1 Sublethal effects of the neonicotinoid insecticide thiamethoxam on 2 the transcriptome of the honeybee (*Apis mellifera*)

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9 Running title: Neonicotinoids in the honeybee

10 **Abstract:** Neonicotinoid insecticides are now the most widely used insecticides in the world.
11 Previous studies have indicated that sublethal doses of neonicotinoids impair learning, memory
12 capacity, foraging and immunocompetence in honeybees (*Apis mellifera*). Despite this, few
13 studies have been carried out on the molecular effects of neonicotinoids. In this study, we focus on
14 the second-generation neonicotinoid thiamethoxam, which is currently widely used in agriculture
15 to protect crops. Using high-throughput RNA-Seq, we investigated the transcriptome profile of
16 honeybees after subchronic exposure to thiamethoxam (10 ppb) over 10 days. In total, 609
17 differentially-expressed genes (DEGs) were identified, of which 225 were up-regulated and 384
18 were down-regulated. The functions of some DEGs were identified, and GO enrichment analysis
19 showed that the enriched DEGs were mainly linked to metabolism, biosynthesis and translation.
20 KEGG pathway analysis showed that thiamethoxam affected biological processes including
21 ribosomes, the oxidative phosphorylation pathway, tyrosine metabolism pathway, pentose and
22 glucuronate interconversions and drug metabolism. Overall, our results provide a basis for
23 understanding the molecular mechanisms of the complex interactions between neonicotinoid
24 insecticides and honeybees.

25 **Key words:** Thiamethoxam; honeybees (*Apis mellifera*); RNA-Seq; differential gene expression

26 **Summary statement:** *NR1*, *Cyp6as5*, *nAChRa9* and *nAChRβ2* were up-regulated in honeybees
27 exposed to thiamethoxam, while *CSP3*, *Obp21*, *defensin-1*, *Mrjp1*, *Mrjp3* and *Mrjp4* were
28 down-regulated.

30 1. Introduction

31 Honeybees (*Apis mellifera*, L.) have a high social and economic value since they produce
32 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 floescence (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 *et al.*, 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 (Blacquire *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 honeybee 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 honeybee 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 honeybees. 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 honeybees after exposure to a sublethal concentration (10 ppb) for 10 days. 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 honeybee populations.

2. Results

2.1. Raw read processing and quantitative gene expression

Using high-throughput RNA-Seq, six libraries (SCTH_1-3 and CK_1-3) were created from the two treatments (SCTH and CK). In total, SCTH_1, SCTH_2, SCTH_3, CK_1, CK_2 and CK_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 (Table S1).

We calculated gene activity by counting the reads that mapped to exon regions (≥ 3 per gene). The average number of genes expressed in SCTH and CK 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 (Table S2). 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 DGE dataset was normal.

Figure 1.

2.2. Differentially-expressed genes between SCTH and CK honeybees

In total, 609 differentially-expressed genes (DEGs) were detected, of which 225 (45.2%) were up-regulated and 334 (54.8%) were down-regulated in the SCTH bees compared with CK bees (Fig. 2, Table S3). A list of the 20 genes with the most significant differential expression is shown in Table S4 and of these, 17 were down-regulated and 3 were up-regulated in SCTH honeybees. The 67 confirmed DEGs are listed in Table S5, 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*, *RpS8*, *RpSA*, *RpL32*, *RpL18A*, *RpS3A*, *RpS6*, *RpS12*, *RpL13* and *RpL19*) have high expression levels, but were down-regulated in SCTH honeybees. In contrast, two nicotinic acetylcholine receptors (nAChRs) subunits, *nAChRa9* and *nAChRβ2*, were up-regulated along with cytochrome P450 6AS5 (*Cyp6as5*).

Some genes, for example *defensin 1*, *vitellogenin*, LOC725387 and LOC406093, all have very high expression levels in both SCTH and CK honeybees, with intensity read copy counts of > 10,000. *Defensin1*, *vitellogenin* and LOC406093 were down-regulated in SCTH honeybees, whereas LOC725387 was up-regulated.

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*, *Obp17*, *Obp21* and *CSP3* all showed significantly decreased

expression in SCTH honeybees. Moreover, three major royal jelly protein (MRJP) coding genes, *Mrjp*, *Mrjp2* and *Mrjp3*, were down-regulated in the SCTH group. Although the expression level of a memory-related gene, *NMDA receptor 1 (NRI)*, was relatively low, it was differentially expressed between SCTH and CK honeybees.

Figure 2.

2.3. Gene ontology (GO) enrichment analysis

A total of 445 DEGs were enriched for GO terms, including 167 up-regulated and 278 down-regulated genes in SCTH bees (Table S6), 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, ribonucleoprotein complex, ribosomal subunit and ribosomal subunit. Most of the genes were down-regulated in SCTH honeybees (Fig 3, Table S6). In terms of molecular function, the DEGs played roles in structural constituent of ribosome, structural molecule activity and oxidoreductase activity (Fig 3).

Figure 3

2.4 KEGG pathway analysis

The KEGG database (<http://www.genome.jp/kegg>) was used to assign functional annotations to the DEGs. A total of 377 DEGs were identified and mapped to 75 pathways in the KEGG pathway database (Table S7), including 104 up-regulated and 273 down-regulated genes. 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.

3. Discussion

The neonicotinoid insecticide thiamethoxam is highly toxic to honeybees with LD_{50} values in the range of a few ng per bee. The sublethal effects of thiamethoxam on honeybees 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 differentially-expressed genes (DEGs) in honeybees on exposure to sublethal concentration of thiamethoxam, including 225 up-regulated genes and 384 down-regulated genes.

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 honeybees.

Nicotinic acetylcholine receptors (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, *nAChR α 9* and *nAChR β 2*. These results are consistent with previous research that thiamethoxam up-regulates expression of *nAChR α 1* and *nAChR α 2* (Christen *et al.*, 2016; Christen *et al.*, 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 honeybee 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 honeybee 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: odorant-binding proteins (OBPs) and chemosensory proteins (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 down-regulation of *CSP3* and *Obp21* seen in our study suggested a reduced chemosensory ability in challenged honeybees. 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 honeybees (Li *et al.*, 2015).

Neonicotinoids could also affect the immunocompetence of honeybees. 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 honeybee innate immunity and include defensin, which is coded by two different defensin genes (*defensin-1* and *defensin-2*) in the honeybee genome (Evans *et al.*, 2006). *Defensin-1* has been found to be up-regulated 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 down-regulation 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 honeybees from oxidative stress (Amdam *et al.*, 2006; Seehuus *et al.*, 2006). Down-regulation of vitellogenin can shorten the lifespan of honeybees (Nelson *et al.*, 2007), so the thiamethoxam-induced decrease in *vitellogenin* expression in our experiment might contribute to reducing honeybee longevity.

Nutrition also plays an important role in honeybee development and longevity. Royal jelly (RJ) is a natural source of nutrients such as essential amino acids, lipids, vitamins and acetylcholine. Major Royal Jelly Proteins (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 honeybees used in our experiments were all young worker bees which can produce RJ in the hypopharyngeal glands. The thiamethoxam-induced down-regulation of *mrjp1*, *mrjp3* and *mrjp4* might decrease MRJP synthesis and indirectly cause a nutrition reduction in the larvae and queen.

However, just like other insects, honeybees 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 over-expressed 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 honeybees.

In summary, using high-throughput RNA-Seq and analysis of differential gene expression, we detected 609 differentially-expressed genes in honeybees (*Apis mellifera*) 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 pesticides and honeybees.

4. Materials and Methods

4.1. Honeybee 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. From July to September 2016 the frames were held in an incubator under the following conditions: $35 \pm 1^\circ\text{C}$, a relative humidity (RH) of $50 \pm 10\%$ and in darkness. We obtained the newly-emerged honeybees and put them into cages ($11 \times 10 \times 8$ cm). They were fed with bee bread collected from the same apiary, sucrose–water solution (1:1 w:v), and maintained for three days at $30 \pm 1^\circ\text{C}$, a RH of $70 \pm 10\%$ and in darkness.

4.2. 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 sublethal concentration (10 ppb) of thiamethoxam (SCTH) was prepared in a 50% sucrose-water solution (1:1 w:v) with a 0.03% final concentration of acetone. A 50% sucrose-water solution (1:1 w:v) with the same concentration of acetone was also prepared as a control check (CK).

Four-day-old bees were used for the bioassays and each treatment (SCTH or CK) involved three cages of 60 bees each, all from the same colony. After ten days, we collected all bees and stored them at -80°C.

4.3. 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).

4.4. Library preparation for transcriptome sequencing

A total of 3 µg RNA was to prepare samples. Sequencing libraries were generated using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, 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, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C. 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.

4.5 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.

4.6 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 honeybee genome (NCBI: Amel_4.5) was built using Bowtie v2.2.3, and paired-end clean reads were aligned to the honeybee 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.

4.7. 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 (DGE) 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, Gene Ontology (GO) enrichment analysis of differentially-expressed genes 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 differentially-expressed genes. In the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, we used KOBAS software to test the statistical

enrichment of differential gene expression in KEGG pathways.

Competing interests

The authors declare no competing or financial interests.

Author contributions

L.S.Y. and T.F.S. conceived and designed the study. T.F.S., Y.F.W and L.Q. performed the experiments. T.F.S. analyzed data and wrote the manuscript. F.L. edited and revised the manuscript. All authors have read and approved the final manuscript.

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References

- Alburaki, M., Boutin, S., Mercier, P.L., Loublier, Y., Chagnon, M. Derome N. (2015). Neonicotinoid-coated zeamays seeds indirectly affect honeybee performance and pathogen susceptibility in field trials. *PLoS One*. **10**, e0125790.
- Aliouane, Y., El Hassani, A.K., Gary, V., Armengaud, C., Lambin, M. Gauthier, M. (2009). Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. *Environ. Toxicol. Chem.* **28**, 113–122.
- Alkassab, A.T., Kirchner, W.H. (2016). Impacts of chronic sublethal exposure to clothianidin on winter honeybees. *Ecotoxicology* **25**, 1000–1010.
- Alptekin, S., Bass, C., Nicholls, C., Paine, M.J., Clark, S.J., Field, L., Moores, G.D. (2016). Induced thiacloprid insensitivity in honeybees (*Apis mellifera* L.) is associated with up-regulation of detoxification genes. *Insect. Mol. Biol.* **25**, 171-180.
- Amdam, G.V., Simoes, Z.L.P., Hagen, A., Norberg, K., Schröder, K., Mikkelsen, Ø., Kirkwood, T.B.L., Omholt, S.W. (2004). Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Experimental Gerontology* **39**, 767–773.
- Aronstein, K., Saldivar, E. (2005). Characterization of a honey bee Toll related receptor gene *Am18w* and its potential involvement in antimicrobial immune defense. *Apidologie* **36**, 3–14.
- Blacquire, T., Smagghe, G., van Gestel, C.A., Mommaerts, V. (2012). Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicol* **21**, 973–992.
- Brandt, A., Gorenflo, A., Siede, R., Meixner, M, Bchler, R. (2016). The neonicotinoids thiacloprid, imidacloprid,

335 and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.). *J. Insect Physiol.* **86**, 40–47

336 Breeze, TD., Bailey, AP., BalcombeK, G., Potts, S.G. (2011). Pollination services in the UK: how important are

337 honeybees? *Agric. Ecosyst. Environ.* **142**, 137–143.

338 Briand, L., Swasdipan, N., Nespoulous, C., Bezirard, V., Blon, F., Huet, J.C., Ebert, P., Penollet, J.C. (2002).

339 Characterization of a chemosensory protein (ASP3c) from honeybee (*Apis mellifera* L.) as a brood pheromone

340 carrier. *Eur. J. Biochem.* **269**, 4586–4596.

341 Brown, L.A., Ihara, M., Buckingham, S.D., Matsuda, K., Sattelle, D.B. (2006). Neonicotinoid insecticides display

342 partial and super agonist actions on native insect nicotinic acetylcholine receptors. *J. Neurochem.* **99**, 608–615.

343 Buttstedt, A., Moritz, R.F., Erler, S. (2014). Origin and function of the major royal jelly proteins of the honeybee

344 (*Apis mellifera*) as members of the yellow gene family. *Biol Rev Camb Philos Soc.* **89**, 255–69.

345 Catae, A.F., Roat, T.C., De Oliveira, R.A., Nocelli, R.C.F., Malaspina, O. (2014). Cytotoxic effects of

346 thiamethoxam in the midgut and malpighian tubules of Africanized *Apis mellifera* (Hymenoptera: Apidae).

347 *Microsc. Res. Tech.* **77**, 274–281.

348 Charreton, M., Decourtye, A., Henry, M., Rodet, G., Sandoz, J.C., Charnet, P., Collet, C. (2015). A locomotor

349 deficit induced by sublethal doses of pyrethroid and neonicotinoid insecticides in the honeybee *Apis mellifera*.

350 *PLoS One* **10**, e0144879.

351 Chauzat, MP., Cauquil, L., Roy, L., Franco, S., Hendrikx, P., Ribiere-Chabert, M. (2013). Demographics of the

352 European apicultural industry. *PLoS One* **8**, e79018.

353 Christen, V., Mittner, F., Fent, K. (2016). Molecular effects of neonicotinoids in honeybees (*Apis mellifera*).

354 *Environ. Sci. Technol.* **50**, 4071–4081.

355 Christen, V., Mittner, F., Fent, K. (2017). Binary mixtures of neonicotinoids show different transcriptional changes

356 than single neonicotinoids in honeybees (*Apis mellifera*). *Environmental Pollution* **220**, 1264–1270.

357 Codling G., Al Naggar, N.Y., Giesy, J.P., Robertson, A.J. (2016). Concentrations of neonicotinoid insecticides in

358 honey, pollen and honeybees (*Apis mellifera* L.) in central Saskatchewan, Canada. *Chemosphere* **144**,

359 2321–2328.

360 Cresswell, J.E. (2011). A meta-analysis of experiments testing the effects of a neonicotinoid insecticide

361 (imidacloprid) on honey bees. *Ecotoxicology* **20**, 149–57.

362 Danihlík, J., Aronstein, K., Petřivalský, M. (2015). Antimicrobial peptides: a key component of honey bee innate

363 immunity. *J. Apicult Res.* **2**, 1–14.

364 De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., Lemaitre, B. (2002). The Toll and Imd pathways are the

365 major regulators of the immune response in *Drosophila*. *EMBO J.* **21**, 2568–2579.

366 Degrandi-Hoffman, G., Hagler, J. (2000). The flow of incoming nectar through a honey bee (*Apis mellifera* L.)

367 colony as revealed by a protein marker. *Insectes Sociaux* **47**, 302–306.

368 Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost M., Thompson G.J., Zou, Z,

369 Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect. Mol.*

370 *Biol.* **15**, 645–656.

371 Fischer, J., Muller, T., Spatz, A.K., Greggers, U., Grunewald, B., Menzel, R. (2014). Neonicotinoids interfere with

372 specific components of navigation in honeybees. *PLoS One* **9**, e91364.

373 Goulson, D. (2013). An overview of the environmental risks posed by neonicotinoid insecticides. *J. Appl. Ecol.* **50**,

374 977–987.

375 Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L. (2015). Bee declines driven by combined stress from

376 parasites, pesticides, and lack of flowers. *Science* **347**, 1255957.

377 Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.F., Aupinel, P. (2012). A common pesticide decreases

378 foraging success and survival in honey bees. *Science* **336**, 348–50.

379 Iovinella, I., Dani, F.R., Niccolini, A., Sagona, S., Michelucci, E., Gazzano, A., Turillazzi, S., Felicioli, A., Pelosi,

380 P. (2011). Differential Expression of odorant-binding proteins in the mandibular glands of the honey bee

381 according to caste and age. *J. Proteome Re.* **10**, 3439-3449.

382 Johnson, R.M., Ellis, MD., Mullin, CA., Frazier, M. (2010). Pesticides and honey bee toxicity—USA. *Apidologie*

383 **41**, 312–31.

384 Johnson, R.M., Mao, W., Pollock, H.S., Niu, G., Schuler, M.A., Berenbaum, M.R. (2012). Ecologically appropriate

385 xenobiotics induce cytochrome P450s in *Apis mellifera*. *PLoS One* **7**, e31051.

386 Kamakura, M. (2011). Royalactin induces queen differentiation in honeybees. *Nature* **473**, 478–483.

387 Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science*

388 **294**, 1030 -1038.

389 Krupke, C.H., Hunt, G.J., Eitzer, B.D., Andino, G., Given, K. (2012). Multiple routes of pesticide exposure for

390 honey bees living near agricultural fields. *PLoS One* **7**, e29268.

391 Kwakman, P.H.S., te Velde, A.A., de Boer, L., Speijer, D., Vandenbroucke-Grauls, C.M.J.E., Zaat, S.A.J. (2010).

392 How honey kills bacteria. *FASEB Journal* **24**, 2576–2582.

393 Kwakman, P.H.S., te Velde, A.A., de Boer, L., Vandenbroucke-Grauls, C.M.J.E., Zaat, S.A.J. (2011). Two major

394 medicinal honeys have different mechanisms of bactericidal activity. *PLoS One* **6**, e17709.

395 Laurino, D., Porporato, M., Patetta, A., Manino, A. (2011). Toxicity of neonicotinoid insecticides to honey bees:
396 laboratory tests. *Bulletin of Insectology* **64**, 107-113.

397 Li, H.L., Wu, F., Zhao, L., Tan, J., Jiang, H.T., Hu, F.L. (2015). Neonicotinoid insecticide interact with honeybee
398 odorant-binding protein: implication for olfactory dysfunction. *Int. J. Biol. Macromol.* **81**, 624–630.

399 Maienfisch, P., Huerlimann, H., Rindlisbacher, A., Gsell, L., Dettwiler, H., Haettenschwiler, J., Sieger, E., Walti,
400 M. (2003). The discovery of thiamethoxam: a second-generation neonicotinoid. *Pest. Manag. Sci.* **57**, 165-176.

401 Mao, W., Rupasinghe, S.G., Johnson, R.M., Zangerl, A.R., Schuler, M.A., Berenbaum, M.R. (2009). Quercetin-
402 metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae). *Comp Biochem*
403 *Physiol B Biochem Mol Biol.* **154**, 427-434.

404 Mengoni, G.C., Farina, W.M. (2015). Effects of sublethal doses of imidacloprid on young adult honeybee
405 behaviour. *PLoS One.* **10**, e0140814.

406 Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., Pettis, J.S. (2010). High
407 levels of miticides and agrochemicals in north American apiaries: implications for honey bee health. *PLoS One.*
408 **5**, e9754.

409 Müssig, L., Richlitzki, A., Rößler, R., Eisenhardt, D., Menzel, R., Lebouille, G. (2010). Acute disruption of the
410 NMDA receptor subunit NR1 in the honeybee brain selectively impairs memory formation. *J. Neurosci.* **30**,
411 7817–7825.

412 Neumann, P., Carreck, N.L. (2010). Honey bee colony losses. *J. Apic. Res.* **49**, 1–6.

413 Nelson, C.M., Ihle, K.E., Fondrk, M.K., Page, R.E., Amdam, G.V. (2007). The gene vitellogenin has multiple
414 coordinating effects on social organization. *PLoS Biology* **5**, e62.

415 Oliveira, R.A., Roat, T.C., Carvalho, S.M., Malaspina, O. (2014). Side-effects of thiamethoxam on the brain and
416 midgut of the Africanized honeybee *Apis mellifera* (Hymenoptera: Apidae). *Environ Toxicol.* **29**, 1122-1133.

417 Pelosi, P., Zhou, J.J., Ban, L.P., Calvello, M. (2006). Soluble proteins in insect chemical communication. *Cell.*
418 *Mol. Life. Sci.* **63**, 1658-1676.

419 Peng, Y.C., Yang, E.C. (2016). Sublethal dosage of imidacloprid reduces the microglomerular density of honey bee
420 mushroom bodies. *Sci. Rep.* **6**, 19298.

421 Pettis, J.S., Lichtenberg, E.M., Andree, M., Stitzinger, J., Rose, R., van Engelsdorp, D. (2013). Crop pollination
422 exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. *PLoS*
423 *One.* **98**, e70182.

424 Potts, S., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E. (2010). Global pollinator

-
- 425 declines: trends, impacts and drivers. *Trends. Ecol. Evol.* **25**, 345–353.
- 426 Richard, F.J., Holt, H.L., Grozinger, C.M. (2012). Effects of immunostimulation on social behavior, chemical
- 427 communication and genome-wide gene expression in honey bee workers (*Apis mellifera*). *BMC Genomics* **13**,
- 428 558.
- 429 Sánchez-Hernández, L., Hernández-Domínguez, D., Martín, M.T., Nozal, M.J., Higes, M., Bernal Yagüe, J.L.
- 430 (2016). Residues of neonicotinoids and their metabolites in honey and pollen from sunflower and maize seed
- 431 dressing crops. *J. Chromatogr A.* **1428**, 220-227.
- 432 Sanchez-Gracia, A., Vieira, F.G., Rozas, J. (2009). Molecular evolution of the major chemosensory gene families
- 433 in insects. *Heredity* **103**, 208-216.
- 434 Schmuck, R., Lewis, G. (2016). Review of field and monitoring studies investigating the role of nitro-substituted
- 435 neonicotinoid insecticides in the reported losses of honey bee colonies (*Apis mellifera*). *Ecotoxicology.* **25**,
- 436 1617–1629.
- 437 Seehuus, S.C., Norberg, K., Gimsa, U., Krekling, T., Amdam, G.V. (2006). Reproductive protein protects sterile
- 438 honeybee workers from oxidative stress. *PNAS.* **103**, 962–967.
- 439 Si, A., Helliwell, P., Maleszka, R. (2004). Effects of NMDA receptor antagonists on olfactory learning and
- 440 memory in the honeybee (*Apis mellifera*). *Pharmacol. Biochem. Behav.* **77**, 191–197.
- 441 Stanley, D.A., Raine, N.E. (2016). Chronic exposure to a neonicotinoid pesticide alters the interactions between
- 442 bumblebees and wild plants. *Functional Ecology* **30**, 1132-1139.
- 443 Stoner, K.A., Eitzer, B.D. (2012). Movement of soil-applied imidacloprid and thiamethoxam into nectar and pollen
- 444 of squash (*Cucurbita pepo*). *PLoS One.* **7**, e39114.
- 445 Tison, L., Hahn, M.L., Holtz, S., Rossner, A., Greggers, U., Bischoff, G., Menzel, R. (2016). Honeybees' behavior
- 446 is impaired by chronic exposure to the neonicotinoid thiacloprid in the field. *Environ. Sci. Technol.* **50**,
- 447 7218-7227.
- 448 Tomizawa, M., Casida, J.E. (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu*
- 449 *Rev. Pharmacol. Toxicol.* **45**, 247-68.
- 450 VanEngelsdorp, D., Speybroeck, N., Evans, J.D., Nguyen, B.K., Mullin, C., Frazier, M., Frazier, J., Cox-Foster, D.,
- 451 Chen, Y., Tarpy, D.R. et al. (2010). Weighing risk factors associated with bee colony collapse disorder by
- 452 classification and regression tree analysis. *J. Econ Entomol.* **103**, 1517–23.
- 453 Zannat, T., Locatelli, F., Rybak, J., Menzel, R., Lebouille, G. (2006). Identification and localisation of the NR1
- 454 sub-unit homologue of the NMDA glutamate receptor in the honeybee brain. *Neurosci Lett.* **398**, 274 –279.

Figure legends

Fig.1. The average number of genes specifically expressed in SCTH and CK libraries, shown as number of genes expressed in each class. Honeybees exposure to sublethal concentration of thiamethoxam (10ppb) over 10 days, with acetone as the control check. Each library (SCTH or CK) involved three biological replicates of 15 bees each. SCTH: sublethal concentration of thiamethoxam; CK: control check.

Fig.2. Volcano plot of differentially-expressed genes between SCTH and CK honeybees (*Apis mellifera*). Each treatment (SCTH or CK) involved three biological replicates of 15 bees each. Genes with an adjusted P -value (padj) ≤ 0.05 (FDR correction method) were considered differentially expressed between SCTH and CK bees. Red points: up-regulated genes in SCTH bees; Green points: down-regulated genes in SCTH bees; Blue points: no significant difference. SCTH: sublethal concentration of thiamethoxam; CK: control check.

Fig.3. GO enrichment analysis of the differentially-expressed genes (DEGs) between SCTH and CK honeybees (*Apis mellifera*). Green bars: DEGs were enriched for biological process; Orange bars: DEGs were enriched for cellular component; Purple bars: DEGs were enriched for molecular function. Asterisk indicates GO terms were significantly enriched by DEGs (corrected P -values ≤ 0.05 , FDR correction method). SCTH: sublethal concentration of thiamethoxam; CK: control check.

Table 1. Information on selected differentially-expressed genes between SCTH and CK honeybees (*Apis mellifera*), corrected P -value ≤ 0.05 .

Gene	Regulation (SCTH VS CK)	Function
<i>NR1</i>	Up	Memory formation (Müßig <i>et al.</i> , 2010)
<i>CSP3</i>	Down	Brood pheromone transportation (Briand <i>et al.</i> , 2002)
<i>Obp21</i>	Down	Solubilization and release of semiochemicals (Iovinella <i>et al.</i> , 2011)
<i>defensin-1</i>	Down	Fight against pathogens (Aronstein and Saldivar, 2005; Kwakman <i>et al.</i> , 2010, 2011; Richard <i>et al.</i> , 2012)
<i>vitellogenin</i>	Down	Regulation of lifespan (Amdam <i>et al.</i> , 2006; Nelson <i>et al.</i> , 2007)

		Nutritional (Buttstedt <i>et al.</i> , 2014); Caste differentiation
<i>Mrjp1</i>	Down	(Kamakura, 2011)
<i>Mrjp3</i>	Down	
<i>Mrjp4</i>	Down	Nutritional (Buttstedt <i>et al.</i> , 2014)
<i>Cyp6as5</i>	Up	Thiacloprid resistance (Alptekin <i>et al.</i> , 2016)
<i>nAChRa9</i>	Up	-
<i>nAChRβ2</i>	Up	-

SCTH: sublethal concentration of thiamethoxam; CK: control check. Up: up-regulated in SCTH bees; Down: down-regulated in SCTH bees.

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

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