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

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Running title: Neonicotinoids in the honeybee

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 honeybees (*Apis mellifera*). Despite this, 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 honeybees after subchronic exposure to thiamethoxam (10 ppb) over 10 days. In total, 609 differentially-expressed genes (DEGs) were identified, of which 225 were up-regulated and 384 were down-regulated. The functions of some DEGs were identified, and GO enrichment analysis showed that the enriched DEGs were mainly linked to metabolism, biosynthesis and translation. KEGG 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 honeybees.

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

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

1. Introduction

Honeybees (*Apis mellifera*, L.) 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

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

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

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 (≥ 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

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.
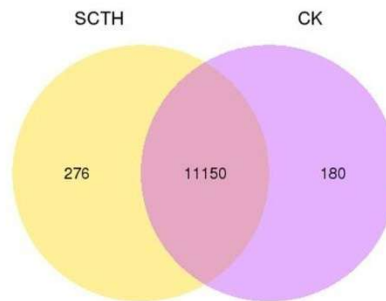
95

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

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.
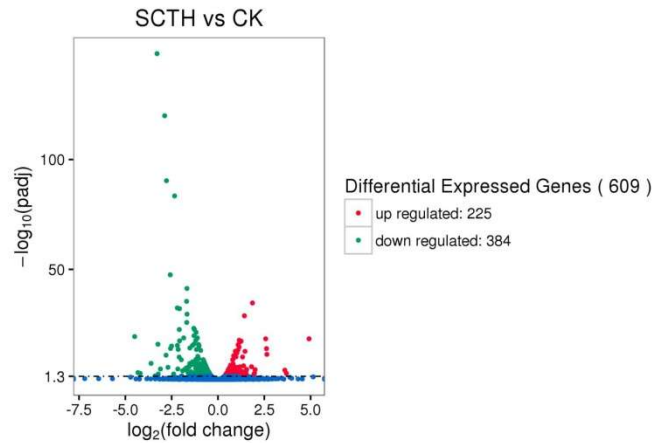
120

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).

The Most Enriched GO Terms (SCTHvsCK)
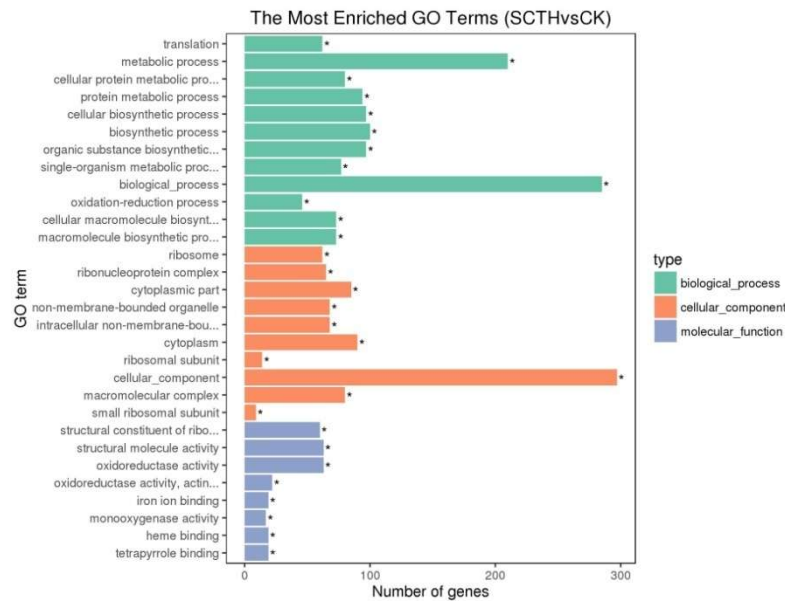
135

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

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

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.*,

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

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: $35 \pm 1°C$, a relative humidity (RH) of $50 \pm 10\%$

231 and in darkness. We obtained the newly-emerged honeybees and put them into cages ($11 \times 10 \times 8$

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 $30 \pm 1°C$, a RH of $70 \pm 10\%$ 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

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

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 ≤ 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 ≤ 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

302   enrichment of differential gene expression in KEGG pathways.

303

315

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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) $\leq$ 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 $\leq$ 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 $\leq$ 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)

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

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