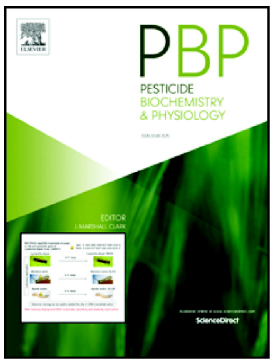

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Differential physiological effects of neonicotinoid insecticides on honey bees: a comparison between *Apis mellifera* and *Apis cerana*

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ABSTRACT

Acute toxicities (LD50s) of imidacloprid and clothianidin to *Apis mellifera* and *A. cerana* were investigated. Changing patterns of immune-related gene expressions and the activities of four enzymes between the two bee species were compared and analyzed after exposure to sublethal doses of insecticides. Results indicated that *A. cerana* was more sensitive to imidacloprid and clothianidin than *A. mellifera*. The acute oral LD50 values of imidacloprid and clothianidin for *A. mellifera* were 8.6 and 2.0 ng/bee, respectively, whereas the corresponding values for *A. cerana* were 2.7 and 0.5 ng/bee. The two bee species possessed distinct abilities to mount innate immune response against neonicotinoids. After 48 h of imidacloprid treatment, carboxylesterase (CCE), prophenol oxidase (PPO), and acetylcholinesterase (AChE) activities were significantly downregulated in *A. mellifera* but were upregulated in *A. cerana*. Glutathione-S-transferase (GST) activity was significantly elevated in *A. mellifera* at 48 h after exposure to imidacloprid, but no significant change was observed in *A. cerana*. AChE was downregulated in both bee species at three different time points during clothianidin exposure, and GST activities were upregulated in both species exposed to clothianidin. Different patterns of immune-related gene expression and enzymatic activities implied distinct detoxification and immune responses of *A. cerana* and *A. mellifera* to imidacloprid and clothianidin.

Keywords:

Apis cerana; *Apis mellifera*; Imidacloprid; Clothianidin; Detoxification; Immunity

1. Introduction

Honey bees are important pollinators of crops and wild plants, and they encounter several biotic and abiotic factors, including pathogens [1, 2], pesticides, and parasites in their whole adult lives [3, 4]. These factors acting alone or in combination have been linked to the decline of the pollinator in some regions worldwide [5]. In particular, honey bees are exposed to lethal and sublethal doses of pesticides during foraging because of the application of pesticides in crops against pests [6]. This phenomenon has led to increased attention to systemic pesticides, such as neonicotinoid insecticides, and their toxicity to honey bees [7].

Physiological and behavioral changes are manifested by honey bees exposed to sublethal concentrations of neonicotinoid insecticides. Previous studies showed that clothianidin and imidacloprid impaired the foraging activity of *Apis mellifera* bees at concentrations as low as 0.5 and 1.5 ng/bee, respectively [6]. Honey bees exhibited the highest mortality and consumed the highest amount of sugar water than the control groups when exposed to combined imidacloprid and *Nosema* [8]. Honey bees from colonies exposed to sublethal doses of imidacloprid had significantly higher levels of *Nosema* spore counts than bees from the control colonies, suggesting that interactive effects between pesticides and pathogens may contribute to colony losses worldwide [9]. Furthermore, transcriptome analysis revealed that the expression of midgut immunity genes and the metabolism of trehalose were altered in honey bees treated with *Nosema* combined with fipronil or imidacloprid [10]. Honey bees exposed to sublethal doses of neonicotinoid clothianidin exhibited high titers of *Deformed wing virus* [7]. In addition, honey bees treated with imidacloprid or clothianidin showed reduced hemocyte density, encapsulation response, and antimicrobial activity [11].

Western honey bees (*A. mellifera*) have been extensively investigated. By contrast, relatively little information is available to evaluate the toxic effects of neonicotinoid insecticides on Eastern honey bees, *A. cerana.* This species is indigenous in Asia and

widely cultivated in China for honey production and pollination services [12]. Imidacloprid at sublethal concentrations caused deficits in learning performances and risk decision-making in *A. cerana* bees [13, 14], but no data are available relative to the biochemical changes in *A. cerana* exposed to neonicotinoid insecticides. In addition, given the distinct differences in response to various biotic stressors between *A. mellifera* and *A. cerana* [15-17], whether differences exist in the physiologic response between the two species of bees exposed to neonicotinoid insecticides clothianidin and imidacloprid should be known. Acute oral toxicities of the neonicotinoid insecticides in the two bee species were therefore determined.

It has been reported that synthetic pesticides interact with insect immunity and may affect insect immunity via oxidative stress [18, 19]. In a previous review of key factors driving honey bees decline, Goulson et al. have also stressed the fact that both detoxification response and immune responses in honey bees can be impaired by pesticide exposure [20]. *Apidaecin*, *hymenoptaecin*, *defensin1*, *defensin2* and *apidaecin* are essential components of the Toll pathway which has been clearly identified in the genome of *A. mellifera* [21]. The Toll pathway is important for innate immunity in insects by regulating the immune-responsive genes [22]. Immune-related gene expression between *A. cerana* and *A. mellifera* during sublethal pesticide exposure were comparatively analyzed. The immune-related genes including *eater*, *vitellogenin*, *hymenoptaecin*, *defensin1*, *apidaecin*, *defensin2* and *abaecin* were measured by RT-qPCR using primer pairs from previous studies [23, 24]. In addition, It has been proven that enzymatic activities are proper biomarkers to assess stress responses in honey bees exposed to xenobiotics [25, 26]. Therefore, we selected four different enzymes involved in immune response, metabolic detoxification and target site insensitivity in this study. Changing patterns of enzymatic activities of acetylcholinesterase (AChE), carboxylesterase (CCE), glutathione-S-transferase (GST), and prophenol oxidase (PPO) were comparatively analyzed to determine whether they play different roles in response to xenobiotics in the two different species of honey bees.

2. Materials and methods

2.1. Acute (24 h) oral toxicity (LD50) testing

The acute oral toxicity testing was performed in three different *A. mellifera* colonies and three different *A. cerana* colonies. Worker honey bees of mixed ages captured from hive combs without brood were used for conducting acute toxicity tests according to previous studies [27, 28]. For each colony, honey bees were collected from the hive, and three cages, with each cage containing 20 bees for a specific dose of pesticides, were replicated. Imidacloprid and clothianidin were purchased from Sigma-Aldrich (Sigma-Aldrich Co.). Acetone was used to dissolve pesticides to make stock solutions (500 ng/μl), which were stored at 4 °C and covered by tinfoil to protect against light. Five test solutions of pesticides were prepared by diluting stock solutions with 30% (w/v) sugar solution. Given the crop content of honey bees would be completely consumed after a 2 h starve period [29], honey bees captured from hives were kept in cages without food in an incubator at 30 °C for approximately 2 h before toxicity testing in the present study [29, 30]. Therefore, the crop content of the bees was equal before toxicity testing in the present study. Experimental honey bees were fed with 2 μl of sugar solution containing each specific dose of pesticides individually using a pipette, and the control groups of bees were fed with 2 μl of 30% sugar solution containing 1.4% acetone. Different pesticide concentrations for each of the *Apis* species were used according to published data and our preliminary experiments. Five tested doses of imidacloprid (6.0, 8.0, 10.0, 12.0, and 14.0 ng/bee) were administered to *A. mellifera* for imidacloprid exposure, and clothianidin doses for this species were 1.6, 1.8, 2.0, 2.2, and 2.4 ng/bee. *A. cerana* were exposed to imidacloprid at 1.0, 2.0, 3.0, 4.0, and 5.0 ng/bee and to clothianidin at 0.2, 0.4, 0.6, 0.8, and 1.0 ng/bee. Each group of 20 bees was kept in a cage equipped with a feeder filled with 30% sugar solution, and the cages were maintained in an incubator at 30 °C and 70%±5% RH. The deaths 24 h after treatment were recorded.

2.2. Exposure of honey bees to sublethal doses of pesticides

Based on the LD50 values obtained in the study, doses approximately equal to

LD50/2 of the pesticides were used to evaluate the effects of the tested pesticides on the immune-related gene expression and activities of CCE, PPO, AChE and GST in the two bee species. Three different colonies of each species were used, and forager honey bees captured at the hive entrance were used. Three cages of bees, with 20 bees each from each colony in each cage, were orally administered with each pesticide using the methods described above. For *A. mellifera*, sublethal doses of imidacloprid (4.3 ng/bee) and clothianidin (1.0 ng/bee) were used. The sublethal doses of imidacloprid (1.0 ng/bee) and clothianidin (0.3 ng/bee) were used for *A. cerana* in the study. The control groups of bees were treated with sugar solution containing acetone. After oral administration of each pesticide, cages of bees were then placed in an incubator at 30 °C and 70%±5% RH before being sampling at three cages per treatment at 2, 24, and 48 h after pesticide administration.

2.3. qRT-PCR for relative gene expression

2.3.1. RNA extraction and cDNA synthesis

Total RNA was extracted from individual honeybees with Trizol reagent (Invitrogen) according to the manufacturer's protocol. For bees sampled at each specific time points for each treatment, nine bees were randomly selected for RNA extraction. Thus, 108 *A. mellifera* bees and 108 *A. cerana* bees were used for RNA extraction.

The cDNA was synthesized using the *TransScript*® First-Strand cDNA Synthesis SuperMix (Transgen) according to the manufacturer's instructions. The reaction mixture consisted of 4 μl of 5×*TransScript*® SuperMix, 1 μl of gDNA Remover, and 1 μg of total RNA. RNase Free dH_2O was added to the reaction mixture for a final volume of 20 μl. The reaction mixture was incubated at 42°C for 15 min, and then at 85 °C for 5 s. The synthesized cDNA was stored at −20 °C for later use.

2.3.2. Real-time quantitative PCR

Real-time qPCR reactions were performed in an ABI 7500 Real-Time PCR system. (Applied Biosystems, Inc.), and 12.5 μl of reaction mixtures consisted of 1 μl of diluted cDNA with three-fold dilution, 6.25 μl of 2×*TransScript*® qPCR SuperMix

(Transgen), 4 μl of RNase free dH_2O, 0.25 μl of Passive Reference Dye II, and 0.5 μl of each 10 μM forward and reverse primers [23, 24]. Nine cDNA samples corresponding to each group of bee samples were analyzed. Each reaction mixture for each cDNA sample was repeated in triplicate, and negative controls (non-template) were also analyzed in parallel. Comparison of amplification efficiency between target and reference genes was performed according to previous studies [31]. The PCR amplification program was as follows: pre-denaturation at 95 °C for 3 min, 40 cycles of 95 °C for 3 s, and 56 °C for 1 min. Then, the melting curve was analyzed from 60 °C to 95 °C. Fluorescence was measured for every 1 °C increase in temperature.

2.4. Determination of the specific activities of enzymes

2.4.1. Extraction and concentration determination of total tissue protein

Tissues from nine honey bees from each group of honey bee samples were dissected. Briefly, the honey bee head was dissected on a plastic plate placed onto an ice bath mixed with dry ice under a stereomicroscope. The chitin covering the brain was dissected with a blade and was carefully removed using fine tweezers. The brain tissue was obtained after removing hypopharyngeal, mandibular and salivary glands. Separation of thorax tissues was conducted by removing the chitin overlying the thorax using the fine tweezers. Brain and thorax tissues from three bees were mixed and put into a 1.5 ml tube, and three tubes were used. Nine midgut tissues dissected from nine honey bees were put into three 1.5 ml tubes with each tube containing three midgut tissues. The tissue samples were rinsed twice with ice-cold phosphate buffered saline (PBS). Supernatant fluid was discarded, and tissue sample was resuspended in 1 ml of total protein extraction buffer containing phenylmethyl sulfonyl fluoride (TransGen Biotech). Tissue samples were ground using sterile pestles on ice, followed by homogenization using a vortex vibrator for 5 min. The process was repeated until no visible tissue fragments were present. The homogenized suspension was transferred to a new 1.5 ml tube and incubated on ice for 30 min, during which the homogenized suspension was vibrated for approximately 30 s every 10 min. The homogenized suspension was then centrifuged for 10 min at 14,000 ×*g* and 4 °C, and

the supernatant, which contained total proteins, was carefully collected. The total proteins were stored at −80 °C for later use.

The bicinchoninic acid (BCA) assay was used to quantify the total proteins extracted from the mixed tissue sample of brain and thorax, as well as from the midgut tissue sample. Briefly, a standard solution of seven different serial dilutions (25, 50, 100, 200, 300, 400, and 500 μg/ml) of bovine serum albumin (BSA) was used to construct the standard curve (TransGen Biotech). Test sample solutions were diluted 5-fold in PBS. Then, 20 μl of serial dilutions of BSA and 20 μl of test sample solutions were added in a 96-well microtiter plate. After adding 200 μl of BCA working solution to each well, the plate was incubated at 37 °C for 60 min. The absorbance was measured at 595 nm using a microplate reader (TECAN Infinite F50). Protein concentrations of test samples were determined from the standard curve.

2.4.2. Detection of enzymatic activity using sandwich ELISA

Enzymatic activities of AChE, CES, and PPO were determined using proteins extracted from the mixed tissue sample of brain and thorax. Enzymatic activities of GST were determined using proteins extracted from midgut tissues. The activities of the four enzymes were quantified by employing a sandwich ELISA (enzyme-linked immunosorbent assay) using the ELISA Kit (Mlbio Shanghai Enzyme-linked Biotechnology, China) according to the manufacturer's instructions. Briefly, the microtiter plate was precoated with anti-AChE, anti-CES, anti-PPO, and anti-GST antibodies before 50 μl of standard enzymes or test sample were added to the wells of the plate. After incubation at 37 °C for 30 min, the reaction mixtures were washed five times in buffer. Then, 50 μl of horseradish peroxidase (HRP)-labeled antibodies were added to the wells of the microtiter plate, and the samples were incubated at 37 °C for 30 min and washed five times in buffer. Chromogenic reaction between tetramethylbenzidine and HRP was conducted at 37 °C for 15 min, and the optical density (OD) value of each well was measured at 450 nm. The activities of the four enzymes were calculated based on the standard curve constructed using standard enzymes of known activities. The relative activities of each enzyme in the specific

tissue from honey bees were expressed as absorbance per milligram of protein.

2.5. Data analysis

The acute LD50 values of imidacloprid and clothianidin in each honey bee species were calculated by probit analysis using SPSS 16.0 (SPSS Inc.). The relative expression levels of immunity genes from the samples were calculated using $2^{-\triangle\triangle Ct}$, and statistical analysis was performed using Student's t-test. Enzymatic activities were compared by one-way ANOVA followed by LSD multiple comparison test. $P < 0.05$ values were considered significant.

3. Results

3.1. Different toxicity levels of imidacloprid and clothianidin in two bee species

The acute oral LD50 values of imidacloprid and clothianidin for *A. mellifera* were 8.6 and 2.0 ng/bee, respectively, whereas the corresponding values for *A. cerana* were 2.7 and 0.5 ng/bee (Table 1). The LD50 values of imidacloprid were approximately 4–5-fold higher than those of clothianidin in the two bee species. Clothianidin was more highly toxic than imidacloprid to honey bees, regardless of bee species. *A. cerana* was more sensitive to imidacloprid and clothianidin than *A. mellifera*.

3.2. Effects of sublethal imidacloprid exposure on gene expression in two bee species

Given the immune gene *defensin2* exhibited the minimal expression in the two bee species we chose *defensin2* as a calibrator. To simplify data presentation, the relative expression levels of immune genes were subjected to log2 transformation. The expression of immune-related genes *eater, vitellogenin, hymenoptaecin, defensin1* and *apidaecin* were significantly upregulated in *A. mellifera* after 2 h of exposure to imidacloprid (Fig. 1A). Moreover, all these genes, *abaecin*, and *defensin2* were significantly upregulated in *A. mellifera* at 24 h after exposure to imidacloprid (Fig. 1B). All genes examined, except *vitellogenin, hymenoptaecin*, and *defensin2*, were significantly elevated in *A. mellifera* after 48 h of imidacloprid exposure (Fig. 1C).

Similar trends were observed for immune-related mRNA levels in *A. cerana*

exposed to imidacloprid than in control bees. Significantly higher expression of *abaecin, eater*, *vitellogenin*, *hymenoptaecin*, *defensin1*, and *apidaecin* was observed in imidacloprid-exposed bees after 2 h of exposure (Fig. 1D). All seven genes were markedly upregulated in *A. cerana* exposed to imidacloprid after 24, and 48 h (Fig. 1E and F).

3.3. Effects of sublethal clothianidin exposure on gene expression in two bee species

All seven genes were significantly increased in *A. mellifera* after 2, and 48 h of exposure to clothianidin (Fig. 2A and B). The mRNA levels of *abaecin*, *eater*, *vitellogenin*, *defensin1*, *defensin2*, and *apidaecin* were significantly upregulated in *A. mellifera* after 48 h of exposure to clothianidin (Fig. 2C).

Significant differences were found in the gene expression profiles between clothianidin-exposed *A. cerana* and control bees after 2, 24, and 48 h. Specifically, significantly higher mRNA levels of *abaecin*, *eater*, *hymenoptaecin*, *defensin1*, *defensin2*, and *apidaecin* were consistently observed in clothianidin-exposed *A. cerana* than in control bees after 2 (Fig. 2D), 24 (Fig. 2E), and 48 h (Fig. 2F). However, the *vitellogenin* gene showed a fluctuating expression pattern. This gene was downregulated after 2 h of exposure but upregulated at 24 h after exposure in clothianidin-exposed *A. cerana*. In addition, no significant difference was found in the relative expression levels of *vitellogenin* between clothianidin-exposed *A. cerana* and control bees at 48 h after exposure.

3.4. Effects of sublethal imidacloprid exposure on enzymatic activities in two bee species

There was no significant difference in the enzymatic activities of control samples collected at three time points, and data from control groups were pooled for comparative analysis with the treated groups. We compared the activities of CCE, PPO, AChE and GST in honey bees at three different time points after pesticide exposure. Imidacloprid-treated *A. mellifera* bees did not exhibit any significant difference in CCE activities over time compared with the control group of bees (Fig.

3A). However, PPO and AChE activities gradually decreased with prolonged time and were significantly depressed in imidacloprid-exposed *A. mellifera* at 48 h after exposure (Fig. 3B and C). However, GST activities gradually increased over time and were significantly elevated in imidacloprid-exposed *A. mellifera* at 48 h after exposure (Fig. 3D).

However, the CCE, PPO and AChE activities in *A. cerana* at 48 h after exposure to imidacloprid showed a contrasting trend compared with *A. mellifera*. The activities of the three detoxifying enzymes were significantly elevated in *A. cerana* exposed to imidacloprid after 48 h of treatment (Fig. 3A, B and C, respectively). Among them, CCE activities were significantly downregulated in imidacloprid-exposed *A. cerana* at 2 h after exposure (Fig. 3A), and AChE activities were markedly downregulated in *A. cerana* after 2 and 24 h exposure (Fig. 3C). By contrast, GST activities showed no significant change in imidacloprid-exposed *A. cerana* during the exposure period (Fig.3D).

3.5. Effects of sublethal clothianidin exposure on enzymatic activities in two bee species

In the comparison of the bees in the control group, a similar pattern of activity changes, including CCE, PPO, AChE and GST, were observed in clothianidin-treated *A. mellifera* and *A. cerana*. CCE and PPO activities exhibited no significant differences in clothianidin-treated *A. mellifera* at the three different time points (Fig. 4A and B). AChE activities were significantly downregulated in honey bees exposed for 48 h compared with those exposed for 2 h (Fig. 4C). However, GST activities in *A. mellifera* exposed for 48 h to clothianidin were significantly higher than honey bees exposed for 2 h (Fig. 4D).

No significant changes were observed in CCE and PPO activities of *A. cerana* at the three different time points after treatment with clothianidin (Fig. 4A and B). Compared with the control group, AChE activities were significantly downregulated in clothianidin-treated *A. cerana*. In addition, AChE activities were significantly higher in honey bees exposed for 48 h than those exposed for 2 and 24 h (Fig. 4C).

However, compared with the control group, GST activities were significantly elevated in *A. cerana* exposed for 24 and 48 h to clothianidin (Fig. 4D).

4. Discussion

Previous studies showed that the acute oral LD50 values of imidacloprid and clothianidin in *A. mellifera* at 24 h varied widely [30, 32]. The oral LD50 values (24 h) of imidacloprid for *A. m. mellifera* and *A. m. caucasica* were both about 5 ng/bee [30], which is similar to the 24 h oral LD50 value of imidacloprid for *A. mellifera* L. in the present study. The oral LD50 values (24 h) of clothianidin for *A. mellifera* L. is about 2.84 ng/bee [29], with which our data fit. However, for three different subspecies of *A. mellifera*, the mean oral LD50 values (24 h) of imidacloprid is 118.74 ng/bee, and that of clothianidin is 3.53 ng/bee [32], which are different from our data. Genetic distinction between different honey bee subspecies, colony differences, and trophallaxis in honey bees probably contributed to the variability in the LD50 values [33]. In addition, the physiological state of honey bees varies over different seasons may also affect toxicity of pesticides to honey bees [34]. Therefore, honey bees were fed individually with sugar solutions containing specific doses of pesticides in the present study to avoid food transfers during trophallaxis between individual honey bees. Previous studies showed that honey bees adjusted the trophallactic behaviors based on their crop volumes, and a positive correlation was found between crop load and food transfers between honey bees [35]. After approximately 2 h of starvation, only 2 μl of 30% sucrose solution containing specific dose of pesticides was fed before they were placed in the cages. Honey bees had extremely low amounts of sucrose solution in the crop, and bees engaging in food transfers would be very low in this study. Of the two pesticides tested, the LD50 values of imidacloprid in the two bee species were approximately 4–5-fold higher than those of clothianidin. This result is consistent with those from previous studies which showed much lower acute toxicity of imidacloprid than clothianidin [32]. Both chemicals are more toxic to *A.*

cerana than to *A. mellifera*, indicating that *A. cerana* is more susceptible to pesticides than *A. mellifera*.

The honey bee *A. cerana* thus should be a more highly sensitive bioindicator of environmental pollution caused by heavy metals, insecticides [36]. In addition, it has been shown that colony losses of *A. mellifera* may be driven by insecticides [3]. However, similar levels of colony losses were not observed in *A. cerana* colonies, which may be explained by differences in colony level traits between the two bee species. The absconding behavior is more frequent in *A. cerana* than *A. mellifera* [37], and *A. cerana* bees may abandon their colonies when the combs are contaminated with multiple pesticide residues [38]. Other traits, such as smaller nest size and foraging at high altitude areas, may contribute to the reduction of insecticide hazard in *A. cerana* bees [37].

Previous reviews have demonstrated that oxidative stress induced by pesticides is one possible mechanism of pesticides toxicity in animals and insects [39, 40]. Vitellogenin plays vital roles in the immune response and regulation of longevity in worker honey bees [41]. Gene expression of *vitellogenin* in worker bees protect against oxidative stress induced by pesticides [40, 42]. At the indicated time points in the study, upregulation of *vitellogenin* in the two bee species exposed to neonicotinoids indicated that this gene may be a defensive response to oxidative stress at a certain period of time. Of the seven immune-related genes investigated in the study, *eater*, *defensin1*, and *apidaecin* were significantly upregulated in the two species of honey bees exposed to neonicotinoids, suggesting that the three genes may be good markers to monitor the immune defense against neonicotinoids in honey bees. *Defensin2* was upregulated in the two species of honey bees exposed to imidacloprid, although with a less consistent pattern of expression. Moreover, expression levels of *defensin2* were significantly upregulated in the two species of honey bees exposed to clothianidin which is more toxic than imidacloprid. The two defensins display distinct immune response to pesticides, and further studies are needed to elucidate interactions

of the two defensins in honey bees exposed to pesticides. The genes encoding antimicrobial peptides abaecin and hymenoptaecin exhibited distinct expression patterns in the two bee species. The two genes were significantly upregulated in neonicotinoid-exposed *A. cerana* but showed no significant upregulation at multiple time points in neonicotinoid-exposed *A. mellifera* throughout the exposure period. Thus, these data suggest that all seven immune-related genes are involved to some extent in the immune response against neonicotinoids irrespective of bee species. The two bee species exhibited different abilities to mount innate immune response against neonicotinoids, and differences in the metabolism and toxicity between the two pesticides may also result in distinct expression patterns of the immune-related genes in the two bee species.

Previous studies showed that GST, CCE are main detoxification enzymes in insects responding to chemical stress [43]. The enzyme CCE is primarily responsible for the phase I detoxification processes, which alter the structure of toxin and deactivate pesticides [43]. Distinct activities of three subtypes of CCE were observed in *A. mellifera* exposed to thiamethoxam [25], while no significant changes in the activity of CCE were observed in imidacloprid-exposed *A. mellifera* in the present study. Thiamethoxam is the precursor of clothianidin [44], and different detoxification mechanisms against neonicotinoids and its metabolites may exist in *A. mellifera* bees. In addition, at three different time points during imidacloprid exposure, sublethal doses of imidacloprid induced elevated CCE and PPO activities in *A. cerana* bees but depressed PPO activities in *A. mellifera* bees. The enzyme PPO is associated with the innate immunity of insects by participating in cellular and humoral defense and plays critical roles in defending against invaders in insects [19, 45]. The opposite trend regarding CCE and PPO activities indicated that the two bee species may have distinct detoxification and immune mechanisms against imidacloprid.

The enzyme AChE plays crucial roles in the nervous system by terminating nerve impulses in the nervous system by catalyzing the hydrolysis of acetycholine [46]. As agonists of the neurotransmitter acetycholine, neonicotinoids bind to the insect nicotinic receptor, resulting in prolonged activation of the receptor until the death of

insects [27]. AChE was depressed in *A. mellifera* but elevated in *A. cerana* during exposure to imidacloprid, suggesting the different potencies of the enzyme in the metabolism of acetylcholine between the two species of bees. GST is the principal enzyme involved in the phase II detoxification process and further catalyzes the conjugate formation generated by phase I detoxification [43]. GST plays a role in preventing oxidative stress induced by thiamethoxam in *A. mellifera* bees [25]. Our studies also suggest that GST may be involved in oxidative stress response caused by imidacloprid in *A. mellifera* bees. However, no significant GST changes were observed in imidacloprid-exposed *A. cerana*, indicating that GST is apparently not required to prevent oxidative stress in *A. cerana* bees exposed to sublethal doses of imidacloprid.

Furthermore, no significant changes in CCE and PPO activities were observed in the two bee species during exposure to clothianidin. Downregulation of AChE was also observed both in *A. mellifera* and *A. cerana* at the three different time points during clothianidin exposure. The ability of honey bees to detoxify xenobiotics may have been compromised when they were exposed to more toxic pesticides, because clothianidin is more toxic to honey bees than imidacloprid irrespective of species. However, the GST activities were both upregulated in clothianidin-exposed *A. mellifera* and *A. cerana* bees. The differences in potencies between the two pesticides indicated the distinct induction of detoxification enzymes in honey bees. Lethal doses of neonicotinoids were used to compare the pesticide sensitivity of the two bee species; however, activities of CCE, PPO, AChE and GST in the two bee species were evaluated at sublethal doses of neonicotinoids. The detoxification mechanisms involved in lethal and sublethal effects of neonicotinoid insecticides on honey bees may be different. In addition, some detoxifying enzymes and genes were not included in this study, for example Cytochrome P450s, *catalase*, and *AmNOS*, which could have key roles in the detoxification of honey bees [47].

Therefore, our data provide further evidence that clothianidin is more highly toxic than imidacloprid to honey bees [48], regardless of bee species, and *A. cerana* is more sensitive to the two insecticides compared with *A. mellifera*. In addition, the results

indicated that changing patterns of immune response and activities of CCE, PPO, AChE and GST differed during the immune and detoxification process between *A. mellifera* and *A. cerana*. Further studies regarding global analysis of enzyme activities and detoxification and immune gene expression profiles will be required to study differential metabolism and detoxification pathways between the two bee species.

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Figure captions

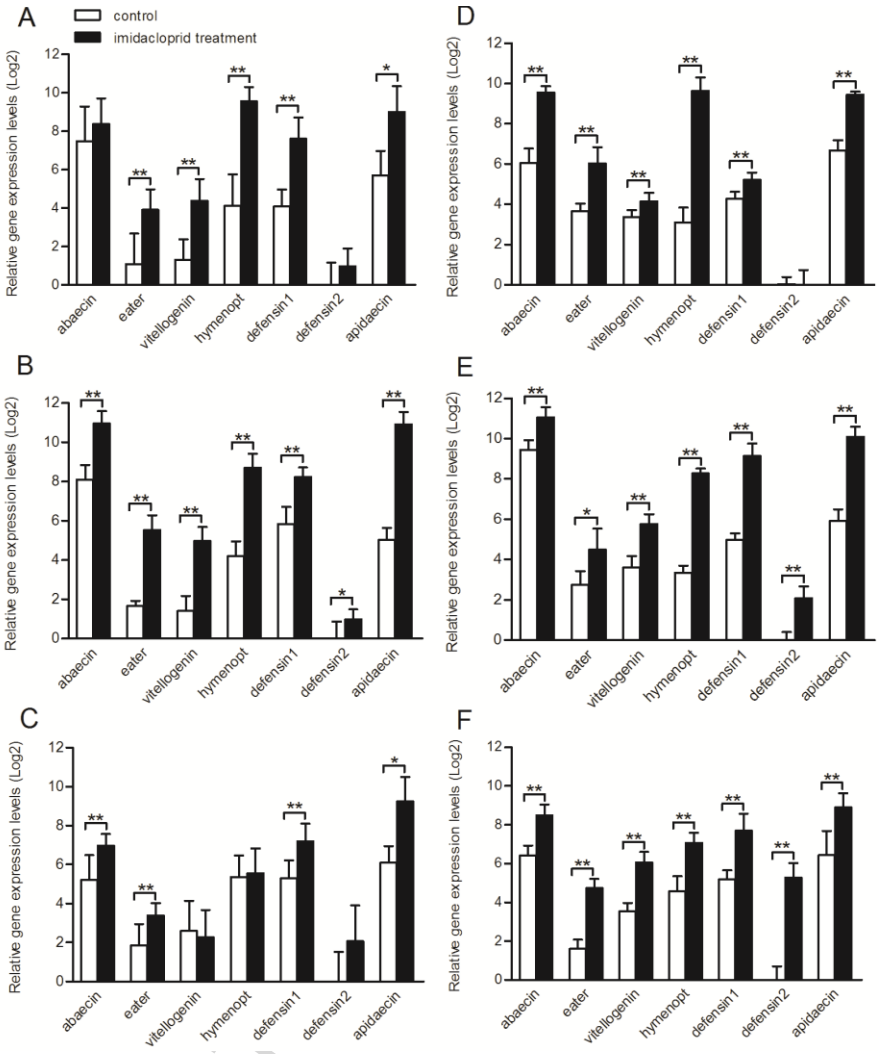
Fig. 1. Relative immune-related gene expression levels in *A. mellifera* treated by imidacloprid after 2h (A), 24h (B) and 48h (C); relative immune-related gene expression levels in *A. cerana* treated by imidacloprid after 2h (D), 24h (E) and 48h (F). Asterisks denote significant differences: one, $P< 0.05$; two, $P< 0.01$.

Fig. 2. Relative immune-related gene expression levels in *A. mellifera* treated by clothianidin after 2h (A), 24h (B) and 48h (C); relative immune-related gene expression levels in *A. cerana* treated by clothianidin after 2h (D), 24h (E) and 48h (F). Asterisks denote significant differences: one, $P< 0.05$; two, $P< 0.01$.

Fig. 3. Enzyme activities of CCE, PPO, AChE and GST in two bee species after exposure to sublethal doses of imidacloprid. Asterisks denote significant differences: one, $P< 0.05$; two, $P< 0.01$.

Fig.4. Enzyme activities of CCE, PPO, AChE and GST in two bee species after exposure to sublethal doses of clothianidin. Asterisks denote significant differences: one, $P< 0.05$; two, $P< 0.01$.

Fig. 1.



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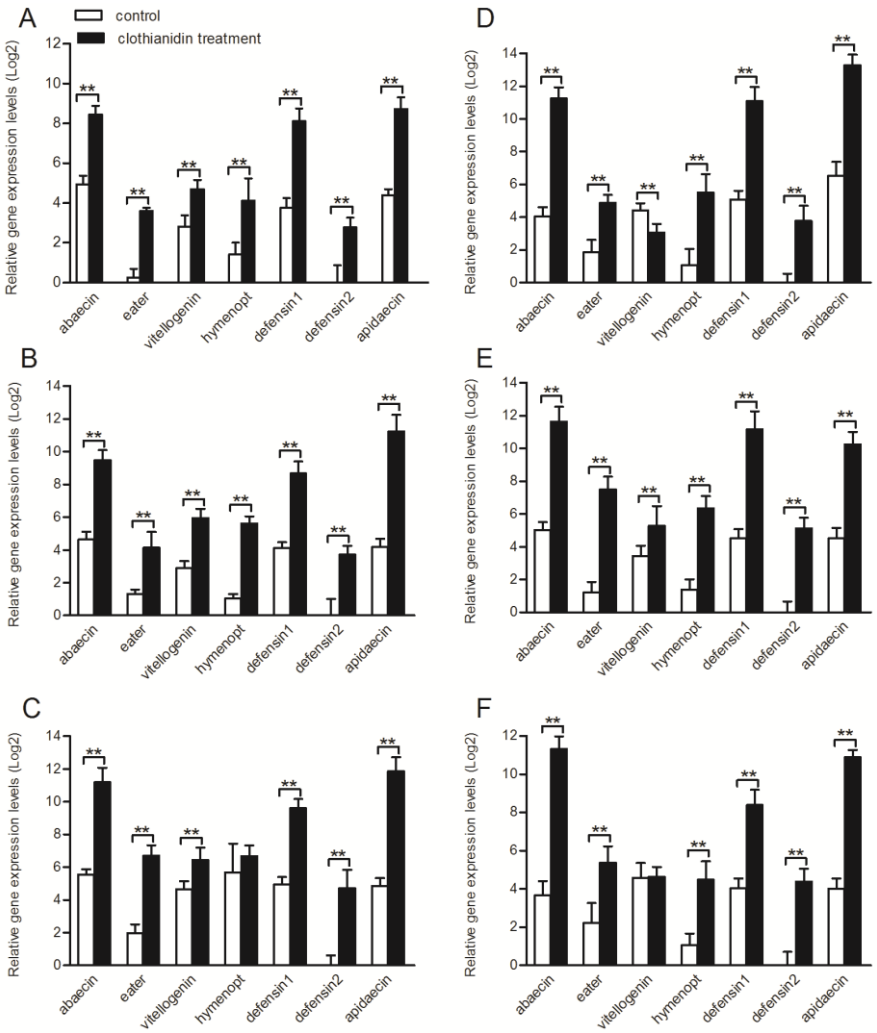

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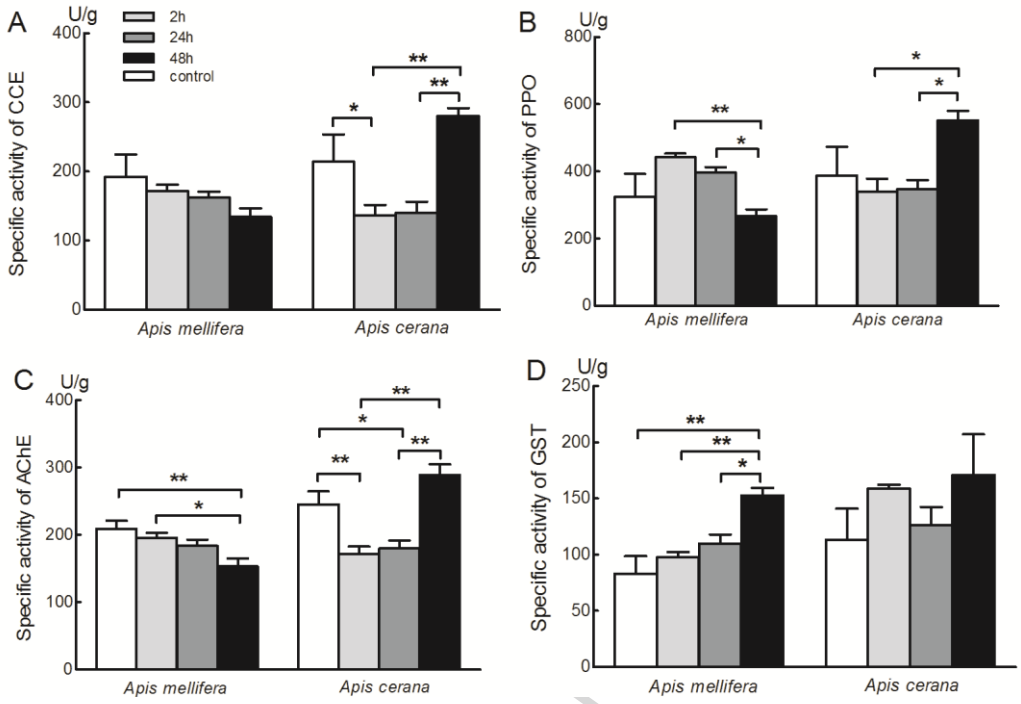

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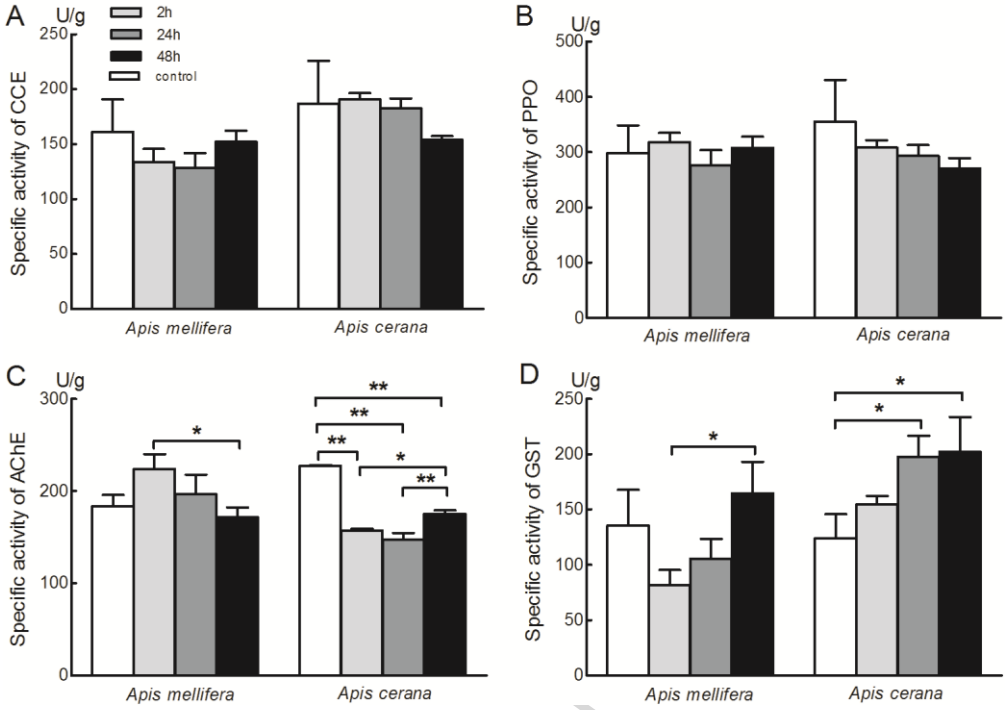
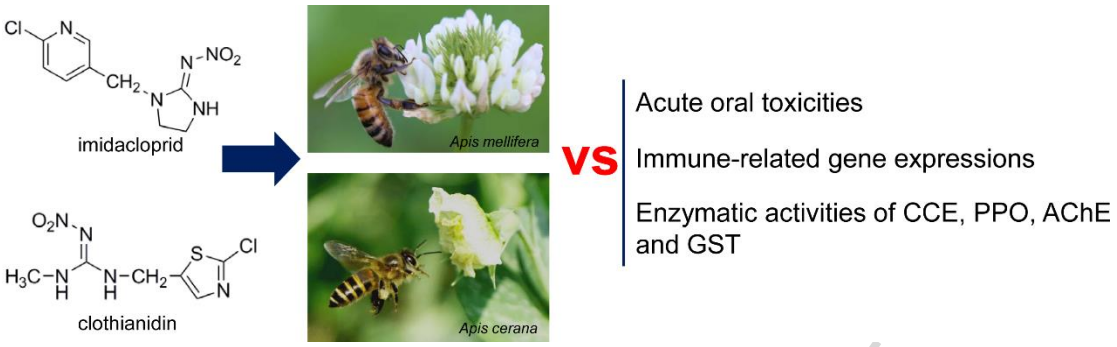


Table 1. The acute (24 h) oral LD50 values of imidacloprid and clothianidin in *Apis mellifera* and *Apis cerana*. The 95% confidence limits are given in square brackets.

Bee species	**imidacloprid**			**clothianidin**		
	LD50 (ng/bee)	Chi-square	P value	LD50 (ng/bee)	Chi-square	P value
A. mellifera	8.6 [7.8-9.3]	1.457	0.692	2.0 [1.9-2.1]	0.285	0.963
A. cerana	2.7 [2.4-3.1]	2.45	0.484	0.5 [0.4-0.6]	0.959	0.811



Graphical abstract

Highlights:

1. *A. cerana* is more sensitive to the two insecticides compared with *A. mellifera*.
2. Activities of detoxification enzymes were different between the two bee species.
3. Neonicotinoids induced distinct immune responses in the two bee species.