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Research Article

Sublethal Effects of the Neonicotinoid Insecticide Thiamethoxam on the Transcriptome of the Honey Bees (Hymenoptera: Apidae)

Teng-Fei Shi,[1] Yu-Fei Wang,[1] Fang Liu,[2] Lei Qi,[2] and Lin-Sheng Yu[1,2,3]

[1]School of Plant Protection, Anhui Agricultural University, Hefei 230036, China, [2]School of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, China, and [3]Corresponding author, e-mail: yulinsheng@ahau.edu.cn

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Abstract

Neonicotinoid insecticides are now the most widely used insecticides in the world. Previous studies have indicated that sublethal doses of neonicotinoids impair learning, memory capacity, foraging, and immunocompetence in honey bees (*Apis mellifera*, Linnaeus) (Hymenoptera: Apidae). Despite these, few studies have been carried out on the molecular effects of neonicotinoids. In this study, we focus on the second-generation neonicotinoid thiamethoxam, which is currently widely used in agriculture to protect crops. Using high-throughput RNA-Seq, we investigated the transcriptome profile of honey bees after subchronic exposure to 10 ppb thiamethoxam over 10 d. In total, 609 differentially expressed genes (DEGs) were identified, of which 225 were upregulated and 384 were downregulated. Several genes, including *vitellogenin*, *CSP3*, *defensin-1*, *Mrjp1*, and *Cyp6as5* were selected and further validated using real-time quantitative polymerase chain reaction assays. The functions of some DEGs were identified, and Gene Ontology–enrichment analysis showed that the enriched DEGs were mainly linked to metabolism, biosynthesis, and translation. Kyoto Encyclopedia of Genes and Genomes pathway analysis showed that thiamethoxam affected biological processes including ribosomes, the oxidative phosphorylation pathway, tyrosine metabolism pathway, pentose and glucuronate interconversions, and drug metabolism. Overall, our results provide a basis for understanding the molecular mechanisms of the complex interactions between neonicotinoid insecticides and honey bees.

Key words: *Apis mellifera*, thiamethoxam, RNA-Seq, qPCR, differential gene expression

Honey bees (*Apis mellifera*, Linnaeus) (Hymenoptera: Apidae) have a high social and economic value since they produce various substances such as honey and also play an important role in pollination and agricultural production (Breeze et al. 2011). In recent years, attention has been paid to the large decrease to global apiculture (Neumann and Carreck 2010, Potts et al. 2010, van Engelsdorp et al. 2010, Chauzat et al. 2013), but the reasons are still poorly understood. Recent studies have however suggested that the decrease could be due to the widespread use of insecticides (Johnson et al. 2010, Goulson et al. 2015, Schmuck and Lewis, 2016).

Recently, there have been far-reaching changes in the insecticide market. Many of the traditional insecticides, e.g., organophosphorus and pyrethroids, have been replaced by systemic insecticides, especially neonicotinoids. Neonicotinoids act on the insect nervous system mainly through agonistic action on nicotinic acetylcholine receptors (nAChRs) (Brown et al. 2006), and since they have low mammalian toxicity (Tomizawa and Casida 2005), they are widely used for controlling insect pests. Neonicotinoids are commonly applied as seed coatings or as foliar sprays on crops. Once absorbed into the plant, neonicotinoids can translocate to dew drops, nectar, and pollen of crops during florescence (Krupke et al. 2012, Stoner and Eitzer 2012). The contaminated nectar and pollen may be consumed by foragers (Goulson 2013) or taken to the nest for long-term storage where they are eaten by the young adults and larvae (DeGrandi-Hoffman and Hagler 2000, Cresswell 2011). Recent studies have detected various neonicotinoids in bee products, e.g., honey, pollen, and beeswax (Stoner and Eitzer 2012, Codling et al. 2016, Sánchez-Hernández et al. 2016), meaning that the neonicotinoids can have chronic effects.

Even though several neonicotinoids, including thiamethoxam, imidacloprid, and clothianidin, have been found to be highly toxic to honeybees (Laurino et al. 2011), they are not acutely lethal at field levels (Blacquière et al. 2012). Nevertheless, there are considerable chronic and sublethal effects, including impairment to the brain, mushroom body, and midgut (Catae et al. 2014; Oliveira et al. 2014; Peng and Yang 2016) and decreased learning and memory

capacity (Aliouane et al. 2009; Mengoni and Farina 2015; Alkassab and Kirchner 2016). Evidence from semi-field or field research indicated that neonicotinoids negatively affect foraging activity and homing flight (Henry et al. 2012; Fischer et al. 2014; Tison et al. 2016). Moreover, neonicotinoids have been found to affect honey bees' immunocompetence (Brandt et al. 2016) and increase the risk of other stressors such as pathogens (Pettis et al. 2013; Alburaki et al. 2015).

Despite the implications for honey bee colonies, little research has so far been carried out into the molecular effects of neonicotinoids. Christen et al. (2016) found that exposure to neonicotinoids changed the transcription of *AChRα1* and 2, *creb*, *pka,* and *vitellogenin* in the brain of honey bees. The latest research from this group (Christen et al. 2017) showed that binary mixtures of neonicotinoids lead to different transcriptional changes in *nAChR* subunits and *vitellogenin* than single neonicotinoids and that transcription was most strongly induced by thiamethoxam.

In the current study, we focused on the second-generation neonicotinoid thiamethoxam (Maienfisch et al. 2003). Using high-throughput RNA-Seq, we investigated the transcriptome profile of honey bees after exposure to a sublethal concentration (10 ppb) for 10 d. The transcriptome profiles were then systematically analyzed by differential gene expression, Gene Ontology (GO) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Our study aims to provide a basis to explore the molecular mechanisms of thiamethoxam and contribute to the understanding of the decline in honey bee populations.

Materials and Methods

Honey Bee Rearing

Two frames with sealed broods near adult emergence were taken from a healthy colony at the Institute of Apiculture Research of Anhui Agriculture University (Hefei, China). The population had not previously been exposed to pesticide. The frames were held in an incubator under the following conditions: 35 ± 1°C, a relative humidity (RH) of 50 ± 10% and in darkness. We obtained the newly emerged honey bees and put them into wooden cages (11 × 10 × 8 cm). They were fed with bee bread collected from the same apiary, 50% (w/v) sucrose–water solution, and maintained for 3 d at 30 ± 1℃, an RH of 70 ± 10%, and in darkness. The dead bees in each cage were removed daily.

Thiamethoxam Preparation and Exposure

The residues of thiamethoxam in trapped pollen generally ranges from 0.6 to 53.3 ppb (Mullin et al. 2010; Krupke et al. 2012; Stoner and Eitzer 2012; Codling et al. 2016; Sánchez-Hernández et al. 2016) and in honey from 2.5 to 17.2 ppb (Codling et al. 2016; Sánchez-Hernández et al. 2016). Based on this, a field-realistic level of 10 ppb (Stanley and Raine 2016) was selected as the sublethal concentration. A stock solution of thiamethoxam (> 99% purity, 1000 ppm) was obtained from J&K (Shanghai, China) and prepared using acetone as a solvent. A 10 ppb of thiamethoxam was prepared in a 50% sucrose–water solution with a 0.03% final concentration of acetone. A 50% sucrose–water solution with the same concentration of acetone was also prepared as a control.

Four-day-old bees were used for the bioassays. Bees received enough bee bread and contaminated sucrose–water solution each day. The bee bread and sucrose–water solution were renewed each day. The experiment was carried out with 60 bees per replicate and three replicates per treatment. The dead bees in each cage were removed daily. After 10 d, we collected all bees and stored them at –80°C. Fifteen bees from each replicate were pooled to obtain one RNA sample.

RNA Extraction

Pools of 15 bees from each sample were prepared for total RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured using a Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA), and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library Preparation for Transcriptome Sequencing

In total, 3-μg RNA from each total RNA sample was to prepare samples. Sequencing libraries were generated using a NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, CA, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under a high temperature in NEBNext First Strand Synthesis Reaction Buffer (5 ×). First strand cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase. After adenylation of the 3' ends of DNA fragments, NEBNext Adaptors with hairpin loop structures were ligated to prepare for hybridization. cDNA fragments of 150–200 bp in length were selected by purifying library fragments with an AMPure XP system (Beckman Coulter, Beverly, USA). Then, 3-μl USER Enzyme (NEB, CA, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C. Polymerase chain reaction (PCR) was then performed using a Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. Finally, PCR products were purified using an AMPure XP system, and library quality was assessed using an Agilent Bioanalyzer 2100 system.

Clustering and Sequencing

Clustering of the index-coded samples were performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 4000 platform and 150 bp paired-end reads were generated.

Read Processing

Raw reads in the FASTQ format were first processed through in-house Perl scripts. Clean reads were obtained by removing low-quality reads or those containing adapters or poly-N. At the same time, the Q20, Q30, and GC contents were calculated. All downstream analyses were based on the high-quality clean reads. The index of the honey bee genome (NCBI: Amel_4.5) was built using Bowtie v2.2.3, and paired-end clean reads were aligned to the honey bee genome using TopHat v2.0.12. HTSeq v0.6.1 was used to count the reads mapped to each gene, and then the FPKM (Fragments Per Kilobase of transcript sequence per Million reads) of each gene was calculated based on the length of the gene and reads count mapped to it.

Differential Expression Analysis

Differential expression analysis was performed using the DESeq R package (2.15.3). DESeq provides statistical routines to determine differential expression in digital gene expression data using a model based on negative binomial distribution. The resulting *P*-values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with an adjusted *P*-value < 0.05 were considered differentially expressed. Finally, GO-enrichment analysis of differentially expressed genes (DEGs) was carried out using the GOseq R package, in which gene length bias was corrected. GO terms with corrected *P*-values < 0.05 were considered significantly enriched by DEGs. In the KEGG database, we used KOBAS software to test the statistical enrichment of differential gene expression in KEGG pathways.

Real-Time Quantitative PCR Analysis

We selected five DEGs (*vitellogenin*, *CSP3*, *defensin-1*, *Mrjp1*, and *Cyp6as5*) for real-time quantitative PCR (qPCR) assays. We started with 0.5-µg total RNA for each sample. *β-actin* was used as reference gene for normalizing the expression levels of the five target genes. ReverTra Ace qPCR RT Master Mix Kit (Toyobo, Osaka, Japan) and SuperReal PreMix Plus (SYBR Green) Kit (TIANGEN, Beijing, China) were used to obtain the cDNA and perform qPCR according to the manufacturer's protocol, respectively. Reverse transcription and qPCR were performed using with PCR system (Bioer, Osaka, Japan) and StepOnePlus Real-Time PCR System (Life Technology, CA, USA), respectively. The relative expression of the target genes was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. All the primers used were given in Supp Table S1 (online only). The differences in *vitellogenin*, *CSP3*, *defensin-1*, *Mrjp1*, and *Cyp6as5* between thiamethoxam-treated bees, and controls were significant by paired-sample *t*-test.

Results

Raw Read Processing and Quantitative Gene Expression

Using high-throughput RNA-Seq, six libraries were created from thiamethoxam-treated and control honey bees. In total, thiamethoxam-1, thiamethoxam-2, thiamethoxam-3, control-1, control-2, and control-3 generated 43,672,706, 42,805,654, 43,630,710, 43,594,286, 44,650,260, and 43,379,868 usable reads, respectively. After mapping to the reference genome (NCBI: Amel_4.5) and the junction database, 38,527,526, 38,026,904, 38,277,904, 39,258,011, 40,153,044, and 37,645,388 total mapped reads were acquired, and the numbers of uniquely mapped reads were 37,673,745, 37,163,425, 37,440,667, 38,121,707, 38,960,068, and 36,686,103. Among these unique reads, 77.58–81.93% were mapped to exon regions (Supp Table S2 [online only]).

We calculated gene activity by counting the reads that mapped to exon regions (≥ 3 per gene). The average number of genes expressed in thiamethoxam and control libraries was 11,426 and 11,330 respectively; and 11,150 genes were expressed in both groups (Fig. 1). We also divided gene expression levels into five grades according to their RPKM (Reads Per Kilo bases per Million reads) values (Supp Table S3 [online only]). In each library, 30.22–31.79% of the reads had RPKM values <1, 11.49–12.56% had RPKM values of 1–3, 28.20–29.17% had RPKM values of 3–15, 18.36–20.86% had values of 15–60 and 8.23–8.95% had RPKM values >60. These results showed that a small number of genes were expressed at very high levels, but the majority were expressed at low levels, indicating that the distribution of our digital gene expression data set was normal.

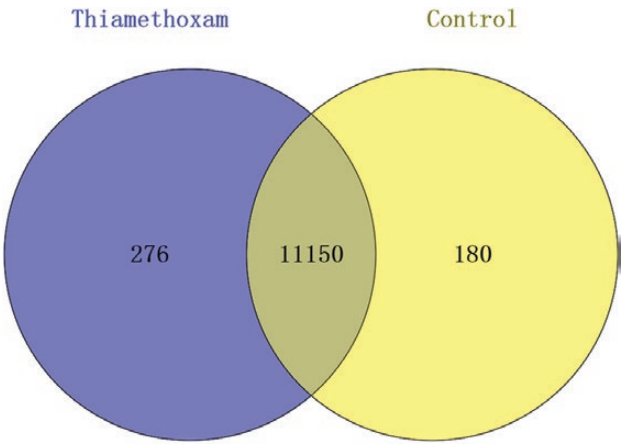

Fig. 1. The average number of genes specifically expressed in thiamethoxam and control libraries, shown as number of genes expressed in each class.

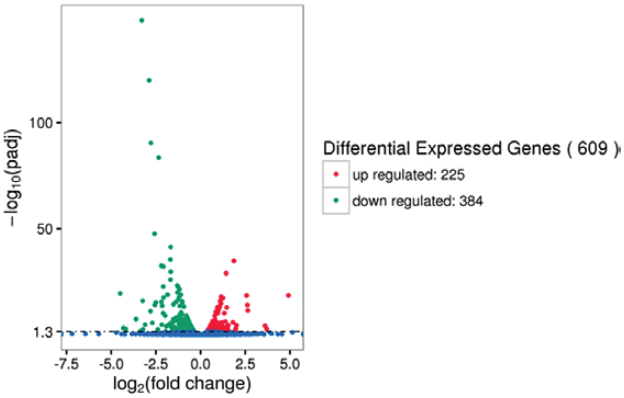

Fig. 2. Volcano plot of differentially expressed genes in honey bees exposed to 10 ppb thiamethoxam over 10 d. Genes with an adjusted *P*-value (padj) < 0.05 (FDR correction method) were considered differentially expressed. Red points: upregulated genes in thiamethoxam-treated bees; green points: downregulated genes in thiamethoxam-treated bees; blue points: no significant difference.

Differentially Expressed Genes

In total, 609 DEGs were detected in honey bees exposed to 10 ppb thiamethoxam, of which, 225 (45.2%) were upregulated and 334 (54.8%) were downregulated (Fig. 2, Supp Table S4 [online only]). A list of the 20 genes with the most significant differential expression were shown in Supp Table S5 (online only), and of these, 17 were downregulated and 3 were upregulated. The 67 confirmed DEGs were listed in Suppl Table S6 (online only), and the others have been designated as hypothetical proteins. We focused here only on those that had previously been confirmed (Table 1).

We found that 10 ribosomal protein (RP) genes, *RpL37* (GB40492), *RpS8* (GB42679), *RpSA* (GB43548), *RpL32* (GB47227), *RpL18A* (GB47433), *RpS3A* (GB49377), *RpS6* (GB50333), *RpS12* (GB51201), *RpL13* (GB54192), and *RpL19* (GB54433), have high expression levels but were downregulated in thiamethoxam-treated bees. In contrast, two nAChRs subunits, *nAChRα9* (GB53427d) and *nAChRβ2* (GB53428), were upregulated along with cytochrome P450 6AS5 (*Cyp6as5*) (GB49890).

Some genes, for example, *defensin1* (GB41428), *vitellogenin* (GB49544), LOC725387 (GB40021), and LOC406093 (GB53576), all have very high expression levels in honey bees, with intensity read copy counts of >10,000. *Defensin1*, *vitellogenin,* and LOC406093 were downregulated in thiamethoxam-treated bees, whereas LOC725387 was upregulated.

Odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) are believed to be involved in odor recognition and chemical communication (Pelosi et al. 2006; Sanchez-Gracia et al. 2009). The genes *Obp3* (GB53371), *Obp17* (GB46226), *Obp21* (GB46230), and *CSP3* (GB52324) all showed significantly decreased expression upon exposure to thiamethoxam. Moreover, three major royal jelly protein (MRJP)-coding genes, *Mrjp1* (GB55205), *Mrjp3* (GB55204), and *Mrjp4* (GB55206), were downregulated. Although the expression level of a memory-related gene, *NMDA receptor 1* (*NR1*) (GB46886), was relatively low, it was differentially expressed in thiamethoxam-treated bees compared with controls.

GO Enrichment Analysis

In total, 445 DEGs were enriched for GO terms, including 167 upregulated and 278 downregulated genes in thiamethoxam-treated bees (Supp Table S7 [online only]), and the top 30 most enriched terms are shown in Fig. 3. The genes were divided into three classes: molecular function, cellular components, and biological process. Based on the GO terms for biological process, we found that most genes were enriched for translation, various metabolic, and biosynthetic processes, such as protein metabolism, cellular protein metabolism, single-organism metabolism, cellular biosynthetic, cellular macromolecule biosynthetic, macromolecule biosynthetic, and organic substance biosynthetic (Fig. 3). The main DEGs that were enriched coded for cellular components, including the ribosome,

Table 1. Information on selected differentially expressed genes in honey bees exposed to 10 ppb thiamethoxam over 10 d, corrected *P*-value < 0.05

Genes	Regulation	Function
NR1	Up	Memory formation (Müßig et al. 2010)
CSP3	Down	Brood pheromone transportation (Briand et al. 2002)
Obp21	Down	Solubilization and release of semiochemicals (Iovinella et al. 2011)
defensin-1	Down	Fight against pathogens (Aronstein and Saldivar 2005; Kwakman et al. 2010, 2011; Richard et al. 2012)
vitellogenin	Down	Regulation of lifespan (Amdam et al. 2006; Nelson et al. 2007)
Mrjp1	Down	Nutritional (Buttstedt et al. 2014); caste differentiation (Kamakura 2011)
Mrjp3	Down	Nutritional (Buttstedt et al. 2014)
Mrjp4	Down	
Cyp6as5	Up	Thiacloprid resistance (Alptekin et al. 2016)
nAChRa9	Up	-
nAChRβ2	Up	-

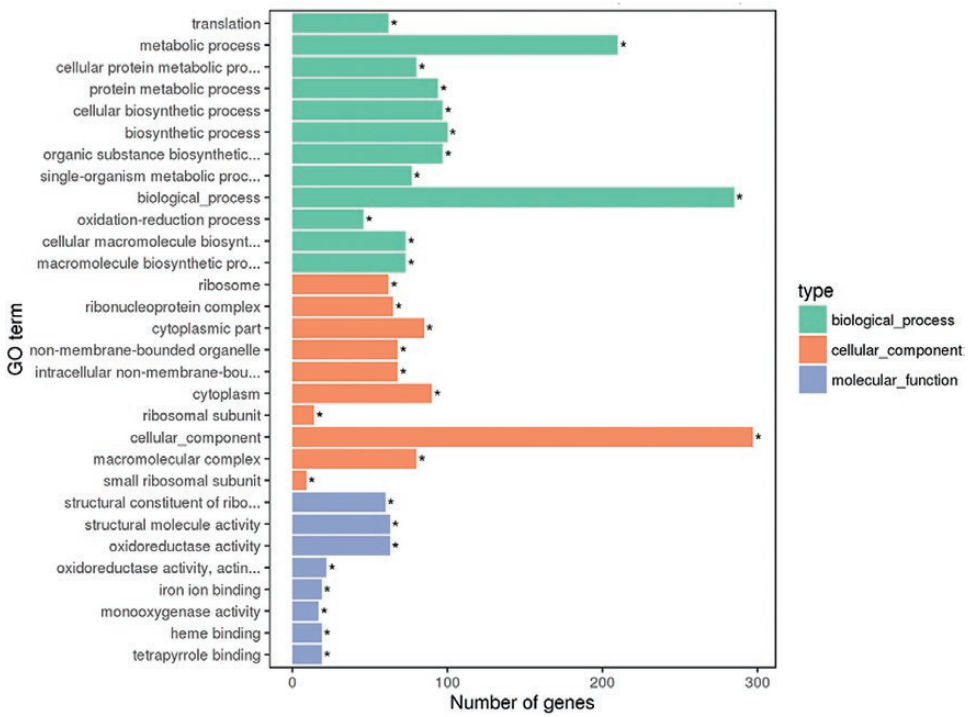

Fig. 3. GO-enrichment analysis of the differentially expressed genes in honey bees exposed to 10 ppb thiamethoxam over 10 d. Green bars: DEGs were enriched for biological process; orange bars: DEGs were enriched for cellular component; purple bars: DEGs were enriched for molecular function. * indicates GO terms were significantly enriched by DEGs (corrected *P*-values < 0.05, FDR correction method).

Table 2. The five significantly enriched pathways, corrected *P*-value < 0.05

Pathways	Pathway ID	Genes number	Corrected *P*-value
Ribosome	ame03010	64	1.1001522981e-25
Oxidative phosphorylation	ame00190	19	0.0286819305139
Pentose and glucuronate interconversions	ame00040	7	0.0286819305139
Tyrosine metabolism	ame00350	6	0.042425360978
Drug metabolism - other enzymes	ame00983	7	0.0369487673874

ribonucleoprotein complex, ribosomal subunit, and ribosomal subunit. Most of the genes were downregulated in thiamethoxam-treated bees (Fig. 3, Supp Table S7 [online only]). In terms of molecular function, the DEGs played roles in structural constitutent of ribosome, structural molecule activity, and oxidoreductase activity (Fig. 3).

KEGG Pathway Analysis

The KEGG database (http://www.genome.jp/kegg) was used to assign functional annotations to the DEGs. In total, 377 DEGs were identified and mapped to 75 pathways in the KEGG pathway database (Supp Table S8 [online only]), including 104 upregulated and 273 downregulated genes in thiamethoxam-treated bees. Among these pathways, five were significantly enriched with a corrected *P*-value < 0.05 (Table 2). These included the regulation of most genes related to ribosomes, oxidative phosphorylation, tyrosine metabolism, pentose and glucuronate interconversion, and drug metabolism.

Real-Time qPCR Analysis

To confirm the results of the RNA-Seq datas, five DEGs (*vitellogenin*, *CSP3*, *defensin-1*, *Mrjp1*, and *Cyp6as5*) were selected for qPCR validation. As shown in Fig. 4, the expression levels of *vitellogenin*, *CSP3*, *defensin-1*, *Mrjp1*, and *Cyp6as5* were all significantly different in thiamethoxam-treated bees compared with controls (*vitellogenin*: $t = -17.886$, df = 2, $P = 0.003$; *CSP3*: $t = -33.777$, df = 2, $P = 0.001$; *defensin-1*: $t = -6.906$, df = 2, $P = 0.02$; *Mrjp1*: $t = -4.409$, df = 2, $P = 0.048$; *Cyp6as5*: $t = 4.777$, df = 2, $P = 0.041$). *CSP3*, *vitellogenin*, *defensin-1*, and *Mrjp1* were downregulated in honey bees exposed to thiamethoxam, while *Cyp6as5* was upregulated. The results were consistent with the sequencing results.

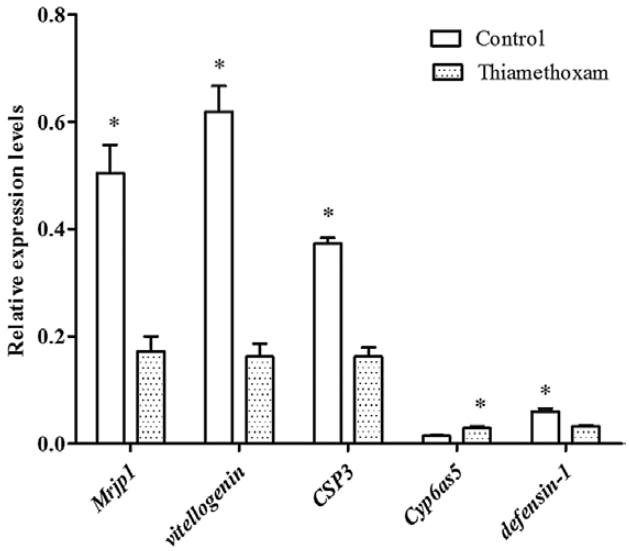

Fig. 4. Real-time quantitative PCR analysis of *vitellogenin*, *CSP3*, *defensin-1*, *Mrjp1*, and *Cyp6as5* in honey bees exposed to 10 ppb thiamethoxam over 10 d. Datas are means ± SEM. * indicates a significant difference compared with controls ($P < 0.05$, paired-samples *t*-test).

Discussion

The neonicotinoid insecticide thiamethoxam is highly toxic to honey bees with LD_{50} values in the range of a few ng per bee. The sublethal effects of thiamethoxam on honey bees have been extensively studied at many different physiological levels (Aliouane et al. 2009; Henry et al. 2012; Catae et al. 2014; Oliveira et al. 2014; Alburaki et al. 2015). Here, we identified 609 DEGs in honey bees on exposure to 10 ppb thiamethoxam, including 225 upregulated genes and 384 downregulated genes. Sevral DEGs, including *vitellogenin*, *CSP3*, *defensin-1*, *Mrjp*, and *Cyp6as5* were selected and further validated using qPCR assays. The results were consistent with the sequencing results. These data demonstrate the reliability of the RNA-Seq results.

The results of GO and KEGG analysis showed that the regulation of many DEGs related to metabolism, biosynthesis, and translation, the ribosome pathway, oxidative phosphorylation pathway, tyrosine metabolism pathway, and pentose and glucuronate interconversions. This suggests that thiamethoxam mainly affects physiological and developmental processes in honey bees.

nAChRs are targets of neonicotinoids, and their induction can produce overt effects such as decreased memory and locomotor capacity (Aliouane et al. 2009; Charreton et al. 2015). Our study showed that subchronic exposure to thiamethoxam increases the expression of two nAChRs subunits, *nAChRα9* and *nAChRβ2*. These results are consistent with previous research that thiamethoxam upregulates expression of *nAChRα1* and *nAChRα2* (Christen et al. 2016, 2017). This indicates a compensatory reaction to the functional loss of nAChRs due to neonicotinoids (Christen et al. 2016).

NMDA glutamate receptors (NMDARs) are composed of NR1 and NR2 subunits and are known to play an important role in memory formation (Kandel, 2001): injection of NMDA receptor antagonists led to an impairment of long-term memory (Si et al. 2004). The NR1 subunit is expressed throughout the honey bee brain and plays a critical role in the functional expression of NMDARs (Zannat et al. 2006). Müßig et al. (2010) reported that inhibiting expression of the *NR1* subunit in the honey bee brain can impair the formation of mid-term and early long-term memory. Thus, the thiamethoxam-induced alteration of *NR1* in our study might partly explain the effects on memory formation.

There are two known classes of small soluble proteins in the chemosensilla of insects: OBPs and CSPs. These are believed to be involved in odor recognition and chemical communication (Pelosi et al. 2006; Sanchez-Gracia et al. 2009), and CSP3 is thought to play a role in brood pheromone transportation (Briand et al. 2002).

OBP21 can bind the main components of queen mandibular pheromone as well as farnesol, a compound used as a trail pheromone (Iovinella et al. 2011). The downregulation of *CSP3* and *Obp21* seen in our study suggested a reduced chemosensory ability in challenged honey bees. This is generally consistent with recent research which indicated that a sublethal dose of the neonicotinoid imidacloprid decreased the binding affinity of OBP2 to a floral volatile, β-ionone, in Asiatic honey bees (Li et al. 2015).

Neonicotinoids could also affect the immunocompetence of honey bees. A recent study reported that exposure to field-realistic concentrations of imidacloprid decreased hemocyte density, the encapsulation response, and antimicrobial action (Brandt et al. 2016). Antimicrobial peptides are a key component of honey bee innate immunity (Danihlík et al. 2015) and include defensin, which is coded by two different defensin genes (*defensin-1* and *defensin-2*) in the honey bee genome (Evans et al. 2006). *Defensin-1* has been found to be upregulated following bacterial challenge (Aronstein and Saldivar, 2005; Richard et al. 2012), suggesting that it is important in pathogen defense. Kwakman et al. (2010, 2011) found that *defensin-1* is a key antimicrobial compound of honey. The thiamethoxam-induced downregulation of *defensin-1* found in our experiment may be involved in reducing the effectiveness of the immune system.

Vitellogenin is also a component of the defense mechanism since it acts as a free-radical scavenger to protect honey bees from oxidative stress (Amdam et al. 2006; Seehuus et al. 2006). Downregulation of vitellogenin can shorten the lifespan of honey bees (Nelson et al. 2007), so the thiamethoxam-induced decrease in *vitellogenin* expression in our experiment might contribute to reducing honey bee longevity.

Nutrition also plays an important role in honey bee development and longevity. Royal jelly (RJ) is a natural source of nutrients such as essential amino acids, lipids, vitamins, and acetylcholine. MRJPs constitute around 90% of total RJ protein. So far, nine MRJPs (MRJP1–MRJP9) and their encoding genes (*Mrjp1-Mrjp9*) have been identified, and MRJPs1–MRJPs5 have been suggested to have a nutritional function due to their abundance in RJ protein (Buttstedt et al. 2014). MRJP1 is the predominant MRJP and is more highly expressed in the hypopharyngeal glands than in other body parts. An MRJP1 monomer, royalactin, was found to drive queen development through an Egfr-mediated signaling pathway (Kamakura 2011). The honey bees used in our experiments were all young worker bees which can produce RJ in the hypopharyngeal glands. The thiamethoxam-induced downregulation of *mrjp1*, *mrjp3*, and *mrjp4* might decrease MRJP synthesis and indirectly cause a nutrition reduction in the lavae and queen.

However, just like other insects, honey bees have systems to detoxify insecticides. Cytochrome P450 monooxygenases (P450s) are the main detoxification enzymes which play an important role in the detoxification and metabolism of xenobiotics and insecticides. A P450 gene, *CYP6as5*, was induced in our experiment. This gene is a member of the CYP6AS subfamily, which have been implicated in the metabolism of xenobiotics in honey and pollen (Mao et al. 2009; Johnson et al. 2012). A recent study showed that *CYP6as5* was also significantly overexpressed in thiacloprid-treated bees compared with untreated controls and induced thiacloprid insensitivity (Alptekin et al. 2016). The P450 gene, *CYP6as5*, seems to play a central role in neonicotinoid insecticide resistance in honey bees.

In summary, using high-throughput RNA-Seq and analysis of differential gene expression, we detected 609 DEGs in honey bees after challenge with a sublethal concentration of thiamethoxam. We identified several genes involved in various physiological functions, but further studies are needed to confirm the results of this analysis. GO terms and KEGG pathways were also used to further understand the function of these genes. Our results provide a reference for understanding the molecular mechanisms of the complex interactions between neonicotinoid insecticides and honey bees.

Acknowledgments

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Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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