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

Zhiguo Li [a,1], Meng Li [a,1], Jingfang He [a], Xiaomeng Zhao [a], Veeranan Chaimanee [b], Wei-Fone Huang [a], Hongyi Nie [a], Yazhou Zhao [c,a], Songkun Su [a,*]

[a] College of Bee Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian, China
[b] Department of Biotechnology, Maejo University Phrae Campus, Rong Kwang, Phrae 54140, Thailand
[c] Institute of Apiculture Research, Chinese Academy of Agricultural Sciences, Beijing 100093, China

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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.

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

* Corresponding author.
E-mail address: susongkun@zju.edu.cn (S. Su).
[1] These authors contributed equally to this work.

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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