bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



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

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

4 [1] School of Plant Protection, Anhui Agricultural University, Hefei 230036, China;
5 [2] School of Animal Science and Technology, Anhui Agricultural University, Hefei 230036,
6 China;
7 * Correspondence: stf0623@163.com; Tel./Fax: +86-0551-65782158

8

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.

29

---

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

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



33 production (Breeze *et al*., 2011). In recent years, attention has been paid to the large decrease to
34 global apiculture (Neumann and Carreck, 2010; Potts et al., 2010; Van Engelsdorp *et al*., 2010;
35 Chauzat *et al*., 2013) but the reasons are still poorly understood. Recent studies have however
36 suggested that the decrease could be due to the widespread use of insecticides (Johnson *et al*.,
37 2010; Goulson *et al*., 2015; Schmuck and Lewis, 2016).

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39 traditional insecticides, *e.g.* organophosphorus and pyrethroids, have been replaced by systemic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

51 Even though several neonicotinoids, including thiamethoxam, imidacloprid and clothianidin,
52 have been found to be highly toxic to honeybees (Laurino *et al*., 2011), they are not acutely lethal
53 at field levels (Blacquière *et al*., 2012). Nevertheless, there are considerable chronic and sublethal
54 effects, including impairment to the brain, mushroom body and midgut (Catae *et al*., 2014;
55 Oliveira *et al*., 2014; Peng and Yang, 2016) and decreased learning and memory capacity
56 (Aliouane *et al*., 2009; Mengoni and Farina, 2015; Alkassab and Kirchner, 2016). Evidence from
57 semi-field or field research indicated that neonicotinoids negatively affect foraging activity and
58 homing flight (Henry *et al*., 2012; Fischer *et al*., 2014; Tison *et al*., 2016). Moreover,
59 neonicotinoids have been found to affect honeybee immunocompetence (Brandt *et al*., 2016) and
60 increase the risk of other stressors such as pathogens (Pettis *et al*., 2013; Alburaki *et al*., 2015).

61 Despite the implications for honeybee colonies, little research has so far been carried out into
62 the molecular effects of neonicotinoids. Christen *et al*. (2016) found that exposure to

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



63 neonicotinoids changed the transcription of *AChRα1* and *2*, *creb*, *pka* and *vitellogenin* in the brain
64 of honeybees. The latest research from this group (Christen *et al.,* 2017) showed that binary
65 mixtures of neonicotinoids lead to different transcriptional changes in *nAChR* subunits and
66 *vitellogenin* than single neonicotinoids, and that transcription was most strongly induced by
67 thiamethoxam.

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

75

## 76 2. Results

77 2.1. Raw read processing and quantitative gene expression

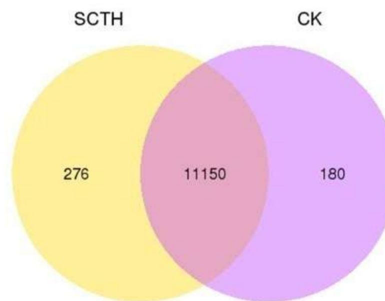
78 Using high-throughput RNA-Seq, six libraries (SCTH_1-3 and CK_1-3) were created from
79 the two treatments (SCTH and CK). In total, SCTH_1, SCTH_2, SCTH_3, CK_1, CK_2 and
80 CK_3 generated 43,672,706, 42,805,654, 43,630,710, 43,594,286, 44,650,260 and 43,379,868
81 usable reads, respectively. After mapping to the reference genome (NCBI: Amel_4.5) and the
82 junction database, 38,527,526, 38,026,904, 38,277,904, 39,258,011, 40,153,044 and 37,645,388
83 total mapped reads were acquired, and the numbers of uniquely mapped reads were 37,673,745,
84 37,163,425, 37,440,667, 38,121,707, 38,960,068 and 36,686,103. Among these unique reads,
85 77.58-81.93% were mapped to exon regions (Table S1).

86 We calculated gene activity by counting the reads that mapped to exon regions ($\geq$ 3 per gene).
87 The average number of genes expressed in SCTH and CK libraries was 11,426 and 11,330
88 respectively; and 11,150 genes were expressed in both groups (Fig. 1). We also divided gene
89 expression levels into five grades according to their RPKM (Reads Per Kilo bases per Million
90 reads) values (Table S2). In each library, 30.22–31.79% of the reads had RPKM values < 1,
91 11.49–12.56% had RPKM values of 1-3, 28.20–29.17% had RPKM values of 3-15, 18.36–20.86%
92 had values of 15-60 and 8.23–8.95% had RPKM values > 60. These results showed that a small

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



93 number of genes were expressed at very high levels but the majority were expressed at low levels,
94 indicating that the distribution of our DGE dataset was normal.




95
96 **Figure 1.**

97 2.2. Differentially-expressed genes between SCTH and CK honeybees

98 In total, 609 differentially-expressed genes (DEGs) were detected, of which 225 (45.2%)
99 were up-regulated and 334 (54.8%) were down-regulated in the SCTH bees compared with CK
100 bees (Fig. 2, Table S3). A list of the 20 genes with the most significant differential expression is
101 shown in Table S4 and of these, 17 were down-regulated and 3 were up-regulated in SCTH
102 honeybees. The 67 confirmed DEGs are listed in Table S5, and the others have been designated as
103 hypothetical proteins. We focused here only on those that had previously been confirmed (Table
104 1).

105 We found that 10 ribosomal protein (RP) genes (*RpL37*, *RpS8*, *RpSA*, *RpL32*, *RpL18A*,
106 *RpS3A*, *RpS6*, *RpS12*, *RpL13* and *RpL19*) have high expression levels, but were down-regulated in
107 SCTH honeybees. In contrast, two nicotinic acetylcholine receptors (nAChRs) subunits, *nAChRα9*
108 and *nAChRβ2*, were up-regulated along with cytochrome P450 6AS5 (*Cyp6as5*).

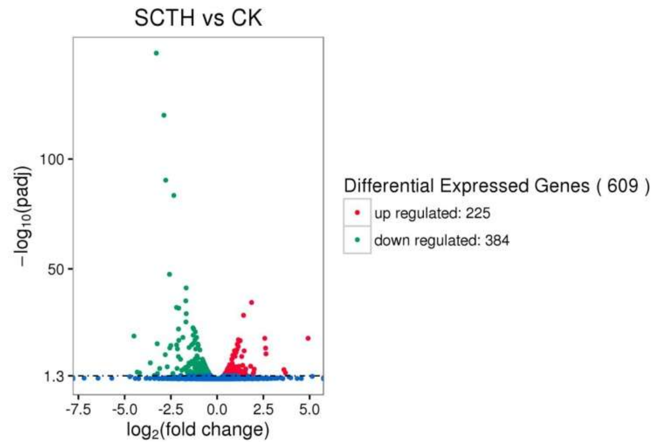
109 Some genes, for example *defensin 1*, *vitellogenin*, LOC725387 and LOC406093, all have
110 very high expression levels in both SCTH and CK honeybees, with intensity read copy counts of >
111 10,000. *Defensin1*, *vitellogenin* and LOC406093 were down-regulated in SCTH honeybees,
112 whereas LOC725387 was up-regulated.

113 Odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) are believed to be
114 involved in odor recognition and chemical communication (Pelosi *et al*., 2006; Sanchez-Gracia *et*
115 *al*., 2009). The genes *Obp3*, *Obp17*, *Obp21* and *CSP3* all showed significantly decreased

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



116 expression in SCTH honeybees. Moreover, three major royal jelly protein (MRJP) coding genes,
117 *Mrjp*, *Mrjp2* and *Mrjp3*, were down-regulated in the SCTH group. Although the expression level
118 of a memory-related gene, *NMDA receptor 1* (*NR1*), was relatively low, it was differentially
119 expressed between SCTH and CK honeybees.




120

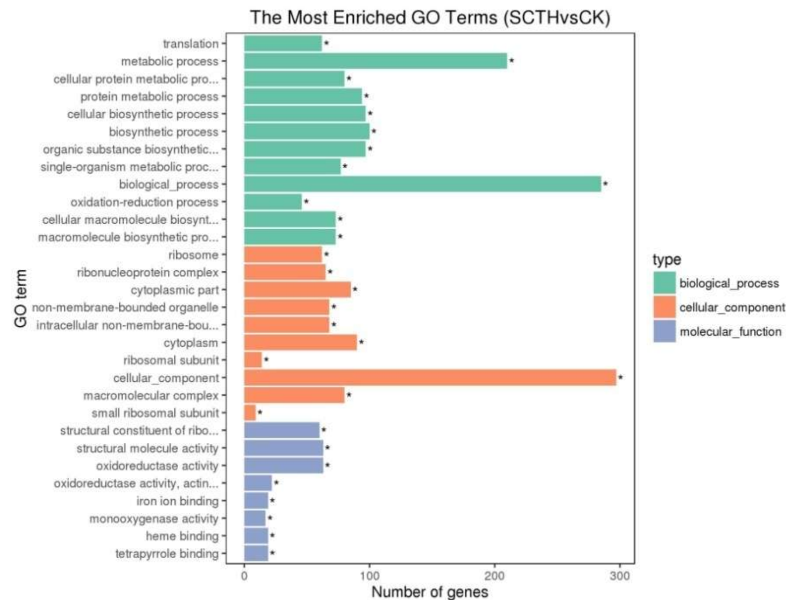
121 **Figure 2.**

122 2.3. Gene ontology (GO) enrichment analysis

123 A total of 445 DEGs were enriched for GO terms, including 167 up-regulated and 278
124 down-regulated genes in SCTH bees (Table S6), and the top 30 most enriched terms are shown in
125 Fig 3. The genes were divided into three classes: molecular function, cellular components and
126 biological process. Based on the GO terms for biological process, we found that most genes were
127 enriched for translation, various metabolic and biosynthetic processes, such as protein metabolism,
128 cellular protein metabolism, single-organism metabolism, cellular biosynthetic, cellular
129 macromolecule biosynthetic, macromolecule biosynthetic and organic substance biosynthetic (Fig
130 3). The main DEGs that were enriched coded for cellular components, including the ribosome,
131 ribonucleoprotein complex, ribosomal subunit and ribosomal subunit. Most of the genes were
132 down-regulated in SCTH honeybees (Fig 3, Table S6). In terms of molecular function, the DEGs
133 played roles in structural constitutent of ribosome, structural molecule activity and oxidoreductase
134 activity (Fig 3).

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.






135

136 **Figure 3**

137 2.4 KEGG pathway analysis

138 The KEGG database (http://www.genome.jp/kegg) was used to assign functional annotations
139 to the DEGs. A total of 377 DEGs were identified and mapped to 75 pathways in the KEGG
140 pathway database (Table S7), including 104 up-regulated and 273 down-regulated genes. Among
141 these pathways, five were significantly enriched with a corrected *P*-value ≤ 0.05 (Table 2). These
142 included the regulation of most genes related to ribosomes, oxidative phosphorylation, tyrosine
143 metabolism, pentose and glucuronate interconversion and drug metabolism.

144

145 ## 3. Discussion

146 The neonicotinoid insecticide thiamethoxam is highly toxic to honeybees with $LD_{50}$ values in
147 the range of a few ng per bee. The sublethal effects of thiamethoxam on honeybees have been
148 extensively studied at many different physiological levels (Aliouane *et al*., 2009; Henry *et al*.,
149 2012; Catae *et al*., 2014; Oliveira *et al*., 2014; Alburaki *et al*., 2015). Here we identified 609
150 differentially-expressed genes (DEGs) in honeybees on exposure to sublethal concentration of
151 thiamethoxam, including 225 up-regulated genes and 384 down-regulated genes.

152 The results of GO and KEGG analysis showed that the regulation of many DEGs related to

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



153 metabolism, biosynthesis and translation, the ribosome pathway, oxidative phosphorylation
154 pathway, tyrosine metabolism pathway and pentose and glucuronate interconversions. This
155 suggests that thiamethoxam mainly affects physiological and developmental processes in
156 honeybees.

157 Nicotinic acetylcholine receptors (nAChRs) are targets of neonicotinoids and their induction
158 can produce overt effects such as decreased memory and locomotor capacity (Aliouane *et al*.,
159 2009; Charreton *et al*., 2015). Our study showed that subchronic exposure to thiamethoxam
160 increases the expression of two nAChRs subunits, *nAChRα9* and *nAChRβ2*. These results are
161 consistent with previous research that thiamethoxam up-regulates expression of *nAChRα1* and
162 *nAChRα2* (Christen *et al*., 2016; Christen *et al*., 2017). This indicates a compensatory reaction to
163 the functional loss of nAChRs due to neonicotinoids (Christen *et al*., 2016).

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

172 There are two known classes of small soluble proteins in the chemosensilla of insects:
173 odorant-binding proteins (OBPs) and chemosensory proteins (CSPs). These are believed to be
174 involved in odor recognition and chemical communication (Pelosi *et al*., 2006; Sanchez-Gracia *et*
175 *al*., 2009), and CSP3 is thought to play a role in brood pheromone transportation (Briand *et al*.,
176 2002). OBP21 can bind the main components of queen mandibular pheromone as well as farnesol,
177 a compound used as a trail pheromone (Iovinella *et al*., 2011). The down-regulation of *CSP3* and
178 *Obp21* seen in our study suggested a reduced chemosensory ability in challenged honeybees. This
179 is generally consistent with recent research which indicated that a sublethal dose of the
180 neonicotinoid imidacloprid decreased the binding affinity of OBP2 to a floral volatile, β-ionone, in
181 Asiatic honeybees (Li *et al*., 2015).

182 Neonicotinoids could also affect the immunocompetence of honeybees. A recent study

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



183 reported that exposure to field-realistic concentrations of imidacloprid decreased hemocyte density,
184 the encapsulation response and antimicrobial action (Brandt *et al*., 2016). Antimicrobial peptides
185 are a key component of honeybee innate immunity and include defensin, which is coded by two
186 different defensin genes (*defensin-1* and *defensin-2*) in the honeybee genome (Evans *et al.*, 2006).
187 *Defensin-1* has been found to be up-regulated following bacterial challenge (Aronstein and
188 Saldivar, 2005; Richard *et al.*, 2012), suggesting that it is important in pathogen defense.
189 Kwakman *et al.* (2010, 2011) found that *defensin-1* is a key antimicrobial compound of honey.
190 The thiamethoxam-induced down-regulation of *defensin-1* found in our experiment may be
191 involved in reducing the effectiveness of the immune system.

192 Vitellogenin is also a component of the defense mechanism since it acts as a free-radical
193 scavenger to protect honeybees from oxidative stress (Amdam *et al.*, 2006; Seehuus *et al.*, 2006).
194 Down-regulation of vitellogenin can shorten the lifespan of honeybees (Nelson *et al.*, 2007), so
195 the thiamethoxam-induced decrease in *vitellogenin* expression in our experiment might contribute
196 to reducing honeybee longevity.

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

208 However, just like other insects, honeybees have systems to detoxify insecticides.
209 Cytochrome P450 monooxygenases (P450s) are the main detoxification enzymes which play an
210 important role in the detoxification and metabolism of xenobiotics and insecticides. A P450 gene,
211 *CYP6as5*, was induced in our experiment. This gene is a member of the CYP6AS subfamily,
212 which have been implicated in the metabolism of xenobiotics in honey and pollen (Mao *et al.*,

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



213 2009; Johnson *et al.*, 2012). A recent study showed that *CYP6as5* was also significantly
214 over-expressed in thiacloprid-treated bees compared with untreated controls, and induced
215 thiacloprid insensitivity (Alptekin *et al.*, 2016). The P450 gene, *CYP6as5*, seems to play a central
216 role in neonicotinoid insecticide resistance in honeybees.

217 In summary, using high-throughput RNA-Seq and analysis of differential gene expression,
218 we detected 609 differentially-expressed genes in honeybees (*Apis mellifera*) after challenge with
219 a sublethal concentration of thiamethoxam. We identified several genes involved in various
220 physiological functions, but further studies are needed to confirm the results of this analysis. GO
221 terms and KEGG pathways were also used to further understand the function of these genes. Our
222 results provide a reference for understanding the molecular mechanisms of the complex
223 interactions between neonicotinoid pesticides and honeybees.

224

225

## 4. Materials and Methods

226 4.1. Honeybee rearing

227 Two frames with sealed broods near adult emergence were taken from a healthy colony at the
228 Institute of Apiculture Research of Anhui Agriculture University (Hefei, China). The population
229 had not previously been exposed to pesticide. From July to September 2016 the frames were held
230 in an incubator under the following conditions: 35 ± 1°C, a relative humidity (RH) of 50 ± 10%
231 and in darkness. We obtained the newly-emerged honeybees and put them into cages (11 × 10 × 8
232 cm). They were fed with bee bread collected from the same apiary, sucrose–water solution (1:1
233 w:v), and maintained for three days at 30 ± 1℃, a RH of 70 ± 10% and in darkness.

234

235 4.2. Thiamethoxam preparation and exposure

236 The residues of thiamethoxam in trapped pollen generally ranges from 0.6 to 53.3 ppb
237 (Mullin *et al.*, 2010; Krupke *et al.*, 2012; Stoner and Eitzer, 2012; Codling *et al.*, 2016;
238 Sánchez-Hernández *et al.*, 2016), and in honey from 2.5 to 17.2 ppb (Codling *et al.*, 2016;
239 Sánchez-Hernández *et al.*, 2016). Based on this, a field-realistic level of 10 ppb (Stanley and
240 Raine, 2016) was selected as the sublethal concentration. A stock solution of thiamethoxam (> 99%
241 purity, 1000 ppm) was obtained from J&K (Shanghai, China) and prepared using acetone as a

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



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

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

249

250 4.3. RNA extraction

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

256

257 4.4. Library preparation for transcriptome sequencing

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

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



272 polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified
273 using an AMPure XP system and library quality was assessed using an Agilent Bioanalyzer 2100
274 system.
275
276 4.5 Clustering and sequencing
277 Clustering of the index-coded samples were performed on a cBot Cluster Generation System
278 using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions.
279 After cluster generation, the library preparations were sequenced on an Illumina Hiseq 4000
280 platform and 150 bp paired-end reads were generated.
281
282 4.6 Read processing
283 Raw reads in the FASTQ format were first processed through in-house Perl scripts. Clean
284 reads were obtained by removing low-quality reads or those containing adapters or poly-N. At the
285 same time, the Q20, Q30 and GC contents were calculated. All downstream analyses were based
286 on the high-quality clean reads. The index of the honeybee genome (NCBI: Amel_4.5) was built
287 using Bowtie v2.2.3, and paired-end clean reads were aligned to the honeybee genome using
288 TopHat v2.0.12. HTSeq v0.6.1 was used to count the reads mapped to each gene, and then the
289 FPKM (Fragments Per Kilobase of transcript sequence per Million reads) of each gene was
290 calculated based on the length of the gene and reads count mapped to it.
291
292 4.7. Differential expression analysis
293 Differential expression analysis was performed using the DESeq R package (2.15.3). DESeq
294 provides statistical routines to determine differential expression in digital gene expression (DGE)
295 data using a model based on negative binomial distribution. The resulting *P*-values were adjusted
296 using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with
297 an adjusted $P$-value $\leq 0.05$ were considered differentially expressed. Finally, Gene Ontology (GO)
298 enrichment analysis of differentially-expressed genes was carried out using the GOseq R package,
299 in which gene length bias was corrected. GO terms with corrected $P$-values $\leq 0.05$ were
300 considered significantly enriched by differentially-expressed genes. In the Kyoto Encyclopedia of
301 Genes and Genomes (KEGG) database, we used KOBAS software to test the statistical

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



302 enrichment of differential gene expression in KEGG pathways.

303

304 Competing interests

305 The authors declare no competing or financial interests.

306

307 Author contributions

308 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.
309 analyzed data and wrote the manuscript. F.L. edited and revised the manuscript. All authors have read and
310 approved the final manuscript.

311

312 Funding

313 This work was supported by the Earmarked Fund for China Agriculture Research System (No.
314 CARS-45-KXJ9).

315

316 References

317 Alburaki, M., Boutin, S., Mercier, P.L., Loublier, Y., Chagnon, M. Derome N. (2015). Neonicotinoid-coated zea
318 mays seeds indirectly affect honeybee performance and pathogen susceptibility in field trials. *PLoS One*. **10**,
319 e0125790.

320 Aliouane, Y., El Hassani, A.K., Gary, V., Armengaud, C., Lambin, M. Gauthier, M. (2009). Subchronic exposure of
321 honeybees to sublethal doses of pesticides: effects on behavior. *Environ. Toxicol. Chem.* **28**, 113–122.

322 Alkassab, A.T., Kirchner, W.H. (2016). Impacts of chronic sublethal exposure to clothianidin on winter honeybees.
323 *Ecotoxicology* **25**, 1000–1010.

324 Alptekin, S., Bass, C., Nicholls, C., Paine, M.J., Clark, S.J., Field, L., Moores, G.D. (2016). Induced thiacloprid
325 insensitivity in honeybees (*Apis mellifera* L.) is associated with up-regulation of detoxification genes. *Insect.*
326 *Mol. Biol*. **25**, 171-180.

327 Amdam, G.V., Simoes, Z.L.P., Hagen, A., Norberg, K., Schrøder, K., Mikkelsen, Ø., Kirkwood, T.B.L., Omholt,
328 S.W. (2004). Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in
329 honeybees. *Experimental Gerontology* **39**, 767–773.

330 Aronstein, K., Saldivar, E. (2005). Characterization of a honey bee Toll related receptor gene *Am18w* and its
331 potential involvement in antimicrobial immune defense. *Apidologie* **36**, 3–14.

332 Blacquière, T., Smagghe, G., van Gestel, C.A., Mommaerts, V. (2012). Neonicotinoids in bees: a review on
333 concentrations, side-effects and risk assessment. *Ecotoxicol* **21**, 973–992.

334 Brandt, A., Gorenflo, A., Siede, R., Meixner, M, Büchler, R. (2016). The neonicotinoids thiacloprid, imidacloprid,

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



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

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



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 dialoguebetween 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.

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



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., Leboulle, 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, RA., Roat, T.C., Carvalho, SM., Malaspina, O. (2014). Side-effects of thiamethoxam on the brain and
416 midgut of the Africanized honeybee *Apis mellifera* (Hymenopptera: 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

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



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., Leboulle, 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.

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



455

456 **Figure legends**

457

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

463

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

470

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

477

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

| Gene | Regulation (SCTH VS CK) | 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) |

bioRxiv preprint doi: https://doi.org/10.1101/114256; this version posted March 6, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



| | | |
|---|---|---|
| *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 | - |

480 **SCTH: sublethal concentration of thiamethoxam; CK: control check. Up: up-regulated in SCTH bees;**
481 **Down: down-regulated in SCTH bees.**
482

483 **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 |

484