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Brain transcriptome of honey bees (*Apis mellifera*) exhibiting impaired olfactory learning induced by a sublethal dose of imidacloprid

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ABSTRACT

Declines in honey bee populations represent a worldwide concern. The widespread use of neonicotinoid insecticides has been one of the factors linked to these declines. Sublethal doses of a neonicotinoid insecticide, imidacloprid, has been reported to cause olfactory learning deficits in honey bees via impairment of the target organ, the brain. In the present study, olfactory learning of honey bees was compared between controls and imidacloprid-treated bees. The brains of imidacloprid-treated and control bees were used for comparative transcriptome analysis by RNA-Seq to elucidate the effects of imidacloprid on honey bee learning capacity. The results showed that the learning performance of imidacloprid-treated bees was significantly impaired in comparison with control bees after chronic oral exposure to imidacloprid (0.02 ng/μl) for 11 days. Gene expression profiles between imidacloprid treatment and the control revealed that 131 genes were differentially expressed, of which 130 were downregulated in imidacloprid-treated bees. Validation of the RNA-Seq data using qRT-PCR showed that the results of qRT-PCR and RNA-Seq exhibited a high level of agreement. Gene ontology annotation indicated that the oxidation-reduction imbalance might exist in the brain of honey bees due to oxidative stress induced by imidacloprid exposure. KEGG and ingenuity pathway analysis revealed that *transient receptor potential* and *Arrestin 2* in the phototransduction pathway were significantly downregulated in imidacloprid-treated bees, and that five downregulated genes have causal effects on behavioral response inhibition in imidacloprid-treated bees. Our results suggest that downregulation of brain genes involved in immune, detoxification and chemosensory responses may result in decreased olfactory learning capabilities in imidacloprid-treated bees.

1. Introduction

The European honey bee, *Apis mellifera* (Hymenoptera: Apidae) plays an important role in natural ecosystems by providing pollination services for crop and wild plants around the world (Klein et al., 2007). In addition to providing agriculture and ecosystem service of pollination, honey bees produce hive products such as honey, royal jelly, wax, venom, propolis, etc. (Moritz et al., 2005). However, losses of honey bee colonies at varying degrees have been occurring in many parts of the world in recent years (Neumann and Carreck, 2010), and the exact causes of honey bee loss remain elusive. Among the abiotic and biotic stress factors, neonicotinoid pesticides have been regarded as one of the major threats to honey bee health (Tsvetkov et al., 2017).

The systemic neonicotinoid insecticides are the most widely used class of insecticides in the world. Neonicotinoids have neurotoxic effects on insects by acting as an agonist on the nicotinic acetylcholine receptors of insect brain (Goulson, 2013). The imidacloprid was the first neonicotinoid introduced to the market and has been used widely in crop protection because of its broad-spectrum activity against pests (Elbert et al., 2008). However, lethal and sublethal effects on non-target insects such as honey bees and bumble bees were observed by varying doses of imidacloprid (Goulson, 2013). Compared with the control worker bees, worker bumble bees (*Bombus terrestris*) from colonies exposed to imidacloprid at 10 parts per billion (ppb) were less likely to perform foraging activities (Mommaerts et al., 2010). Additionally, dose-dependent effects of imidacloprid were observed on worker bumble bees exposed to field-realistic dosages of imidacloprid, which reduced the production of brood in worker bumble bees (Laycock et al., 2012). It was also reported that sublethal doses of neonicotinoid pesticides adversely affected foraging behaviors and homing ability of

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honey bees, and might finally result in the collapse of the colony (Henry et al., 2012). This abnormal foraging behavior was also observed in honey bees exposed to imidacloprid with concentrations as low as 50 ppb (Yang et al., 2008).

The proboscis extension response (PER) has become a model for studying the neural basis of learning and memory in insects (Menzel, 2012). The associative odor-conditioning assay consists of pairing an odor (conditioned stimulus, CS) with sucrose solution (unconditioned stimulus, US) (Felsenberg et al., 2011), and has been used extensively to study the influence of biotic and abiotic factors on learning and memory performances in honey bees (Iqbal and Mueller, 2007; Decourtye et al., 2003). Previous studies showed that honey bees orally exposed to sublethal doses of imidacloprid (12 ng/bee) exhibited significantly lower learning performances compared to control bees (Decourtye et al., 2004a). Further, honey bees fed a sucrose solution containing 24 ppb imidacloprid were found to exhibit impaired olfactory learning performance under both laboratory and semi-field conditions (Decourtye et al., 2004b). It has also been shown that aversive short-term learning and memory performances of honey bees could be significantly impaired by imidacloprid with a concentration of approximately 20 ppb (Zhang and Nieh, 2015). Finally, honey bees from colonies exposed to 20 ppb imidacloprid were found to become more susceptible to *Nosema* infections than honey bees from control colonies (Pettis et al., 2012).

While there has been a considerable amount of research on the effects of sublethal doses of imidacloprid exposure on learning performance of honey bees, no information is available about gene expression profile associated with impaired olfactory learning in the honey bee brain. The honey bee brain is composed of about 950,000 neurons and is approximately one mm^3 in size (Menzel and Giurfa, 2001), and the learning-related structural and functional changes of the brain have been clearly demonstrated in a previous study (Faber et al., 1999). In the present study, we provide evidence that learning performance of honey bees can be impaired by chronic oral exposure to sublethal concentrations of imidacloprid. Furthermore, we carried out RNA-Seq transcriptome analysis in brain samples of bees exposed to sublethal doses of imidacloprid versus control bees. The expression of genes involved in immune, detoxification, and chemosensory responses were significantly downregulated and were altered as a possible result of neonicotinoid imidacloprid impaired olfactory learning. Our findings provide significant insight into the possible molecular mechanisms involved in impaired conditional learning performance in honey bees exposed to imidacloprid.

2. Material and methods

2.1. Honey bees

Four capped brood combs were removed from four different colonies individually and placed in an incubator at 34 °C with approximately 70% relative humidity (RH). About 500 newly emerged bees were collected daily and marked on the thorax using paint markers. The marked newly emerged bees were then returned to their field colonies. After spending 11 days in the hive, these same-aged marked bees were collected using soft forceps. The collected bees were transferred to eight laboratory rearing cages (45 bees per cage). The caged honey bees were placed in an incubator (30 °C, 70% RH) and used for subsequent chronic exposure to imidacloprid.

2.2. Chronic exposure by imidacloprid

Imidacloprid stock solutions at 400 ng/μl were prepared by dissolving imidacloprid (Sigma-Aldrich, USA) in acetone (Sinopharm Chemical Reagent Co., China) and stored at 4 °C. A sublethal dose of imidacloprid at 20 ppb (0.02 ng/μl) was prepared by diluting the stock solutions with 30% sugar water (wt/wt). The eight cages were divided into two groups: four cages of bees were assigned as the treatment group or the control group. Caged honey bees in treatment groups were fed ad libitum with 30% sugar water containing 20 ppb of imidacloprid. Caged honey bees in control groups were fed ad libitum with 30% sugar water containing acetone. Sugar water consumption was recorded and compared in the first three days during 11 days of imidacloprid exposure between imidacloprid-treated and control bees. Cages were checked every day, and dead bees were removed during the exposure period. After 11 days of chronic oral exposure to imidacloprid, honey bees were used for behavioral studies.

2.3. PER behavioral experiments

For both treatment and control groups, each cage of honey bees was used for an associative learning test and tests were repeated four times using four different cages of honey bees respectively. The associative learning test consisting of three conditioning trials was performed in accordance with previous studies (Felsenberg et al., 2011; Müller, 2002), and the interval between the three conditioning trials was 10 min. Honey bees were immobilized on ice individually before they were separately harnessed in each copper tube with cloth tape. The harnessed bees could move their proboscis freely and were then kept in an incubator (30 °C, 70% RH) for 2 h before the associative learning test. The antenna of each harnessed honey bee was touched with a drop of 50% sugar water (wt/wt), and the honey bee not extending its proboscis to sugar water stimulation was discarded from the test. A round filter paper (2.5 cm diameter) soaked with 10 μl of lemon essence was put into a 50 ml syringe, and the lemon-scented air (CS) was continuously delivered to the antenna of harnessed bees for 4 s using a modified air pump device described in our previous studies (Li et al., 2017a). Immediately after the 2 s odor exposure, honey bees were allowed to suck the 50% sugar water (US) for 4 s. After stimulation with the lemon odor during both the second and third conditioning trial, control honey bees able to extend their proboscis and imidacloprid-treated honey bees unable to extend their proboscis were used for gene expression analysis respectively. The honey bee samples from both groups were snap frozen in liquid nitrogen immediately after the third conditioning trial and stored at −80 °C until brain dissection.

2.4. RNA extraction and RNA-Seq library preparation

Total RNA was extracted from each sample with ten pooled dissected brains using TRIZOL reagent (Invitrogen) per the manufacturer's specification. Three repetitive samples were used for control and imidacloprid-treated bees, respectively. The degradation and contamination of total RNA were checked by 1.0% denaturing gel electrophoresis. Purity and quantity of total RNA were determined using the NanoPhotometer spectrometer and Qubit RNA Assay kit in Qubit 2.0 Fluorometer respectively. Additionally, RNA integrity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

The mRNA was purified from three micrograms total RNA using magnetic oligo (dT) beads and was then fragmented at elevated temperature in a fragmentation buffer consisting of divalent cations plus NEBNext First-Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized from the fragmented mRNA with random hexamer primers using M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA was synthesized using DNA Polymerase I, dNTPs, and RNase H. The purified double-stranded cDNA was converted into blunt ends, followed by adenylation of the 3′ ends and ligation to the NEBNext Adapter. Ampure XP beads were then used for selecting cDNA fragments of around 200 bp. The adaptor-ligated/size-selected cDNA fragments were then mixed with 3 μl USER enzyme (NEB) which were incubated at 37 °C for 15 min followed by 5 min at 95 °C. The enriched cDNA libraries were made via PCR using Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primers. Before sequencing, PCR products were purified with AMPure XP beads, and the

quality of cDNA libraries was assessed using the Agilent Bioanalyzer 2100.

2.5. RNA sequencing and analysis

Clustering of the index-coded samples was carried out in a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) per manufacturer's specifications. The paired-end reads ranging from 125 bp to 150 bp in length were generated on the Illumina platform according to manufacturer's specifications. Clean reads were filtered by removing the adaptor sequence, reads with unknown base N accounting for > 10% of the entire read sequence, and low-quality reads from the raw reads. The clean reads were mapped to the *Apis mellifera* L. reference genome (Amel_4.5) using Hisat v2.0.5 (Kim et al., 2015). The number of clean reads mapped to each gene was counted through featureCounts (v1.5.0-p3) (Liao et al., 2013). Expression levels of each gene were normalized in RPKM (Reads per kilobase of exon model per million mapped reads) values based on the gene length and the number of reads mapped to each gene (Mortazavi et al., 2008).

Differential gene expression between the two different samples was carried out using DESeq2 (R package, version 1.16.1) (Love et al., 2014). Annotation and functional enrichment analysis of differentially expressed genes were performed with the clusterProfiler R package based on the Gene Ontology terms (GO) database (Ashburner et al., 2000; Yu et al., 2012). Analysis of enriched differentially expressed pathways in which differentially expressed genes involved was performed using the clusterProfiler R package based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Yu et al., 2012; Kanehisa et al., 2006). To further investigate functional effects of the downregulated genes in brains of imidacloprid-treated bees, Ingenuity Pathway Analysis software (IPA; QIAGEN, CA) was employed to perform downstream effects analysis based on the Ingenuity Knowledge Base (Krämer et al., 2013).

2.6. Quantitative real-time PCR

To validate the RNA sequencing results, the immune, detoxification response-related genes, chemosensory-related genes, and genes from enriched GO categories and KEGG pathways were further assayed by quantitative real-time PCR (qPCR). The cDNA was generated from RNA samples obtained as above using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instructions. Total RNA (1 μg) was mixed with 2 μl of 5× gDNA Eraser buffer, 1 μl of gDNA Eraser, and RNase-free water was added to a final volume of 10 μl. The reaction mixtures were incubated at 42 °C for 2 min to remove genomic DNA. The reaction mixtures were mixed with 1 μl PrimeScript RT enzyme mix, 1 μl RT primer mix, 4 μl 5 × PrimeScript buffer and 4 μl RNase-free water were incubated at 37 °C for 2 min and at 85 °C for 5 s. The synthesized cDNA was stored at −20 °C until later use.

Each qPCR reactions of 10 μl consisted of 5 μl of 2× SYBR Premix Ex Taq II (Tli RNaseH Plus, TaKaRa), 0.8 μl of each PCR primer (10 μM) (Table S1) (Evans, 2006), 1 μl of cDNA (1:3 dilution) and 3.2 μl of RNase-free water. The qPCR was carried out using the Bio-Rad CFX 384 Real-time system, and all reactions were performed in triplicate. The reaction conditions were 95 °C for 30s, and 40 cycles of 95 °C for 5 s and 60 °C for 30s, followed by melting curve analysis. The Ct value of each target gene was normalized to the geometric mean of the Ct values of two reference genes *rp49* and *actin* (Villar and Grozinger, 2017; Lourenco et al., 2008). Approximately equal amplification efficiencies of the target and reference genes were compared and confirmed as previously described (Chen et al., 2005), and relative expression levels of each target gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

For RNA-Seq data analysis, the adjusted P value (padj) < 0.05 was considered statistically significant (Benjamini and Hochberg, 1995). The unpaired Student's *t*-test was used to examine differences in mean survival rates and syrup consumption of control and imidacloprid-treated bees and to compare relative expression levels of target genes in the two groups of bees. Differences in learning performance between control and imidacloprid-treated bees were determined by the Chi-squared test. The Fisher's exact test was used for comparing the overlap between regulators and function/disease dataset molecules in downstream effects analysis (Krämer et al., 2013). Data are presented as mean ± SD.

3. Results

3.1. Mortality and syrup consumption analysis

Mortality was analyzed based on the number of remaining bees each day in three control cages versus three treatment cages. The mean survival rate of control bees ranged from 86%–99%, and the mean survival rate of imidacloprid-treated bees ranged from 87%–100% during the 11 days' exposure (Fig. 1). We observed no significant adverse effects of 20 ppb imidacloprid on the survival rate of honey bees (*t*-test, P > .05), and the imidacloprid concentration used in the study was sublethal. The average of sugar water consumption per honey bee was 87 ± 7 μl and 90 ± 5 μl in the treatment and control group, respectively. There was no significant difference in sugar water consumption between the two groups (*t*-test, P > .05), indicating that 30% sugar water containing 20 ppb of imidacloprid had no significant repellent effect on honey bees.

3.2. Olfactory learning performances

We performed the PER test to observe learning performance in imidacloprid-bees (n = 139) compared to control bees (n = 135). During the first learning test, we observed no bees in either treatment engaging in PER with respect to the lemon odor stimulant. However, the percentage of control bees showing PER was 85% in both the second and third learning test versus imidacloprid-treated bees showing 65% and 63% in the second and third learning test respectively (Fig. 2). There was a significant difference in learning performance between the two groups of bees (χ^2 test, P < .01), and control bees exhibited better learning performance than bees exposed to a sublethal dose of imidacloprid at 20 ppb.

3.3. Analysis of RNA-Seq data

After filtering low-quality reads, the average numbers of clean reads

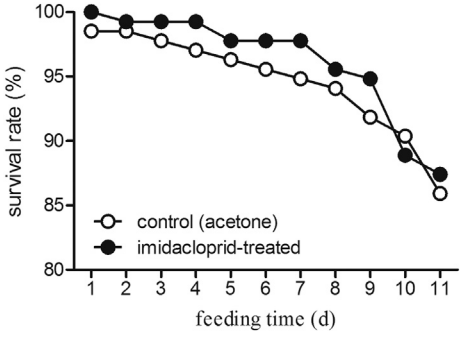

Fig. 1. Analysis of survival rate between imidacloprid-treated (solid circles) and control (empty circles) bees. Curves do not significantly differ statistically, P > .05, unpaired Student's *t*-test.

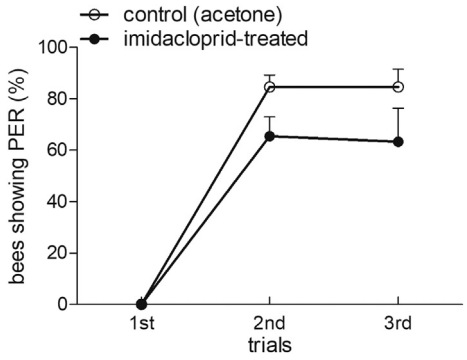

Fig. 2. Significant differences in learning performance between imidacloprid-treated (solid circles, n = 139) and control bees (empty circles, n = 135). The Chi-squared test, P < .01, Error bars indicate SD.

were 56.9 and 52.7 million for the sequencing libraries of control bees and imidacloprid-treated bees respectively. The whole sequence reads were deposited in the NCBI Sequence Read Archive (SRA, accession number: SRP133690). Approximately 87% of the clean reads were mapped to the honey bee genome assembly (Amel_4.5) for the sequencing libraries of control bees and imidacloprid-treated bees.

The clean read mapping results showed that the total numbers of mapped genes were 22,566, and a total of 15,403 unique genes were comparatively analyzed in the two groups of honey bees. A total of 15,272 genes exhibited no significant differences in gene expression levels between the two group of honey bees (padj > 0.05). A total of 131 genes that were significantly differentially expressed between control bees and imidacloprid-treated bees (padj < 0.05) were identified. Among the 131 differentially expressed genes (DEGs) (Table S2), 130 genes were downregulated and one gene was upregulated in brains of imidacloprid-treated bees compared with that of control bees (Fig. 3).

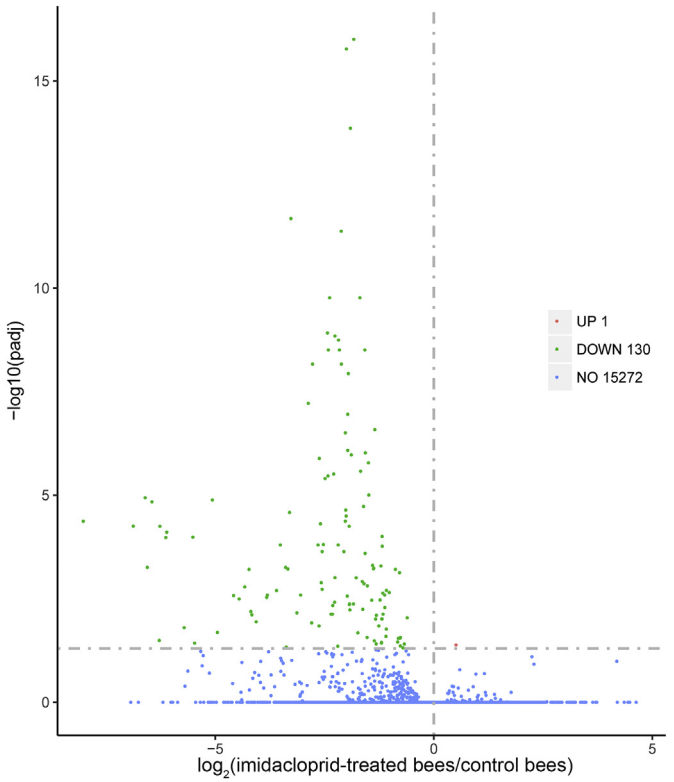

Fig. 3. Differentially expressed genes in the brain of the imidacloprid-treated and control bees. The number of significantly upregulated, and downregulated genes in brains of imidacloprid-treated bees were shown in red and green respectively (padj < 0.05). No differential expression between the two group of honey bee genes was shown in blue (padj > 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Down-regulation of immune, detoxification, and antioxidant response genes

Of genes with significantly down-regulated expression in the brain of imidacloprid-treated bees, immune genes encoding abaecin (GB18323), apisimin (GB53576), defensin 1 (GB41428), glucose dehydrogenase (GB43007), glucose dehydrogenase-like (GB51446), leucine-rich repeat-containing protein 26-like (GB44192), phenoloxidase subunit A3 (GB43738), serine protease easter (GB45700) and tyrosine aminotransferase (GB45969) exhibited a 1.5-fold to 84.7-fold change in expression levels compared to control bees (padj < 0.05, Fig. 4). In addition, seven insecticide resistance-related genes encoding cuticular protein 14 (GB46297), cytochrome *b*561 (GB40148), cytochrome P450 6a2 (GB49876), cytochrome P450 9e2 (GB43713), esterase A2 (GB43571), cytochrome P450 6a17 (GB49885) and UDP-glucuronosyltransferase 2C1 (GB52179) exhibited a 1.6-fold to 23.2-fold downregulation in imidacloprid-treated bees (padj < 0.05, Fig. 4). Imidacloprid-induced immune dysfunction has previously been demonstrated in honey bees (James and Xu, 2012; Li et al., 2017b; Wu et al., 2017), and immune response induction could exert inhibitory effects on associative learning performances in honey bees (Mallon et al., 2003).

Moreover, functional annotation of DEGs revealed that ten of the 130 significantly downregulated genes in imidacloprid-treated bees were significantly enriched for three GO categories (padj < 0.05), and the number of genes assigned to oxidation-reduction process (GO:0055114), iron ion binding (GO:0005506) and oxidoreductase activity (GO:0016491) was 10, 5, and 10 respectively (Table 1). Given there was only one upregulated gene, and no GO category associated with the upregulated gene was identified in imidacloprid-treated bees. All ten unique genes involved in the oxidation-reduction process and oxidoreductase activity were significantly downregulated in the exposed bees when compared to controls (padj < 0.05). The down-regulation of these classes of genes may lead to dysfunction in the oxidative-reductive mechanism within the honey bee, and an oxidation-reduction imbalance may decrease olfactory learning in honey bees (Farooqui, 2014).

3.5. Pathway analysis of DEGs

KEGG Ontology (KO) enrichment analysis revealed that two downregulated genes (GB41297, GB51068) in imidacloprid-treated bees were significantly enriched in the phototransduction pathway (padj < 0.05). The transient receptor potential protein (TRP) encoded by GB41297 involves in Ca^{2+} entry and expression of the gene induce permeation of Ca^{2+} into the cell in *Drosophila* (Hu et al., 1994). A threshold concentration of intracellular Ca^{2+} is necessary for memory formation during olfactory learning in honey bees, and honey bees with Ca^{2+} blocked in the brain exhibit impaired long-term memory (Perisse et al., 2009). The downregulation of the gene may disrupt calcium balance in the brain of honey bees. In addition, arrestins are required for maintenance of normal olfactory function in *Drosophila* (Elaine Merrill et al., 2005), and a sublethal dose of imidacloprid may affect the olfactory sensitivity of honey bees via downregulation of the gene GB51068 encoding arrestin 2 in the brain.

IPA analysis indicated that five orthologs in the honey bee have indirect causal effects on behavioral response inhibition in imidacloprid-treated bees (P < .05, Fig. 5). The downregulation of the gene GB50003 encoding brain synaptic vesicular amine transporter

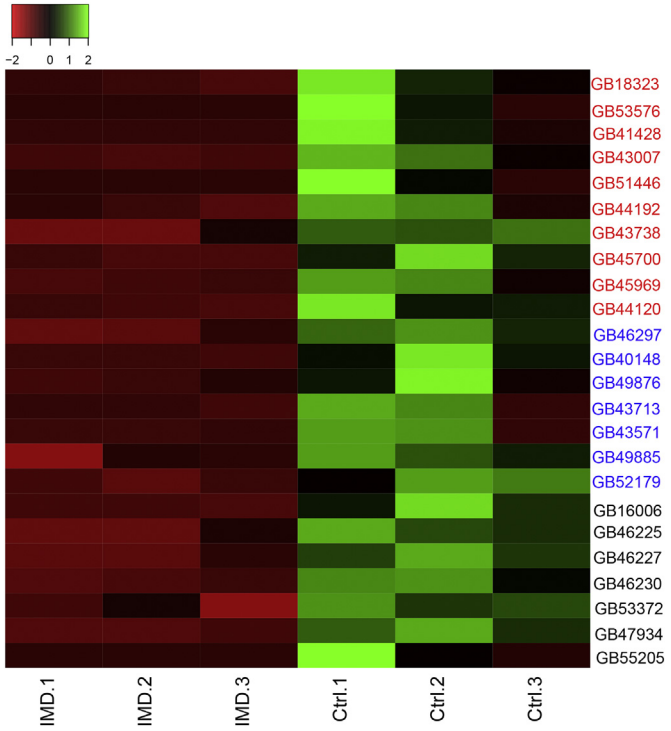

Fig. 4. Heatmap generated based on RPKM values from RNA-Seq. The accession numbers marked in red, blue, and black represent genes involved in immune, detoxification, and chemosensory responses in brains of imidacloprid-treated (IMD) and control bees (Ctrl) respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(SLC18A2) leaded to inhibition of conditioning, olfactory discrimination, anxiety-like behavior and coping response. The downregulation expression of OPN4 (melanopsin, GB41643), AQP4 (aquaporin 4, GB41240) and NT5E (apyrase, GB52756) suppress behavioral responses of honey bees through inhibitory processes. In addition, the downregulation expression of NT5E has inhibitory effects on working memory (short-term memory) of honey bees, and downregulation expression of cAMP-dependent protein kinase (PRKACB, GB48362) may also have inhibitory effects on conditioning response of honey bees.

3.6. Validation of DEGs by qPCR

The relative expression levels of 23 DEGs involved in immune, detoxification, and chemosensory responses were further validated by qPCR (Fig. 6). The expression patterns of 23 DEGs by qPCR were in good agreement with the RNA-seq transcriptome data. The relative expression levels of the 20 DEGs were significantly lower in imidacloprid-exposed honey bees than control bees ($P < .05$; $P < .01$) (Fig. 6). A lower gene expression level of the remaining three genes (GB43738, GB49876, and GB16006), albeit not significantly, was found in imidacloprid-exposed honey bees. The expression levels of immune and detoxification genes including *abaecin* (GB18323), *easter* (GB45700) and

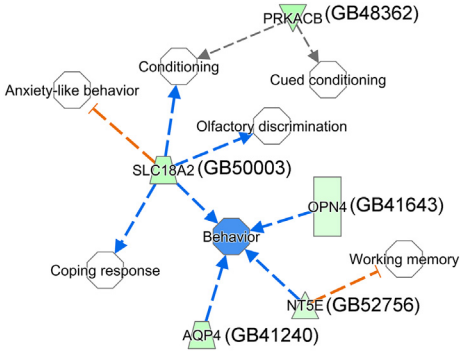

Fig. 5. Functional effects of the downregulated genes in brains of imidacloprid-treated bees. The accession numbers of five orthologs in honey bees are shown in parentheses. The dashed blue line indicates downregulation of the gene leads to inhibition of the downstream function; the dashed orange line indicates downregulation of the gene leads to activation-mediated inhibition of the downstream function; the dashed gray line indicates effect not predicted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CYB561 2-like (GB40148) in control bees were significantly higher than in imidacloprid-treated honey bees; the expression levels of chemosensory-related genes including *odorant-binding protein 16* (*Obp 16*, GB46225), *Obp 18* (GB46227), *Obp 21* (GB46230), *Obp 4* (GB53372), and *SLC18A2* (GB50003) in control bees were significantly higher than in imidacloprid-treated honey bees; the genes from enriched GO categories and KEGG pathways including *Trp* (GB41297) and *Arr2* (GB51068) in control bees were significantly higher than in imidacloprid-treated honey bees. The validation results of the 23 DEGs indicated that the qPCR data correlate well with the transcriptome data and further confirm the significance imidacloprid induced downregulation of genes involved in immune, detoxification and chemosensory responses in the brains of honey bees.

4. Discussion

Residual levels of imidacloprid in fields vary broadly and are associated closely with environmental conditions, pesticide application methods and crops (Bonmatin et al., 2005; Dively and Kamel, 2012). Imidacloprid residues in sunflower pollen were 13 ppb (Laurent and Rathahao, 2003), and the maximum concentration of imidacloprid residues in maize pollen was 18 ppb (Bonmatin et al., 2005). It is generally assumed that field-realistic doses of imidacloprid range from 0.7 to 10 ppb in the pollen and nectar of flowering crops (Whitehorn et al., 2012). Contaminated pollen and nectar foraging by honey bees from pesticides-exposed crops during a period of two to four weeks of flowering could result in the accumulation of pesticides in hive components including wax, honey and bee bread (Whitehorn et al., 2012; Pettis et al., 2004). Therefore, even though imidacloprid residue levels in the field is lower than sublethal levels from laboratory studies, the accumulated imidacloprid in the hive may reach chronic toxicity levels which can interfere with the normal behavioral performance of honey bees. The data presented in this study showed that gene expression associated with the olfactory learning performances in honey bees

Table 1
GO enrichment analysis for the significantly downregulated genes in imidacloprid-treated bees.

GO ID	Enriched GO terms	The assigned genes associated with each GO term					padj
GO:0055114	Oxidation-reduction process	GB52785	GB44549	GB43007	GB55515	GB49876	0.045
		GB50655	GB43713	GB51446	GB50178	GB49885	
GO:0005506	Iron ion binding	GB55515	GB49876	GB50655	GB43713	GB49885	0.039
GO:0016491	Oxidoreductase activity	GB52785	GB44549	GB43007	GB55515	GB49876	0.039
		GB50655	GB43713	GB51446	GB50178	GB49885	

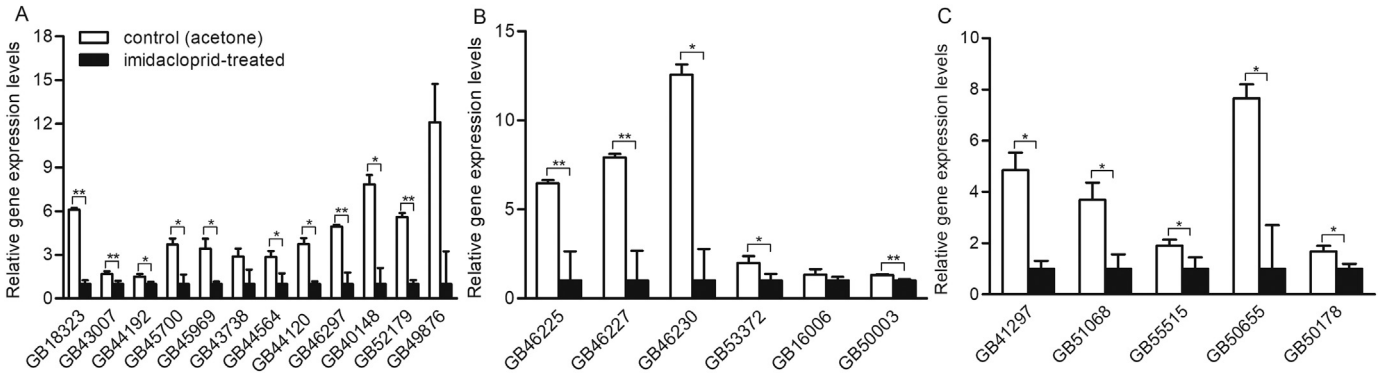

Fig. 6. qPCR analysis of differentially expressed genes between imidacloprid-treated and control bees. (A) Immune and detoxification response related genes, (B) chemosensory-related genes, and (C) genes from enriched GO categories and KEGG pathways. Unpaired Student's *t*-test, *P < .05, **P < .01. Error bars indicate SD.

chronically exposed to 20 ppb imidacloprid were significantly impaired. The behavioral results are in accordance with previous reports in which imidacloprid was shown to impair memory and brain metabolism of honey bees under both laboratory and semi-field conditions (Decourtye et al., 2004a; Decourtye et al., 2004b). Impairment of the cognitive function in the brain affects the foraging activity of honey bees, and may ultimately affect colony development (Belzunces et al., 2012). In addition to the obvious behavioral changes induced by imidacloprid exposure in honey bees, changes in gene expression in response to physiological stress associated with the imidacloprid exposure were also reported (James and Xu, 2012). To our knowledge, this is the first study to investigate genome-wide expression patterns in the brain of honey bees exhibiting impaired olfactory learning behaviors induced by a sublethal dose of imidacloprid. The gene expression profiles underlying the behavioral responses clearly show that genes involved in immunity, detoxification, and chemosensation were significantly downregulated in brains of the exposed bees.

It has been demonstrated that pesticides affect detoxification and immune response of insects. Xenobiotics including pesticides are molecularly detoxified through oxidation processes in insects (James and Xu, 2012). Annotation of enriched GO terms revealed that genes associated with the oxidation-reduction process were significantly down-regulated, which may lead to the generation of reactive oxidative species (ROS). Increased oxidative stress was observed in neonicotinoid-exposed bees due to excessive production of ROS (James and Xu, 2012). Iron plays important roles in redox reactions and down-regulation of genes involved in iron ion binding may indicate altered iron homeostasis in the brain of imidacloprid-treated bees. Elevated levels of iron can promote ROS production which induces further oxidative stress (Farooqui, 2013). In addition, the immune genes including *abaecin*, *apisimin*, *defensin 1*, *prophenoloxidase* and detoxification genes including *CYP450 6a2*, *CYP450 9e2*, *CYP450 6a17*, and *esterase A2* were significantly down-regulated in brains of imidacloprid-treated honey bees exhibiting impaired olfactory learning. Xenobiotic detoxification in honey bees is closely related to detoxifying enzymes including cytochrome P450s, and esterases (Yu et al., 1984; Berenbaum and Johnson, 2015). Cytochrome P450s are involved in oxidative metabolism of imidacloprid in many organisms including humans, fruit flies and honey bees (Tomizawa and Casida, 2005; Zhu et al., 2017), though different cytochrome P450 subfamilies may display differential efficiency of imidacloprid metabolism in bees (Manjon et al., 2018). Although esterases exhibit negligible roles in imidacloprid detoxification of honey bees (Zhu et al., 2017), esterases can function as antimicrobial proteins and induce immune response in pesticide-exposed insects (James and Xu, 2012). Olfactory learning deficits can probably be caused by oxidative stress and immune response in the brain of imidacloprid-treated bees.

The brain is extremely vulnerable to oxidative damage (Farooqui, 2008). Previous studies indicated that oxidative metabolism of the mushroom body calyces was increased in honey bees exhibiting impaired learning abilities after 30 min of treatment with imidacloprid (Decourtye et al., 2004a). Reduced density of the synaptic units was observed in the mushroom body calyces of adult bees treated with a sublethal dosage of imidacloprid during stages of larval development (Peng and Yang, 2016). The olfactory sensilla in *Drosophila* have been shown to express the *obp* genes (Galindo and Smith, 2001), which play important roles in insect olfaction function (Galindo and Smith, 2001; Zhou, 2010). Our studies revealed that *obp4*, *obp21*, *obp18* and *obp 16* were significantly downregulated in imidacloprid-treated bees compared with controls. In addition, the worker-enriched antennal transcript (GB47934, *Amwat*), possibly involved in olfactory processing in *A. mellifera* bees (Kamikouchi et al., 2004), was significantly downregulated in brains of the imidacloprid exposed bees. The downregulation of the *arrestin*, *Amwat*, and four other *obp* genes may indicate an impaired olfactory function in the brain of honey bees chronically exposed to imidacloprid, leading to dysfunctional behaviors.

The major royal jelly proteins (MRJPs), a family of multifunctional proteins, are involved in the regulation of physiology, development, and behavior of honey bees (Buttstedt et al., 2014). Previous studies showed that the expression of *mrjps* including *mrjp1*, *mrjp4* were repressed in heads of honey bees treated with a sublethal dose of imidacloprid (Wu et al., 2017). Furthermore, it has been shown that *mrjp1* was expressed in Kenyon cells of the mushroom bodies, which are critically involved in learning and memory abilities of honey bees (Kucharski et al., 1998). Honey bees with reduced learning ability exhibited low expression levels of *mrjp1* (Hojo et al., 2010). The *mrjp1* (GB55205), *mrjp4* (GB55206), and *mrjp 2-like* (GB55211) genes were significantly down-regulated in the brain of imidacloprid-treated honey bees exhibiting impaired olfactory learning in this study. This finding provides further evidence for the pivotal roles played by *mrjp1* in the development of the learning capacity of the honey bee. In addition, it should be noted that the expression level of the gene encoding synaptic vesicular amine transporter (SLC18A2, GB50003) in imidacloprid-treated honey bees was 2.4-fold lower than control bees. The brain vesicular amine transporter plays key roles in storing and releasing neurotransmitters including dopamine and serotonin (Wimalasena, 2011), which are essential for memory maintenance in *Drosophila* (Sitaraman et al., 2012). The downregulation of the gene may influence neurotransmitter homeostasis in the brain and ultimately affect memory retrieval of honey bees exposed to a sublethal dose of imidacloprid (Decourtye et al., 2004a). IPA analysis further confirmed that the causal relation between downregulation of the SLC18A2 gene and inhibited behaviors including olfactory discrimination in the present study. Additionally, previous studies showed that AQP4 (GB41240) null mice exhibited deficient learning ability in comparison to wide-type mice (Szu and Binder, 2016). The gene encoding apyrase (GB52756), a phosphatase involved in the hydrolysis of ATP, has been proved to be associated with acquisition and memory in rats (Bonan et al., 1998).

Melanopsin (OPN4, GB41643) plays important roles in behavioral responses to light and expression levels of melanopsin affect learning acquisition in mice (LeGates et al., 2012). Activation of the cAMP-dependent protein kinase during the olfactory conditioning process plays crucial roles for memory formation in honey bees (Müller, 2000), therefore, downregulation of the gene (GB48362) encoding the kinase possibly affect the conditioning response of honey bees. The inhibited behaviors revealed by IPA analysis may be mediated in part through downregulated expression of the five orthologs in honey bees.

Taken together, these findings demonstrate that the majority of differentially expressed genes were significantly down-regulated in honey bee brains chronically exposed to imidacloprid. Downregulation of brain genes involved in immune, detoxification, and chemosensory responses which may result in decreased olfactory learning capabilities in imidacloprid-treated honey bees. More research is needed to investigate the interactions between imidacloprid, the nervous system and the immune system in honey bees, and the effects of imidacloprid-induced impaired olfaction learning on the orientation and navigation of honey bees under the field conditions.

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References

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., 2000. Gene Ontology: tool for the unification of biology. Nat. Genet. 25, 25.

Belzunces, L.P., Tchamitchian, S., Brunet, J.-L., 2012. Neural effects of insecticides in the honey bee. Apidologie 43, 348–370.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Stat Methodol. 289–300.

Berenbaum, M.R., Johnson, R.M., 2015. Xenobiotic detoxification pathways in honey bees. Curr. Opin. Insect Sci. 10, 51–58.

Bonan, C.D., Dias, M.M., Battastini, A.M.O., Dias, R.D., Sarkis, J.J.F., 1998. Inhibitory avoidance learning inhibits ectonucleotidases activities in hippocampal synaptosomes of adult rats. Neurochem. Res. 23, 977–982.

Bonmatin, J., Marchand, P., Charvet, R., Moineau, I., Bengsch, E., Colin, M., 2005. Quantification of imidacloprid uptake in maize crops. J. Agric. Food Chem. 53, 5336–5341.

Buttstedt, A., Moritz, R.F., Erler, S., 2014. Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. Biol. Rev. 89, 255–269.

Chen, Y.P., Higgins, J.A., Feldlaufer, M.F., 2005. Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). Appl. Environ. Microbiol. 71, 436–441.

Decourtye, A., Lacassie, E., Pham-Delègue, M.H., 2003. Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. Pest Manag. Sci. 59, 269–278.

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M., Pham-Delègue, M.-H., 2004a. Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). Pestic. Biochem. Physiol. 78, 83–92.

Decourtye, A., Devillers, J., Cluzeau, S., Charreton, M., Pham-Delègue, M.-H., 2004b. Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. Ecotoxicol. Environ. Saf. 57, 410–419.

Dively, G.P., Kamel, A., 2012. Insecticide residues in pollen and nectar of a cucurbit crop and their potential exposure to pollinators. J. Agric. Food Chem. 60, 4449–4456.

Elaine Merrill, C., Sherertz, T.M., Walker, W.B., Zwiebel, L., 2005. Odorant-specific requirements for arrestin function in *Drosophila* olfaction. Dev. Neurobiol. 63, 15–28.

Elbert, A., Haas, M., Springer, B., Thielert, W., Nauen, R., 2008. Applied aspects of neonicotinoid uses in crop protection. Pest Manag. Sci. 64, 1099–1105.

Evans, J.D., 2006. Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. J. Invertebr. Pathol. 93, 135–139.

Faber, T., Joerges, J., Menzel, R., 1999. Associative learning modifies neural representations of odors in the insect brain. Nat. Neurosci. 2, 74–78.

Farooqui, T., 2008. Iron-induced oxidative stress modulates olfactory learning and memory in honeybees. Behav. Neurosci. 122, 433.

Farooqui, T., 2013. A potential link among biogenic amines-based pesticides, learning and memory, and colony collapse disorder: a unique hypothesis. Neurochem. Int. 62, 122–136.

Farooqui, T., 2014. Oxidative stress and age-related olfactory memory impairment in the honeybee *Apis mellifera*. Front. Genet. 5, 60.

Felsenberg, J., Gehring, K.B., Antemann, V., Eisenhardt, D., 2011. Behavioural pharmacology in classical conditioning of the proboscis extension response in honeybees (*Apis mellifera*). J. Vis. Exp. 47, 2282.

Galindo, K., Smith, D.P., 2001. A large family of divergent Drosophila odorant-binding proteins expressed in gustatory and olfactory sensilla. Genetics 159, 1059–1072.

Goulson, D., 2013. REVIEW: an overview of the environmental risks posed by neonicotinoid insecticides. J. Appl. Ecol. 50, 977–987.

Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., Decourtye, A., 2012. A common pesticide decreases foraging success and survival in honey bees. Science 336, 348–350.

Hojo, M., Kagami, T., Sasaki, T., Nakamura, J., Sasaki, M., 2010. Reduced expression of *major royal jelly protein 1* gene in the mushroom bodies of worker honeybees with reduced learning ability. Apidologie 41, 194–202.

Hu, Y., Vaca, L., Zhu, X., Birnbaumer, L., Kunze, D.L., Schilling, W.P., 1994. Appearance of a novel Ca^{2+} influx pathway in Sf9 insect cells following expression of the transient receptor potential-like (*trpl*) protein of *Drosophila*. Biochem. Biophys. Res. Commun. 201, 1050–1056.

Iqbal, J., Mueller, U., 2007. Virus infection causes specific learning deficits in honeybee foragers. Proc. Biol. Sci. 274, 1517–1521.

James, R., Xu, J., 2012. Mechanisms by which pesticides affect insect immunity. J. Invertebr. Pathol. 109, 175–182.

Kamikouchi, A., Morioka, M., Kubo, T., 2004. Identification of honeybee antennal proteins/genes expressed in a sex-and/or caste selective manner. Zool. Sci. 21, 53–62.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K.F., Itoh, M., Kawashima, S., Katayama, T., Araki, M., Hirakawa, M., 2006. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. 34, D354–D357.

Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357.

Klein, A.M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. Lond. B Biol. Sci. 274, 303–313.

Krämer, A., Green, J., Pollard Jr., J., Tugendreich, S., 2013. Causal analysis approaches in ingenuity pathway analysis. Bioinformatics 30, 523–530.

Kucharski, R., Maleszka, R., Hayward, D., Ball, E., 1998. A royal jelly protein is expressed in a subset of Kenyon cells in the mushroom bodies of the honey bee brain. Naturwissenschaften 85, 343–346.

Laurent, F.M., Rathahao, E., 2003. Distribution of [^{14}C] imidacloprid in sunflowers (*Helianthus annuus* L.) following seed treatment. J. Agric. Food Chem. 51, 8005–8010.

Laycock, I., Lenthall, K.M., Barratt, A.T., Cresswell, J.E., 2012. Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*). Ecotoxicology 21, 1937–1945.

LeGates, T.A., Altimus, C.M., Wang, H., Lee, H.-K., Yang, S., Zhao, H., Kirkwood, A., Weber, E.T., Hattar, S., 2012. Aberrant light directly impairs mood and learning through melanopsin-expressing neurons. Nature 491, 594.

Li, Z.G., Li, M., Huang, J.N., Ma, C.S., Xiao, L.C., Huang, Q., Zhao, Y.Z., Nie, H.Y., Su, S.K., 2017a. Effects of Sublethal Concentrations of Chlorpyrifos on Olfactory Learning and memory Performances in two Bee Species, *Apis mellifera* and *Apis cerana*. Sociobiology 64, 174–181.

Li, Z., Li, M., He, J., Zhao, X., Chaimanee, V., Huang, W.-F., Nie, H., Zhao, Y., Su, S., 2017b. Differential physiological effects of neonicotinoid insecticides on honey bees: a comparison between *Apis mellifera* and *Apis cerana*. Pestic. Biochem. Physiol. 140, 1–8.

Liao, Y., Smyth, G.K., Shi, W., 2013. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 25, 402–408.

Lourenco, A.P., Mackert, A., Cristino, A.D., Simoes, Z.L.P., 2008. Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. Apidologie 39, 372–385.

Love, M., Anders, S., Huber, W., 2014. Differential analysis of count data–the DESeq2 package. Genome Biol. 15, 550.

Mallon, E.B., Brockmann, A., Schmid-Hempel, P., 2003. Immune response inhibits associative learning in insects. Proc. R. Soc. Lond. B Biol. Sci. 270, 2471–2473.

Manjon, C., Troczka, B.J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G., Singh, K.S., Zimmer, C.T., Homem, R.A., Lueke, B., 2018. Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides. Curr. Biol. 28 (1137-1143. e1135).

Menzel, R., 2012. The honeybee as a model for understanding the basis of cognition. Nat. Rev. Neurosci. 13, 758–768.

Menzel, R., Giurfa, M., 2001. Cognitive architecture of a mini-brain: the honeybee. Trends Cogn. Sci. 5, 62–71.

Mommaerts, V., Reynders, S., Boulet, J., Besard, L., Sterk, G., Smagghe, G., 2010. Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior. Ecotoxicology 19, 207–215.

Moritz, R.F., Härtel, S., Neumann, P., 2005. Global invasions of the western honeybee (*Apis mellifera*) and the consequences for biodiversity. Ecoscience 12, 289–301.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628.

Müller, U., 2000. Prolonged activation of cAMP-dependent protein kinase during conditioning induces long-term memory in honeybees. Neuron 27, 159–168.

Müller, U., 2002. Learning in honeybees: from molecules to behaviour. Zoology 105,

313–320.
Neumann, P., Carreck, N.L., 2010. Honey bee colony losses. J. Apic. Res. 49, 1–6.
Peng, Y.-C., Yang, E.-C., 2016. Sublethal dosage of imidacloprid reduces the microglomerular density of honey bee mushroom bodies. Sci. Rep. 6, 19298.
Perisse, E., Raymond-Delpech, V., Néant, I., Matsumoto, Y., Leclerc, C., Moreau, M., Sandoz, J.-C., 2009. Early calcium increase triggers the formation of olfactory long-term memory in honeybees. BMC Biol. 7, 30.
Pettis, J.S., Collins, A.M., Wilbanks, R., Feldlaufer, M.F., 2004. Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35, 605–610.
Pettis, J.S., Johnson, J., Dively, G., 2012. Pesticide exposure in honey bees results in increased levels of the gut pathogen *Nosema*. Naturwissenschaften 99, 153–158.
Sitaraman, D., LaFerriere, H., Birman, S., Zars, T., 2012. Serotonin is critical for rewarded olfactory short-term memory in Drosophila. J. Neurogenet. 26, 238–244.
Szu, J.I., Binder, D.K., 2016. The role of astrocytic aquaporin-4 in synaptic plasticity and learning and memory. Front. Integr. Neurosci. 10, 8.
Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268.
Tsvetkov, N., Samson-Robert, O., Sood, K., Patel, H.S., Malena, D.A., Gajiwala, P.H., Maciukiewicz, P., Fournier, V., Zayed, A., 2017. Chronic exposure to neonicotinoids reduces honey bee health near corn crops. Science 356, 1395–1397.
Villar, G., Grozinger, C.M., 2017. Primer effects of the honeybee, *Apis mellifera*, queen pheromone 9-ODA on drones. Anim. Behav. 127, 271–279.
Whitehorn, P.R., O'connor, S., Wackers, F.L., Goulson, D., 2012. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science 336, 351–352 1215025.
Wimalasena, K., 2011. Vesicular monoamine transporters: Structure-function, pharmacology, and medicinal chemistry. Med. Res. Rev. 31, 483–519.
Wu, M.-C., Chang, Y.-W., Lu, K.-H., Yang, E.-C., 2017. Gene expression changes in honey bees induced by sublethal imidacloprid exposure during the larval stage. Insect Biochem. Mol. Biol. 88, 12–20.
Yang, E.C., Chuang, Y.C., Chen, Y.L., Chang, L.H., 2008. Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). J. Econ. Entomol. 101, 1743–1748.
Yu, S., Robinson, F., Nation, J., 1984. Detoxication capacity in the honey bee, *Apis mellifera* L. Pestic. Biochem. Physiol. 22, 360–368.
Yu, G., Wang, L.-G., Han, Y., He, Q.-Y., 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 16, 284–287.
Zhang, E., Nieh, J.C., 2015. The neonicotinoid imidacloprid impairs honey bee aversive learning of simulated predation. J. Exp. Biol. 218, 3199–3205.
Zhou, J.-J., 2010. Odorant-binding proteins in insects. Vitam. Horm. 83, 241–272.
Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). PLoS One 12, e0176837.