoid insecticides are a class of neurotoxic insecticides, which are applied most widely in agricultural production compared with other agrochemicals (Decourtye and Devillers, 2010; Abrol, 2013), due to the lower toxicity to vertebrates and higher specificity to insects (Tomizawa and Casida, 2011). Unluckily, the beneficial pollinators as invertebrates are similarly poisoned like target pests when we are applying the neonicotinoid insecticides on planting crops. Honey bees are critical commercial pollinators that play a significant role in maintaining ecological balance (Potts et al., 2010). In recent years, neonicotinoid insecticides have been raising global concern due to growing evidence that they have generated a series of adverse effects on honey bees (Sánchez-Bayo et al., 2016), including shortening the lifespan (Iwasa et al., 2004; Shi et al., 2019a), impairing immunocompetence (Brandt et al., 2016) and foraging behavior (Colin et al., 2019; Shi et al., 2020) of worker bees, influencing colony development and queen reproduction (Wu-Smart and Spivak, 2016).

Previous studies demonstrated the lower acute toxicities of the cyano-substituted neonicotinoids for acetamiprid and thiacloprid compared with the nitro-substituted neonicotinoids for imidacloprid, clothianidin, thiamethoxam, dinotefuran, and nitenpyram (Iwasa et al., 2004). However, risk assessment of pesticides on bees should be paid more attention on its sublethal effects instead of the acute toxicity, because bees are usually indirectly exposed to pesticides at sublethal doses. Till now, researchers found that neonicotinoid insecticides have posed adversely sublethal effects on foraging and homing ability, olfactory memory, immune and detoxification function, and development of bees (Hassani et al., 2008; Henry et al., 2012; Brandt et al., 2016; Colin et al., 2019; Qi et al., 2019; Yang et al., 2019). Acetamiprid is used worldwide in the tea, vegetables, fruit trees, flowers, and other plants for pest control (Zhou et al., 2006). Further studies frequently reported sublethal effects of acetamiprid on honey bees, which not only affects the olfactory memory and cognitive ability (Hassani et al.,

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2008; Aliouane et al., 2010), but also adversely influences the queen rearing (Shi et al., 2019b). Furthermore, a recent study has found that acetamiprid, in combination with nearly half of other pesticides, exhibited synergistic effects on honey bees (Wang et al., 2020). In most cases, influences of external factors on the phenotype of organisms can be reflected with genetic change, and previous studies have used changes in gene expression levels to assess exposure risk of bees to pesticides (Shi et al., 2018; Qi et al., 2019). For acetamiprid, researchers have found that it caused obvious memory decline associated with lower expression of memory-related genes in honey bees (Shi et al., 2019a), while its impacts on immune and detoxification related genes in bees are rarely investigated. In this study, dynamic processes of effects of continuously acetamiprid exposure on gene expression in honey bees at different developmental stages have been systematically investigated for the first time.

Metamorphosis development of bee species consists of four distinct life cycle phases, including eggs, larvae, pupae, and adults (Qi et al., 2019). Bees may be exposed to pesticides environment throughout their life cycle either by direct or indirect exposure. From this point of view, it is essential to investigate the effects of honey bees exposed to pesticides at different life cycle phases, especially for larvae and young bees, which are the foundation for colony development and renewal. Previous studies confirmed that honey bee larvae treated with pesticides could obviously delay the growth (Grillone et al., 2017; Wang et al., 2017), induce significant mortality (Tan et al., 2016; Tavares et al., 2017; Qi et al., 2019), and change gene expression related with immunity and detoxification (Wang et al., 2017; Qi et al., 2019; Tesovnik et al., 2019). In addition, honey bees from emerged to pre-harvest stage are also susceptive to pesticides (Liao et al., 2018; Shi et al., 2019a; Wang et al., 2020). Therefore, it has been increasingly clear that bees in larvae and early adult stages are susceptible to pesticides, respectively. Remarkably, bees' daily food limited in the inside of the hive from the larva to the pre-harvest stage; during this period, they are in the same diet environment. For this reason, more attention should be paid on the effect of external and/or internal factors on the continuous development process of a bee from larva to adult. For example, recent research systematically explored the honey bees, which were persistently exposed to pesticide first in larval and later in the adult stage (Tesovnik et al., 2019).

The field-realistic concentrations of acetamiprid provided by the manufacturer is 50–500 mg/L. A recent research has detected the LC_{50} value of honey bee larvae exposed to acetamiprid within 72 h, which was 188.49 mg/L (Yang et al., 2019), and the maximum acetamiprid residues in fruits or vegetables were limited within 5 mg/kg (Zhang et al., 2014). In addition, the maximum residue of acetamiprid in pollen, nectar, and beeswax was 134, 13.3, and 61 μg/kg, respectively (Sanchez-Bayo; Goka, 2014; Jabot et al., 2015). In the current study, two sublethal acetamiprid concentrations (5 and 25 mg/L) plus a control (0 mg/L) were set to investigate effects of acetamiprid on the development and survival ability of worker bees from larvae to adult. In fact, pesticide residues in honey/pollen/beeswax are extremely low exposure scenario (Yang et al., 2019), which are diluted by raw materials in the beehive. Additionally, pesticide residues detected in the beehive could change with season and management, and will accumulate over the years (Daniele et al., 2017). Further, foraging bees don't persistently collect food containing pesticide residues because of their widely scope of foraging. Moreover, when farmers are spraying pesticides on crops, the pesticides will diffuse in the air and may transport into beehives via wind. Thus, bees are usually under the alternate environments of low and high pesticide exposure scenario. Here, the treatment concentrations of acetamiprid between the residual concentrations in beehive and environmental application concentrations were set. The development of worker bees from larvae to adult stage involving the larvae weight, capped worker cells, birth weight, and emergence rate were completely documented. We further evaluated the effect of acetamiprid on the immune genes (*Hymenoptaecin* and *Abaecin*) and detoxification genes (*GSTS3* and *CYP450*) of larvae, 1-day-old and 7-day-old worker bees, and the expression of acetylcholinesterase (AChE) genes (*Ace1*and *Ace2*) and memory genes (*Nmdar1* and *Tyr1*) were examined in larvae and adult worker bees, respectively. Meanwhile, the survival ability of adult worker bees exposed to acetamiprid from 1 to 14 days were further analyzed. To date, the effect of acetamiprid on honey bee larvae is rarely known, especially for the whole development process of honey bees from larvae to adult workers. This study adds new evidence that acetamiprid treatment inhibits development and survival ability due to gradually reduced regulation of immunity and detoxification during the long-term exposure to acetamiprid from larval to adult stage in honey bee workers.

2. Materials and methods

2.1. Chemicals and reagents

The acetamiprid, 70% water dispersible granule, was provided by Jiangxi Heyi Chemical Co., Ltd, which containing high water solubility and permeability. The composition of the formulation of acetamiprid includes 70% acetamiprid (principal component), 12% bentonite (packing materials), 10% ammonium chloride (disintegrating agent), 4% ZX-D9 (dispersing agents), 2% naphthalenesulfonic acid formaldehyde condensate (dispersing agents), and 2% M (wetting agent) (Zhang, 2008). Acetamiprid was dissolved in 50% (w/v) sucrose water. The prepared acetamiprid solutions were stored in the −4 °C refrigerator and used within one week. The total RNA was extracted using an RNA extraction kit (TransZol Up Plus RNA Kit), which was from the TransGen Biotech Co., Ltd. Reverse transcription kit and RT-qPCR kit (SYBR® Premix Ex Taq™ II) were both purchased from TaKaRa company.

2.2. Honey bee rearing

Experimental bee colonies (*Apis mellifera* L.) were provided from the apiary at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.77° N, 115.83° E), which were reared according to standard beekeeping practices (Shi et al., 2019a). Selected bee colonies were healthy that were not threatened by pathogens (foulbrood), parasitic mites, and had no prior exposure to pesticides. An empty frame was artificially divided into three parallel regions which were inserted into the comb foundations, then put into the beehive for comb building. After that, the honey bee queen was restricted in the empty comb to lay eggs for 12 h. About 96 h later, the eggs were incubated into 2-day-old larvae (marked as D2), and the experimental larvae comb had been taken out from the colony, then each larva in the cell was fed with 1.5 μL acetamiprid solution using a pipette and put back into the source colony. Each bee larva from D3 to D5 were fed 2, 2.5, and 3 μL acetamiprid solution, respectively. The feed amount was similar to that reported by Dai et al. (2018).

On D16, treated comb with capped brood was removed from the colony and transferred into an incubator at 34 °C, 70 ± 5% relative humidity for emergence. Newly emerged worker bees in each group continuously fed adequate acetamiprid solution for 14 days. This experiment was repeated three times by using three different colonies.

2.3. Experimental design

Experiments were conducted in April and finished in July 2019. Two concentrations of acetamiprid (5 and 25 mg/L) were designed to expose larvae and adult worker bees, and 50% sucrose water served as a control (0 mg/L). On D7, the larvae began to seal and entered into the pupal stage, and the capped worker cells in each treatment group were counted. Meanwhile, we gently removed the larvae with fine tweezers, and the weight of approximately 16 larvae from each treatment was individually weighed. Then, the remaining capped larvae on the comb were put into the source colony to continue to develop. On D16, removing the treated comb and three parallel regions on the comb were individually covered with fine wire enclosures. The comb was maintained at 34 °C, 70 ± 5% relative humidity, and most adult worker bees emerged on D19. The birth weight of each bee was measured within 2 h after emergence, and the emergence rate of worker bees in each treatment was calculated when the worker bees no longer continued to emerge.

After emergence, 50 newly emerged bees from each group were put into a wooden box for rearing at 34 °C, 65 ± 5% relative humidity, which was used to count the lifespan of bees. The mortality of bees in each group was recorded every day until the 14th day, due to worker bees start the foraging activity usually on 15–20 days old. The remaining freshly worker bees were reared at the same conditions for later RT-qPCR experiments. All the above experiments were repeated three times using three source colonies.

$$\text{Proportion of capped worker cells} = \frac{\text{Capped worker cells}}{\text{Total treated larvae}} \times 100\%$$

$$\text{Emergence rates} = \frac{\text{Emerged worker bees}}{\text{Capped worker cells}} \times 100\%$$

2.4. Sample collection and gene expression analysis

The larvae, 1-day-old and 7-day-old worker bees were sampled on D7, D19, and D25, respectively, while the larvae samples were collected after weighing. A total of nine larvae or bees in each group from a single colony were sampled at each point time, and three complete larvae or three heads of bees were pooled as a sample (each treatment in a single colony contained three samples). Using three colonies run three replicates. All the samples were temporarily froze with liquid nitrogen and subsequently stored in refrigerator at −80 °C.

Total RNA was extracted from each sample according to the manufacturer's protocol. RNA purity was checked using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and based on the OD260/280 ratio, values between 1.8 and 2.0 met the standards. Then, first-strand cDNA was synthesized for RT-qPCR reactions. In this study, expression of the immune genes (*Hymenoptaecin* and *Abaecin*) and detoxification genes (*GSTS3* and *CYP450*) were quantified in larvae, 1-day-old and 7-day-old worker bees, and the expression of acetylcholinesterase (AChE) genes (*Ace1*and *Ace2*) and memory genes (*Nmdar1* and *Tyr1*) were detected in larvae and adult worker bees, respectively. Gene-specific primers were designed according to the primer information reported in the literature (Ratnieks and Carreck, 2010; Boncristiani et al., 2012; Yuan et al., 2016; Shi et al., 2018; Liao et al., 2018). Paired primers for AChE, immune, detoxification and memory related genes of *A. mellifera* were listed in Table 1, and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control. RT-qPCR were accomplished on an Applied Biosystems ABI 7500 machine. The PCR reaction mixture (10 μL) contained 1 μL cDNA, 5 μL SYBR® Premix ExTaq™ II, 0.2 μL Rox correction fluid, 0.4 μL each of the forward and reverse primers, and 3 μL ddH2O. The reaction thermal procedure included an initial denaturation (95 °C 30 s), quantification for 40 cycles (95 °C 10 s, 60 °C 1 min), and a dissociation from 50 °C to 90 °C (elevated by 1 °C every 6 s). All samples were calculated in triplicate, and the standard deviation of three technical replicates' values of each reaction was validated within 0.5. Then the relative expression of each target gene was calculated with methods reported by Huang et al. (2012).

Table 1
The forward and reverse primers of genes used in real-time quantitative PCR.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
Ace1	CAAGTTCGAGGTGCTGATGG	CGTGATGTCTGCTTCTGTGG
Ace2	CTCGATCTGTTGAGGGAAGC	TGTACACCTCCTCCCAGTCC
Hymenoptaecin	ATATCCCGACTCGTTTCCGA	TCCCAAACTCGAATCCTGCA
Abaecin	TGTCGGCCTTCTCTTCATGG	TGACCTCCAGCTTTACCCAAA
CYP450	CAAAATGGTGTTCTCCTTACCG	ATGGCAACCCATCACTGC
GSTS3	TGCATATGCTGGCATTGATT	TCCTCGCCAAGTATCTTGCT
Nmdar1	GTATTTCCGTCGCCAAGTC	TGTAAACCAATCCCATAGCCA
Tyr1	CGTCGGGCGAGCGAGATA	GCCAAACCAACCAGCAAAT
GAPDH	GCTGGTTTCATCGATGGTTT	ACGATTTCGACCACCGTAAC

2.5. Statistical analysis

Firstly, the boxplot method in descriptive statistics of SPSS17.0 was used to remove the abnormal values (values over mean ± 3 times standard deviation were abnormal) of the larvae weight, birth weight of newly emerged bees and gene expression levels among different groups to meet normality, then performed the One-way ANOVA analysis. While the data of the proportion of capped cells and emergence rate was conducted arcsine substitution to perform ANOVA analysis. Meanwhile, when data from three groups was consistent with homogeneity of variances, Fisher's LSD test was used to perform multiple comparative analysis. Finally, the survival among different groups was analyzed based on the Log Rank (Mantel-Cox) test of Kaplan-Meier method in SPSS17.0 software.

3. Results

3.1. Acetamiprid exposure disturbed the development of A. mellifera workers

There was no significant difference in larvae weight among three groups (0, 5, and 25 mg/L) ($F_{2,149} = 0.897$, df = 2, $P > 0.05$). However, the proportion of capped worker cells in 25 mg/L group was significantly lower than the 0 mg/L group (LSD test: $P < 0.05$), while there was no significant change between 25 mg/L group and 5 mg/L group (LSD test: $P > 0.05$; Table 2). Remarkably, the toxic effect of acetamiprid on the growth of worker bees is gradually emerged with the development process of larvae. Compared to 0 mg/L group, the birth weight and emergence rate in two acetamiprid treatments were significantly decreased (Birth weight: $F_{2,139} = 2.958$, df = 2, $P = 0.055$, $P_{(0,\ 5)} = 0.019$, $P_{(0,\ 25)} = 0.049$; Emergence rate: $F_{2,6} = 12.214$, df = 2, $P < 0.01$, $P_{(0,\ 5)} = P_{(0,\ 25)} = 0.005$; Table 3). Differences between acetamiprid treatments were not significant (LSD test: $P > 0.05$).

Data in the table are mean ± SE (standard error). a and b are different letters used to represent significant differences ($P < 0.05$,

Table 2
Effects of acetamiprid on the weight and capped rate of *A. mellifera* larvae.

Concentration of acetamiprid (mg/L)	Larvae weight (mg)	Proportion of capped worker cells (%)
0	167.12 ± 1.57[a]	98.58 ± 0.52[a]
5	166.98 ± 1.51[a]	97.99 ± 0.35[ab]
25	169.69 ± 1.69[a]	97.00 ± 0.11[b]

Table 3
Effects of acetamiprid on the birth weight and emergence rate of *A. mellifera* workers.

Concentration of acetamiprid (mg/L)	Birth weight (mg)	Emergence rate (%)
0	122.36 ± 1.93[a]	98.92 ± 0.15[a]
5	116.22 ± 1.80[b]	97.22 ± 0.08[b]
25	117.22 ± 1.97[b]	97.20 ± 0.57[b]

Fisher's LSD test) following the data in the same column. The same for Table 3.

3.2. Acetamiprid induced gene expression at larval stage

The relative expression of immune genes (*Hymenoptaecin* and *Abaecin*), detoxification genes (*GSTS3* and *CYP450*) and AChE genes (*Ace1*and *Ace2*) in bee larvae were quantified on D7 (Fig. 1). Relative expression of *CYP450* and *Ace2* were both gradually up regulated with the increasing acetamiprid concentrations, in comparison with 0 mg/L group, the 25 mg/L group exhibited significant up-regulation (*CYP450*: $P_{(0,\ 25)} = 0.028$; *Ace2*: $P_{(0,\ 25)} = 0.007$). However, relative expression of *Hymenoptaecin* in 5 mg/L group was significantly up regulated than the 0 mg/L group and 25 mg/L group ($F_{2,22} = 4.684$, df = 2, $P < 0.05$). Therefore, larvae exposed to acetamiprid in the early life stage activated the expression of some related genes to resist toxins.

3.3. Gene expression of freshly worker bees returned to the control level

The relative expression of memory genes (*Nmdar1* and *Tyr1*), immune genes (*Hymenoptaecin* and *Abaecin*) and detoxification genes (*GSTS3* and *CYP450*) in 1-day-old worker bees were quantified on D19 (Fig. 2). No significant change of gene expression was found among three treatment groups (*Nmdar1*: $F_{2,24} = 0.364$, df = 2, $P > 0.05$; *Tyr1*: $F_{2,24} = 0.015$, df = 2, $P > 0.05$; *Hymenoptaecin*: $F_{2,24} = 0.326$, df = 2, $P > 0.05$; *Abaecin*: $F_{2,24} = 0.198$, df = 2, $P > 0.05$; *GSTS3*: $F_{2,24} = 0.973$, df = 2, $P > 0.05$; *CYP450*: $F_{2,24} = 1.009$, df = 2, $P > 0.05$). Specifically, after the pupal stage, expression of immune- and detoxification-related genes in 1-day-old worker bees returned to the control level.

3.4. Gene expression of 7-day-old worker bees were down regulated

Hosts in most cases choose to up regulate related genes, such as immune and detoxification genes, for improving the resistance to toxins (Mao et al., 2011; Wang et al., 2017), as the larvae did after exposure to acetamiprid (Fig. 1). Nevertheless, how honey bees regulated after a long-term exposure to pesticides has not yet been elucidated. In this study, the relative expression of memory genes (*Nmdar1* and *Tyr1*), immune genes (*Hymenoptaecin* and *Abaecin*) and detoxification genes (*GSTS3* and *CYP450*) in 7-day-old worker bees had been further detected on D25 (Fig. 3). The relative expression levels of memory-related genes among three groups in 7-day-old worker bees were not significantly different (*Nmdar1*: $F_{2,23} = 0.449$, df = 2, $P > 0.05$; *Tyr1*: $F_{2,23} = 0.395$, df = 2, $P > 0.05$), which were similar to 1-day-old worker bees (Figs. 2 and 3). However, the expression of immune gene (*Abaecin)* and detoxification genes (*GSTS3* and *CYP450*) were all significantly down regulated in 25 mg/L group compared with 0 mg/L group (LSD test: *Abaecin*: $P = 0.041$; *GSTS3*: $P = 0.040$; *CYP450*: $P = 0.038$), and expression of *Abaecin* in 5 mg/L group was significantly down regulated than 0 mg/L group (LSD test: $P < 0.05$). In addition, expression of *Hymenoptaecin* was not significantly affected ($F_{2,19} = 0.930$, df = 2, $P > 0.05$). Results indicated that long-term exposure to acetamiprid gradually loses the immunity and detoxification functions of bees, and bees have a tolerance to metabolize the pesticides.

3.5. Continuous exposure to acetamiprid reduced the lifespan of adult worker bees

The survival rate of worker bees from day 1 to day 14 were calculated (Table 4, Fig. 4). During this period, the lifespans of 25 mg/L group were 1.344 and 1.463 days earlier, on average, than those of 0 mg/L group and 5 mg/L group respectively ($X^2 = 58.199$, df = 2, $P < 0.001$), while there was no significant difference between 0 mg/L group and 5 mg/L group ($X^2 = 1.612$, df = 1, $P > 0.05$). Furthermore, the survival rate of worker bees in the 25 mg/L group began to decrease significantly at the 7th day, which was significantly lower than the other groups.

4. Discussion

This study investigated effects of acetamiprid on the development and survival ability of worker bees (*A. mellifera*) from larval to pre-harvest stage in actual rearing conditions. Additionally, it would also provide new insights into the regulating characteristics of genes related to immune and detoxification in host when exposed to pesticides.

Results showed that exposure to acetamiprid at 25 mg/L concentration significantly reduced the larvae capping rate than 0 mg/L group (Table 2), indicating that acetamiprid at 25 mg/L would have an adverse effect on the survival ability of the larvae, then unsuccessfully metamorphose to pupae. However, acetamiprid treatment did not significantly affect the larvae weight, which was identical to the results reported by Yang et al. (2019). It seems that acetamiprid in this dose range will not affect the food intake of larvae. The birth weight and emergence rate were documented after worker bee emerged, and honey bee larvae treated with acetamiprid induced significantly lower birth weight and emergence rate in comparison with the 0 mg/L group (Table 3), indicating that acetamiprid may interfere with the growth and metabolism of pupal larvae and subsequently affect their survival rate. These findings indicate that acetamiprid can suppress the development of honey bee larvae due to the long-term sublethal effect.

How acetamiprid affect the physiology of honey bees was further explored at the gene expression level. Neonicotinoids insecticides display selective actions on insect nicotinic acetylcholine receptors (nAChRs), including acetamiprid (Matsuda et al., 2001). AChE is a key enzyme in the process of nerve conduction in insects

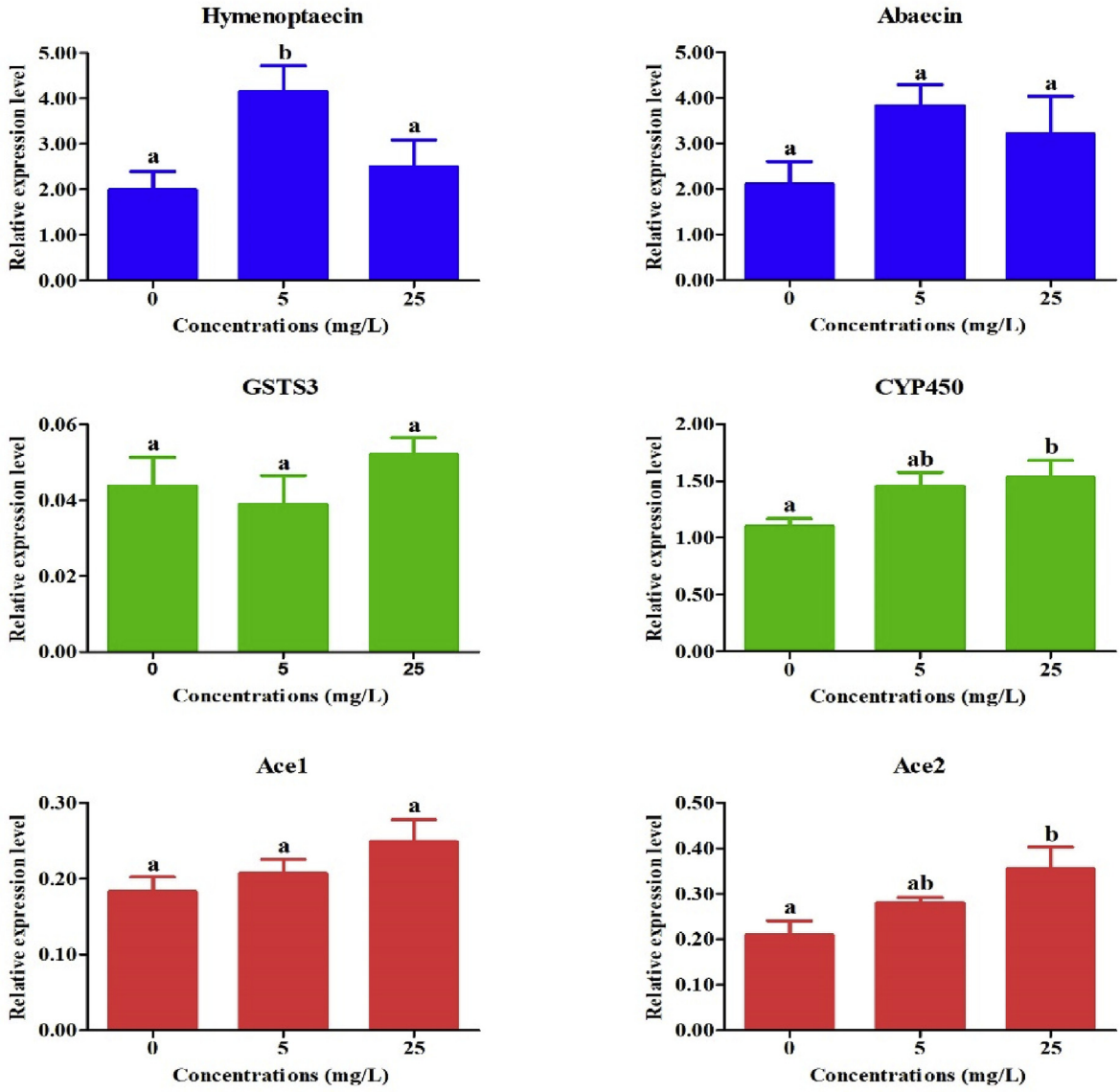

Fig. 1. Effects of acetamiprid on the relative expression levels of immune genes (*Hymenoptaecin* and *Abaecin*), detoxification genes (*GSTS3* and *CYP450*) and AChE genes (*Ace1* and *Ace2*) in *A. mellifera* larvae. Data in the figure are mean ± SE (standard error). Different letters above bars mean significant difference ($P < 0.05$, Fisher's LSD test). Three pooled samples per colony in each group (a total of nine samples) were used for gene expression analysis. The histogram of immune genes, detoxification genes and AChE genes was highlighted with blue, green and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

not only involves in hormone synthesis but also relates to insect resistance and regulates insect physiological behaviors through the nervous system (Soreq and Seidman, 2001; Jin et al., 2015). Genes of *Ace1* and *Ace2* are two important genes encoding AChE in bees (Ho et al., 2012). A previous study has reported that neonicotinoid can seriously reduce the activity of AChE (Jin et al., 2015). In the present study, larvae treated with acetamiprid at 25 mg/L induced up-regulation of *Ace2* (Fig. 1), which may be explained to the compensation mechanism for remedying the lack of AChE activity in larva body. Furthermore, combining with the results of larvae capping rate, whether the expression of AChE-related genes linked with worker bees capping behavior through regulating the synthesis of larval hormones deserves further attentions. Immune- and detoxification-related genes are involved in regulating the immune and detoxification functions of the whole development process of the host, which is very important for the development and survival of the host. Then, immune- (*Hymenoptaecin* and *Abaecin*) and detoxification-related genes (*GSTS3* and *CYP450*) throughout the larval, 1-day-old and 7-day-old adult stage were quantified, respectively. *Hymenoptaecin* and *Abaecin* are important immune genes in bees, which are involved in the regulation of host immune function (Casteels et al., 1990, 1993). The glutathione S-transferase (*GST*) and cytochrome P450 (*CYP450*) are the two major groups of detoxifying enzymes (Boncristiani et al., 2012). At the larval stage, expression of *CYP450* in 25 mg/L group exhibited up-regulation than 0 mg/L group, while *Hymenoptaecin* in 5 mg/L group expressed highest levels than other two groups, and no significant change was found among three groups in *GSTS3* and *Abaecin* genes (Fig. 1), suggesting that larvae exposed to acetamiprid in the early life stage can activate the expression of specific genes rather than all genes to resist toxin, and different immune genes have different response mechanisms to exposure concentrations. Along with development, expression of immune and detoxification genes firstly returned to the control level in freshly worker bees, then

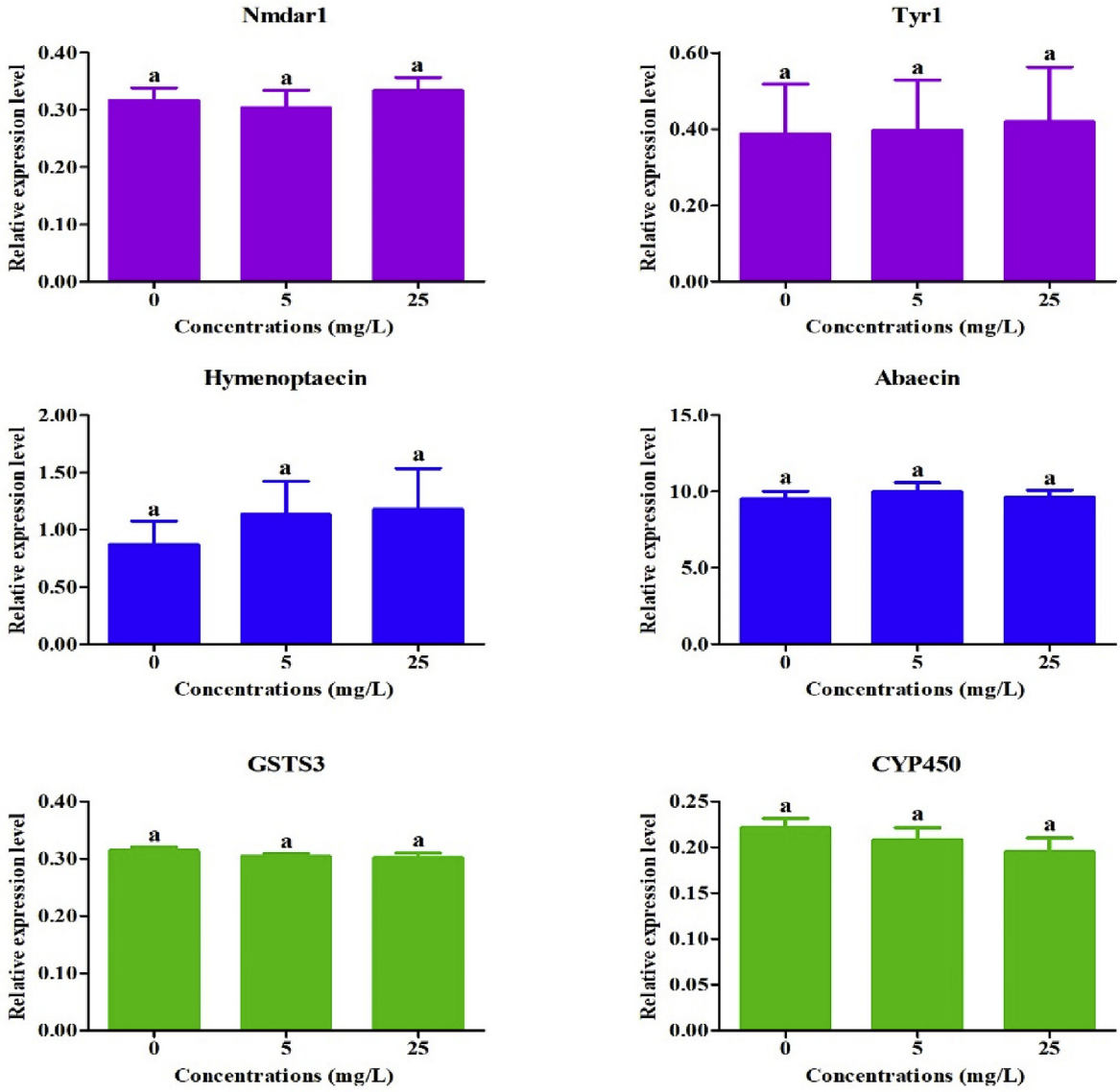

Fig. 2. Effects of acetamiprid on the relative expression levels of memory genes (*Nmdar1* and *Tyr1*), immune genes (*Hymenoptaecin* and *Abaecin*) and detoxification genes (*GSTS3* and *CYP450*) in 1-day-old worker bees. Data in the figure are mean ± SE (standard error). Same letters above bars mean no significant difference ($P > 0.05$, Fisher's LSD test). Three pooled samples per colony in each group (a total of nine samples) were used for gene expression analysis. The histogram of memory genes, immune genes and detoxification genes was highlighted with purple, blue and green, respectively, the same as below. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

gradually loses the immunity and detoxification functions in the late adult stage (Figs. 2 and 3). These may reflect that honey bee's response to pesticide stress exists a limitation and subsequently caused weakness along with long-term exposure. However, the immune- and detoxification-related genes in honey bees that response to other pesticides is unclear, suggesting us to explore whether exists a universal principle by investigating the effects for various pesticides applied on honey bees with different exposure time and doses. Moreover, we'd like to explore effects of acetamiprid exposure on early memory-related properties in bees. Memory-related genes (*Nmdar1* and *Tyr1*) which are confirmed containing regulatory effects on memory ability of *A. mellifera* bees (Blenau et al., 2000; Kucharski et al., 2007; Zachepilo et al., 2008), were examined from new emergence to the late adult stage. In this study, the expression of memory-related genes among the three groups in 1-day-old and 7-day-old worker bees were not significantly different (Figs. 2 and 3). This may be explained that the brain of a bee has not fully developed until 7 days old, and conducts little cognitive activity, thus causing inactive in the expression of genes involving in learning and memory.

The survival of worker bees in 25 mg/L group was significantly decreased than 0 mg/L group within 14 days (Table 4, Fig. 4), indicating that the residue of acetamiprid over 25 mg/L in food will generate negatively influences on the viability of bees. In addition, the difference in the survival rate of 25 mg/L group was more pronounced after the seventh day compared with other groups, and it was in harmony with the down-regulation of immune- and detoxification-related genes due to continuous exposure to acetamiprid. There was no significant difference between 5 mg/L group and 0 mg/L group within 14 days. It is possible that acetamiprid at a relatively low concentration (5 mg/L) may also have an impact on the viability of worker bees along with more exposure time (more

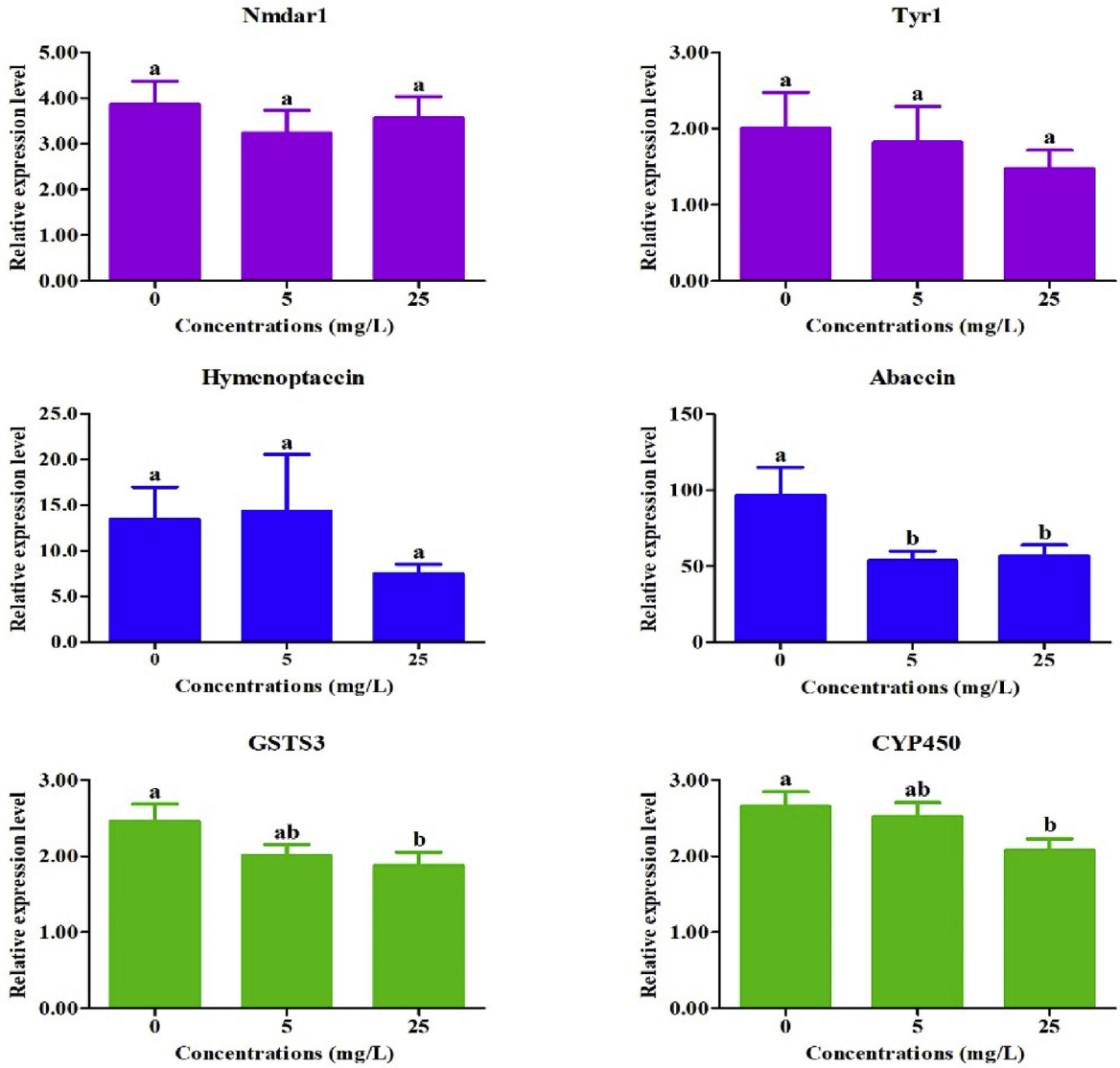

Fig. 3. Effects of acetamiprid on the relative expression levels of memory genes (*Nmdar1* and *Tyr1*), immune genes (*Hymenoptaecin* and *Abaecin*) and detoxification genes (*GSTS3* and *CYP450*) in 7-day-old worker bees. Data in the figure are mean ± SE (standard error). Different letters above bars mean significant difference ($P < 0.05$, Fisher's LSD test). Three pooled samples per colony in each group (a total of nine samples) were used for gene expression analysis.

Table 4
Effects of acetamiprid on the average lifespans of *A. mellifera* workers.

Concentration of acetamiprid (mg/L)	Average lifespan (days)	Sample size
0	13.601 ± 0.139^{a}	143
5	13.720 ± 0.128^{a}	150
25	12.257 ± 0.238^{b}	148

Data in the table are mean ± SE (standard error). Different letters following the data in the same column mean significant differences ($P < 0.05$, Log Rank test).

than 14 days). However, recent research has reported the potential risk of adjuvants to bees, such as nonylphenol polyethoxylates, organosilicone surfactants and the solvent N-methyl-2-pyrrolidone (NMP) (Mullin et al., 2015). Therefore, the compounds containing in acetamiprid or other pesticides also need to be further evaluated for determining environment-friendly adjuvants relative to pollinators. Furthermore, due to the fact that only a small part of drugs entering the animal body can be absorbed, while most drugs will be discharged out of the body with the animal excretion activities; hence, it is needed to measure the residues of acetamiprid and its metabolites in honey bees after exposure in further study that we can know the utilization rate of honey bees to acetamiprid. Moreover, metabolites of acetamiprid such as N-methyl-6-chloropyridine methylamine, 6-pyridine niacin and 6-chloropyridine formyl are produced in soil and other environmental media, which display toxicity to environment (Yu et al., 2007). Hence, it is of importance to pay more attention on the effects of metabolites of acetamiprid on honey bees. Effective acetamiprid concentrations (5 and 25 mg/L) in the present study are between the residual concentrations in beehive and environmental application concentrations. It is robustly recommended that bees should be moved away when crops are sprayed with acetamiprid. In all, findings in the current study enrich the knowledge of host response to external stress and highlight the risks of honey bees exposed to pesticides for a long time in the development stage.

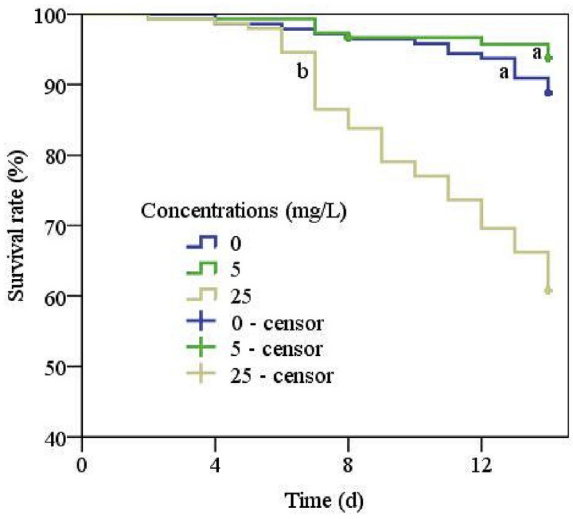

Fig. 4. Effects of acetamiprid on the average lifespan of *A. mellifera* worker bees. Adult worker bees were continuously fed the acetamiprid solutions (0, 5 and 25 mg/L) for 14 days after emergence, and death of bees were counted every day. Different letters along the curve lines denote significant differences (Log Rank test, $P < 0.05$).

5. Conclusion

In this study, acetamiprid over 5 mg/L disturbed the development of honey bee larvae; the expression of immune- and detoxification-related genes in honey bees were significantly down-regulated after a long-term exposure to acetamiprid, indicating that exposure to acetamiprid in the continuous development process may destroy the immune and detoxification functions of honey bees; the lifespan of bees treated with acetamiprid at 25 mg/L was significantly reduced. These data reflect the potential risk for honey bees continuously exposed to acetamiprid, and the present study contributes to the understanding of regulating law of host to deal with external stress at the gene expression level. However, the tolerance of honey bees response to different pesticides associated exposure time and doses needs to be further investigated, which may contribute to protect bees from poisoning.

Author contributions

In this work, J Shi carried out the laboratory work and wrote the manuscript. X Wu conceived this research and designed experiments. R Zhang and Y Pei contribute to the laboratory work. X Wu, J Shi and C Liao performed the experimental analysis and participated in the revisions of this manuscript. All authors read and approved the final manuscript.

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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