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Effects of Sublethal Concentrations of Chlorpyrifos on Olfactory Learning and Memory Performances in Two Bee Species, *Apis mellifera* and *Apis cerana*

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Abstract

Chlorpyrifos is a widely used organophosphorus insecticide. The acute oral 24 h median lethal dose (LD50) value of chlorpyrifos in *Apis mellifera* and in *Apis cerana* was estimated to assess differential acute chlorpyrifos toxicity in both bee species. The LD50 values of chlorpyrifos in *A. mellifera* and in *A. cerana* are 103.4 ng/bee and 81.8 ng/bee, respectively, which suggests that *A. cerana* bees are slightly more sensitive than *A. mellifera* bees to the toxicity of chlorpyrifos. Doses half the acute LD50 of chlorpyrifos were selected to study behavioral changes in both bee species using proboscis extension response assay. *A. mellifera* foragers treated with chlorpyrifos showed significantly lower response to the 10% sucrose solution compared to control bees after 2, 24 and 48 h. Chlorpyrifos significantly impaired the olfactory learning abilities and 2 h memory retention of forager bees regardless of honey bee species, which may affect the foraging success of bees exposed to chlorpyrifos.

Introduction

Insecticides are widely used worldwide and play an important role in protecting crops from damage caused by all kinds of pests, including aphids, sap-sucking insects and leaf-eating caterpillars, etc. (Henry et al., 2012; Mpumi et al., 2016). At the same time, a growing body of evidence shows that insecticides have inevitably caused adverse behavioral and physiological effects on individual bees and colonies (Henry et al., 2012; Di Prisco et al., 2013). Honey bees are important pollinating insects for wild plants and crops worldwide, and much attention has been paid to declines in pollinators. Colony losses in some parts of the world are due to pathogens (Neumann & Carreck, 2010), insecticides (Henry et al., 2012), weather, habitat loss (Potts et al., 2010; Vanengelsdorp & Meixner, 2010), or interactions among these factors (Goulson et al., 2015).

Neonicotinoid insecticides induce chronic oral toxicity that alters the gustatory responsiveness of adult honey bees and impairs their olfactory learning performances (Decourtye et al., 2003; Goñalons & Farina, 2015). Both the type of insecticides exposure in honey bees and dose of insecticides administered to honey bees affect their behaviors based on proboscis extension response (PER) (Goñalons & Farina, 2015). In addition, it was found that sublethal doses of neonicotinoid insecticides decrease the synaptic density of the mushroom body calyx of honey bees and therefore honey bees exposed to 0.04 ng imidacloprid per bee larvae in the larval stage exhibit an impaired olfactory associative behavior in the adult stage (Yang et al., 2012; Peng & Yang, 2016). Similar to neonicotinoid insecticides, organophosphorus pesticides, such as chlorpyrifos (CPF), are widely used to control pests infesting agricultural crops (Qin et al., 2014). However, chlorpyrifos elicits toxic effects on beneficial insects, aquatic organisms and

amphibians by inhibiting the activities of acetylcholinesterase (AChE) in their central nervous systems (Pope, 1999; Levin et al., 2004; Dimitrie & Sparling, 2014). Chlorpyrifos is also ranked as the third most frequent pesticide residue detected in hives; this pesticide has also been detected in hive products, such as pollen, nectar, wax, and propolis (Chauzat et al., 2006; Mullin et al., 2010). The colony residue levels of chlorpyrifos has significantly increased larval mortality (Zhu et al., 2014). Apoptotic cells have also been found in the midgut of honey bee larvae treated with sublethal levels of chlorpyrifos compared with those in untreated larvae (Gregorc & Ellis, 2011). Upon exposure to sublethal chlorpyrifos concentrations, adult honey bees suffer from impaired learning and recall performances (Urlacher et al., 2016). In addition, few queens also emerge when larvae reared in colonies are exposed to sublethal chlorpyrifos levels, and emerged queens become infected with Deformed wing virus; this finding indicates that sublethal chlorpyrifos levels impair the development of queens (DeGrandi-Hoffman et al., 2013).

Olfactory learning and memory test based on PER has been performed to investigate the cellular and molecular mechanisms of the learning and memory processes mainly in honey bees *Apis mellifera* (Menzel & Muller, 1996). Behavioral tests have also been conducted to evaluate the behavioral and physiological responses of honey bees to exogenous factors, such as chemicals and pathogens (Decourtye et al., 2004; Iqbal & Mueller, 2007). This classic learning paradigm consists of two elements: an odor (conditioned stimulus, CS) and a sucrose solution (unconditioned stimulus, US) (Menzel & Muller, 1996). Until recently the learning paradigm has also been applied to examine olfactory learning of *Apis cerana* (Wang & Tan, 2014). *A. cerana*, a honey bee species native to Asia, is an important crop and flora pollinator in the Asian region. About two million *A. cerana* colonies are kept in hives in China (Li et al., 2012), and these colonies are used to produce honey and pollinate crops, especially those in hilly and mountainous areas (Hepburn & Radloff, 2011). Previous studies showed that subtheal doses of imidacloprid impair learning acquisition and decision-making abilities of *A. cerana* (Tan et al., 2014; Tan et al., 2015). However, although the acute toxicity and sublethal effects of insecticides on *A. mellifera* have been extensively explored, the toxic effects of pesticides on *A. cerana* have been rarely examined. It is therefore necessary to determine whether differences existed in the acute and chronic toxicity of chlorpyrifos to the two honey bee species. In our study, 50% oral lethal dose (LD50) was established for *A. cerana*, and differences in the acute toxicity of chlorpyrifos were compared between *A. mellifera* and *A. cerana*. The effects of sublethal chlorpyrifos concentrations on the sucrose responsiveness, olfactory learning, and memory performances of the two honey bee species were further evaluated on the basis of acute oral toxicity. Our study provides evidence that *A. cerana* is more sensitive than *A. mellifera* to chlorpyrifos. The learning and memory performances of *A. cerana* treated with sublethal chlorpyrifos doses are more impaired than those of *A. mellifera*.

Material and Methods

Acute oral toxicity tests

Acute oral toxicity testing in honey bees were performed following standard protocols (OECD 213 1998) and previous studies (Suchail et al., 2000; Iwasa et al., 2004). Briefly, chlorpyrifos (Sigma-Aldrich Co.) was dissolved with acetone (Sinopharm Chemical Reagent Co.) to prepare stock solutions (500 ng/μl). The stock solutions were stored at 4 °C and covered with tin foil to protect from light prior to testing. Five test concentrations of the pesticide were obtained by diluting the stock solutions with 30% (w/v) sucrose solution to determine oral LD50 of chlorpyrifos at 24 h for *A. mellifera* and *A. cerana*. Three *A. mellifera* colonies and three *A. cerana* colonies were used in our study. Adult honey bees were randomly collected from hives and starved for 2 h before the toxicity testing in an incubator at 30 °C, 70% ± 5% RH. For each test concentration of chlorpyrifos, three cages, with each cage containing 20 bees, were used for the tests. Sixty adult workers from each colony were subjected to acute oral toxicity test. The experiments were repeated thrice for all the colonies. Using a 2.5 μl pipette, adult workers were fed with 2 μl of 30% sucrose solution with a specific dose of chlorpyrifos and were assigned as treatment groups. *A. mellifera* were fed with 72, 88, 104, 120, and 136 ng chlorpyrifos/bee. *A. cerana* were fed with 60, 70, 80, 90, and 100 ng chlorpyrifos/bee. The control adult workers were individually fed with 2 μl of 30% sucrose solution containing acetone. The treatment and control groups were kept in an incubator at 30 °C and 70% ± 5% RH and fed with 30% sucrose solution ad libitum. The number of dead honey bees was recorded, and different acute oral LD50 were determined 24 h after of the five test concentrations of chlorpyrifos were administered orally.

Sucrose responsiveness

A. mellifera and *A. cerana* foragers collected at the entrance of the hives were exposed to sublethal doses of chlorpyrifos by using the same procedure to perform acute oral toxicity tests on the basis of the acute oral LD50 determined in this study. Doses equal to LD50/2 were selected to assess the behavioral changes in the two honey bee species exposed to chlorpyrifos using the PER assay.

Twenty foragers were captured at the hive entrance of each colony during the peak foraging time at day time. Sixty foragers from three different colonies were used for sucrose responsiveness test. The honey bees were immobilized individually in an ice bath in a vial and were confined in a plastic tube with cloth adhesive tape. Chlorpyrifos (52 ng/bee) was administered orally to *A. mellifera*. Chlorpyrifos (41 ng/bee) was also orally administered to *A. cerana* bees. The control bees were orally treated with acetone alone. The sucrose responsiveness test was performed at 2, 24, and 48 h after exposure in accordance with previously described

methods (Li et al., 2013). Harnessed bees were placed and kept in an incubator (30 °C, 70% ± 5% RH) until the bees were subjected to sucrose responsiveness test at each time point. Serial concentrations of sucrose solution [0.1%, 0.3%, 1%, 3%, 10%, and 30% (wt/wt)] were used in the study. A wooden tooth pick was immersed in the sucrose solution and was placed in contact with the antenna of the confined bees. Honey bees were tested by using increasing concentrations of sucrose and with two minute intervals between tests. The number of bees responding to each concentration was recorded. After the test, a drop of water was applied to touch the antenna of the bees between two consecutive tests of the sucrose solution and to prevent sensitization in honey bees as a result of repeated stimulation. The honey bees were further examined with 50% (wt/wt) sucrose solution, and the honey bees that were unresponsive to 50% sucrose solution were excluded from the analysis (Li et al., 2013).

Olfactory learning and memory test

The responsiveness to 30% sucrose solution, which was used to elicit PER in honey bees during this test, was not significantly different between the treated honey bees and the control bees. Forager bees were immobilized and harnessed in accordance with the same methods described above. About 80-90 foragers collected from three different colonies for each bee species and were used for olfactory learning and memory test. Chlorpyrifos and acetone treated foragers were subjected to olfactory learning tests 2 and 24 h after the treatment was administered. The olfactory learning processes involved six training trials, and each training trial consisted of lemon odor (CS) paired with 30% sucrose solution (US). The interval between training trials was 10 min. The honey bees were acclimated for approximately 30 min in the conditioning site after they were removed from the incubator. We accurately controlled the timing and duration of CS by using a modified air pump device. The two branches of a Y-shaped tube were connected via the two outlets of the air pump, and one of the two branches contained a round paper (2.5 cm in diameter) soaked with 10 µl of lemon oil. After the air pump was switched on, the common branch of the Y-shaped tube constantly delivered lemon-scented air toward the antenna of the harnessed bees. The common branch of the Y-shaped tube delivered a constant unscented airflow when the switch was off. Odor associative conditioning learning was conducted in accordance with previously described methods (Müller, 2002). After six training trials were completed, the retention capacities of the honey bees were examined at 2 and 24 h, respectively. The memory test was performed using lemon odor alone (Felsenberg et al., 2011). The number of bees responding to the odor was recorded. The proboscis of the honey bees included in the analysis extended fully during the test; after the test was completed, the honey bees unresponsive to 50% sucrose solution were excluded from the analysis (Felsenberg et al., 2011).

Statistical analysis

LD50 of chlorpyrifos for *A. mellifera* and *A. cerana* were estimated through probit regression analysis of the survival data by using SPSS 16.0 software. Sucrose responsiveness, olfactory learning, and memory performances were statistically compared between the chlorpyrifos-treated bees and the control bees by using Fisher's exact test. Differences were considered significant at p <0.05.

Results

Differential acute toxicity of chlorpyrifos in A. mellifera and A. cerana

The different oral acute toxic effects of chlorpyrifos on *A. mellifera* and *A. cerana* were observed in the study. The acute (24 h) oral LD50 of chlorpyrifos were 103.4 and 81.8 ng/bee for *A. mellifera* and *A. cerana*, respectively (Table 1). *A. cerana* was more sensitive to chlorpyrifos than *A. mellifera*.

Table 1. The LD50 values and its 95% confidence limits of chlorpyrifos at 24 h in *Apis mellifera* and *Apis cerana*.

Bee species	Oral LD50 (ng/bee)	95% confidence limits
Apis mellifera	103.4	96.2-110.9
Apis cerana	81.8	76.0-88.8

Sucrose responsiveness of honey bees exposed to sublethal chlorpyrifos doses

Chlorpyrifos-treated bees and acetone-treated bees were compared for each concentration of sucrose solution separately. The responses to 10% sucrose solution of *A. mellifera* treated with chlorpyrifos were significantly lower (p <0.05) after 2, 24 and 48 h (Fig 1A, 1B and 1C, respectively) than those of the control bees treated with acetone. The two groups did not significantly differ in terms of their responses to other concentrations of sucrose solution. The responses of chlorpyrifos-treated *A. cerana* and acetone-treated bees to serial concentrations of sucrose solution did not significantly differ after 2 (Fig 1D), 24 (Fig 1E), and 48 h (Fig 1F) of exposure to sublethal chlorpyrifos doses.

Chlorpyrifos-induced impairment of learning and memory performances of the two honey bee species

The tested bees did not show conditioned PER in the first trial. The percentage of acquisition rate 2 h after exposure of the chlorpyrifos-treated *A. mellifera* ranged from 47.8% to 58.9% from the second trial to the sixth trial. By contrast, the percentage of acquisition rate of the corresponding control bees ranged from 74.4% to 84.4%. For each trial, the percentage of PER significantly differed between the two groups (p<0.01; Fig 2A). The memory retention at 2 and 24 h was significantly
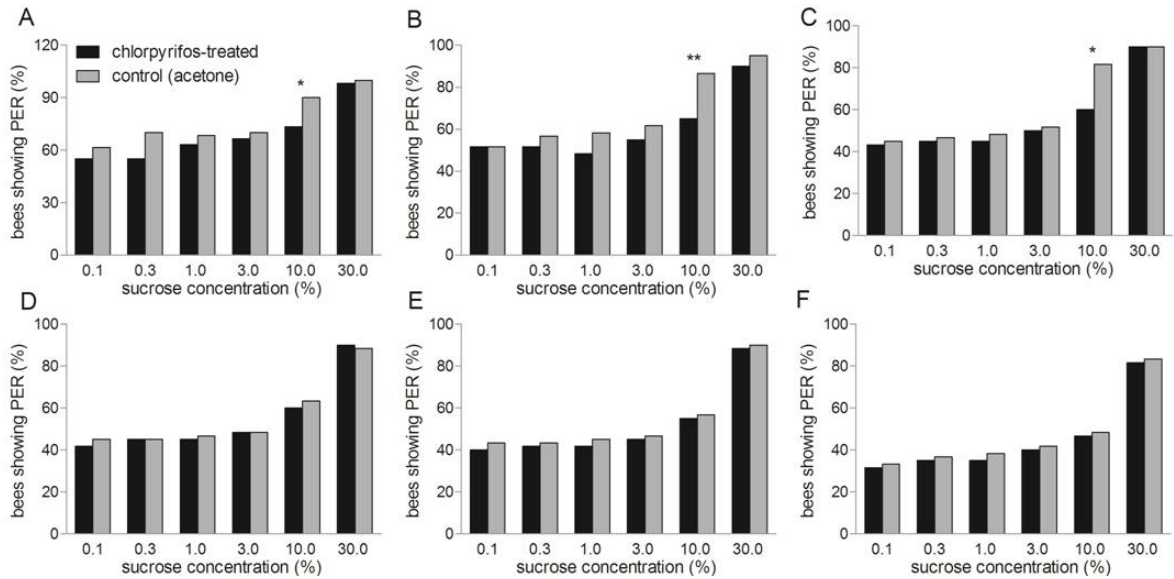
Fig 1. Analysis of sucrose responsiveness in honey bees exposed to sublethal doses of chlorpyrifos. Response of honey bees (Top: *A. mellifera*; bottom: *A. cerana*) treated with sublethal oral doses of chlorpyrifos to serial sucrose solutions after 2 h (A and D), 24 h (B and E) and 48 h (C and F) exposure. Control bees received acetone. Asterisks denote significant differences: one, p<0.05; two, p<0.01.

lower in the chlorpyrifos-treated *A. mellifera* than in the control bees (p<0.01; Fig 2C).

The percentage of acquisition rate 24 h after exposure of the chlorpyrifos-treated *A. mellifera* ranged from 28.9% to 41.1% from the second trial to the sixth trial. The percentage of acquisition rate of the corresponding control bees ranged from 58.9% to 74.4%. For each trial, the percentage of PER significantly differed between the two groups (p<0.01; Fig 2B). Memory retention at 2 and 24 h was significantly lower in the chlorpyrifos-treated *A. mellifera* than in the control bees (Fig 2D).
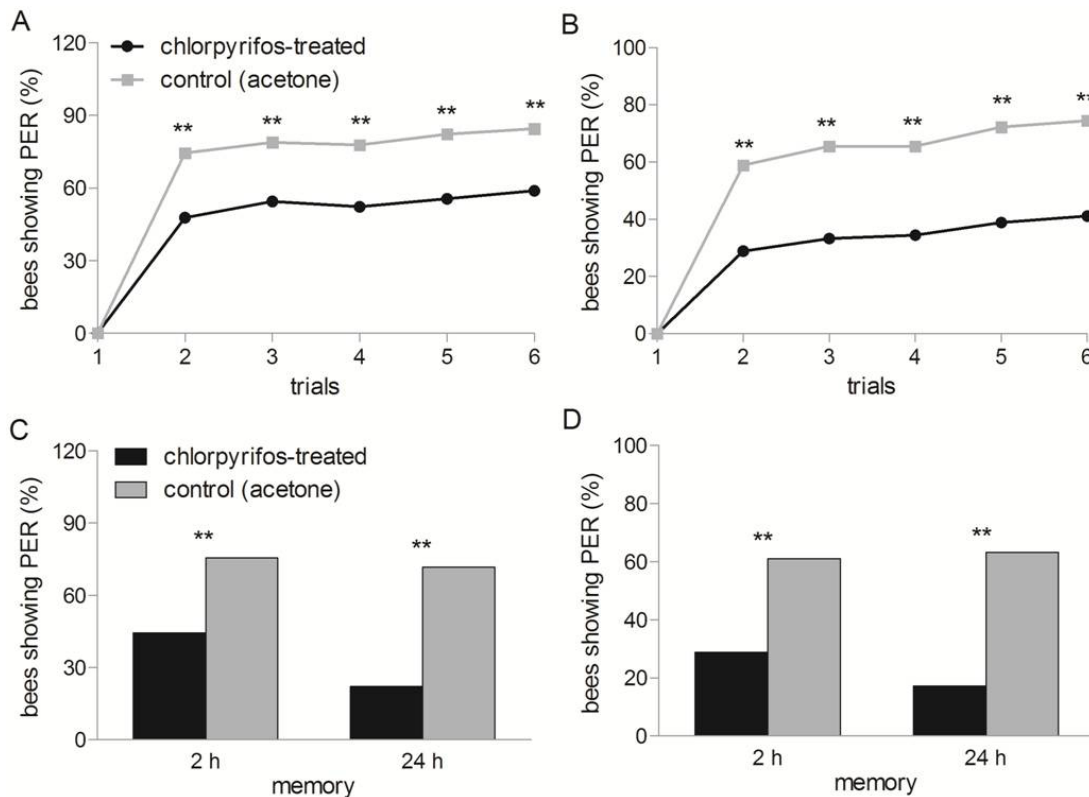

Fig 2. Sublethal doses of chlorpyrifos impair learning and memory performances in *A. mellifera*. Learning performances were significantly impaired in chlorpyrifos treated *A. mellifera* honey bees after 2 h (A), and 24 h (B) exposure. Among honey bees exposed for 2 h, honey bees showed significantly impaired memory performances 2 and 24 h after six training trials (C); among honey bees exposed for 24 h, honey bees showed significantly impaired memory performances 2 and 24 h after six training trials as well (D). Control bees received acetone. Asterisks denote significant differences: two, p<0.01.

For *A. cerana*, the acquisition rates ranged from 8.0% to 46.6% in the chlorpyrifos-treated honey bees from the second trial to the sixth trial 2 h after exposure. By comparison, the acquisition rates ranged from 27.8% to 70.0% in the acetone-treated honey bees. Similarly, the chlorpyrifos-treated honey bees significantly differed from the acetone-treated honey bees in each trial (p <0.01; Fig 3A). The retention capacities were significantly higher in the acetone-treated honey bees than in the chlorpyrifos-treated honey bees 2 and 24 h after the training test was completed (p<0.01 and p<0.05, respectively; Fig 3C).

The acquisition rates ranged from 7.6% to 51.5% in the chlorpyrifos-treated honey bees from the second trial to the sixth trial 24 h after exposure. By contrast, the acquisition rates ranged from 33.8% to 74.6% in the acetone-treated honey bees. The chlorpyrifos-treated bees significantly differed from the acetone-treated honey bees for each trial (p<0.01) except in the fifth trial (p<0.05; Fig 3B). The retention capacities were significantly higher in the acetone-treated honey bees than in the chlorpyrifos-treated honey bees 2 h after the training test was completed (p<0.01). Conversely, the retention capacities between the acetone-treated honey bees and the chlorpyrifos-treated honey bees were not significantly different 24 h after the test was completed (Fig 3D).
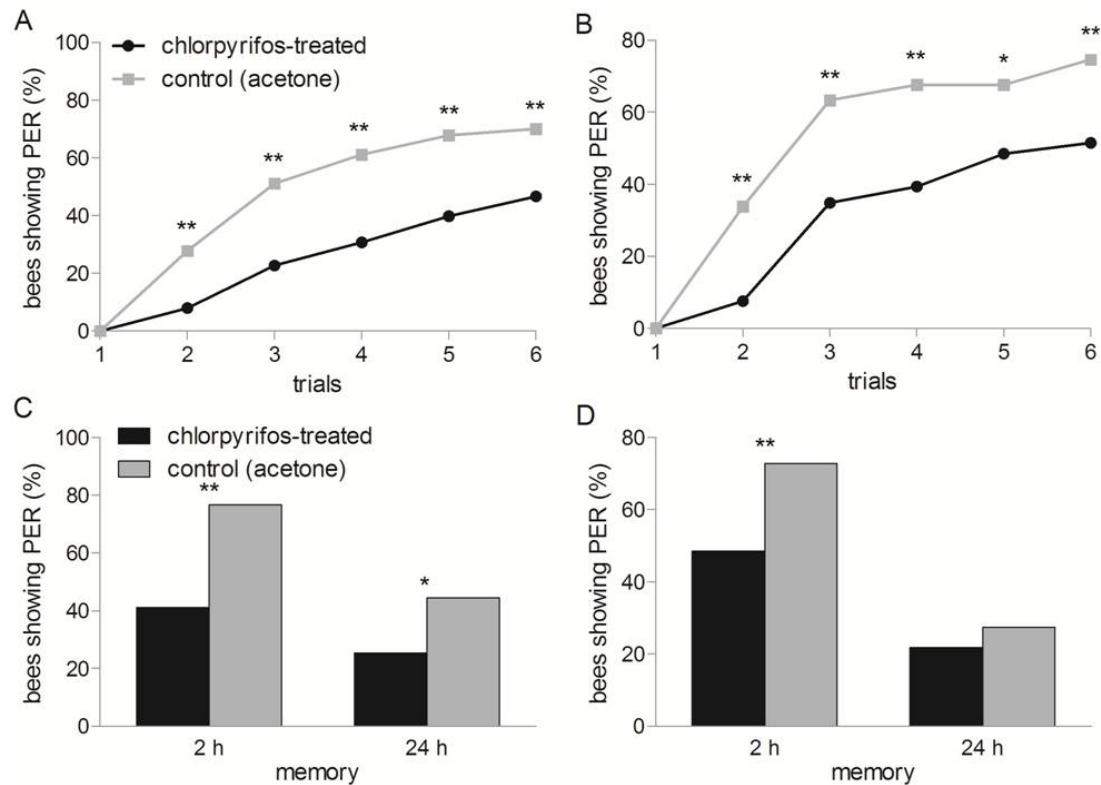

Fig 3. Sublethal doses of chlorpyrifos impair learning and memory performances in *A. cerana*. Learning performances were significantly impaired in chlorpyrifos treated *A. cerana* honey bees after 2 h (A), and 24 h (B) exposure. Among honey bees exposed for 2 h, honey bees showed significantly impaired memory performances 2 and 24 h after six training trials (C); among honey bees exposed for 24 h, honey bees showed significantly impaired memory performances 2 h, but not 24 h after six training trials (D). Control bees received acetone. Asterisks denote significant differences: one, p<0.05; two, p<0.01.

Discussion

The sucrose responsiveness of the control *A. mellifera* and *A. cerana* revealed that the percentage of *A. cerana* showing PER ranged from 45% to 88%. By contrast, the percentage of *A. mellifera* exhibiting PER ranged from 68% to 100%. Consistent with previous studies (Yang et al., 2013), our study revealed that *A. mellifera* was more responsive to sucrose than *A. cerana*. In the six consecutive acquisition trials, both honey bee species were unresponsive to CS in the first acquisition trial, indicating that there were no sensitization responses in the honey bees. The acquisition rates of control *A. mellifera* ranged from 74% to 84%. By comparison, the acquisition rates of control *A. cerana* ranged from 28% to

70%. *A. cerana* possibly required more time to acclimate to the experimental confinement used in the PER assay and thus might influence the behavioral motivation of *A. cerana* during the sucrose responsiveness test and olfactory learning test.

The insecticide is one of the factors responsible for the impaired homing abilities, defective immune responses, delayed larval development, reduced adult bee longevity, and *A. mellifera* colony collapse (Wu et al., 2011; Henry et al., 2012; Di Prisco et al., 2013). Honey bees were fed individually with test concentrations of chlorpyrifos, the real dose administration in each honey bee can therefore be obtained in the study. In our cage studies, *A. cerana* is more sensitive to oral acute toxicities of chlorpyrifos than *A. mellifera*. However, there are few reports of collapse due to applications

of insecticides in *A. cerana* colonies (Park et al., 2015). This finding may be correlated with differential physiological and behavioral characteristics between the two honey bee species. The responses to higher or lower concentrations of sucrose solutions between chlorpyrifos-and acetone-treated honey bees were not significantly different. This finding suggested that sublethal chlorpyrifos concentrations may not damage the gustatory receptors of the antennae in honey bees. Chlorpyrifos-treated *A. mellifera* exhibited a significantly lower response to 10% sucrose solution than the corresponding control bees did. Sublethal chlorpyrifos concentrations may induce a dynamic disorder in the normal function of the gustatory receptors of *A. mellifera*. Therefore, this pesticide may elicit a dynamic response to a moderate sucrose concentration (10% sucrose solution).

Nicotinic acetylcholine receptors (nAChRs) in the brain of honey bees participate in the olfactory learning and memory performances of honey bees (Jones et al., 2006). Acetylcholine (ACh) is a potent neurotransmitter during this process (Goldberg et al., 1999). Chlorpyrifos, an important organophosphate, exert its mode of action by inhibiting AChE activity (Williamson et al., 2013). This enzyme cannot hydrolyze ACh and terminate excitatory neurotransmission in the central nervous systems of forager bees exposed to sublethal chlorpyrifos concentrations (Palmer et al., 2013). Therefore, the acquisition and retention capacities of chlorpyrifos-treated forager bees were impaired. Chlorpyrifos oxon is a chlorpyrifos metabolite more potent than the parent compound in terms of inhibiting AChE activities (Williamson et al., 2013). Acquisition tests were performed 2 and 24 h after the honey bees were exposed to chlorpyrifos in the test. In our study, chlorpyrifos oxon possibly impaired the acquisition capacities of honey bees after 24 h of exposure. Chlorpyrifos oxon may also elicit adverse effects on the recall process 24 h after the acquisition tests were completed. On the basis of these findings, we concluded that sublethal chlorpyrifos concentrations significantly impaired the olfactory learning abilities and 2 h memory retention of honey bees regardless of species. Associative recall in honey bees can be used as an alternative strategy for navigation and successful search for food resources (Reinhard et al., 2004). Therefore, impaired associative recall performances may affect the successful foraging of honey bees exposed to chlorpyrifos and may eventually influence the growth and survival of honey bee colonies. In this study, the retention capacities of chlorpyrifos-treated *A. cerana* and acetone-treated bees did not significantly differ 24 h after the six trials which were performed 24 h after exposure to chlorpyrifos. This result is possibly due to the differential effects of chlorpyrifos on the physiological and molecular processes underlying the learning and memory functions of the two honey bee species.

Acknowledgements

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