The Effects of Sub-lethal dose of Insecticides on Honeybee Behavior and Transcriptome

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Research Article

Keywords: Apis mellifera, insecticides, imidacloprid, deltamethrin, transcriptome

Posted Date: February 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-175430/v1

Abstract

Exposure to insecticides is the main cause of honeybee (*Apis mellifera*) population decline. At sub-lethal doses, these chemicals have been shown to negatively affect honeybee physiological development and behavior. Previously, we found the insecticide imidacloprid and deltamethrin significant reduced honeybee dancing and foraging efficiency. As a follow up, we performed a deep RNA-seq analysis to reveal the gene regulatory mechanisms underlying the altered foraging behavior. Genes involved in detoxification were up-regulated in both imidacloprid and deltamethrin treatment groups. Gene members in immune pathways, odorant receptors and major royal jelly protein families showed significant up or down regulation in treatment groups compared with controls. This fluctuating gene expression profile reflects that multifaceted aspects of honeybee physiology were affected by the two insecticides, leading to inaccurate communication and impaired learning and memory. Our findings reveal candidate molecular mechanisms under impaired dance performance in honeybees exposed to insecticides.

Introduction

Honeybees (*A. mellifera*) are eusocial insect that play an important role in natural ecosystems by providing global pollination services for crop and wild plants (Klein et al. 2007). However, the honeybee colonies have been declining in recent years (Neumann and Carreck 2010). Multiple factors have been associated with colony losses, including the parasitic mite *Varroa destructor* (Rosenkranz et al. 2010), the fungal parasites *Nosema ceranae* (Antunez et al. 2009), viruses (McMenamin and Genersch 2015), malnutrition, environmental degradation, climate change, queen quality decline (Wei et al. 2019) and widespread insecticide application (Budge et al. 2015; Sanchez-Bayo et al. 2016).

Although the causes of honeybee loss are multifactorial, the large-scale use of systemic insecticides, such as imidacloprid, deltamethrin, and other insecticide types, has been implicated as a major contributing factor (Henry et al. 2012; Farooqui 2013; Woodcock et al. 2016). Imidacloprid is the most commonly used insecticide and has a highly specific affinity to the nicotinic acetylcholine receptors (nAChRs) in the honeybee's nervous system. Deltamethrin is a nerve agent, which disturbs the normal physiological function and signal transmission of nerve cells by interfering with the calcium channel of nerve cells. Accumulating evidence indicates that at sub-lethal doses insecticides cause honeybee brain dysfunction and reduce immune competence, leading to impaired navigation and olfactory learning and memory, and susceptibility to pathogens (Decourtye et al. 2004a; Desneux et al. 2007; Yang et al. 2008; Di Prisco et al. 2013; Matsumoto 2013; Palmer et al. 2013; Williamson and Wright 2013; Brandt et al. 2016).

Previously, we found insecticides disturb the dance communication system of bees. Honeybees fed with insecticide significantly increased the divergence angle, return phases in waggle dances, as well as the crop content of the dance followers. The data suggest that sub-lethal doses of deltamethrin or imidacloprid impaired the honeybees' learning and memory and resulted in cognitive disorder (Zhang et al. 2020a, b).

However, the global gene expression profile associated with impaired learning and memory in the dance bee brain remains unclear. As a follow up, the aim of this study is to analyze the gene expression in brain samples of bees fed sub-lethal doses of imidacloprid or deltamethrin. We found that genes involved in immune, detoxification, and chemosensory responses were significantly altered as a result of insecticide impaired foraging and dancing.

Material And Methods

Sublethal Dose of Insecticides Preparation

Imidacloprid of 99% purity was provided by J&K scientific and chemical company. Imidacloprid was dissolved in acetone and stock solutions of 24 mg/kg in water, were then diluted to final concentrations of 24 µg/kg with 50% sucrose solution. The final concentration of acetone in sucrose solutions was equal to 1% (vol./vol.) (Decourtye et al. 2004b). Treatment with imidacloprid at concentration of 24 µg/kg was chosen because this concentration corresponds to the lowest observed effect concentration (LOEC) affecting the olfactory learning performances of bees after chronic oral treatment and under laboratory conditions (Decourtye et al. 2003). Deltamethrin of 98% purity (J&K scientifc and chemical company) was dissolved in acetone and stock solutions of 235 mg/kg in water, then diluted to final concentrations of 235 µg/kg with 50% sucrose solution. The final concentration of acetone in the sucrose solutions was equal to 1% (vol./vol.). Deltamethrin administered at a concentration of 235µg/kg was chosen because it is half the no-observed-effect concentration (NOEC) for the conditioned proboscis extension reflex (PER) (Decourtye et al. 2005).

Behavioral Experiments

Experiments were conducted in October 2018 at Jiangxi Agricultural University, Nanchang, China (28.46 N, 115.49 E) during a period of constant, warm temperatures and floral dearth, which facilitates feeder training of bees.

Three honeybee colonies (*A. mellifera*) were sequentially used for testing, each with four frames of honeybees and brood in an observation hive. The colonies were healthy and free of pests and diseases. On each experimental day, about 100 foragers at the entrance were captured and placed into individual opaque tubes. The honeybees were released at a feeder placed about 500 meters from the hive. If a released honeybee began to imbibe food, it was marked with color until there were 75 individually marked honeybees. For each colony, 50 % sucrose water (as control group) or 50 % sucrose water with sub-lethal doses of imidacloprid or deltamethrin was offered at the feeder on the alternate days. For about 10 marked honeybees in each group, the duration of imbibing food and returning period from the hive to feeder was recorded with a stopwatch, each honeybee was recorded 10 times. Marked honeybees performed waggle dances after they returned into their observation hive. About 75 dancing honeybees were collected and preserved in liquid nitrogen until later brain dissection grouped by colony.

RNA Sequencing and Analysis

Total RNA was extracted from each sample of 25 pooled dissected brains on dry ice according to the standard protocol of TRIZOL Reagent (Life Technologies, Carlsbad, CA, USA). Three replicates were used for control, imidacloprid-treated and deltamethrin-treated bees respectively. In total 9 pooled samples were collected. RNA libraries were prepared and sequenced using Illumina Hiseq 4000 following standard protocol by Gene Denovo Biotechnology Co. (Guangzhou, China) (Wei et al. 2019).

Read Mapping and Bioinformatic Analysis

The raw reads produced by the sequencing instrument were filtered to remove adaptors, low-quality sequences with unknown nucleotides N, and reads with more than 20% low quality bases (base quality < 10). The high-quality clean reads were assembled into unigenes using the short read assembling program Trinity with default parameters (version2.0.6). Gene functions and classification were analyzed based on searches against the NCBI non-redundant database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Blast2 GO was used to obtain a GO annotation and enrichment analysis.

Differentially Expressed Genes (DEGs)

The expression level of each gene was calculated and normalized by RPKM (reads per kb per million reads) to calculate significantly differentially expressed ones (Audic and Claverie 1997; Trapnell et al. 2014). The false discovery rate (FDR) was used to adjust multiple comparisons. The FDR ≤ 0.05 threshold and an absolute value of $\log_2$Ratio ≥ 1 were used to determine the significance of the gene expression differences in the analysis. All DEGs were mapped to terms in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and we looked for significantly enriched pathways in DEGs using the hypergeometric test. Pathways with $Q \leq 0.05$ were defined as the significantly changed KEGG pathways.

Statistical Analysis

An unpaired Student's t-test was used to examine differences in mean duration of imbibing food and returning period of control, imidacloprid-treated and deltamethrin-treated bees.

Results

The Foraging Time Analysis

The duration of honeybees imbibing food in imidacloprid group and deltamethrin group was significantly lower than the control group ($p < 0.05$). The returning period was significantly higher in imidacloprid group and deltamethrin group compared with the control group ($p < 0.05$) (Fig.1).

Differentially Expressed Genes Detected in all Samples

A total of 85 DEGs were identified in the imidacloprid-treatment group versus the control group, and 122 DEGs were identified in the deltamethrin-treatment group versus the control group. Additionally, 27 DEGs were shared in both treatment groups, including apidaecin, hymenoptaecin, CYP 450 6a14, CYP450 303a1, TpnCI, TpnCIIb, Mrjp1, calmodulin-like protein and other uncharacterized proteins (Fig.2). There were 58 DEGs specific to the imidacloprid-treatment group and 95 DEGs were specific to the deltamethrin-treatment group (Fig.2). In the imidacloprid-treatment group, 60 genes were up-regulated, including 10 detoxification immune genes, 6 metabolization-related genes, 6 sensory and signal transduction genes, and 38 other genes. There were 25 down-regulated differential genes, including 2 involved with detoxification and immunity, 1 metabolism-related gene, 2 for sensory and signaling, and 20 for other genes (Fig.3). In the deltamethrin-treatment group, 76 genes were up-regulated, 10 for detoxification and immunity, 7 for metabolization-related genes, 6 for sensory and signal transduction, and 53 for

other genes. There were 46 down-regulated differentially expressed genes, including 5 detoxification genes, 2 metabolically related genes, 11 sensory and signaling genes, and 28 other genes (Fig.3).

Interestingly, 35 genes of the DEGs between insecticide-treated and control bees were involved in detoxification, immunity, sensation and signal transduction. There were 17 genes with significant differences in the imidacloprid-treatment group and 25 genes in the deltamethrin-treatment group (Table S1, Fig.4). There were eight commonly up-regulated genes in both groups, including cytochrome P450 6a14, peroxisomal multifunctional enzyme type 2-like, apidaecins type 73, hymenoptaecin preproprotein, troponin C type I, troponin C type Iib and calmodulin-like protein 4. The significantly down-regulated gene was major royal jelly protein 1 in both groups.

GO Enrichment Analysis

In the imidacloprid-treatment group and deltamethrin-treatment group, the DEGs were significantly enriched in 7 categories of biological function, namely response to biotic stimulus (GO: 0009607), response to bacterium (GO: 0009617), response to external biotic stimulus (GO: 0043207), response to other organisms (GO: 0051707), glucose metabolic process (GO: 0006006), defense response (GO: 0006952) and response to external stimuli (GO: 0009605). These categories are closely related to biological activities such as metabolism, defense response, chemical sensing and response to external stimuli.

Furthermore, three categories in deltamethrin group reached significant enrichment level (adjusted P value < 0.05) for molecular functions, including nucleotide binding (GO: 0000166), oxidoreductase activity (GO: 0016491) and nucleoside phosphate binding (GO: 1901265). In addition, biological function analysis also found ten significant enrichment terms. These categories are related to biological activities such as cytoskeleton, signal transduction, receptor activity, ion balance, defense response, metabolism, and biosynthesis processes.

Pathway Analysis of DEGs

Fifteen DEGs were enriched into 32 pathways in the imidacloprid group compared with the control group. Three pathways reached significantly enrichment, including Lysine biosynthesis (ko00300), Lysine degradation (ko00310) and Phosphatidylinositol signaling system (ko04070) (Table 1). These pathways are mainly involved in metabolism, amino acid metabolism, drug metabolism, detoxification and immunity. Eighteen DEGs were enriched to 35 pathways in the deltamethrin group compared with the control group. Three pathways reached significantly enrichment, namely Glycine, serine and threonine metabolism (ko00260), Metabolic pathways (ko01100) and Hippo signaling pathway-fly (ko04391) (Table 1). These pathways are mainly related to biological activities such as metabolism, amino acid metabolism, signal transduction, detoxification, drug metabolism and endocytosis.

Table 1
Pathway enrichment analysis for the significantly expressed genes in imidacloprid-treated bees and deltamethrin-treated bees.

Control *vs* Imidacloprid Pathway Enrichment				**Control *vs* Deltamethrin Pathway Enrichment**			
Pathway ID	Pathway	Differentially expressed genes	P-value	Pathway ID	Pathway	Differentially expressed genes	P-value
ko00300	Lysine biosynthesis	724239	0.017	ko00260	Glycine, serine and threonine metabolism	551044, 552457	0.020
ko00310	Lysine degradation	102654572, 724239	0.020	ko01100	tMetabolic pathways	102653682, 102655219, 412843, 551044, 551726, 552457, 725017, 725215, 727387, 727584	0.023
ko04070	Phosphatidylinositol signaling system	551726	0.031	ko04391	Hippo signaling pathway-fly	552637, 727368	0.048

Discussion

In our study, the duration of honeybees imbibing food at feeder was declined and the returning period from the hive to the feeder was increased in both insecticide groups compared with the control groups. The insecticide significantly reduced honeybee foraging duration, possibly because the honeybees dislike the food containing insecticides. As the insecticide disrupt the cognitive system, the honeybees may deviate the normal flying route from feeder to hive (Henry et al. 2012; Matsumoto 2013). Honeybees may deliver defective recruitment communication to hive mates, resulting in longer returning time, as we observed. Recruitment declines more rapidly if dances are disoriented, and it takes up to hours for the next foraging trip (Dornhaus 2002; Matsumoto 2013). The average waiting time for receiver bees thus provides returning foragers with an indication of nectar abundance in the colony's environment (Anderson and Ratnieks 1999). When waiting times are long, the forager may perform a tremble dance, which discourages foraging (Seeley 1992). The foragers' waiting time for receiver bees from a feeder provided with *T. hypoglaucum* honey was higher than when the feeder had common vetch honey (Tan et al. 2012).

In our data, both up- and down-regulation of genes were found involved in detoxification, immune, sensory processing, signaling pathways, and metabolism. These complex changes in gene expression reflected that multifaceted aspects of honeybee physiology might have been affected, leading to dysfunction of the dance communication of honeybees. For example, 6 genes in CYP450 family are involved in xenobiotic detoxification (Claudianos et al. 2006). In our study, the up-regulated expression of CYP450s may have rendered honeybees more sensitive to environmental xenobiotics and potentially impacted the detoxification metabolic process of the two insecticides. In addition, esterase, multidrug resistance-associated protein and peroxisomal multifunctional enzyme were up-regulated after both insecticide treatments. We also observed that genes encoding odorant

binding and cuticular proteins were up-regulated in response to insecticide treatments, suggesting that barrier defense was initiated upon insecticide exposure. These results are consistent with those of a previous study reporting that cuticular proteins are up-regulated when honeybees are exposed to parasite and insecticide treatments (Aufauvre et al. 2014).

In the immunity category, 5 genes-encoding antimicrobial peptides, namely hymenoptaecin, abaecin, apidaecin, apisimin, and defensin were found to be altered at the expression level. The expression levels of hymenoptaecin, apidaecin and apisimin was significantly up-regulated after imidacloprid treatment (Fig. 4, Table S1). The expression of hymenoptaecin, apidaecin and abaecin was significantly up-regulated after deltamethrin treatment, while the expression of defensin was significantly down-regulated (Fig. 4, Table S1). With respect to immunity, these results disagree with those of previous studies, which found that insecticides down-regulate immunity-related genes, such as lysozyme- and hymenoptaecin-encoding genes (Aufauvre et al. 2014; Brandt et al. 2016). This might be due to the length of the insecticide exposure time. In addition, proteins involved in pathogen recognition and signaling proteins in upstream immunity pathways were not significantly altered after imidacloprid and deltamethrin treatment.

Calmodulin-like protein (CaM) is a major Ca^{2+}-binding protein found in the central nervous system (Malenka et al. 1989; Margrie et al. 1998). Insecticide are reports to inhibite of CaM and affect the cellular immune response, signal transduction pathway, and biological function (Enan and Matsumura 1992). CaM may directly act on CaMKII, which has been repeatedly related to long time memory (LTM) formation (Nakazawa et al. 1995; Limback-Stokin et al. 2004). In our study, the calmodulin-like protein 4 in two treatment groups was both up-regulated in expression (Fig. 4, Table S1). So the insecticide may impair the honeybees' memory.

Conclusions

Honeybees' brain systems can be easily disrupted, especially because of the insecticides found in floral resources directly target key neural pathways (Palmer et al. 2013; Peng and Yang 2016; Klein et al. 2017), and interfere with the foraging behavior and dance communication (Menzel and Greggers 1985; Menzel 1990). The decreased duration of collecting food and the increased returning period of dance honeybees after insecticide treatment can affect foraging behavior. We observed changes in expression of genes involved in immune, detoxification, learning or memory and chemosensory responses. These changes may be a result of insecticide impaired foraging and dancing.

Declarations

Ethics Approval and Consent to Participate

All animal procedures were performed in accordance with guidelines developed by the China Council on Animal, Care and protocols were approved by the Animal Care and Use Committee of Jiangxi Agricultural University, China.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

Author contribution statement

ZJZ and ZYZ conceived and designed the experiments. ZYZ and ZL performed the experiments. ZYZ and QH analyzed the data. ZYZ wrote the paper. All authors read and approved the final manuscript.

Acknowledgments

We thank Dr. Frederick Partridge for improving the English of this manuscript. This work was supported by the National Natural Science Foundation of China (31872432), the Earmarked Fund for China Agriculture Research System (CARS-44-KXJ15).

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Figures

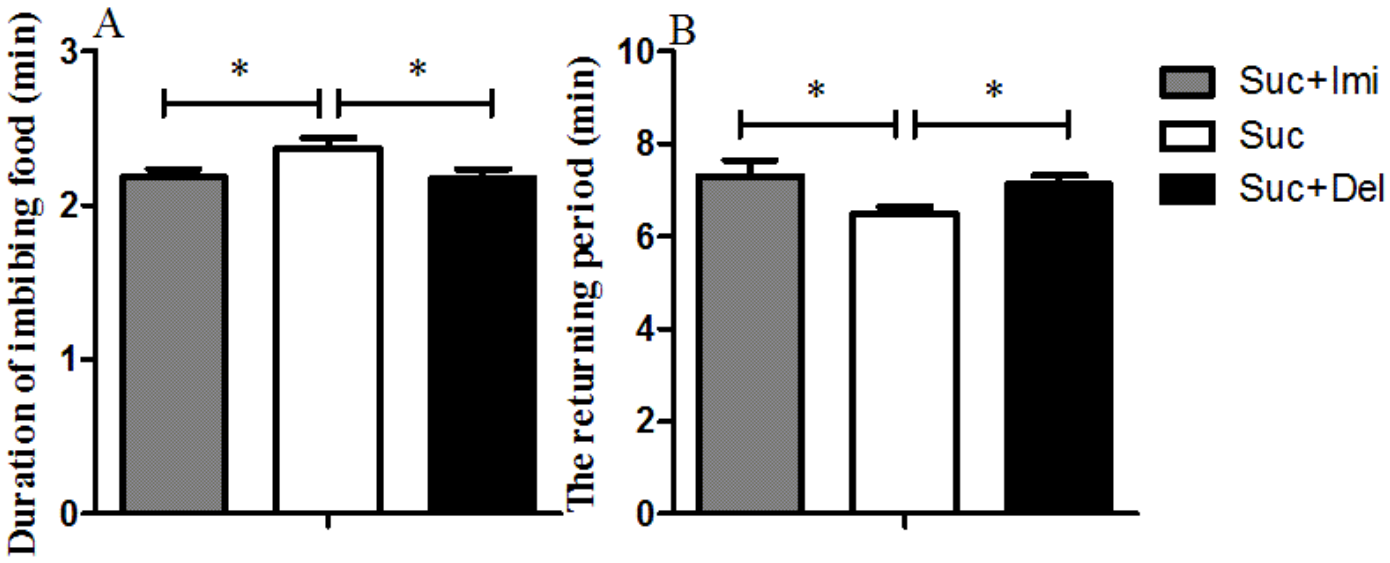

Figure 1

Comparison of the duration of imbibing food and returning period between the insecticide groups and control group

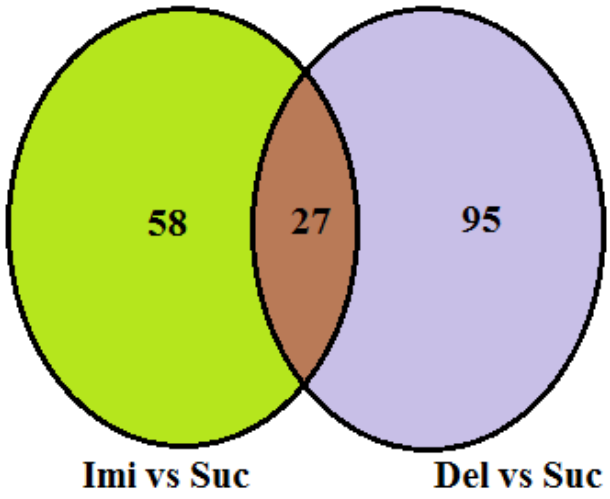

Figure 2

The Venn diagram of DEGs between groups

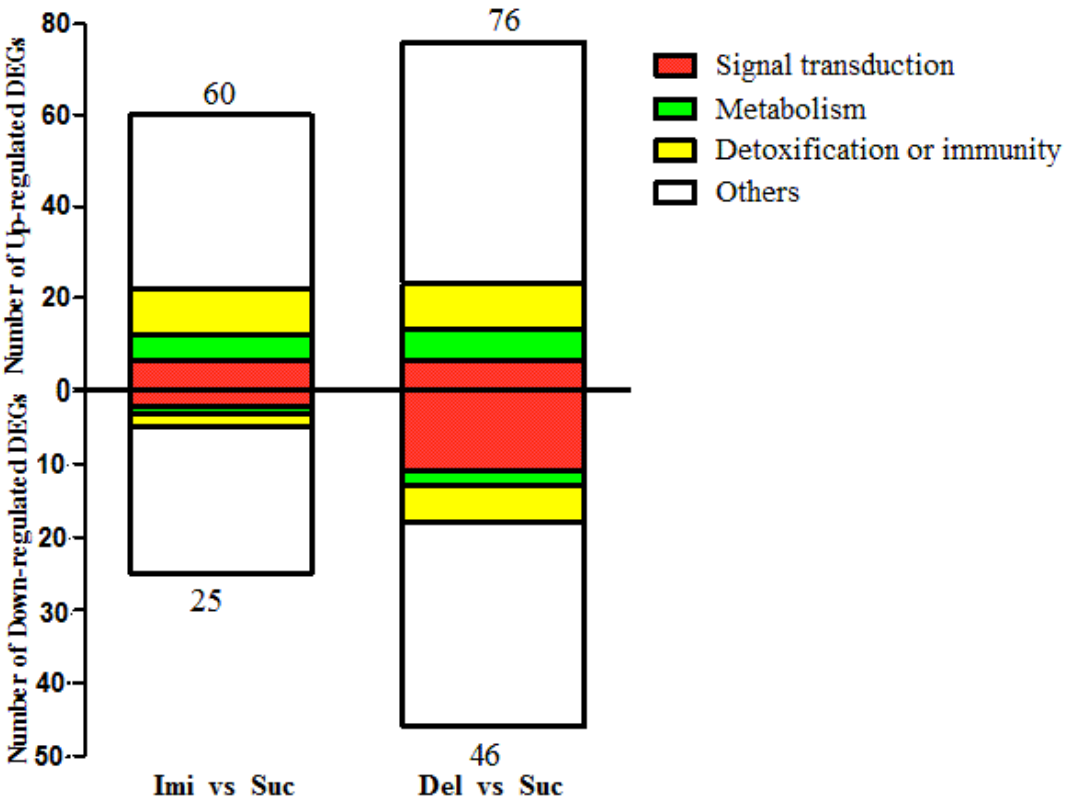

Figure 3

The number of up-regulated or down-regulated DEGs between groups

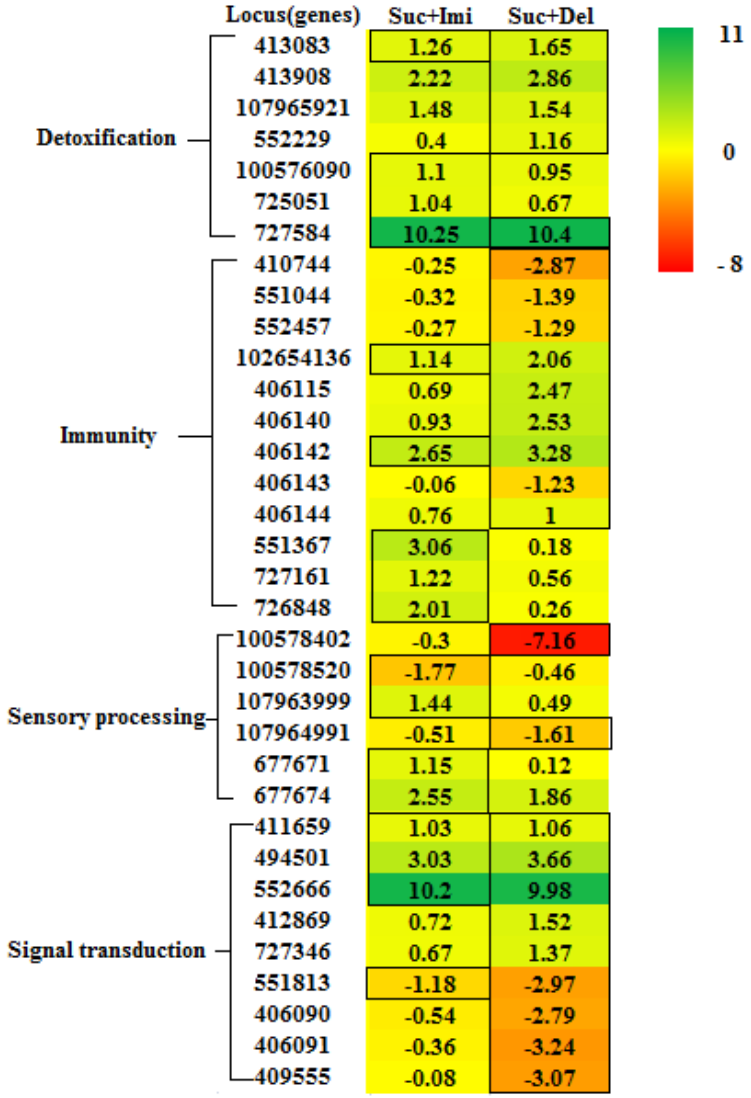

Figure 4

Levels heat map of DEGs between groups. Right column represents the control group, middle column represents the imidacloprid-treated group compared with the control group, and left column represents deltamethrin-treated group compared with the control group. Each row represents a gene, and the log2 ratio of the normalized transcript content is relative to the control, which is shown using different colors: red to green represent the gradation of gene expression abundance from low to high. The data with a border represents a significant difference (the FDR $\leq$ 0.05 threshold and an absolute value of the log2 Ratio $\geq$ 1).

Supplementary Files

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- TableS1.docx