

Enantioselective Olfactory Effects of the Neonicotinoid Dinotefuran on Honey Bees (*Apis mellifera* L.)

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ABSTRACT: Sublethal exposure to neonicotinoids affects honey bee olfaction, but few studies have investigated the sublethal effects of the enantioselective neonicotinoid dinotefuran on honey bee olfaction. This study assessed the sublethal olfactory toxicity of dinotefuran enantiomers to honey bees. Compared to R-dinotefuran, S-dinotefuran had higher acute oral toxicity, sucrose sensitivity effects, octopamine concentrations, lower learning ability, and memory effects on honey bees. High-throughput circular RNA sequencing of the honey bee brain revealed that R-dinotefuran caused more gene regulatory changes than S-dinotefuran. Gene ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathway analyses demonstrated that the SERCA, Kca, and Maxik genes may be related to the enantioselective effects of dinotefuran isomers on honey bee olfaction. These results indicated that the current ecotoxicological safety knowledge about chiral dinotefuran effects on honey bees should be amended.

KEYWORDS: *dinotefuran, honey bees, sublethal olfaction effects, enantioselective toxicity*

1. INTRODUCTION

Honey bees are an indispensable member of natural ecological communities; they provide substantial economic, social, and ecological benefits; they are pollinators of flowering plants; and their pollination activities have increased the amount of researcher attention to foraging.^{1,2} Some evidence suggests that neonicotinoids (thiamethoxam, clothianidin, imidacloprid, and dinotefuran) affect the nontargeted honey bees by binding to nicotinic acetylcholine receptors (nAChRs) to affect nervous system information transmission.^{3–6} The effects of sublethal doses of neonicotinoid insecticides on honey bee olfaction behavior, such as their sensitivity to sucrose as well as their ability to learn and remember, have also been studied by proboscis extension reflex (PER).^{7–10} Sensitivity to sucrose and the ability to learn and remember are related to honey bee olfaction, which may contribute to honey foraging by helping bees to distinguish among different odor trail information. Octopamine was shown to trigger an increase in a specific subset of dopaminergic neurons in the mushroom body, the direct activation of which can substitute for sugar to form appetitive olfactory memories in honey bees; thus, the concentrations of octopamine in the mushroom body are associated with honey bee olfactory memory.¹¹ Chronic exposure to neonicotinoids was found to change gene transcription.¹² In addition, transcriptomic analyses revealed that foraging memories are related to brain gene expression. Because of the negative impacts of these pesticides on insect pollinators, the European Union have banned the use of three neonicotinoid (thianmethoxam, clothianidin, and imidacloprid) on all crops grown outdoors.¹⁴ When foraging, honey bees can be exposed to dinotefuran, which may be found in bee pollen or syrup at concentrations ranging from 0.03 to 0.1 ng/g.^{15,16} However, until now, there have been no studies on the olfactory effects of dinotefuran on honey bees.

The chiral neonicotinoid insecticide dinotefuran has an asymmetrically substituted carbon (C) atom and consists of a pair of enantiomers, and it is used to control the growth of *Delphacidae*, *Aphidoidea*, *Cicadellidae*, stink bugs, and other pests on rice, vegetables, and fruit.^{17–19} The stereoisomers of chiral dinotefuran may exhibit different behaviors in terms of their environmental, physical, and chemical properties, such as bioactivity, toxicity, and metabolism within organisms.^{20–22} Some data have suggested that R-dinotefuran could minimize the hazard to honeybees when dinotefuran is used to control target pests.²³ Consequently, a study on the toxicity of chiral dinotefuran enantiomers to honey bees is required.

The purpose of this study was to investigate the enantioselective effects of dinotefuran on honey bee olfaction. The medial lethal concentrations (LC_{50} s) of rac-, R-, and S-dinotefuran were determined by measuring acute oral toxicity. The sucrose sensitivity, learning ability, and memory of honey bees were evaluated at sublethal doses using the PER method, and then the different effects of R- and S-dinotefuran on honey bees were compared. To explore the enantioselective toxicity effects of the two dinotefurans on honey bee learning and memory retention, the octopamine levels were determined. Moreover, we studied gene expression by using transcriptomics to provide a better understanding of the differences in sublethal R- and S-dinotefuran olfaction effects on honey bees.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents.

Dinotefuran racemate standards (99.8% purity) were purchased from Dr. Ehrenstorfer GmbH

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(Augsburg, Germany); technical dinotefuran racemate (95% purity) was obtained from Jiangsu KWIN Co., Ltd. (Yancheng, China); and the dinotefuran enantiomer was made by Daicel Chiral Technologies (Shanghai, China) Co., Ltd. Geraniol (enantiomeric excess 99%) and octopamine hydrochloride (0.1 g) standards were obtained from ANPEL Co., Ltd., Shanghai, China. The purity of the enantiomers was measured by Daicel Chiral Technologies (Shanghai, China) Co., Ltd., and the configuration (*R* and *S*) of the dinotefuran enantiomers was based on the optical rotation (+ and −) as reported by Kiriyama.²⁴ The specific rotation of the two enantiomers was determined at Guangxi Boce Test Technical Services Co., Ltd. Acetonitrile, HPLC-grade acetonitrile, and ammonia were purchased from Merck KGaA (Germany). The water was purified using a Milli-Q system from Millipore (Bedford, MA). Stock solutions of *rac*-, *R*-, and *S*-dinotefuran (100 mg L^{−1}) were prepared in water. The standard solutions (50 mg L^{−1}) of *R*- and *S*-dinotefuran were prepared by diluting of the stock solutions (100 mg L^{−1}) with water. The standard solutions were used to examine the optical purity of the dinotefuran enantiomers by ACQUITY UltraPerformance Convergence Chromatography (UPC²) (Waters AMY1_3.0 (150 mm × 2.1 mm, 2.5 μm) column). All solutions were stored at 4 °C. There was no interconversion in the dinotefuran standard solutions, and the solutions did not degrade to their metabolites during the 1-week storage period.

2.2. Honey Bee Preparation. Young and adult honey bee (*Apis mellifera*) workers were collected from three healthy colonies that were maintained at Guangxi University, Nanning, China. Every colony consisted of approximately 6 000 workers and a fertile 1-year-old queen. The bees were adequately fed (50% sugar solution and water) and were not treated with chemical substances. The bees were collected using a plastic bottle and treated with nitrogen until they stopped moving. The immobile bees were immediately transferred to small plastic boxes (250 mL) in groups of 15 individuals and maintained in the dark under controlled conditions (29 ± 1 °C and 60 ± 10% relative humidity) for 24 h.

2.3. Acute Oral Exposure. The acute oral exposure experiment was performed based on the guidelines from the Organization for Economic Co-operation and Development.²⁵

Preliminary experiments were carried out to determine the dinotefuran concentrations that produced 0–100% mortality at 48 h after exposure. After acute oral preliminary experiments, six desired concentrations of dinotefuran treatments and a control treatment for every tested chemicals (*R*-dinotefuran, *S*-dinotefuran, and *rac*-dinotefuran) were used for the oral exposure. The six desired concentrations of *S*-dinotefuran, *R*-dinotefuran, and *rac*-dinotefuran were 0.10, 0.17, 0.28, 0.48, 0.82, 1.39 mg/kg, 1.70, 2.89, 4.91, 8.35, 14.19, 24.14 mg/kg, and 0.53, 0.90, 1.53, 2.61, 4.43, 7.54 mg/kg, respectively. Three boxes (15 bees per box) per treatment were used for the oral exposure. The sucrose solution (50%, w/v) contained *rac*-dinotefuran, *R*-dinotefuran, *S*-dinotefuran or solvent alone (control check). The honey bees were then fasted for 2 h and exposed to *rac*-dinotefuran, *R*-dinotefuran, or *S*-dinotefuran for 6 h. According to the test guidelines for environmental safety assessments of chemical pesticides in China, 10 bees should be treated with 100–200 μL source solutions, so 300 μL of source solutions were needed for our acute toxicity experiment. During the 6 h test, if 300 μL of dinotefuran-contained solutions was consumed, the feeder solutions were replaced with a sufficient sucrose-only solution. In addition, the weight of the treatment diet per test group was recorded before being administered and at the end of the exposure. The mortality of the bees was recorded after 24 and 48 h. The weight of the dinotefuran consumed by the bees was evaluated by measuring the weights of the treated solutions and the dose in μg of active ingredient/bee during the mortality analysis. Then, the mortality curves and the LD₅₀ values of each insecticide at 24 and 48 h postexposure, respectively, were created. The experiment was repeated three times, and it was assumed that each bee only consumed 20 μL of the solutions to calculate the average LC₅₀ values.

2.4. Sucrose Sensitivity. The proboscis extension reflex (PER) test was used to evaluate the sucrose sensitivity levels of the bees according to the method reported by Alkassab et al.²⁶

Here, the sensitivities of the sample honey bees to solutions containing different sucrose concentrations were measured after each bee was fed 20 μL of *rac*-dinotefuran, *R*-dinotefuran, *S*-dinotefuran at LC₅ (0.13 mg/kg, 1.89 mg/kg, and 0.06 mg/kg), LC₂₀ (0.43 mg/kg, 3.60 mg/kg, and 0.12 mg/kg), or LC₃₀ (0.68 mg/kg, 4.65 mg/kg, and 0.17 mg/kg) or only the solvent (control check). The sucrose sensitivity of the honey bees was determined at 24 h after a 6 h exposure. During the 6 h period, if the dinotefuran-contained solutions were consumed, the feeder solutions were replaced with sufficient sucrose-only solution. Before the experiment, the bees were starved for 2 h to adapt them to the environment. Then, the honey bee antennae were stimulated at 3 min intervals with sucrose solutions of increasing concentrations at 0 (water), 10%, 30%, and 50% (w/v) (*n* = 50). The PERs of the bees were recorded in response to the sucrose stimulation for each treatment. The experiment was repeated three times.

2.5. Olfactory Learning and Memory Assays. The experimental procedures were based on the work of Alkassab et al.²⁶ PER assays were used to study learning and memory in worker honey bees after their exposure to *rac*-dinotefuran, *R*-dinotefuran, *S*-dinotefuran or a pure sucrose solution. Exposures to sublethal doses of pesticide were set up by adding *rac*-dinotefuran, *R*-dinotefuran, *S*-dinotefuran (LC₅: 0.13 mg/kg, 1.89 mg/kg, 0.06 mg/kg or LC₂₀: 0.43 mg/kg, 3.60 mg/kg, 0.17 mg/kg) or solvent only (control) to sucrose solutions (50%, w/v). Then, the solutions were fed to adult workers for 6 h. There were 20 bees per treatment, and each treatment was repeated five times. During the 6 h period, if 20 μL/bee of dinotefuran-contained solutions was consumed, the feeder solutions were replaced with sufficient sucrose-only solution. The learning ability and memory of the honey bees was tested after 2 and 24 h. Before the test, the bees were starved for 2 h to adapt them to the environment, and then they were mounted individually in glass tubes with only their antennae and mouthparts left free.

The honey bees were trained during two trials (each trial contained 30 bees, and the 60 test bees were selected from 100 treated honey bees) with 20–30 min intertrial intervals. Three experiments were repeated per treatment. The bees were subjected to five test trials (the first to fifth time points) in which the conditioned stimulus (CS) was provided. Bees that responded to the CS before the first time point were not included in the experiment to follow. A sucrose solution (50%, w/v) was used as the unconditioned stimulus (US), and a geraniol odor was used as the CS.²⁷ A filter paper (4 cm²) was soaked with a drop of geraniol (10 μL) and placed in a glass Pasteur pipet. During each trial, a commanded solenoid valve was used to control a 6 s pulse of airflow into the pipet, which was guided toward the bee's antennae. After a 4 s pulse of geraniol odor, the bees were rewarded with a sucrose solution for 3 s. In addition, because of the possibility of mechanosensory stimulation from the changing airflow, a gas cylinder provided a constant flow of air through an additional glass Pasteur pipet to the bee's antennae, and the same air pumps were used at the same flow rate approximately 30 s before and after the CS pulse. During this experimental procedure, the bees that responded positively in the first time were not further tested the following times. The number of trained honey bees that responded to the geraniol odor during the 4 s pulse was recorded, and the number was totaled every test time to follow. The entire experiment was repeated three times.

2.6. Octopamine Collection and Analysis. The role of octopamine in honey bee learning was investigated in reference to the method reported by Rix.²⁸ The method for hemolymph octopamine collection was modified from that of Bateson et al.²⁹ At 2 or 24 h after treatment (the treatment concentrations were LC₂₀ s of *R*-, *S*-, and *rac*-dinotefuran), the bees were treated with nitrogen until immobile. Then, hemolymph was collected from 5 to 10 bee heads per treatment using flame-stretched capillary tubes. This Hemolymph (10 μL) was collected from a puncture made in the head capsule near the median ocellus, and it was immediately placed in a micro-

Table 1. Acute Toxicity of Dinotefuran in Honey Bee at 24 and 48 h after Treatment

		LD ₅₀ ($\mu\text{g}/\text{bee}$)	LC ₅₀ (mg/L)	95% fiducial limits	slope \pm SE	χ^2
S-dinotefuran		0.11	0.29	0.20–0.42	2.27 \pm 0.53	0.25
R-dinotefuran	24 h	3.08	7.71	5.40–10.04	2.87 \pm 0.60	1.06
rac-dinotefuran		0.59	1.48	0.65–3.35	1.56 \pm 0.53	0.01
S-dinotefuran		0.11	0.28	0.20–0.39	2.38 \pm 0.54	0.22
R-dinotefuran	48 h	2.57	6.42	5.00–8.62	3.24 \pm 0.64	1.83
rac-dinotefuran		0.51	1.28	0.82–5.65	1.65 \pm 0.53	0.25

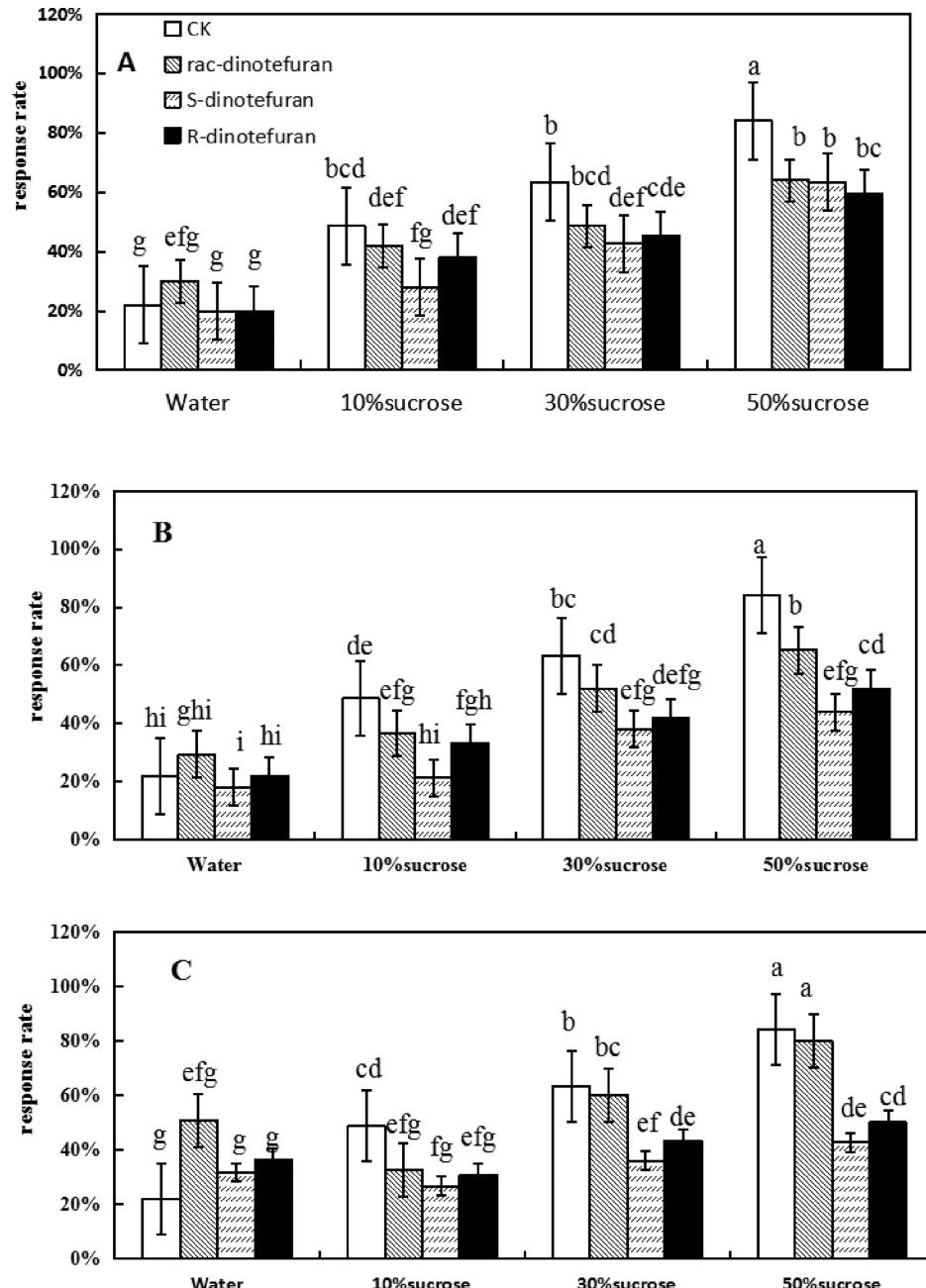


Figure 1. Beak stretch response rates of honey bees treated with different concentrations of rac-, R-, and S-dinotefuran: (A) LC₅, (B) LC₂₀, and (C) LC₃₀. The data represent the means \pm standard deviations. Data with different letters are significantly different ($p < 0.05$).

centrifuge tube that contained 1 mL of 0.1% perchloric acid. The solutions were centrifuged at 7100g for 5 min at 4 °C. The supernatant was collected and stored in amber-colored scintillation vials at 4 °C until analysis.

The hemolymph octopamine concentrations were determined using HPLC-UV (high-performance liquid chromatography-ultra-

violet and visible spectrum) with a C-18 column and a Waters 2489 UVD (ultraviolet/visible detector) set to 270 nm. The mobile phase consisted of 7:3 (v/v) 0.02 M citric acid and 0.02 M sodium dihydrogen phosphate-acetonitrile.

2.7. RNA Sequencing and Analysis. A total of 100 randomly chosen adult worker bees were placed inside 10 bottles (10 bees per

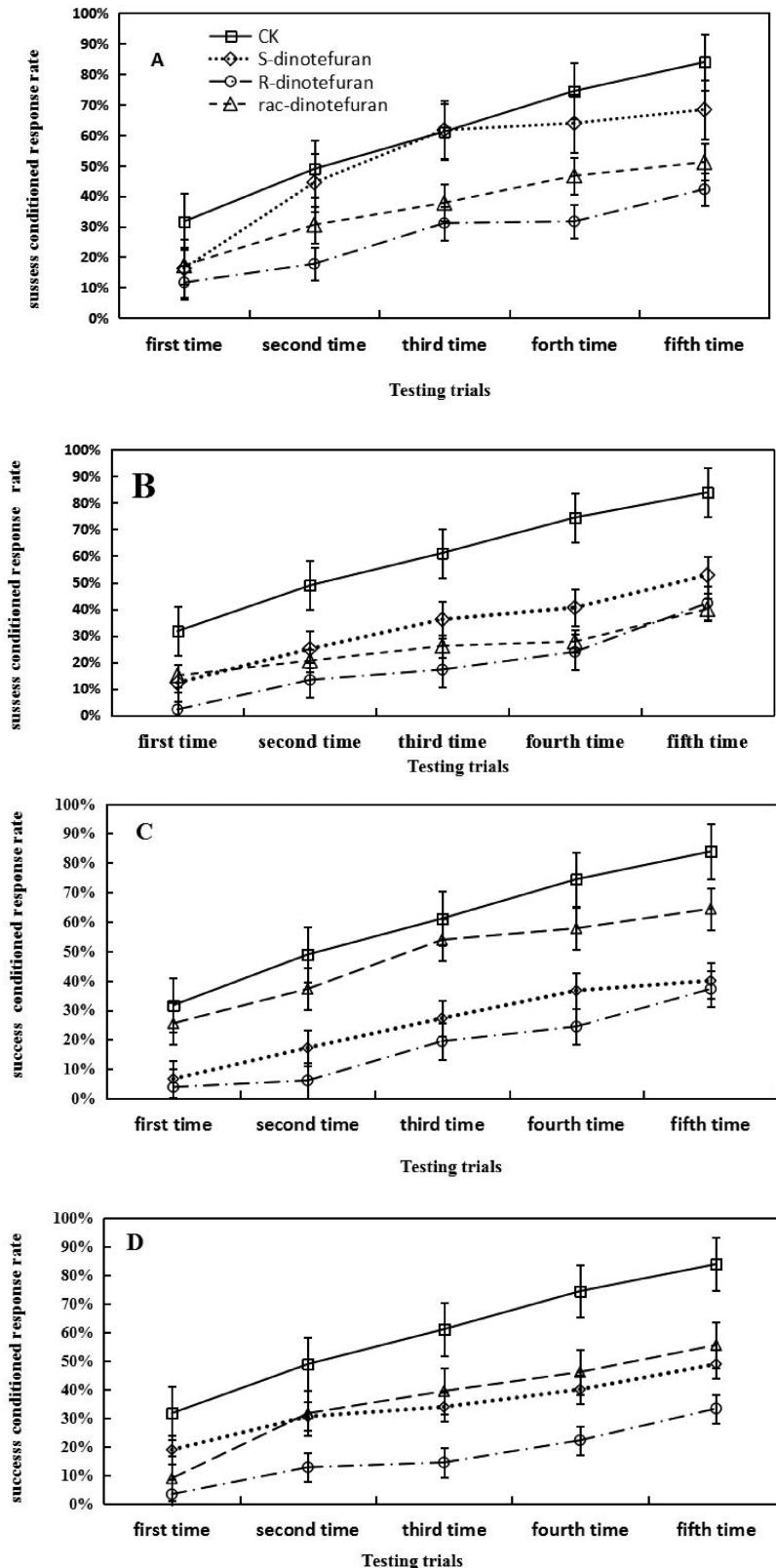


Figure 2. Learning curve of honey bee. Successful conditioned response rates for honey bee learning and memory retention. (A and B) LC₅ at 2 and 24 h, respectively, (C and D) LC₂₀ at 2 and 24 h, respectively. The data represent the means \pm standard deviations.

bottle) and placed in an appropriate environment (25–28 °C, 50–70 humidity) for every tested groups. The bees were fed *rac*-, *R*-, and *S*-dinotefuran at LC₂₀ concentrations for 6 h. The heads of honey bees among the treated bees that were still alive at 24 h after the exposure were collected for a transcriptomic analysis. Every dinotefuran

treatment group had a minimum of 60 live bees. There were three biological replicates each for the control and dinotefuran-treated groups. The total RNA from the honey bee heads were extracted by the GENEWIZ Company (Suzhou, China), and RNA quality tests were conducted using an Agilent Eukaryote Total RNA (Agilent

2100). The rRNA (rRNA) was then removed with Ribo-Zero rRNA Removal Kits, and an RNA library was constructed using a NEBNext Ultra Directional RNA Library Prep kit (E7420L). The library was purified using an AxyPrep Mag PCR Clean-up Kit, and the purity was determined using an Agilent High Sensitivity DNA Kit (Agilent 2100). The RNA concentration was evaluated by Qubit and Agilent High, and RNA was enriched using a TruSep PE Cluster Kit V4 in cBot. Then, Illumina HiSeq sequencing was conducted using a Truseq SBS Kit v4-HS.

The raw reads were preprocessed to remove low-quality regions and adapter sequences. The reads with these fragments and base quality values below 20 were removed (quantified by Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, CA) with Cutadapt (version 1.9.1). Clean data were mapped to the *A. mellifera* genome (version Amel_4.5) from NCBI (National Center for Biotechnology Information) and aligned to a reference genome using the software BWA (Burrows-Wheeler Alignment, version 0.7.12). CircRNA was identified with CIRI software (v 2.9), and the junction reads at the back-splicing loci of circRNA were used to calculate the expression. The DESeq Bioconductor package (<http://www.bioconductor.org>), a model based on the negative binomial distribution, was used to analyze the differential expression. To control the false discovery rate, *p*-values were set at <0.05 to indicate differentially expressed genes.

To identify differentially expressed genes, the Gene Ontology (GO) tool TermFinder was used with a significant *p*-value of less than 0.05. House scripts (GENEWIZ, Inc.) were used to enrich for significantly differentially expressed genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

2.8. Statistical Analyses. Statistical analysis of acute oral exposure was performed using the SPSS 17.0 software (Statistical Package for Social Sciences). The mortality ratio (the ratios of surviving honeybee numbers to total experimental honeybee numbers) was corrected using solvent control mortality in the Abbott formula. The log-consumed dinotefuran quality to mortality ratio allowed for the determination of the LD₅₀ values in the honey bees according to a probit analysis. The LC₅₀ was calculated on the basis of the LD₅₀, with the assumption that 20 μL sugar solutions were fed to every honeybee. Differences between the control and treatments were analyzed using the least significant difference (LSD) test. Significance was established at *p* < 0.05.

3. RESULTS

3.1. Acute Oral Toxicity. The acute LD₅₀ value obtained at 24 h after oral exposure to S-dinotefuran was 0.11 μg/bee, which was the lowest value among the three dinotefuran LD₅₀ values, while there was not a significant change in the acute LD₅₀ values after 48 h of S-dinotefuran treatment (0.11 μg/bee). Although the LD₅₀ values of the R-dinotefuran-treated groups decreased from 3.08 μg/bee to 2.57 μg/bee from 24 to 48 h, the highest LD₅₀ values were found among these treated groups (Table 1). The LD₅₀ values of rac-dinotefuran was between that of S-dinotefuran and R-dinotefuran, at 0.59 μg/bee at 24 h and 0.51 μg/bee at 48 h. The LD₅₀ values exhibited a decreasing trend over time in the 48-h and 24-h exposure groups.

In this study, we estimated the LC₅₀ according to the LD₅₀ values with the assumption that each bee consumed 20 μL of solution during their exposure. The LC₅₀ values were used to calculate the LC₅, LC₂₀, and LC₃₀ values of dinotefuran for the following experiment. The enantioselective toxicity effects of R- and S-dinotefuran in honey bees were observed during the experiments, and the LC₅₀ values of S-dinotefuran were lower than those of R-dinotefuran at 24 and 48 h.

3.2. Sucrose Responsiveness. Preliminary experiments using three different concentrations of each pesticide were performed to determine the sublethal concentrations for the sucrose responsiveness experiments. Based on the results of the

preliminary experiment, the LC₅, LC₂₀, and LC₃₀ doses were selected for the subsequent sucrose responsiveness experiment. The enantioselective toxicity of dinotefuran on honey bee sucrose responsiveness was calculated by measuring the beak stretch rate, which is dependent on the honey bee PER. The beak stretch rate is the number of responding bees divided by the total test bees during the PER. The higher the beak stretch rate is, the lower the inhibition effects of dinotefuran on honey bees.

The beak stretch rates of the control group in the 10%, 30%, and 50% sucrose solutions were 49%, 63%, and 84%, respectively. The same trend was observed in the groups treated with rac-dinotefuran, S-dinotefuran, and R-dinotefuran; the beak stretch rates increased with the increasing sucrose concentrations. It was observed that sublethal doses of rac-dinotefuran, S-dinotefuran, and R-dinotefuran reduced the response rates of honey bees to solutions containing different sucrose concentrations (Figure 1A–C) (*p* < 0.05). The beak stretch rates for rac-dinotefuran were greater than those for R- and S-dinotefuran. At the oral exposure concentrations, the average beak stretch rates of the honey bees treated with rac-, R-, and S-dinotefuran decreased as the pesticide concentration increased.

At the LC₅ concentration, the rac-dinotefuran-treated bees exhibited the highest beak stretch rates, and the R-dinotefuran-treated group rates were higher than those of the S-dinotefuran treated groups when the sucrose concentration was below 50%. When the sucrose concentration was 50%, the values for the R-dinotefuran-treated groups were lower than those for the S-dinotefuran-treated groups, and there was no significant difference between these two treatment groups. The same trend was observed in the treated groups when the dinotefuran concentrations were LC₂₀ and LC₃₀, and rac-dinotefuran-treated bees had the highest beak stretch rates followed by the R-dinotefuran and S-dinotefuran-treated groups.

The effects of the enantioselective toxicity of R- and S-dinotefuran were observed in the experiments; S-dinotefuran led to greater inhibition of honey bee sucrose responsiveness than did R-dinotefuran, except for the LC₅ concentration in the 50% sucrose solution.

3.3. Olfactory Learning and Memory Assays. Based on the results of the preliminary experiment, the LC₅ and LC₂₀ doses were selected for the subsequent sucrose responsiveness experiment. Bees were exposed only to the applied geraniol, and the presence of the PER was considered a successful response. In this study, the success rate of bee responses to the CS was used to assess the honey bee's learning ability and memory. In Figure 2A–D (*p* < 0.05), the olfactory learning performance is represented as the percentage of conditioned PER during the testing phase in bees that were fed the two concentrations of each pesticide and in control honey bees that were fed sucrose only. Dinotefuran had a significant effect on PER-based learning success, with an 83% success rate in the control group and a success rate below 70% in the dinotefuran-treatment groups.

A reduction in the olfactory learning performance was noted in honey bees during the conditioning trials. The success rates (fifth time point) of the LC₅ and LC₂₀ trials for rac-dinotefuran were 51% and 64% after 2 h, and the values reduced to 40% and 56% after 24 h. The success rates after 2 and 24 h (fifth time point) of the LC₅ R-dinotefuran treatment were both 42%, which are greater than the success rates of the LC₂₀ R-dinotefuran treatment (fifth time point) (37% after 2 h and

33% after 24 h). R-dinotefuran produced a greater negative influence on learning and memory in honey bee than S-dinotefuran as reflected by the success rate values of S-dinotefuran in the LC₅ and LC₂₀ trial results of 68%, 40% after 2 h and 53%, 49% after 24 h, respectively.

3.4. Octopamine Levels in Honey Bees. Dinotefuran had a significant effect on hemolymph octopamine concentrations. The octopamine concentrations in dinotefuran-exposed honey bees were lower than those in the control (Table 2). In the control treatment, the octopamine

Table 2. Hemolymph Octopamine Concentrations of Dinotefuran-Treated Honey Bee Groups

	2 h (mg/L)	24 h (mg/L)
control	0.23 ± 0.015	0.24 ± 0.010
S-dinotefuran	0.18 ± 0.005	0.19 ± 0.010
R-dinotefuran	0.15 ± 0.020	0.14 ± 0.010
rac-dinotefuran	0.21 ± 0.015	0.20 ± 0.010

concentrations in the honey bee hemolymph were 0.23 mg/kg and 0.24 mg/kg after 2 and 24 h, respectively. The octopamine concentrations in the dinotefuran-treatment groups (0.18–0.21 mg/kg at 2 h and 0.14–0.20 mg/kg at 24 h) were lower than in the control group. Different effects of R- and S-dinotefuran on the octopamine levels were also observed, but there were no significant differences in octopamine concentrations at 2 and 24 h after honey bees were treated with dinotefuran. The octopamine concentration in the heads of honey bees treated with S-dinotefuran (0.18–0.19 mg/kg) was 1.36-fold greater than that in honey bees exposed to R-dinotefuran (0.14–0.15 mg/kg) ($p = 0.038$, $p < 0.05$). In addition, there were no significant differences in the octopamine concentrations between the rac-dinotefuran and S-dinotefuran treatment groups ($p = 0.152$, $p > 0.05$).

3.5. Transcriptomic Responses to Sublethal rac-, R-, and S-Dinotefuran Concentrations. Circular RNAs (circRNAs) are a large class of noncoding molecules observed over a diverse range of organisms; they have been found in brain tissues and may participate in various biological processes.^{30,31} Three biological replicates were performed using RNA-seq (circular RNA sequencing) of samples from the treatment and control groups. The average length of

transcriptome sequences from a sample of mixed honey bee brains was 149 Mbp. The number of clean reads obtained from the 12 libraries for rac-, R-, and S-dinotefuran and the control after filtering and mapping them to the honey bee genome (*A. mellifera* genome version Amel_4.5 downloaded from NCBI) was 88–113 Mbp. The average total mapped reads after filtering of the control, S-, R-, and rac-dinotefuran treated groups were approximately 81, 80, 85, and 88 Mbp, respectively (Table 3). We then used a *p*-value ≤ 0.05 and an absolute value for fold changes of >1.5 as the criteria for expressed genes. There were 11 upregulated and 7 downregulated genes between the S-dinotefuran-treated and control groups, 10 upregulated and 13 downregulated genes between the R-dinotefuran-treated and control groups, and 13 upregulated and 21 downregulated genes between rac-dinotefuran-treated and control groups (Figure 3).

To understand the functions of these differently expressed genes, GO term enrichment analysis of circRNA was performed. For the up- and downregulated genes, the most significantly enriched GO term was “catalytic activity” in the molecular function (Figure 4). KEGG analysis (Table 4) found that, among the three treated groups, the most enriched terms were in the cGMP-PKG signaling pathway, axon guidance, and pancreatic secretion.

Our study shows that the cGMP-PKG signaling pathway (pathway ID, Ko04022) in environmental information processing and axon guidance (pathway ID, ko04360) and pancreatic secretion (pathway ID, Ko04972) in organismal systems were the important terms that were enriched in the KEGG pathway analysis. There were differences between the S-dinotefuran and R-dinotefuran treatments; the same pathway-regulated genes were different, although the pathway IDs were the same. For the Ko04022 pathway (Figure 5), the secretion gene (SERCA gene) in the S-dinotefuran-treated groups was upregulated (Figure 5a), while the upregulated gene in the S-dinotefuran-treated groups (Figure 5b) was the K⁺-channel gene (Kca gene), which was downregulated in the rac-dinotefuran-treated groups (Figure 5c). The Ablim gene in the Ko04360 pathway (Figure 6) was downregulated in the S-dinotefuran (Figure 6a) and R-dinotefuran-treated groups (Figure 6b), although this gene was upregulated in the rac-dinotefuran treatments (Figure 6c). Regarding the Ko04972

Table 3. Clean Dinotefuran-Treated Honey Bee Library Reads Compared with Reference Genomes

samples ^a	total reads ^b	total mapped ^c	multiple mapped ^d	uniquely mapped ^e	reads mapped in proper pairs ^f
S-dinotefuran-1	96725699	8236944 (85.1578%)	14053597	68315847 (70.6284%)	62341559
S-dinotefuran-2	91899223	79539161 (86.5504%)	11713776	67825385 (73.8041%)	61962933
S-dinotefuran-3	87346975	78382689 (89.7372%)	8724717	69657972 (79.7486%)	64408677
R-dinotefuran-1	92564614	80327726 (86.7802%)	9985083	70342643 (75.993%)	64711295
R-dinotefuran-2	113386807	101191838 (89.2448%)	11147700	90044138 (79.4132%)	83270860
R-dinotefuran-3	94242663	83244587 (88.33%)	10045478	73199109 (77.6709%)	67249864
rac-dinotefuran-1	92668368	81865286 (88.3422%)	10260902	71604384 (77.2695%)	66003147
rac-dinotefuran-2	97582500	86968093 (89.1226%)	10371404	76596689 (78.4943%)	70588207
rac-dinotefuran-3	97374490	85092574 (87.3869%)	10677694	74414880 (76.4213%)	68356164
CK-1	92746720	80730955 (87.0445%)	9657187	71073768 (76.6321%)	65441845
CK-2	97084613	85446930 (88.0128%)	9319095	76127834 (78.4139%)	70420042
CK-3	88700139	77009034 (86.8195%)	8654204	68354830 (77.0628%)	63109570

^aSamples were treated with LC₂₀ concentrations of rac-, R-, and S-dinotefuran and control check (CK) groups. ^bThe number of quantitative statistics after sequence filtering. ^cThe number of reads located on the genome. If there is no contamination of the genome, then the total mapped percentage should be greater than 70%. ^dThe number of reads with multiple alignment positions on the reference sequence. ^eThe number of reads with unique alignment positions on the reference sequence. ^fThe number of reads in proper pairs when the sequence is aligned to the chromosome.

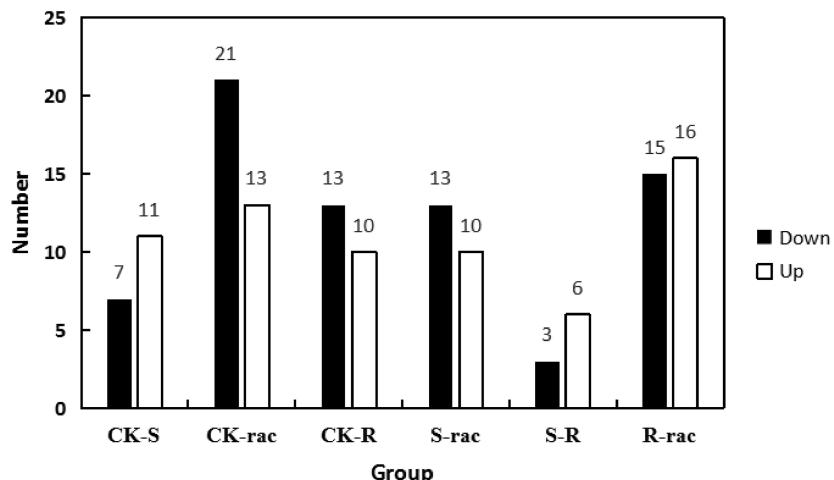


Figure 3. Number of significantly (p -value ≤ 0.05 and fold changes >1.5) up- and downregulated circRNAs for differentially expressed unigenes of *A. mellifera* treated with dinotefuran enantiomers compared with one another or the control. (CK-S is the control check groups versus the S-dinotefuran/treated groups, CK-rac is the control check groups versus the rac-dinotefuran-treated groups, CK-R is the control check groups versus the R-dinotefuran-treated groups, S-rac is the S-dinotefuran-treated groups versus the rac-dinotefuran-treated groups, S-R is the S-dinotefuran-treated groups versus the R-dinotefuran-treated groups, and R-rac is the R-dinotefuran-treated groups versus the rac-dinotefuran-treated groups.)

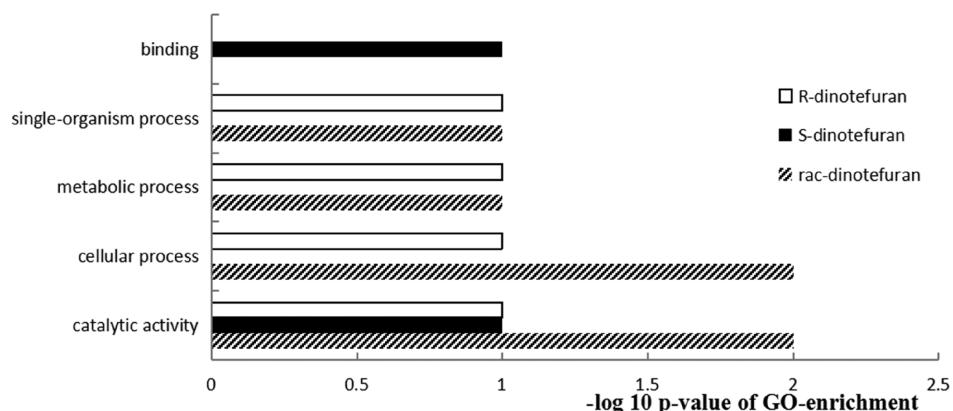


Figure 4. Enriched GO terms of circRNA for differentially expressed unigenes from the brains of *Apis mellifera* L. between the control and dinotefuran-treated groups.

pathway (Figure 7), the SERCA gene was upregulated in the S-dinotefuran treatments (Figure 7a), the voltage-activated potassium channels gene (Maxik gene) was upregulated in the R-dinotefuran treatments (Figure 7b), and the Maxik gene was downregulated in the rac-dinotefuran treatments (Figure 7c).

4. DISCUSSION

Honey bees rely on olfaction to distinguish among pollen types and for learning and memory-making about foraging. Dinotefuran, a type of neurotoxic pesticide, is highly toxic to honey bees.³² In this study, the LD₅₀ value of rac-dinotefuran was 0.59 µg/bee, which was similar to previous reports that also noted its high toxicity to honey bees. Differences between the toxicity levels of S-dinotefuran and R-dinotefuran occurred, with S-dinotefuran being 22.9-fold more toxic than R-dinotefuran at 48 h. The dinotefuran racemate in this study, which consisted of a 1:1 mixture of S-dinotefuran and R-dinotefuran, had LC₅₀ values between those of S-dinotefuran and R-dinotefuran. The previously reported oral ingestion of LD₅₀ values in honey bees treated with rac-dinotefuran was 0.328 µg/bee, and the S-dinotefuran was 41.1- to 128.4-fold more toxic than R-dinotefuran and rac-dinotefuran to honey

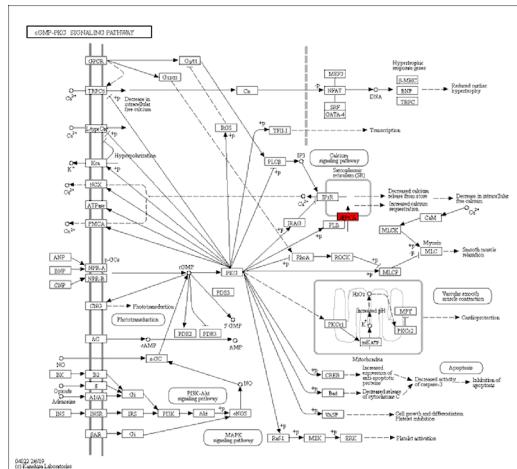
bees.²³ A consistent order of toxicity to *Apis mellifera* between this study and a previous study was as follows: S-dinotefuran>rac-dinotefuran \gg R-dinotefuran. A previous study about the molecular docking of dinotefuran enantiomers showed that S-dinotefuran binds the α 8 subunit of nAChR of *Apis mellifera* with more hydrogen bonds than does R-dinotefuran.²³ Thus, the toxicity of racemic dinotefuran cannot be simply attributed to S-dinotefuran or R-dinotefuran toxicity alone, and there is a certain degree of antagonism between the two enantiomers.

Sucrose responsiveness is a sensory function of honey bee olfaction, which is necessary for honey bees to make foraging decisions. In addition, sucrose-triggered PER and olfactory-conditioned PER can be used to assess the sublethal effects of pesticides on sucrose sensitivity. Under laboratory conditions, high levels of neonicotinoids may kill honey bees, while sublethal concentrations of neonicotinoids may affect honey bee behavior. The sublethal effects of clothianidin on the studied behavioral functions, including the sensory and cognitive functions, indicate that clothianidin has limited effects on honey bees.⁸ Acetamiprid increases the locomotor activity (0.1 and 0.5 µg/bee) and water-induced PER (0.1, 0.5, and 1 µg/bee) in honey bee.³³ Evidence has shown that there

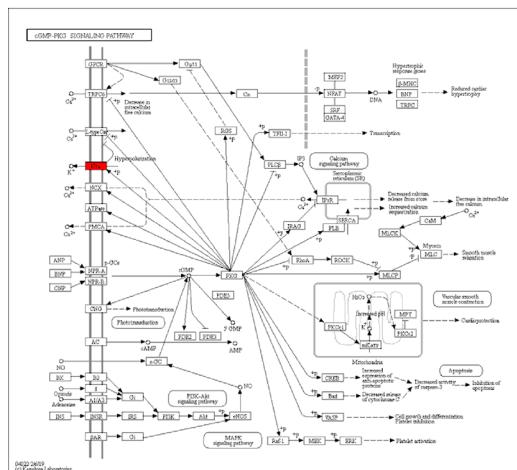
Table 4. KEGG Enrichment Pathway Analysis of the Differentially Expressed Unigenes of Different Dinotefuran-Treated Honey Bee Groups^a

treatment	pathway ID	pathway	category	DEGs with pathway annotation	all genes with pathway annotation
S-dinotefuran	Ko05203	viral carcinogenesis	human diseases	1(25.00%)	67(2.74%)
	Ko04360	axon guidance	organismal systems	1(25.00%)	46(1.88%)
	Ko04922	glucagon signaling pathway	organismal systems	1(25.00%)	35(1.43%)
	Ko01200	carbon metabolism	metabolism	1(25.00%)	70(2.86%)
	Ko00620	pyruvate metabolism	metabolism	1(25.00%)	22(0.90%)
	Ko04972	pancreatic secretion	organismal systems	1(25.00%)	27(1.10%)
	Ko05230	central carbon metabolism in cancer	human diseases	1(25.00%)	22(0.90%)
	Ko00010	glycolysis/gluconeogenesis	metabolism	1(25.00%)	27(1.10%)
	Ko04022	cGMP-PKG signaling pathway	environmental information processing	1(25.00%)	41(1.68%)
	Ko04020	calcium signaling pathway	environmental information processing	1(25.00%)	38(1.55%)
	Ko04930	type II diabetes mellitus	human diseases	1(25.00%)	11(0.45%)
	Ko04744	phototransduction	organismal systems	1(25.00%)	3(0.12%)
	Ko01230	biosynthesis of amino acids	metabolism	1(25.00%)	43(1.76%)
	Ko05010	Alzheimer's disease	human diseases	1(25.00%)	92(3.76%)
	Ko00230	purine metabolism	metabolism	2(50.00%)	100(4.09%)
rac-dinotefuran	Ko00240	pyrimidine metabolism	metabolism	1(11.11%)	66(2.70%)
	Ko04721	synaptic vesicle cycle	organismal systems	1(11.11%)	32(1.31%)
	Ko04970	salivary secretion	organismal systems	1(11.11%)	22(0.90%)
	Ko04724	glutamatergic synapse	organismal systems	1(11.11%)	34(1.39%)
	Ko04911	insulin secretion	organismal systems	3(33.33%)	24(0.98%)
	Ko00500	starch and sucrose metabolism	metabolism	1(11.11%)	17(0.69%)
	Ko04972	pancreatic secretion	organismal systems	1(11.11%)	27(1.10%)
	Ko04022	cGMP-PKG signaling pathway	environmental information processing	1(11.11%)	41(1.68%)
	Ko00220	arginine biosynthesis		1(11.11%)	10(0.41%)
	Ko05220	chronic myeloid leukemia	human diseases	1(11.11%)	21(0.86%)
	Ko05221	acute myeloid leukemia	human diseases	1(11.11%)	13(0.53%)
	Ko00250	alanine, aspartate and glutamate metabolism	metabolism	1(11.11%)	22(0.90%)
	Ko05206	microRNAs in cancer	human diseases	1(11.11%)	44(1.80%)
	Ko04659	Th17 cell differentiation		1(11.11%)	16(0.65%)
R-dinotefuran	Ko04270	vascular smooth muscle contraction	organismal systems	1(11.11%)	30(1.23%)
	Ko04727	GABAergic synapse	organismal systems	1(11.11%)	26(1.06%)
	Ko04745	phototransduction-fly	organismal systems	1(11.11%)	14(0.57%)
	Ko00471	D-glutamine and D-glutamate metabolism	metabolism	1(11.11%)	2(0.08%)
	Ko05230	central carbon metabolism in cancer	human diseases	1(11.11%)	22(0.90%)
	Ko05202	transcriptional misregulation in cancer	human diseases	1(11.11%)	35(1.43%)
	Ko04964	proximal tubule bicarbonate reclamation	organismal systems	1(11.11%)	8(0.33%)
	Ko04360	axon guidance	organismal systems	1(11.11%)	46(1.88%)
	Ko04924	renin secretion		1(11.11%)	16(0.65%)
	Ko04530	tight junction	cellular processes	1(11.11%)	49(2.00%)
	Ko04270	vascular smooth muscle contraction	organismal systems	1(25.00%)	30(1.23%)
	Ko04924	renin secretion		1(25.00%)	16(0.65%)
	Ko04970	salivary secretion	organismal systems	1(25.00%)	22(0.90%)
	Ko04022	cGMP-PKG signaling pathway	environmental information processing	1(25.00%)	41(1.68%)
	Ko04744	phototransduction	organismal systems	1(25.00%)	3(0.12%)
S-dinotefuran	Ko04360	axon guidance	organismal systems	1(25.00%)	46(1.88%)
	Ko04972	pancreatic secretion	organismal systems	1(25.00%)	27(1.10%)
	Ko04911	insulin secretion	organismal systems	2(50.00%)	24(0.98%)
	Ko00230	purine metabolism	metabolism	1(25.00%)	100(4.09%)

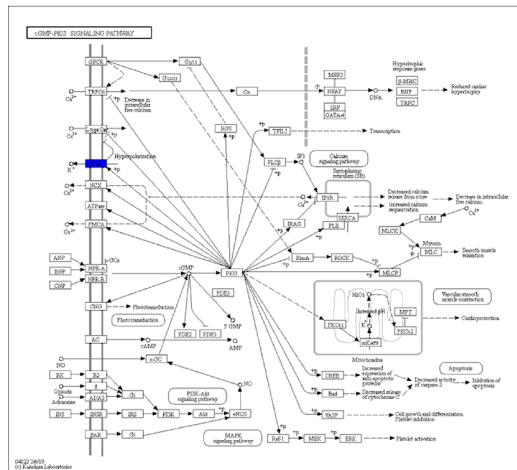
^aSamples were treated with LC₂₀ values of R-, S-, and rac-dinotefuran.



a Control check vs S-dinotefuran treatments



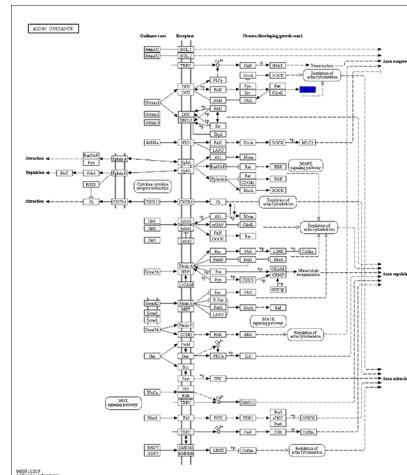
b Control check vs R-dinotefuran treatments



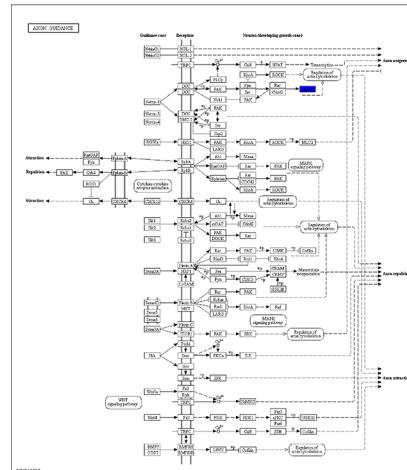
c Control check vs rac-dinotefuran treatments

Figure 5. KEGG enrichment pathway analysis of the Ko04022 pathway. (The red colored gene boxes show that this gene expression is upregulated; the blue colored gene boxes show that this gene expression is downregulated.)

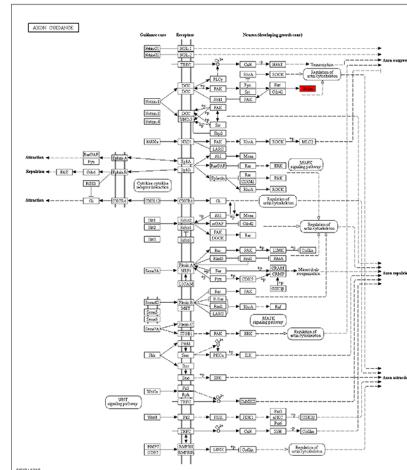
is selective bioaccumulation behavior between *R*-dinotefuran and *S*-dinotefuran in earthworm.²² Therefore, we hypothesized that dinotefuran would have an enantioselective effect on the



a Control check vs S-dinotefuran treatments



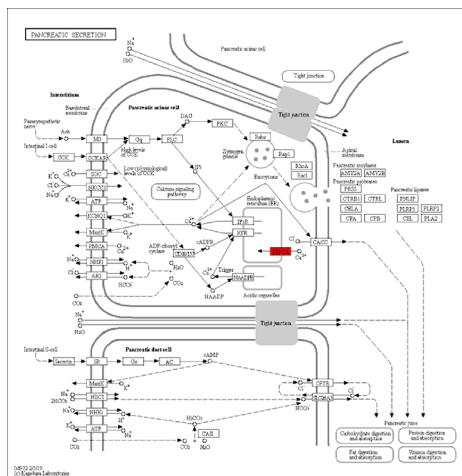
b Control check vs R-dinotefuran treatments



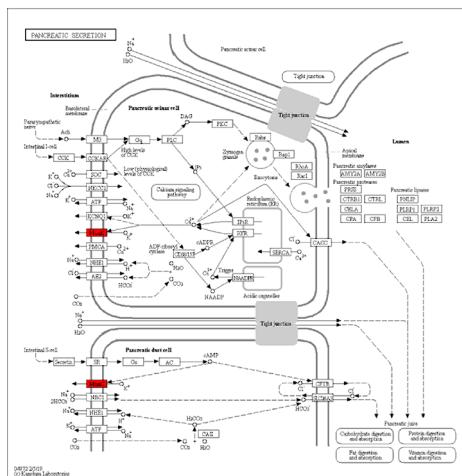
c Control check vs rac-dinotefuran treatments

Figure 6. KEGG enrichment pathway analysis of the Ko04360 pathway. (The red colored gene boxes show that this gene expression is upregulated; the blue colored gene boxes show that this gene expression is downregulated.)

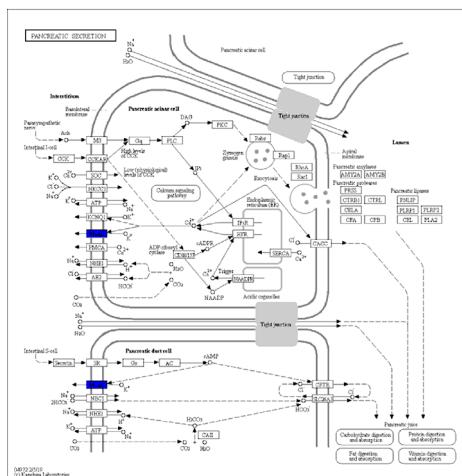
ability of honey bees to respond to sucrose. Here, oral exposure to *rac*-, *R*-, and *S*-dinotefuran at LC₅ and LC₂₀ induced significant reductions in sucrose responsiveness, and



a Control check vs S-dinotefuran treatments



b Control check vs R-dinotefuran treatments



c Control check vs rac-dinotefuran treatments

Figure 7. KEGG enrichment pathway analysis of the Ko04972 pathway. (The red colored gene boxes show that this gene expression is upregulated; the blue colored gene boxes show that this gene expression is downregulated.)

the beak stretch rate observed for *R*-dinotefuran was greater than that observed for *S*-dinotefuran at different sucrose concentrations. With respect to responsiveness to water, only

with LC₃₀ *S*-dinotefuran and *R*-dinotefuran treatments did the water responsiveness of the honey bees increase. The *rac*-dinotefuran may be less toxic than the other two treatments, as assessed by the honey bee sucrose responsiveness at LC₅, LC₂₀, and LC₃₀, which may indicate the effects of biological enzymes. Further biochemical research, such as the determinations of the specific activities of acetylcholinesterase, carboxylesterase, cytochrome P450 monooxygenases, and polyphenoloxidase should be conducted.^{34–37}

Learning and memory in honey bee is associated with olfaction, which is the main sense used for honey bee foraging, communication, and orientation. Dinotefurans have been found in honey bees and hive matrixes,¹⁵ but there have been no studies examining the potential effects of dinotefuran on honey bee behavior, particularly on their learning ability and memory. Our experiments showed that based on the concentrations determined in this study, sublethal levels of dinotefuran have significant effects on learning and memory in honey bees, as measured by PER. Oral treatments with sublethal doses of dinotefuran (LC₅ and LC₂₀) lowered short- and long-term honey bee memory performance. Learning and memory retention in bees exposed to dinotefuran decreased in the *R*-dinotefuran, *S*-dinotefuran, and *rac*-dinotefuran treatment groups compared with those in the control group. This result is similar to those obtained for other neonicotinoid insecticides. Sublethal doses of imidacloprid or clothianidin can also impair honey bee learning and memory.³⁸ Moreover, unlike acetamiprid, which is specifically harmful to long-term memory in honey bees,³³ dinotefuran was harmful to not only long-term memory but also short-term memory. In this study, the success rate obtained after the *S*-dinotefuran treatment was greater than that obtained after the *R*-dinotefuran treatment ($p < 0.05$), and the adverse effects of *rac*-dinotefuran on honey bee learning and memory retention were between those observed for *R*-dinotefuran and *S*-dinotefuran.

Octopamine is released into the insect hemolymph in response to stress, and it is related to appetitive conditioning memory formation. In the honey bee, octopamine mediates at least part of the reinforcing capacity of sugar rewards in an associative olfactory discrimination task.^{11,39} The octopamine levels were determined in bee heads 2 and 24 h after dinotefuran treatments. Honey bees with high levels of octopamine also had high rates of successful learning, and the hemolymph octopamine concentrations were approximately 140–240 $\mu\text{g/L}$ in the control and treated bees. These levels are comparable to those reported in honey bees by other researchers, who found hemolymph octopamine concentrations of approximately 220–320 $\mu\text{g/L}$ and 100–250 $\mu\text{g/L}$ in bee brains.²⁸ Our results demonstrate that sublethal levels of dinotefuran had negative impacts on short-term and long-term learning as well as memory in honey bees, as measured by PER, and had a significant impact on the hemolymph octopamine levels. Moreover, a high level of octopamine inhibition was correlated with low success rates in learning and memory retention. The *S*-dinotefuran-treated groups exhibited greater success rates for learning and memory retention and higher octopamine concentrations than the *R*-dinotefuran-treated groups.

Some synaptic functions of circRNA-generated genes were found to be upregulated in response to altered neuronal activity.⁴⁰ However, until now, there have been limited reports on circRNA expression in the honey bee brain. To increase our understanding of the different effects of sublethal doses of

dinotefuran enantiomers, circRNA transcriptome sequencing of honey bee heads was performed. In this study, the numbers of up- and down-regulated genes in *R*-dinotefuran-treated groups were greater than in *S*-dinotefuran-treated groups. A global transcriptomic study of the different unigenes following treatment with sublethal concentrations of other neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) in honey bee brains revealed 2–234 downregulated and 4–36 upregulated genes.¹² According to the GO term analysis, catalytic activity-related genes were enriched in our study, which differed from the results of a study in which muscle- and ribosomal protein-related genes were linked to the sublethal effects of imidacloprid. Our results also differed from a report in which unfolded protein binding-related genes were linked to the sublethal effects of clothianidin and amylase activity-related genes were linked to the sublethal effects of thiamethoxam in the honey bee *A. mellifera* L.^{12,41} Naeger performed a transcriptomic analysis of learning reward-related behaviors in honey bees. Mushroom body responses to difference in various foods included the molecular pathways involved in the bee response to a food reward.¹³ Transcriptomic profiles of the mushroom bodies generated by RNA sequencing show that genes responsive to only food and food value were enriched for the regulation of neuronal signaling.²⁵ In the present study on the KEGG enrichment pathways, there were three genes related to *S*-dinotefuran and *R*-dinotefuran treatments, and these gene were upregulated in three different pathways. SERCA genes have been found to be related to the sequestration of cytosolic Ca²⁺ in the endoplasmic reticulum. Intracellular Ca²⁺ plays an important role in β-cell function; it is the key intracellular mediator of fuel-stimulated insulin secretion and directly affects insulin biosynthesis. Studies showed that insulin can affect honey bee chemosensory responsiveness and their abilities to discriminate odors.⁴² Kca genes can cause a loss or impairment of channel activity (“loss-of-function” mutations) in vascular biology, and there has been no study showing that Kca gene expression can inhibit or promote learning and remembering abilities in honey bees. This study only showed that Kca genes may have something to do with the honey bee olfaction ability. Maxik is a key regulator of vital body functions and is associated with cell signaling and metabolism, and it is associated with insect survival. Therefore, SERCA genes up regulated may cause chronic toxicity and Maxik up regulated may cause acute toxicity. This study has laid the foundation for investigating circRNA expression mechanisms of olfaction in honey bees.

In conclusion, the acute oral toxicity of *S*-dinotefuran was greater than that of *R*-dinotefuran. However, exposure to sublethal doses of *S*-dinotefuran had fewer negative effects on honey bee learning and memory retention than exposure to sublethal doses of *R*-dinotefuran. The enantioselectivity observed during acute toxicity and the sublethal behavioral effects on honey bee imply that the ecotoxicological effects of enantiomers must be individually considered. The enantioselectivity observed during acute toxicity and the sublethal behavioral effects on honey bees imply that the ecotoxicological effects of enantiomers must be individually considered. The transcriptome results in honey bee brains showed that the SERCA, Kca, and Maxik genes may be related to the enantioselective effects of dinotefuran isomers on honey bee olfaction. The tests of the sublethal behavioral effects on honey bees provided more information than the acute test and thus offered more choices for comparing the toxicities of individual

enantiomers and pesticides racemates. These results may improve our understanding of the ecotoxicological safety of chiral dinotefuran on honey bees.

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Notes

The authors declare no competing financial interest.

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