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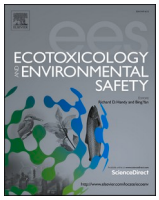

# Juvenile hormone and transcriptional changes in honey bee worker larvae when exposed to sublethal concentrations of thiamethoxam

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ABSTRACT

Thiamethoxam, an insecticide with high usage and large amounts of environmental residues, has been reported to affect the pupation and survival of honey bee larvae at sublethal concentrations. The molecular mechanisms are not fully understood. In this study, we measured the response of juvenile hormone (JH) to environmental concentrations of thiamethoxam using liquid chromatography-tandem mass spectrometry (LC-MS/MS), monitored the dynamic changes in the transcription of genes encoding major JH metabolic enzymes (CYP15A1, FAMET, JHAMT and JHE) using RT-qPCR, and analysed the transcriptome changes in worker larvae under thiamethoxam stress using RNA-seq. Thiamethoxam significantly increased the levels of JH3 in honey bee larvae, but no significant changes in the transcript levels of the four major metabolic enzymes were observed. Thiamethoxam exposure resulted in 140 differentially expressed genes (DEGs). P450 CYP6AS5 was upregulated, and some ion-related, odourant-related and gustatory receptors for sugar taste genes were altered significantly. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that amino acid metabolism and protein digestion and absorption were influenced by thiamethoxam. These changes may do harm to honey bee caste differentiation, foraging behaviour related to sensory perception and nutrient levels of bee colonies. These results represent the first assessment of the effects of thiamethoxam on JH in honey bee larvae and provides a new perspective and molecular basis for the study of JH regulation and thiamethoxam toxicity to honey bees.

## 1. Introduction

As one of the most important pollinators worldwide (Gallai et al., 2009), honey bees (*Apis mellifera*) not only contribute to pollinating plants in nature but also provide pollination services for global crop production for human food (Morse et al., 2000). Approximately half (52/115) of the major global food commodities depend on bee pollination (Klein et al., 2007). In 2006, however, beekeepers in the US noted a mysterious and rapid colony loss, which was later reported as colony collapse disorder (CCD) (Oldroyd et al., 2007). This phenomenon has resulted in a loss of 50% of colonies since the 1950s in North America (Spivak et al., 2011). Furthermore, different degrees of managed colony declines occurred on different continents from 1990 to 2011 (Anstalt, 2013). This pollinator decline leads to decreased crop production (Bauer and Wing, 2010) and plant biodiversity (Biesmeijer et al., 2006; Thomas et al., 2004; Pauw et al., 2011). The trend of declining honey bee populations also dramatically increased the prices of honey bee colony rentals for pollination services (Allsopp et al., 2008). These negative ecological and economic effects have made the government and scientists eager to identify potential drivers of CCD. Yagya Prasad Paudel et al. (Paudel et al., 2015) and Zoran Stanimirović (Stanimirović et al., 2019) reviewed the possible drivers of this unexpected decline and listed diseases in beekeeping, the quality of diet (bee bread and honey) and environmental chemical pollution as important factors. In addition to parasites (*Varroa destructor*) (Paxton, 2010; Berthoud et al., 2010; Carreck et al., 2010; Martin et al., 2010) and diseases caused by microorganisms (foulbrood, nosemosis (Higes et al., 2009) and ascospherosis), an increasing number of studies have reported the effects of pesticides on bees (Henry et al., 2012; Bernal et al., 2010), especially neonicotinoids, which are the most widely used insecticides (Simon-Delso et al., 2015). A global survey of neonicotinoids in honey showed that most honey was contaminated with at least one type of neonicotinoid (Mitchell et al., 2017). Initially, the controversy over the effects of neonicotinoids on bees centred on imidacloprid (Maxim et al., 2007) and then spread to other neonicotinoid insecticides used across the world (Decourtye et al., 2010). Thiamethoxam, a second-generation neonicotinoid, was reported to exhibit unfriendly oral and contact toxicity to honey bees (Iwasa et al., 2004). Papach found that a low dose

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of thiamethoxam (0.6 ng/bee) applied to larvae impaired the learning and memory abilities of emerging adult bees (Papach et al., 2017). Moreover, thiamethoxam was also reported to affect phototropic climbing and motor abilities of bees (Samuelson et al., 2016; Tosi et al., 2017). According to the study by Tavares, thiamethoxam reduces the survival of larvae and pupae and subsequently decreases the percentage of emerging honey bees (Tavares et al., 2017). Abundant toxicological studies of the effects of thiamethoxam on bees have been completed, but the mechanism still requires further study.

JH is secreted by the *corpora allata* (CA), which regulates growth, metamorphosis and reproductive organs in arthropods (Riddiford, 1994). It was reported as one of the key hormones involved in metamorphosis regulation in holometabolous insects (Riddiford, 2012). Insect metamorphosis is only triggered when JH is metabolised by JHE. Does thiamethoxam affect metamorphosis by altering JH levels in the larvae?

Evidently, JH level in insects are related to its synthesis and degradation. The biosynthesis of JH is usually divided into two steps. The first step is the process from acetyl coenzyme A (acetyl-CoA) to farnesyl pyrophosphate (FPP), which exists in all higher eukaryotes and some viruses to synthesise steroids and terpenoids (Goldstein et al., 1990). In the second step, which is unique to arthropods, FPP is hydrolysed to farnesol (FA) and then methylated and epoxidated to JH3 (JH type in bees). Two reactions in this step are catalysed by CYP15 family members of cytochrome P450 (CYP15A1) and juvenile hormone methyltransferase (JHAMT) (Bellés et al., 2005; Shinoda et al., 2003). In addition, farnesoic acid *O*-methyltransferase (FAMET) may also be involved in the terminal synthesis of JH and may be a rate-limiting enzyme in the biosynthetic process (Williamson et al., 2001). Juvenile hormone, on the other hand, is degraded by the enzyme JH esterase (JHE) (Bomtorin et al., 2014). With the rapid development of chromatography, HPLC, GC-MC and LC-MC have been used to detect JH in insects (Bede et al., 2000). Meanwhile, due to its high sensitivity, large data volume and wide sequencing depth, RNA-seq sequencing technology has become an important research method for accurately assessing differences in the regulation of transcription levels (Wang et al., 2009).

In this study, JH3 levels in honey bee worker larvae reared in vitro were measured using LC-MC/MS after exposure to thiamethoxam at sublethal concentrations, and dynamic monitoring was implemented to determine whether changes occurred in several JH3-related key terminal synthases (JHAMT, CYP15A1 and FAMET) and metabolic enzymes (JHE). Finally, transcriptome sequencing was performed to study the changes in larval transcriptional abundance 2 days after exposure to thiamethoxam.

## 2. Materials and methods

The thiamethoxam standard, JH3, HPLC-grade methanol, acetonitrile and isooctane were purchased from ANPEL Laboratory Technologies (Shanghai) Inc. Cell culture plates, D-glucose, D-fructose, yeast extract and other reagents (analytical reagents) were obtained from Sangon Bioengineering (Shanghai) Co., Ltd. Royal jelly were supplied by a bee keeper in Nanning.

### 2.1. Larval rearing and thiamethoxam exposure in vitro

Honey bee *(A. mellifera)* colonies were kept on the campus of Guangxi University. Three colonies with no *Varroa* infestation and other health issues were selected to obtain worker larvae. The queen was locked in an excluder with an empty frame for egg laying to obtain larvae of known ages. Three days later, 1st instar larvae were transferred to 48-well cell culture plates, which were placed in sterile queen cell cups containing 25 μL of diet A, as shown in Table 1. All plates were placed in an incubator (34.5 °C, 95% RH), and the day of larval removal was defined as day 1. Rearing methods were based on research by Tavares and colleagues (Tavares et al., 2017). No diet was served on day 2. Afterwards, 25 μL of diet B were provided on day 3, and 30 and 40 μL of diet C were provided on days 4 and 5, respectively.

**Table 1**
Diets used for larvae rearing.

| Component | Diet A (day1) | Diet B (day3) | Diet C (day4, 5) |
|---|---|---|---|
| Royal jelly | 50% | 50% | 50% |
| Glucose | 6% | 7.5% | 9% |
| Fructose | 6% | 7.5% | 9% |
| Yeast extract | 1% | 1.5% | 2% |
| $H_2O$ | 37% | 33.5% | 30% |

Thiamethoxam was added directly to diet C to concentrations of 0.001 ng/μL, 0.01 ng/μL and 0.1 ng/μL (0.03, 0.3 and 3 ng/bee, respectively) on day 4. The control group was provided with non-thiamethoxam diet C. Three concentrations were set according to the study by Gaelle Daniele in 2018 (Daniele et al., 2018), which reported the residue of thiamethoxam in honey bee (LOQ-0.001 ng/μL), bee bread (LOQ-0.01 ng/μL) and wax (Up to 0.1065 ng/μL). And they were far below the $LC_{50}$ concentration (14.34 ng/μL of diet) (Tavares et al., 2015).

### 2.2. Extraction and detection of JH3 after thiamethoxam exposure

The method for extracting JH3 was based on the method described by Furuta K (Furuta et al., 2013). On day 6, about 1.00 g (about 11 larvae, the actual weight of each repeat was recorded for later JH3 level calculation) of the larvae was weighed and ground with liquid nitrogen. Five biological replicates in both treatment and control were performed. Afterwards, it was oscillated for 3 min with 0.2 mL of acetonitrile and 1 mL of methanol. Two millilitres of isooctane were added, oscillated for 3 min for extraction, and then collected by centrifugation. Isooctane extraction was repeated three times to obtain a total of 6 mL of supernatant, which were then dried with a Termovap sample concentrator. One millilitre of acetonitrile was used for elution by eddy oscillation for 2 min. After nitrogen drying, 0.2 mL of acetonitrile (HPLC) was used for elution by oscillation to complete the concentration step. The solutions were filtered with a 0.2 μm membrane filter and analysed directly using LC-MS/MS.

JH3 was analysed using an Agilent (1290) instrument coupled to a Triple Quadrupole G6460C mass spectrometer from Agilent Technology (USA) equipped with an electrospray ionisation (ESI) source. According to the characteristics of optical isomers, JH3 was separated on a CHIRALPAK AY-3R chiral column (150 mm × 2.1 mm, 3 μm, Daicel Chiral Technologies, China). The mobile phase was 0.1% formic acid and acetonitrile (v:v = 55:45) and used at a flow rate of 0.3 mL/min. The temperature of the column was 30 °C, and the injection volume was 3 μL. The capillary voltage was 4000 V, while the drying gas in the ionisation source was 330 °C. The nebuliser pressure was 103 kPa, and detection was performed in multiple reaction monitoring (MRM) mode (ESI mode: positive, precursor ion: 267 *m/z* (cone voltage = 60 v), quantifying ion: 71 *m/z* (collision energy = 15 eV), quantification ion: 147 (collision energy = 15 eV)).

### 2.3. Transcription of JH3-related enzyme genes after thiamethoxam exposure

Larvae for this experiment were collected at 12 h, 24 h, 36 h and 48 h after exposure to 0.01 ng/μL thiamethoxam (0.3 ng/bee), while the control group was provided non-thiamethoxam diet C. Three specimens were collected for each sample, and three replicates were performed. Total RNA was extracted from each sample using the RNAiso Plus kit (TAKARA BIO INC.), and the quantity and quality of RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The reverse transcription kit was Hifair® III 1st Stand cDNA Synthesis Supermix for qPCR (gDNA digester plus) (Yeasen Biotechnology

(Shanghai) Co., Ltd.). Quantitative real-time PCR was carried out using a QuantStudio 6 Flex instrument (Thermo Fisher Scientific) at 95 °C for 30 s for predenaturation and 40 cycles of 95 °C for 5 s and 60 °C for 30 s for PCR. The total reaction volume was 10 μL. Primer sequences for genes encoding JH3-related enzymes and internal controls were designed using Primer-BLAST (NCBI). We analysed the differences in relative gene transcription between the control and treatment groups using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001): $\Delta\Delta Ct = (Ct_{Target} - Ct_{Actin})_{treat} - (Ct_{Target} - Ct_{Actin})_{control}$. RT-qPCR results are presented as means ± standard errors. Standard errors were calculated and analysis of variance was performed using SPSS 26.0 software.

### 2.4. RNA-seq, library preparation and analysis

Larval samples were collected 2 days after exposure to 0.01 ng/μL thiamethoxam (0.3 ng/bee), and 3 replicates each of the control and treatment groups were analysed. Total RNA extraction steps were same as those described in Section 2.3. Library preparation and whole transcriptome sequencing were performed on the Illumina sequencing platform by Genedenovo Biotechnology Co., Ltd. (Guangzhou, China).

The differential expression analysis was performed using edgeR among replicates and using DESeq2 software between control and thiamethoxam treatment groups. We initially assessed sequence quality using Fastp to determine the quality of the raw reads. We aligned clean reads to the *A. mellifera* reference genome assembly (ensembl-release49, http://metazoa.ensembl.org/Apis_mellifera/Info/Index) using HISAT2. We reconstructed the transcript using Stringtie and calculated the transcript levels of all genes in each sample using RSEM. The transcripts with the parameters of $p < 0.05$ and absolute $\log_2$(fold change) $> 1$ were considered differentially expressed transcripts. A Gene Ontology (GO) enrichment analysis and pathway-based analysis (KEGG) were performed with FDR $< 0.05$ as the threshold to recognise the main biological functions of differentially expressed genes (DEGs). We validated the RNA-seq results by performing RT-qPCR for several randomly selected DEGs, and primers were designed using Primer-BLAST (NCBI).

## 3. Results

### 3.1. Thiamethoxam exposure increased the levels of JH3

JH3 has two optical antipodes because one chiral carbon is present in its structure. The two optical antipodes of JH3 were separated by a chiral column in this study, and the retention times were 8.8 min and 10.7 min. Only one of them (10.7 min) was detected in bee larvae (Fig. 1.A). In the previous JH3 addition and recovery tests, the recovery rates were 87.9 ± 3.2% (mean ± relative standard deviation) and 90.5 ± 5.1% when the added concentrations were 0.005 ng/μL and 0.010 ng/μL, respectively. Thus, the extraction method was reliable. Two days after thiamethoxam exposure, juvenile hormone was extracted from the larvae, and the results are shown in Fig. 1.B. The levels of JH3 were significantly increased in larvae exposed to all three concentrations ($p < 0.05$). A significant difference in the increase in juvenile hormone levels was not observed between larvae exposed to 0.01 and 0.1 ng/μL thiamethoxam.

### 3.2. Dynamic changes in the transcription of four JH3-related synthetic and degradation enzymes after thiamethoxam exposure

Fold changes ($2^{-\Delta\Delta Ct}$) in the mRNA levels of the four monitored enzymes fluctuated at each time point compared with the control, but all of them ranged from 0.5 to 2, indicating that transcription of these genes was upregulated or downregulated less than 2-fold (Fig. 2). The statistical analysis with SPSS software showed that the analysis of variance in each group was consistent with a normal distribution. An analysis with independent sample T tests between the control and treatment groups showed that the p values were all greater than 0.05, indicating no statistically significant differences. This result does not suggest that the increase in juvenile hormone levels after exposure to thiamethoxam is due to the regulation of these metabolic enzymes, at least not at the transcriptional level.

### 3.3. RNA-seq and analysis

All transcription data were uploaded to the NCBI SRA database (PRJNA734139). The raw data obtained from transcriptome sequencing of 6 samples of thiamethoxam-exposed worker larvae and control larvae were 54880474–65303934 reads. The proportion of clean data obtained after filtering was 99.84–99.87%. The total number of bases in each sample was greater than 8.23 GB, Q20 was greater than 97.67%, and Q30 was greater than 93.13%. The aforementioned quality control results will ensure the reliability of the subsequent analysis.

Based on the results of the differential expression analysis ($p < 0.05$ and absolute $\log_2$(fold change) $> 1$), thiamethoxam exposure resulted in a significant difference of 140 genes in exposed worker larvae (Fig. 3). Among these genes, 90 (64.3%) were upregulated after exposure. As expected, nAChRa6 (GeneID_551010) was significantly activated by thiamethoxam, consistent with the mechanism of action of neonicotinoids. One P450 gene (CYP6AS5) was upregulated after exposure.

The Gene Ontology (GO) enrichment analysis with GOSeq resulted in nine significantly enriched GO terms in larvae exposed to thiamethoxam (FDR $< 0.05$). Among them, seven were associated with biological processes, which were mainly described as the response to stimuli involved in sensory perception (Fig. 4). The other two were assigned to molecular functions. Both of them were related to signalling receptor activity.

An analysis of enriched KEGG pathways showed that the two most significantly enriched pathways were maturity onset diabetes of the

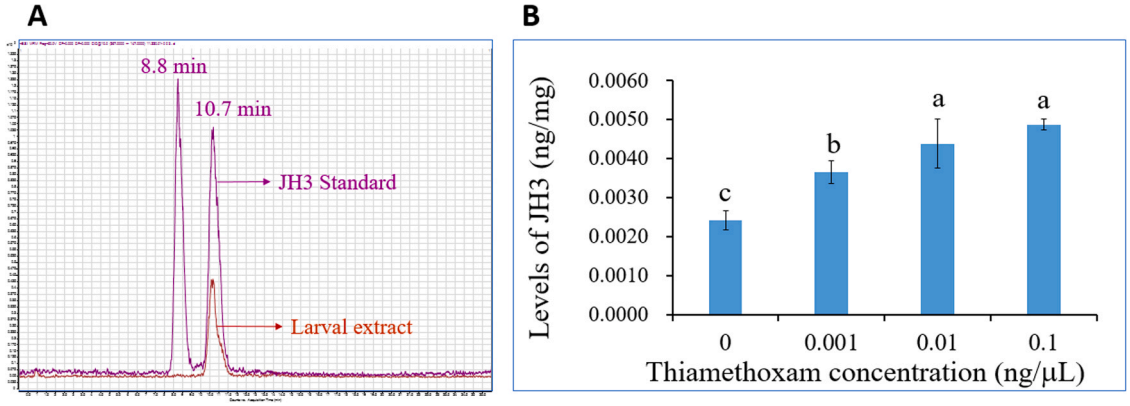



**Fig. 1.** Chromatogram of the JH3 and changes in JH3 levels after 2 days of thiamethoxam exposure.

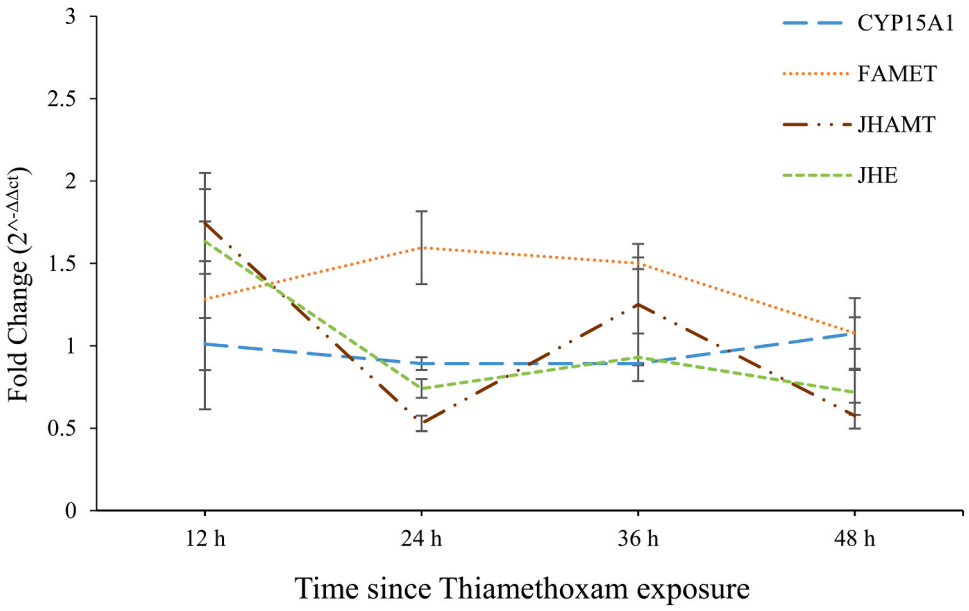



**Fig. 2.** Dynamic changes in the transcription of four JH3-related synthetic and degradation enzymes after thiamethoxam exposure (0.01 ng/μL).

young and pancreatic secretion, which belong to endocrine and metabolic diseases and the digestive system according to KEGG B class, respectively. The top ten KEGG pathways are listed in Table 2. Most of them were involved in amino acid metabolism, including D-glutamine, D-glutamate, glycine, serine, threonine and arginine metabolism. The pancreatic secretion pathway and protein digestion and absorption pathway were related to the digestive system. One steroid biosynthesis pathway and one nitrogen metabolism pathway were also identified.

Several DEGs were randomly selected for RT-qPCR analysis to verify the transcriptome results. The results showed a similar trend of expression changes (and $p > 0.05$) for RT-qPCR and RNA-Seq data, indicating that the RNA-Seq results were reliable.

## 4. Discussion

JH is a very important hormone in honey bees that regulates metamorphosis and caste differentiation in honeybee larvae. JH accelerates the development or integration of the circadian system in young bumble bee (*Bombus terrestris*) workers (Pandey et al., 2020). As early as 1998, researchers discovered differences in juvenile hormones between queen bees and worker bees during larval stages (Hartfelder et al., 1998). A subsequent study by Angel R Barchuk proved that JH was a key component of the developmental determination of queen-like characters (Barchuk et al., 2007). In our experiment, the administration of thiamethoxam at environmental concentrations not only is toxic to bees through nicotinic acetylcholine receptors but also affects bee hormones. These hormonal changes most likely produce abnormal queens and workers in the colony that have undergone unknown changes, which may explain why neonicotinoids cause CCD in bees. In addition, we do not know how long the negative effects of this elevated juvenile hormone level will last and whether it will continue to affect bees after metamorphosis. More work is needed to study the effects of JH3 abnormalities induced by thiamethoxam in the larval stage on the life cycle of adults.

The synthesis and metabolism of JH in insects are well understood, but the mechanism regulating its level remains unclear. In our study, when thiamethoxam exposure increased JH3 levels, the dynamic transcription of related key metabolic enzymes did not change significantly to support this process as expected. Transcriptome results also did not show changes in the transcription of these enzymes. Enzyme titre and activity are not only related to the transcriptional level but are also affected by posttranscriptional modifications, translation regulation, the enzymatic reaction environment, etc., which may explain the results described above. In addition, hormone regulation in an organism is complex and involves a large number of factors. Ion levels, insulin signalling (Tu et al., 2005) and allatostatins (Stay et al., 2007) are also considered influencing factors for JH synthesis.

Ion channels and receptors may be involved in the regulation of JH synthesis in CA. The biosynthetic activity of $Ca^{2+}$ is considered the main factor regulating the JH titter (Tobe et al., 1985). In our study, 12 (8.6%) DEGs were associated with ions (Table 3). Most were involved in ion binding, and the others were related to ion channels. Ions are an important component of organisms and an important participant in biological metabolism and material metabolism. The changes in these genes likely affect organisms.

After thiamethoxam exposure, the transcription of several odourant-related (Or50, Obp1 and LOC100577955) and gustatory receptor for sugar taste genes (LOC413684) was significantly altered in worker larvae. The GO analysis showed that significantly differentially expressed genes were mainly enriched in terms related to sensory perception. In this experiment, the only variable between the treatment and control groups was thiamethoxam in the diet. A few studies have shown that neonicotinoids affect the ability of bees to respond to sugar and odourant associative memory (Mengoni Goñalons and Farina, 2015; Alkassab et al., 2016). Fipronil-treated bees lost the ability to distinguish between known and unknown odourants. Thiamethoxam (1 ng/bee) significantly decreases the responsiveness to antennal sucrose stimulation (Aliouane et al., 2009). During the experiment, decreased food intake was observed, and the individual larvae were smaller than those in the control group. However, these indicators were not measured and recorded to minimise the stimulation of the larvae. Transcriptional changes in these odourant-related genes and sensory perception genes may alter the response of bee larvae to food, thereby affecting food intake and changing nutrient levels. Malnourished mosquitoes significantly reduce their ability to synthesise JH (Perez-Hedo et al., 2014), which suggests that transcriptional alterations in odourant-related and gustatory receptors for sugar taste genes may be the potential molecular mechanism of thiamethoxam-induced increases in JH levels.

Amino acids are common components of floral nectars, and thus the metabolism and function of amino acids in bees should be examined. Hrassnigg speculated that a high amino acid concentration in the haemolymph is the basis for the high protein synthesis activity of laying

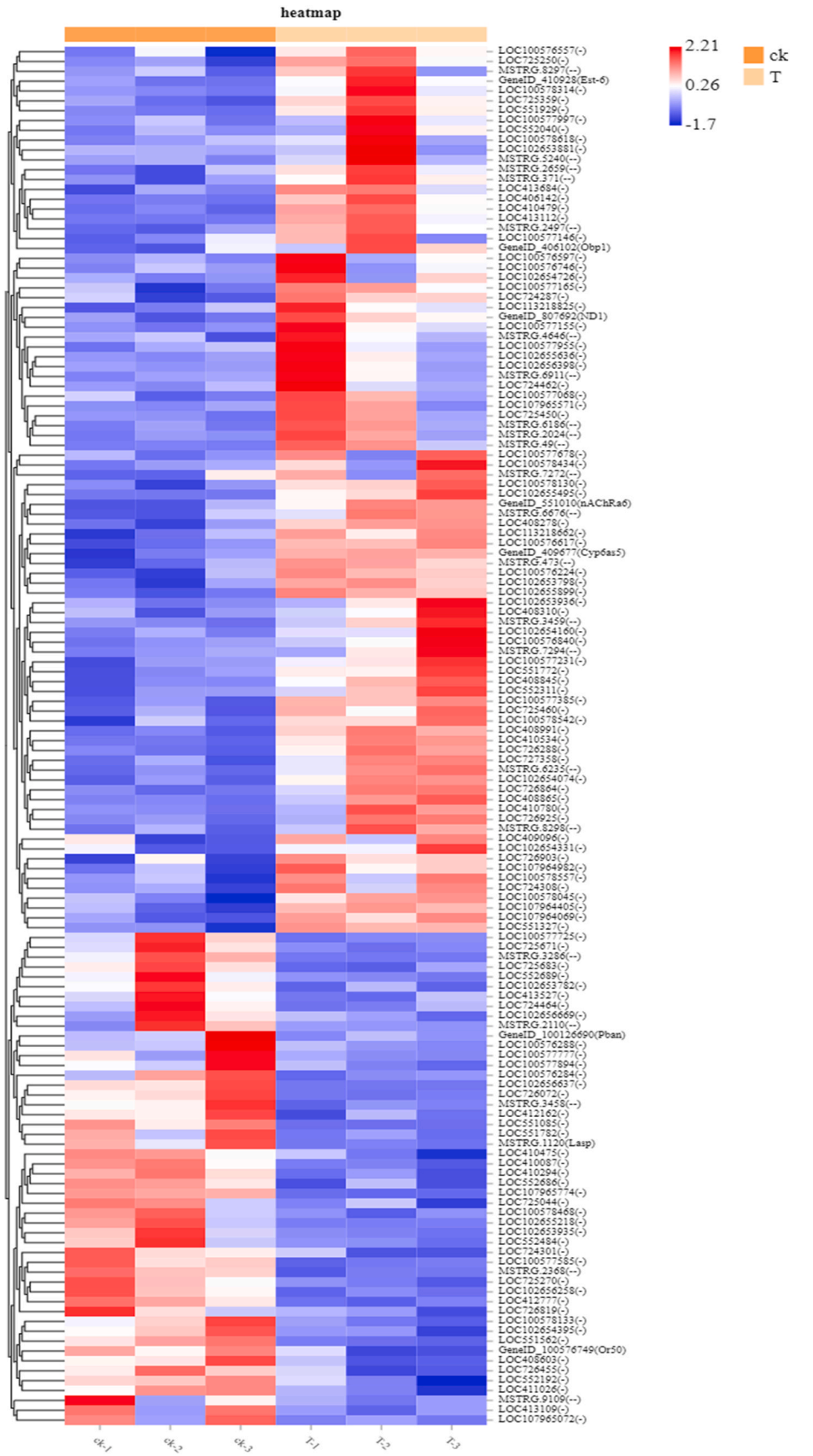
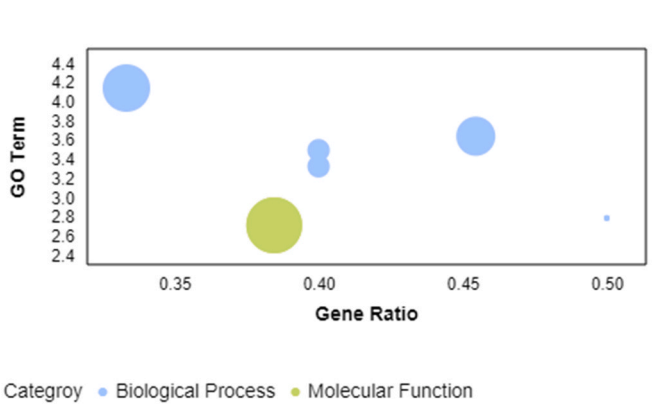



**Fig. 3.** Thiamethoxam exposure leads to gene transcription changes in worker larvae (ck-1, ck-2 and ck-3 were control samples; T-1, T-2 and T-3 were thiamethoxam treatment samples).




GO term (fdr < 0.05)

| ID | Descrption |
|---|---|
| GO:0050877 | nervous system process |
| GO:0007606 | sensory perception of chemical stimulus |
| GO:0007600 | sensory perception |
| GO:0009593 | detection of chemical stimulus |
| GO:0050907 | detection of chemical stimulus involved in sensory perception |
| GO:0050906 | detection of stimulus involved in sensory perception |
| GO:0004888 | transmembrane signaling receptor activity |
| GO:0038023 | signaling receptor activity |
| GO:0003008 | system process |

**Fig. 4.** Nine significantly enriched GO terms identified after thiamethoxam exposure (FDR < 0.05).

**Table 2**
Top 10 enriched KEGG pathways identified after thiamethoxam exposure.

| Pathway | KEGG_B_class | p value | Q value | Pathway ID | Genes |
|---|---|---|---|---|---|
| Maturity onset diabetes of the young | Endocrine and metabolic diseases | 0.002482 | 0.126597 | ko04950 | LOC413112<br>LOC725671 |
| Pancreatic secretion | Digestive system | 0.011645 | 0.237034 | ko04972 | GeneID_410928<br>LOC551327<br>LOC725250 |
| D-Glutamine and D-glutamate metabolism | Metabolism of other amino acids | 0.013943 | 0.237034 | ko00471 | LOC408991 |
| Glycine, serine and threonine metabolism | Amino acid metabolism | 0.026910 | 0.343098 | ko00260 | LOC102653881<br>LOC408603 |
| Steroid biosynthesis | Lipid metabolism | 0.054664 | 0.367867 | ko00100 | GeneID_410928 |
| Protein digestion and absorption | Digestive system | 0.055609 | 0.367867 | ko04974 | LOC551327<br>LOC725250 |
| Mannose type O-glycan biosynthesis | Glycan biosynthesis and metabolism | 0.061292 | 0.367867 | ko00515 | LOC727358 |
| Influenza A | Infectious diseases | 0.068760 | 0.367867 | ko05164 | LOC102656258<br>LOC725250 |
| Arginine biosynthesis | Amino acid metabolism | 0.074415 | 0.367867 | ko00220 | LOC408991 |
| Nitrogen metabolism | Energy metabolism | 0.074415 | 0.367867 | ko00910 | LOC100576224 |

queens (Hrassnigg et al., 2003). Maciej A Pszczolkowski found that L-glutamate stimulates JH synthesis by inducing calcium influx (Pszczolkowski et al., 1999). After thiamethoxam treatment, the D-glutamine and D-glutamate metabolic pathways were significantly enriched. The general understanding is that only one of the optically active isomers has a biological function in an organism. The amino acids that comprise proteins in living organisms are L-type amino acids. D-type amino acids only exist in valinomycin, bacitracin and a few other oligopeptides. Further studies are needed to determine whether D-glutamate exists in bees and has a biological function. However, the metabolism of other amino acids and the digestion and absorption of proteins, as well as the effects of thiamethoxam on other digestive systems and nitrogen metabolism, are likely to affect the nutritional status and development of bees.

Cytochrome P450 enzymes are well known for their metabolism of pesticides (Mao et al., 2011). As early as 1998, P450 (CYP4C7) was found to be involved in the regulation of JH synthesis (Sutherland et al., 1998). CYP15A1 was reported to catalyse the second step of JH synthesis (Bellés et al., 2005). The CYP6AS subfamily is unique to hymenopterans. Sublethal doses of sulfoxaflor (Shi et al., 2020) and thiacloprid (Alptekin et al., 2016), the target of which is the same as thiamethoxam, results in the upregulation of Cyp6AS5. Another study showed that carbendazim decreases JH levels in bee larvae, accompanied by the downregulation of CYP6AS5 (Wang et al., 2018). In our study, larvae exposed to thiamethoxam exhibited increased JH levels, accompanied by the upregulation of CYP6AS5. Thus, a positive correlation was observed between the level of JH in insect larvae and CYP6AS5 transcription in honey bees. A reasonable speculation is that CYP6AS5 may play a role in regulating the level of JH, similar to CYP4C7.

Neonicotinoids target nicotinic acetylcholine receptors (nAChRs) to which they bind and subsequently activate, which can result in paralysis, convulsions and death (Matsuda et al., 2001). Transcriptome results showed that thiamethoxam, a nicotinic insecticide, activated the reaction at very low concentrations (0.01 ng/μL), thus causing nonlethal toxicity to bees.

## 5. Conclusion

In conclusion, the results in this study showed that sublethal concentrations of thiamethoxam significantly increased the levels of JH3 in honey bee worker larvae, which may do harm to honey bee caste differentiation. This also might explain why thiamethoxam reduces the survival of larvae and pupae and subsequently decreases the percentage of emerging honey bees (Tavares et al., 2017). Data of RNA-seq revealed that thiamethoxam altered the expression of ion-related genes, some sensory perception-related genes, and P450 CYP6AS5, and affected multiple amino acid metabolism pathways and protein digestion and absorption pathways. We speculate that some of these changes are related to the regulation of JH level. At the same time, our result provides a new perspective and molecular basis for the study of thiamethoxam toxicity to honey bees.

## CRediT authorship contribution statement

**Honghong Li**: Conceptualization, Methodology, Data curation, Resources, Validation Verification, Writing – original draft. **Sheng Liu**: Methodology, Resources. **Lichao Chen**: Resources. **Jie Luo**: Data

**Table 3**
DEGs associated with ions according Gene Ontology (GO) database.

| DEGs (symbol) | log2 (fc) | p Value | Description | GO function |
|---|---|---|---|---|
| LOC410087 | -1.25 | 0.000000000 | Uncharacterised LOC410087 | GO:0046872// metal ion binding |
| LOC551327 | 1.13 | 0.000000000 | Carboxypeptidase | GO:0046872// metal ion binding |
| MSTRG.1120 (Lasp) | -1.52 | 0.000000032 | PREDICTED: LIM and SH3 domain protein F42H10.3-like [Trachymyrmex cornetzi] | GO:0046914// transition metal ion binding |
| LOC412777 | -1.71 | 0.000018768 | Sodium/ potassium/ calcium exchanger Nckx30C, transcript variant | GO:0008273// calcium, potassium: sodium antiporter activity |
| GeneID_409677 (Cyp6as5) | 1.16 | 0.000039399 | Cytochrome P450 6AS5 | GO:0046872// metal ion binding |
| LOC100578434 | 1.32 | 0.000731757 | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1, transcript variant X1 | GO:0005509// calcium ion binding |
| LOC100576224 | 1.04 | 0.001208628 | Carbonic anhydrase 2, transcript variant X1 | GO:0008270// zinc ion binding |
| LOC725359 | 1.01 | 0.003120747 | Uncharacterised LOC725359 | GO:0005272// sodium channel activity |
| LOC552192 | -1.00 | 0.010412794 | Uncharacterised LOC552192, transcript variant X1 | GO:0046872// metal ion binding |
| LOC413112 | 6.44 | 0.012549229 | Pyruvate kinase-like | GO:0046872// metal ion binding |
| GeneID_551010 (nAChRa6) | 2.93 | 0.016591544 | Nicotinic acetylcholine receptor alpha6 subunit | GO:0005216// ion channel activity |

curation. **Dongqiang Zeng**: Editing, Supervision. **Xuesheng Li**: Writing – review & editing, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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