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Transcriptomic and metabolomic landscape of the molecular effects of glyphosate commercial formulation on *Apis mellifera ligustica* and *Apis cerana cerana*

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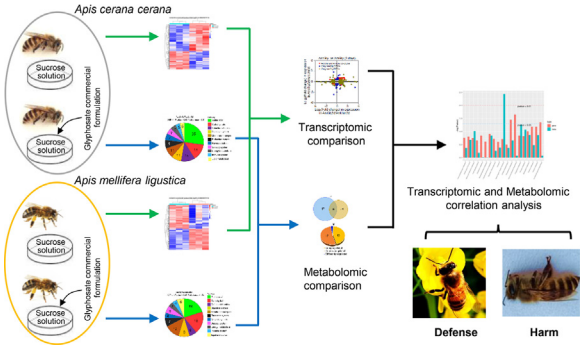
HIGHLIGHTS

- Bees were fed with glyphosate commercial formulation (GCF).
- GCF had adverse impacts on the digestive system and development of bees.
- Bees regulate detoxification and immune system to mitigate pernicious effects.
- *A. cerana cerana* might be better able to withstand GCF than *A. mellifera ligustica*.

GRAPHICAL ABSTRACT



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ABSTRACT

Understanding the causes of the decline in bee population has attracted intensive attention worldwide. The indiscriminate use of agrochemicals is a persistent problem due to their physiological and behavioural damage to bees. Glyphosate and its commercial formulation stand out due to their wide use in agricultural areas and non-crop areas, such as parks, railroads, roadsides, industrial sites, and recreational and residential areas, but the mode of action of glyphosate on bees at the molecular level remains largely unelucidated. Here, we found that the numbers of differentially expressed genes and metabolites under glyphosate commercial formulation (GCF) stress were significantly higher in *Apis cerana cerana* than in *Apis mellifera ligustica*. Despite these differences, the number of differentially expressed transcripts increased following an increase in the GCF treatment time in both *A. cerana cerana* and *A. mellifera ligustica*. GCF exerted adverse impacts on the immune system, digestive system, nervous system, amino acid metabolism, carbohydrate metabolism, growth and development of both bee species by influencing their key genes and metabolites to some extent. The expression of many genes involved in immunity, agrochemical detoxification and resistance, such as antimicrobial peptides, cuticle proteins and cytochrome P450 families, was upregulated by GCF in both bee species. Collectively, our results indicate that both *A. cerana cerana* and *A. mellifera ligustica* strive to mitigate the pernicious effects caused by GCF by regulating detoxification and immune systems. Moreover, *A. cerana cerana* might be better able to withstand the toxic effects of GCF with lower fitness costs than *A. mellifera ligustica*. Our work will contribute to elucidating the deleterious physiological and behavioural impacts of GCF on bees.

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1. Introduction

Insect pollination services are vitally important to crop production, wild plants and global biodiversity (Ashman et al., 2004; Potts et al., 2010). The oft-quoted statistics are that $215 billion of food production, 75% of our cultivated crop species, and 80% of wild plant species are benefitted by insect pollinators (Burd, 1994; Gallai et al., 2009; Klein et al., 2003). Bees are one of the most economically important and prominent insect pollinators worldwide and co-evolved with many flowering plants (Ashman et al., 2004; Klein et al., 2007; Potts et al., 2010). However, bees have suffered substantial population declines and colony losses worldwide (Potts et al., 2010; Powney et al., 2019; Theisen-Jones and Bienefeld, 2016; Vanengelsdorp et al., 2009), which is a worrying phenomenon that has received global attention.

One main cause of the decreases in bee populations is the indiscriminate use of different agrochemicals, such as herbicides and insecticides (Goulson et al., 2015; Johnson, 2015; Potts et al., 2010; Tsvetkov et al., 2017). Historically, most attention has been paid to the impacts on bees exerted by the insecticides used in crop protection, but the relationship between the bee population decline and herbicides remains unclear.

The primary herbicide used globally is glyphosate (Benbrook, 2016; Survey, 2012), which inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in plants and some microorganisms (Kruger et al., 2013; Schrodl et al., 2014; Shilo et al., 2016). Animals lack the shikimate pathway related to EPSPS, which explains why glyphosate is considered a low-toxicity agrochemical (Duke and Powles, 2008). Nonetheless, some evidence indicates that glyphosate exerts effects on nontarget organisms, such as humans, rats, earthworms and bees (Camacho and Mejia, 2017; Gaupp-Berghausen et al., 2015; Motta et al., 2018; Zanardi et al., 2020). Foraging bees are directly exposed to glyphosate on the surface of a flower, in the air or in water while foraging in the natural environment, and these bees also carry glyphosate-contaminated water, pollen and nectar to their beehive, which might result in the exposure of the queen, larvae, pupae, and nurse bees to glyphosate (Sponsler and Johnson, 2017).

Glyphosate exposure exerts negative effects on the physiology and development of the bee brood. Chronic glyphosate exposure during the larval stage reduces *Apis mellifera* brood survival (Dai et al., 2018), and glyphosate induces cell death in the midgut of *A. mellifera* larvae reared in an incubator (Gregorc and Ellis, 2011). An *A. mellifera* brood fed food containing glyphosate exhibits a prolonged duration of early larval stadia and a higher proportion of larvae with reduced weight and delayed moulting. However, these impacts show marked variations with regard to differences in susceptibility of the colony to glyphosate (Vazquez et al., 2018). In addition, the exposure of *A. mellifera* larvae to glyphosate alter the expression levels of some hexameric larval storage proteins, detoxifying genes and immune genes (Gregorc et al., 2012; Vazquez et al., 2018; Vazquez et al., 2020).

Glyphosate also exerts adverse effects on the behaviour and physiological state of adult bees. Chronic glyphosate exposure has a detrimental impact on the midgut bacterial community of newly emerged bees (Dai et al., 2018) and leads to deformed antennae formation in newly emerged workers (Tome et al., 2020). The environmental level of glyphosate disturbs the gut microbiota of adult workers and renders young adult workers of *A. mellifera* more susceptible to *Serratia marcescens* (Motta et al., 2018). Although glyphosate is the active component altering the *A. mellifera* gut microbiota, its main metabolite, aminomethylphosphonic acid, fails to modify bee gut communities (Blot et al., 2019). Glyphosate has impacts on the cognitive, olfactory learning, associative learning and sensory abilities of *A. mellifera* adults and reduces adult food uptake, sucrose responsiveness and sensitivity to nectar reward (Farina et al., 2019; Herbert et al., 2014; Mengoni and Farina, 2018). In addition, *A. mellifera* fed sucrose solution containing 10 mg l^{-1} glyphosate spend more time flying from the release site to the hive than *A. mellifera* fed 2.5 or 5 mg l^{-1} glyphosate, and *A. mellifera* that have ingested glyphosate show more indirect homing flights after the second release than control *A. mellifera* (Balbuena et al., 2015).

Despite the detrimental impacts of glyphosate on bee growth, physiology and behaviour, the mode of action of glyphosate and its commercial formulation on adult bees at the genome-wide and metabolic level remains unclear. Besides, the response of *Apis cerana* to glyphosate and the similarities and differences in the molecular responses of *A. cerana* and *A. mellifera* to glyphosate stress are unclear. We aimed to use molecular techniques, such as transcriptomics and metabolomics, to understand the detailed mode of action of glyphosate commercial formulation (GCF) on *A. cerana cerana* and *A. mellifera ligustica* foraging adults and to identify which genes and metabolic pathways are affected by GCF. In addition, we investigated the differences and similarities among the responses of these two bee species to GCF.

2. Materials and methods

2.1. Chemicals and solutions

GCF solution containing 30% w/v glyphosate and 41% N-(phosphonomethyl)glycine was purchased from YongNong BioSciences Co., Ltd. (China). The product standard number and pesticide registration number of this GCF are GB/T20684–2017 and PD20096301, respectively. According to the manufacturer's instructions, 1 mL of formulation should be diluted using 150 mL of water to obtain field-realistic doses for agricultural purposes. For our experiments with bees, GCF was diluted to the above field-realistic doses using 30% (W/V) sterile sucrose solution. The diluted GCF solution was stored at 4 °C until fed to the bees. In previous studies, glyphosate was used in its unadulterated formulation or as an analytical standard (Balbuena et al., 2015; Dai et al., 2018; Herbert et al., 2014; Vazquez et al., 2018). However, we used a GCF because this is the common form of glyphosate applied in crop areas, such as corn, cotton, and soybean fields and orchards, and non-crop areas, such as industrial sites, roadsides, railroads, parks, and recreational and residential areas, in China.

2.2. Honey bees and treatments

The *A. mellifera ligustica* and *A. cerana cerana* used in this study were maintained at a location with no pesticide use or agricultural activity at Shandong Agricultural University (Taian, China). Foraging *A. mellifera ligustica* of mixed ages were collected (Christen et al., 2016) from six healthy outdoor colonies at the entrance to the hive. The colonies were established by sister queens, and each colony represented an independent replicate. Fifty foraging adults were collected from each colony and then randomly divided into two groups ($n = 25$/group). Each group was maintained in a wooden cage ($12 \times 12 \times 5$ cm) with transparent plastic covers, and each wooden cage was placed in a feed trough. Every day, each treatment group was fed 5 mL of fresh 30% sucrose solution containing field-realistic doses of GCF, and each control group was fed 5 mL of fresh 30% sucrose solution without GCF. All the groups were reared in an incubator at 33 °C with 70% relative humidity and 24 h of darkness. *A. mellifera ligustica* was sampled during GCF treatment, and we waited 3 and 5 days after finishing treatment to sample the *A. mellifera ligustica*. Specifically, samples from each cage of each group were sampled, and each sample, which included four bees, was flash frozen in liquid nitrogen and then stored at −80 °C until used for RNA sequencing (RNA-seq) and metabolomic assays. The sample collection process and times were based on a previously described method (Motta et al., 2018) with some modifications, and the GCF treatment of *A. cerana cerana* was performed using the protocol used for the treatment of *A. mellifera ligustica*.

To reduce variability due to behaviour and age as much as possible, we included the following measures during the sample collection. First, the bee colonies used in the experiment were managed by our

experienced beekeeper every day to ensure that they remained healthy and strong, and only bees from healthy and strong colonies were collected. Second, we performed our experiment during the nectar-rich season, and highest numbers of foraging bees are found during this season compared with other seasons. Therefore, we can collect sufficient numbers of foraging bees within a short time. Third, we collected foraging bees at the entrance to the hive, and only bees that carried pollen in their pollen basket when they returned to the hive were collected.

2.3. RNA-seq and data analysis

Three independent biological replicates of *A. cerana cerana* and *A. mellifera ligustica* treated with GCF for 3 and 5 days were subjected for RNA-seq analysis, and untreated *A. cerana cerana* and *A. mellifera ligustica* samples were used as a control. Each biological replicate was a pool of four bee individuals. RNA from whole bees was extracted. To ensure the RNA stability, the bees were ground to powder using liquid nitrogen and a mortar, and 0.07 g of the resulting powder was added to 1 mL of RNAiso Plus (TaKaRa, China) for the extraction of RNA following the manufacturer's instructions. RNase-free centrifuge tubes and pipet tips were used, and the experimenter wore disposable masks and gloves throughout the entire process of RNA extraction. The RNA integrity was evaluated before sample libraries were constructed, and only undegraded RNA was used for building the sample libraries.

The sample libraries were prepared using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, USA) according to the supplier's instructions and then sequenced on a HiSeq platform by Shanghai OE Biotech. Co., Ltd. (China). The raw reads were processed using Trimmomatic to obtain clean reads (Bolger et al., 2014), and the *A. cerana cerana* and *A. mellifera ligustica* clean reads were mapped to the *A. cerana* and *A. mellifera* reference genomes, respectively, in the NCBI database (https://www.ncbi.nlm.nih.gov/gene/?term=Apis+cerana and https://www.ncbi.nlm.nih.gov/gene/?term=Apis+mellifera, respectively), using HISAT2 (Kim et al., 2015). The read counts and fragments per kilobase of transcript per million mapped reads (FPKM) values of each gene were calculated using HTSeq-count (Anders et al., 2015) and cufflinks (Roberts et al., 2011), respectively. The differential expression analysis was performed using DESeq2 (Love et al., 2014), which was based on negative binomial regression model, and genes were considered differentially expressed if $P < 0.05$ and fold change (FC) > 1.5 (Huai et al., 2018).

The Gene Ontology (GO) database (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) were used for the GO analysis and KEGG analysis of differentially expressed genes (DEGs), respectively. The enrichment analysis of GO terms was performed using topGO (Alexa A., 2010). The *P* value of GO enrichment analyses and KEGG enrichment analyses were calculated by hyper-geometric test, and then were corrected by Benjamini-Hochberg for multiple hypotheses to acquire false discovery rate (FDR). The homology genes of *A. cerana cerana* in *A. mellifera ligustica* and their corresponding gene symbol were searched using blastn (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.6.0/ncbi-blast-2.6.0+-x64-linux.tar.gz). Then, the overlapped DGEs of *A. cerana cerana* and *A. mellifera ligustica* were found using custom R script and Venny 2.1. Hierarchical clustering was perdormed using pheatmap in R package.

2.4. Reverse transcription and reverse transcription quantitative PCR

Each RNA sample (a pool of four bee individuals) was diluted to 200 ng/μL, and 1000 ng of RNA was then reverse transcribed to first-strand cDNA using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (TaKaRa, China) following the manufacturer's instructions. Reverse transcription quantitative PCR (RT-qPCR) based on SYBR green fluorescence was performed using SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (TaKaRa, China). The samples were run on a CFX96 Real-Time Detection System (Bio-Rad, USA) using the following temperature cycling conditions: 30 s at 95 °C followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A final melt cycle was ramped from 65 °C to 95 °C at a rate of 5 °C/5 s to ensure the absence of any non-specific amplification. Each RT-qPCR experiment comprised at least three independent biological replicates with three technical replicates. *β-actin* (Gene symbol: 406122 in *A. mellifera* and LOC107999330 in *A. cerana*), which is commonly used as a reference due to its stable expression in bees (Amdam et al., 2004; Li et al., 2012; Lourenco et al., 2008; Scharlaken et al., 2008; Wang et al., 2012), was used as the reference gene in this study. The gene expression levels were analysed using the $2^{-\Delta\Delta CT}$ method. Student's *t*-test was used to statistically compare the GCF treatment group and control group, and $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered to indicate statistical significance.

The gene symbols of the *A. cerana cerana* genes used for RT-qPCR were the following: LOC107992838, LOC107992885, LOC107994729, LOC107996050, LOC107999482, LOC108000468, LOC108004046, LOC107993492, LOC107993589, LOC107993596, LOC107996301, LOC107998246 and LOC107999827. The gene symbols of *A. mellifera ligustica* genes used for RT-qPCR were the following: LOC100576555, LOC113219028, LOC410747, LOC100577537, LOC408295, Apid1 (Gene description: apidaecin 1), Pgrp-s2 (Gene description: peptidoglycan recognition protein s2), LOC406142, Apd-3 (Gene description: apidermin 3), Apd-1 (Gene description: apidermin 1), LOC410626, LOC725400 and LOC727243. The primers used for RT-qPCR are listed in Table S1.

2.5. Metabolomics

Widely targeted metabolomics (Chen et al., 2013) was carried out on six independent biological replicates of *A. cerana cerana* and *A. mellifera ligustica* treated with GCF for 5 days, and untreated *A. cerana cerana* and *A. mellifera ligustica* were used as controls. Each biological replicate consisted of a pool of four whole bees. The samples were ground to powder using liquid nitrogen and a mortar, and 0.05 g of powder was combined with 1000 μL of ice-cold methanol/water (70%, v/v), which contained 1 μg/mL 2-chlorophenylalanine as an internal standard, and mixed well. The samples were maintained on ice for 15 min and then centrifuged at 4 °C and 12,000 rpm for 10 min. The supernatant was analysed using the ultra-performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS) (UPLC-MS/MS) platforms offered by Wuhan Metware Biotechnology Co., Ltd. (China).

The UPLC-MS/MS analysis was performed using the following conditions. The UPLC column was a Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 × 100 mm). The column temperature was 40 °C, the injection volume was 2 μL, and the flow rate was 0.4 mL/min. The solvent system contained ultrapure water (0.04% acetic acid) and acetonitrile (0.04% acetic acid). The gradient program of ultrapure water/acetonitrile was 95:5 (vol/vol) at 0 min, 5:95 (vol/vol) at 11 min, 5:95 (vol/vol) at 12 min, 95:5 (vol/vol) at 12.1 min and 95:5 (vol/vol) at 14 min. The mass spectrometry (MS) voltages were 5500 V (positive) and − 4500 V (negative). The electrospray ionisation temperature was 500 °C. The ion source gas I, ion source gas II and curtain gas were set to 55 psi, 60 psi and 25 psi, respectively, and collision-activated dissociation was set to a high level. Each ion pair was scanned based on the optimised collision energy and declustering potential in the triple quadrupole MS. The qualitative analysis of metabolites was performed using the self-built database MetWare (MWDB, Wuhan Metware Biotechnology Co., Ltd., China). The subsequent analyses were performed using Analyst 1.63 software (Sciex).

The differentially expressed metabolites were identified by variable importance in projection (VIP) > 1, $P < 0.05$ and FC > 1.5. The abundance of the portion-changed compounds was then normalised using "Range scaling" (Wilinski et al., 2019). The principal component analysis (PCA) was based on a covariance matrix. PCA and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were performed using base package and MetaboAnalystrR in R package, respectively. Functional annotation and enrichment analysis of the differentially expressed

metabolites were performed using the KEGG database. The *P* value of KEGG enrichment analyses was calculated by hyper-geometric test, and then was corrected by Benjamini-Hochberg for multiple hypotheses.

2.6. Correlation analysis of transcriptomic and metabolomic data

Three independent biological replicates were used for the RNA-seq analysis, and widely targeted metabolomics was performed using six independent biological replicates. We selected six colonies; the samples from three of these colonies were subjected to both transcriptomic and metabolomic analyses, and the samples from the other three colonies were only subjected to metabolomic analysis. Each of the colonies represented an independent replicate. When performing a correlation analysis of transcriptomic and metabolomic data, the number of biological replicates should be the same. We thus performed a correlation analysis of transcriptomic and metabolomic data using the data from samples that were subjected to both transcriptomic and metabolomic analyses to explore the correlation between differentially expressed genes and metabolites. The Pearson correlation coefficient (PCC) of the differentially expressed genes and metabolites was calculated using the cor function in the R package. A nine-quadrant graph was used to show the FC of genes and metabolites in each group with PCC > 0.8 among different groups using ggplot2 and getopt. Genes and metabolites with PCC > 0.8 in KEGG pathway were used to prepare a correlation network diagram with Cytoscape.

3. Results

3.1. Transcriptome-wide identification of differentially expressed genes in A. cerana cerana and A. mellifera ligustica under GCF exposure

In our experiment, GCF treatment for 3 and 5 days exerted no significant effects on the survival rate of *A. cerana cerana* and *A. mellifera ligustica* compared with the control treatment (data not shown). To determine the molecular effects of GCF on bees, we performed an RNA-seq assay of *A. cerana cerana* and *A. mellifera ligustica* after GCF exposure for 3 and 5 days. After mapping the clean reads (Table S2) of *A. cerana cerana* to its reference genome, we identified 10,500 and 10,515 expressed genes under GCF stress for 3 days and its control, respectively, and identified 10,508 and 10,502 expressed genes under GCF stress for 5 days and its control, respectively (Fig. 1A). After mapping the clean reads (Table S2) of *A. mellifera ligustica* to its reference genome, we identified 9749 and 9764 expressed genes under GCF stress for 3 days and its control, respectively, and identified 9771 and 9755 expressed genes under GCF stress for 5 days and its control (Fig. 1A).

Based on FC > 1.5 and $P < 0.05$, 319 differentially expressed genes (DEGs, 188 upregulated and 131 downregulated) were identified from the comparison of *A. cerana cerana* treated with GCF for 3 days and untreated *A. cerana cerana* (AccGCF3d/AccUnt3d), and 338 DEGs (158 upregulated and 180 downregulated) were obtained from the comparison of *A. cerana cerana* treated with GCF for 5 days and untreated *A. cerana cerana* (AccGCF5d/AccUnt5d) (Figs. 1A, S1 and Table S3_Sheet 1–2). Eighty-six DEGs (38 upregulated and 48 downregulated) were obtained from the comparison of *A. mellifera ligustica* treated with GCF for 3 days and untreated *A. mellifera ligustica* (AmGCF3d/AmUnt3d), and 141 DEGs (53 upregulated and 88 downregulated) were identified from the comparison of *A. mellifera ligustica* treated with GCF for 5 days and untreated *A. mellifera ligustica* (AmGCF5d/AmUnt5d) (Figs. 1A, S1 and Table S3_Sheet 3–4).

To explore the differences between bees treated with GCF for 3 and 5 days, we performed a comparison analysis of their corresponding DEGs. Seventy-six DEGs overlapped between AccGCF3d/AccUnt3d and AccGCF5d/AccUnt5d, and these included 36 co-upregulated genes, 17 co-downregulated genes and 23 oppositely regulated genes (Fig. 1B and Table S4_Sheet 1). Seven DEGs overlapped between AmGCF3d/AmUnt3d and AmGCF5d/AmUnt5d, and one and six of these genes were co-upregulated and co-downregulated, respectively (Fig. 1B and Table S4_Sheet 2). Notably, members of many gene families, particularly antimicrobial peptides (apidaecin, abaecin, defensin and hymenoptaecin), cuticular proteins (*CPR3*, *CPR14*, and *CPR19*) and cytochrome P450 [CYP450, including *CYP15A1* (LOC107999579, LOC551179), *CYP9E2* (LOC108003159) and *CYP6A14* (LOC114576772, LOC727598)], were upregulated in *A. cerana cerana* or *A. mellifera ligustica* under GCF stress, whereas heat shock protein (Hsp), such as *Hsp83*, *Hsp90*, and *DnaJC17*, and odourant-binding protein (OBP), such as *OBP3*, *OBP9*, and *OBP15*, were downregulated (Table S3). We also found that the number of DEGs was increased in both *A. cerana cerana* (increased 5.6%) and *A. mellifera ligustica* (increased 39%) exposed to GCF for 5 days compared with those exposed to GCF for 3 days, which indicated that increases in the treatment time increased the effect of GCF on bees (Fig. 1A).

To understand the function of DEGs in bees subjected to GCF treatment, we performed a GO analysis. The results showed that DEGs in both *A. cerana cerana* and *A. mellifera ligustica* under GCF stress were enriched in many biological processes, cellular components and molecular functions (Fig. S2 and Table S5). Interestingly, the proportion of downregulated genes associated with metabolic process, growth and developmental process, response to stimulus, reproduction and immune system was markedly higher than that of the upregulated genes in both bee species after exposure to GCF for 5 days than that after exposure for 3 days (Fig. 1C and Table S5). For example, 36.9% and 41.2% DEGs related to growth and development were downregulated in AccGCF3d/AccUnt3d and AmGCF3d/AmUnt3d, respectively (Fig. 1C), whereas 61.6% and 86.5% DEGs connected to growth and development were downregulated in AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d, respectively (Fig. 1C). These results indicate that GCF impairs key pathways in bees, such as growth and development, reproduction and immune system processes, and this detrimental impact can accumulate with increases in the GCF exposure time.

To further determine the roles of the DEGs, we filtered the top 10 GO terms in which DEGs were enriched. We found that the DEGs in both *A. cerana cerana* and *A. mellifera ligustica* were related to many essential pathways (Fig. S3 and Table S5_Sheet 5–8). For example, the analysis of the top GO term in AccGCF5d/AccUnt5d showed that the DEGs were associated with the biological process category “adult feeding behaviour” (Fig. S3). The analysis of the top GO terms in AmGCF5d/AmUnt5d identified DEGs related to various biological processes, such as nervous system development and defence response to bacteria (Fig. S3). These results further indicate that GCF exerts effects on many signalling pathways in bees.

To further explore the functions of DEGs in bees after exposure to GCF, we performed a KEGG enrichment analysis. We found many DEGs in AccGCF3d/AccUnt3d and AccGCF5d/AccUnt5d related to carbohydrate metabolism, energy metabolism, immune system and lipid metabolism (Figs. S4A, S5 and Table S6_Sheet 1–4). We also found that the DEGs in AmGCF3d/AmUnt3d and AmGCF5d/AmUnt5d were related to many important pathways, such as amino acid metabolism, carbohydrate metabolism and lipid metabolism (Fig. S4B and Table S6_Sheet 5–6). These results further indicate that GCF influences the metabolism of many important pathways in bees.

3.2. Comparative analysis of the response of A. cerana cerana and A. mellifera ligustica to GCF at the transcriptome level

To compare the responses of *A. cerana cerana* and *A. mellifera ligustica* to GCF, we performed a comparative analysis of their RNA-seq data. The number of DEGs in *A. cerana cerana* was greater than that in *A. mellifera ligustica* after exposure to GCF for 3 days (increased 270%) and for 5 days (increased 140%) (Figs. 1A and 2A). In addition, the number of DEGs from the antimicrobial peptide, cuticular protein, OBP, odourant receptor, CYP450 and Hsp families was clearly higher in GCF-treated *A. cerana cerana* than in GCF-treated *A. mellifera ligustica* (Table S3). For example, 16 DEGs in AccGCF3d/AccUnt3d and 11 DEGs

in AccGCF5d/AccUnt5d belonged to the cuticular protein family, including apidermin [novel cuticular proteins revealed by the honey bee genome (Kucharski et al., 2007)], and these numbers were markedly higher than those identified in AmGCF3d/AmUnt3d (four DEGs) and AmGCF5d/AmUnt5d (two DEGs) (Table S3). In addition, DEGs related to energy metabolism and the heme binding system were detected in *A. cerana cerana* after exposure to GCF for 3 and 5 days, but these were detected in *A. mellifera ligustica* only after exposure to GCF for 5 days (Figs. S3 and S4). These results indicate that *A. cerana cerana* might be more sensitive to GCF than *A. mellifera ligustica*.

We subsequently performed a Venn analysis to identify the co-regulated DEGs in *A. cerana cerana* and *A. mellifera ligustica* after exposure to GCF. We found that 13 genes, including eight co-upregulated genes, two co-downregulated genes and three oppositely regulated genes, were coregulated in AccGCF3d/AccUnt3d and AmGCF3d/AmUnt3d (Fig. 2A–B and Table S7_Sheet 1). In addition, 52 genes were co-regulated in AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d, and among these, 20, 27 and five genes were co-upregulated, co-downregulated and oppositely regulated, respectively (Fig. 2A–B and Table S7_Sheet 2). The DEGs that overlapped between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d presented a stronger positive correlation ($R = 0.7712$) than those that overlapped between AccGCF3d/AccUnt3d and AmGCF3d/AmUnt3d ($R = 0.3839$) (Fig. 2C). These results suggest that many

A

Species	Samples	The number of all expressed genes	Samples	The number of DEGs	Up-regulated	Down-regulated
	AccUnt3d	10500	AccGCF3d/AccUnt3d	319	188	131
	AccGCF3d	10515				
	AccUnt5d	10508	AccGCF5d/AccUnt5d	338	158	180
	AccGCF5d	10502				
	AmUnt3d	9749	AmGCF3d/AmUnt3d	86	38	48
	AmGCF3d	9764				
	AmUnt5d	9771	AmGCF5d/AmUnt5d	141	53	88
	AmGCF5d	9755				

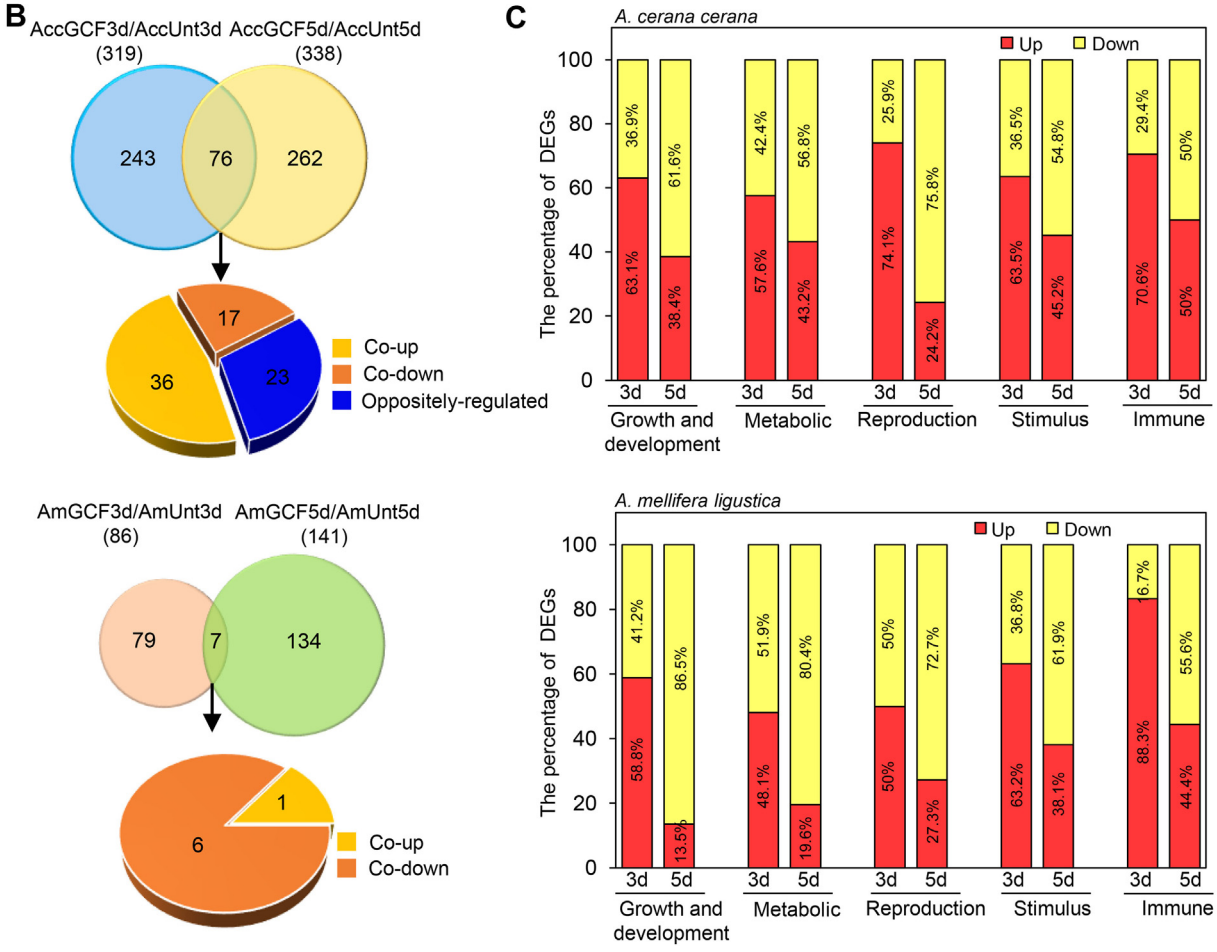
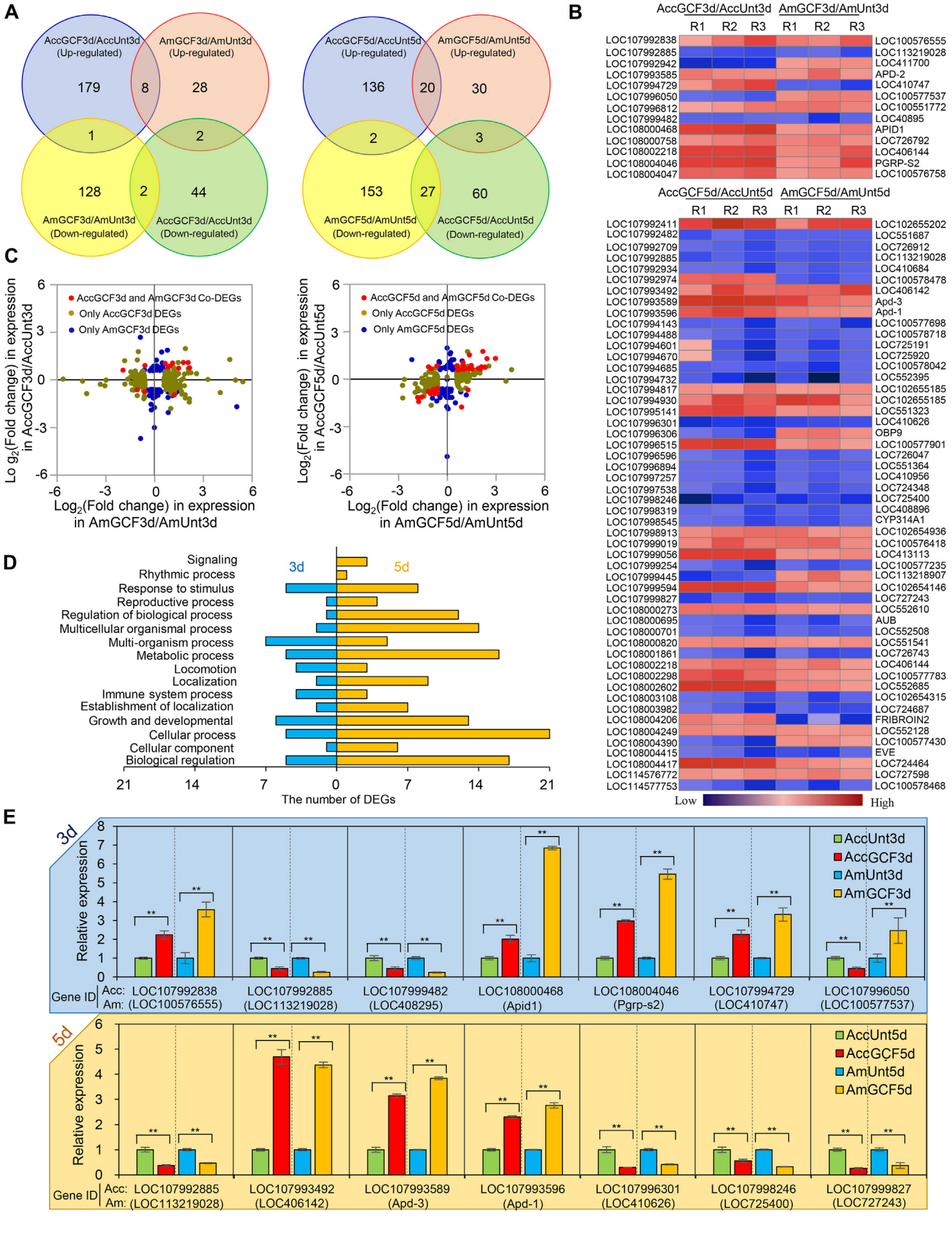


Fig. 1. Transcriptomic profiles of *Apis cerana cerana* and *Apis mellifera ligustica* upon glyphosate commercial formulation (GCF) exposure. A, Statistics of the expressed genes and differentially expressed genes (DEGs) in *A. cerana cerana* after 3 days of exposure to GCF compared with untreated *A. cerana cerana* (AccGCF3d/AccUnt3d), *A. cerana cerana* exposed to GCF for 5 days compared with untreated *A. cerana cerana* (AccGCF5d/AccUnt5d), *A. mellifera ligustica* exposed to GCF for 3 days compared with untreated *A. mellifera ligustica* (AmGCF3d/AmUnt3d), and *A. mellifera ligustica* exposed to GCF for 5 days compared with untreated *A. mellifera ligustica* (AmGCF5d/AmUnt5d). B, Venn diagram showing the overlapping DEGs between AccGCF3d/AccUnt3d and AccGCF5d/AccUnt5d (upon panel) and between AmGCF3d/AmUnt3d and AmGCF5d/AmUnt5d (down panel). C, Percentages of downregulated and upregulated genes involved in growth and development, response to stimulus, reproductive processes, metabolic processes and immune system processes in AccGCF3d/AccUnt3d, AccGCF5d/AccUnt5d (upon panel), AmGCF3d/AmUnt3d and AmGCF5d/AmUnt5d (down panel).


A
AccGCF3d/AccUnt3d (Up-regulated)
AmGCF3d/AmUnt3d (Up-regulated)
179
8
28
1
2
128
2
44
AmGCF3d/AmUnt3d (Down-regulated)
AccGCF3d/AccUnt3d (Down-regulated)
AccGCF5d/AccUnt5d (Up-regulated)
AmGCF5d/AmUnt5d (Up-regulated)
136
20
30
2
3
153
27
60
AmGCF5d/AmUnt5d (Down-regulated)
AccGCF5d/AccUnt5d (Down-regulated)
B
AccGCF3d/AccUnt3d AmGCF3d/AmUnt3d
R1 R2 R3 R1 R2 R3
LOC107992838 LOC100576555
LOC107992885 LOC113219028
LOC107992942 LOC411700
LOC107993585 APD-2
LOC107994729 LOC410747
LOC107996050 LOC100577537
LOC107996812 LOC100551772
LOC107999482 LOC40895
LOC108000468 APID1
LOC108000758 LOC726792
LOC108002218 LOC406144
LOC108004046 PGRP-S2
LOC108004047 LOC100576758
AccGCF5d/AccUnt5d AmGCF5d/AmUnt5d
R1 R2 R3 R1 R2 R3
LOC107992411 LOC102655202
LOC107992482 LOC551687
LOC107992709 LOC726912
LOC107992885 LOC113219028
LOC107992934 LOC410684
LOC107992974 LOC100578478
LOC107993492 LOC406142
LOC107993589 Apd-3
LOC107993596 Apd-1
LOC107994143 LOC100577698
LOC107994488 LOC100578718
LOC107994601 LOC725191
LOC107994670 LOC725920
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LOC107994732 LOC552395
LOC107994817 LOC102655185
LOC107994930 LOC102655185
LOC107995141 LOC551323
LOC107996301 LOC410626
LOC107996306 OBP9
LOC107996515 LOC100577901
LOC107996596 LOC726047
LOC107996894 LOC551364
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LOC107997538 LOC724348
LOC107998246 LOC725400
LOC107998319 LOC408896
LOC107998545 CYP314A1
LOC107998913 LOC102654936
LOC107999019 LOC100576418
LOC107999056 LOC413113
LOC107999254 LOC100577235
LOC107999445 LOC113218907
LOC107999594 LOC102654146
LOC107999827 LOC727243
LOC108000273 LOC552610
LOC108000695 AUB
LOC108000701 LOC552508
LOC108000820 LOC551541
LOC108001861 LOC726743
LOC108002218 LOC406144
LOC108002298 LOC100577783
LOC108002602 LOC552685
LOC108003108 LOC102654315
LOC108003982 LOC724687
LOC108004206 FRIBROIN2
LOC108004249 LOC552128
LOC108004390 LOC100577430
LOC108004415 EVE
LOC108004417 LOC724464
LOC114576772 LOC727598
LOC114577753 LOC100578468
Low High
C
Lo g2(Fold change) in expression in AccGCF3d/AccUnt3d
AccGCF3d and AmGCF3d Co-DEGs
Only AccGCF3d DEGs
Only AmGCF3d DEGs
Log2(Fold change) in expression in AmGCF3d/AmUnt3d
Log2(Fold change) in expression in AccGCF5d/AccUnt5d
AccGCF5d and AmGCF5d Co-DEGs
Only AccGCF5d DEGs
Only AmGCF5d DEGs
Log2(Fold change) in expression in AmGCF5d/AmUnt5d
D
3d
5d
Signaling
Rhythmic process
Response to stimulus
Reproductive process
Regulation of biological process
Multicellular organismal process
Multi-organism process
Metabolic process
Locomotion
Localization
Immune system process
Establishment of localization
Growth and developmental
Cellular process
Cellular component
Biological regulation
The number of DEGs
E
3d
Relative expression
AccUnt3d
AccGCF3d
AmUnt3d
AmGCF3d
Gene ID
Acc: LOC107992838 LOC107992885 LOC107999482 LOC108000468 LOC108004046 LOC107994729 LOC107996050
Am: (LOC100576555) (LOC113219028) (LOC408295) (Apid1) (Pgrp-s2) (LOC410747) (LOC100577537)
5d
Relative expression
AccUnt5d
AccGCF5d
AmUnt5d
AmGCF5d
Gene ID
Acc: LOC107992885 LOC107993492 LOC107993589 LOC107993596 LOC107996301 LOC107998246 LOC107999827
Am: (LOC113219028) (LOC406142) (Apd-3) (Apd-1) (LOC410626) (LOC725400) (LOC727243)

genes are co-employed by *A. cerana cerana* and *A. mellifera ligustica* to defend against GCF, and this phenomenon was more obvious in both species after exposure to GCF for 5 days than after exposure for 3 days.

GO analysis showed that the DEGs that overlapped between *A. cerana cerana* and *A. mellifera ligustica* exposed to GCF stress were enriched in many key processes, such as developmental process, immune system process and response to stimulus (Figs. 2D, S6 and Table S8). These pathways might play important roles in the responses of both bee species to GCF to a certain degree. In addition, to verify the RNA-seq results, we performed an RT-qPCR assay and found that many genes were co-regulated in *A. cerana cerana* and *A. mellifera ligustica* under GCF stress (Fig. 2E), which was consistent with the RNA-seq results (Fig. 2B).

3.3. Relative metabolite abundances in A. cerana cerana and A. mellifera ligustica exposed to GCF

To further investigate the molecular effects of GCF on bees, we performed a widely targeted metabolomic analysis. PCA showed large variances between AccGCF5d and AccUnt5d and between AmGCF5d and AmUnt5d, and distinct groups were observed, which indicated that the stability of the instrument was good during the sample analysis process (Fig. S7A). OPLS-DA also revealed good separation between the GCF-treated bees and the corresponding control bees (Fig. S7B), which suggested that a VIP analysis could be performed for screening differential metabolites. A total of 595 metabolites were identified, and 120 metabolites showed changes in abundance (68 upregulated and 52 downregulated), as defined by VIP > 1, $P < 0.05$ and FC > 1.5, in AccGCF5d/AccUnt5d (Fig. S7C–D and Table S9_Sheet 1). We also quantified 596 metabolites in *A. mellifera ligustica* through a widely targeted metabolomic analysis (Fig. S7C), and among these metabolites, the abundance of 72 metabolites, including 37 upregulated and 35 downregulated compounds, showed significant changes as defined by VIP > 1, $P < 0.05$ and FC > 1.5 (Fig. S7C–D and Table S9_Sheet 2).

We performed a KEGG functional analysis to explore the functions of the metabolites in bees that were altered by exposure to GCF stress. The differential metabolites in AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d were enriched in many pathways, such as amino acid metabolism, carbohydrate metabolism, digestive system, energy metabolism, immune system, lipid metabolism, nervous system, sensory system and membrane transport (Fig. 3, Table S10 and Table S11). In addition, the compounds in AccGCF5d/AccUnt5d reflected increased levels of vitamin B6 metabolism and long-term depression (Table S9_Sheet 1 and Table S10_Sheet 1). Lower levels of many metabolites, including those related to vitamin digestion and absorption, sulphur metabolism, ATP-binding cassette (ABC) transporters, synaptic vesicle cycle and neuroactive ligand-receptor interaction, were observed in AccGCF5d/AccUnt5d (Table S9_Sheet 1 and Table S10_Sheet 1). The metabolites involved in thermogenesis and vitamin B6 metabolism were significantly increased in AmGCF5d/AmUnt5d (Table S9_Sheet 2 and Table S10_Sheet 2). The abundances of compounds associated with protein and carbohydrate digestion and absorption, ABC transporters, and starch, sucrose, arginine and proline metabolism, were decreased in AmGCF5d/AmUnt5d (Table S9_Sheet 2 and Table S10_Sheet 2). These findings indicate that GCF disrupts the normal metabolism of bees.

To investigate the similarities and differences among the responses of *A. cerana cerana* and *A. mellifera ligustica* to GCF at the metabolic level, we performed a comparative analysis of their differential metabolites. The results revealed 33 compounds that overlapped between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d, and these included 15 co-upregulated compounds, 17 co-downregulated compounds and one oppositely regulated compound (Fig. 4A and Table S12). The correlation analysis revealed a significant positive correlation in the co-regulated compounds between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d ($R = 0.918$) (Fig. 4B). These coregulated metabolites were enriched in many key pathways, such as amino acid and carbohydrate metabolomics (Fig. 4C). These results suggest that although some differences were found, *A. cerana cerana* and *A. mellifera ligustica* employ some defence strategies when exposed to GCF stress.

3.4. Correlation analysis of the transcriptomes and metabolomes of bees under GCF stress

To further understand the regulatory network of bees in response to GCF stress, we performed a correlation analysis of the transcriptomes and metabolomes using the samples that were subjected to both transcriptomic and metabolomic assays. We found that many genes and metabolites exhibited strong positive correlations (with $R > 0.8$) in *A. cerana cerana* and *A. mellifera ligustica* (Fig. 5A and Table S13). For example, the DEG with the symbol LOC107996515 showed a strong positive correlation ($R = 0.969$) with N-lactoyl-phenylalanine (MEDN579, enriched in the pathways related to organic acids and their derivatives) in *A. cerana cerana* (Table S13_Sheet 1). The DEG with the symbol LOC550965 presented a significant correlation ($R = 0.996$) with hexanoyl glycine (MEDN041, involved in amino acid metabolism pathways) in *A. mellifera ligustica* (Table S13_Sheet 2). These results indicate that these DEGs might play direct or indirect regulatory roles in the alterations in the corresponding metabolites.

We also found that many DEGs and differentially expressed metabolites were enriched in the same KEGG pathways in both *A. cerana cerana* and *A. mellifera ligustica*, and these pathways included tyrosine metabolism, phenylalanine metabolism and neuroactive ligand-receptor interaction (Fig. 5B and Table S14). For example, the DEG with gene symbol LOC107998542 was enriched in the same neuroactive ligand-receptor interaction pathway with five metabolites (MEDP006, MEDP367, MEDN034, MEDP047, MEDN180 and MEDP185) in *A. cerana cerana* (Table S14_Sheet 1). The DGE with gene symbol LOC411188 was enriched in the same glycolysis pathway with one compound (MEDN220) in *A. mellifera ligustica* (Table S14_Sheet 2). We also used correlation network diagrams to further show the connection between DEGs and altered metabolites in *A. cerana cerana* and *A. mellifera ligustica* (Fig. 5C and Table S15). These results further indicate that GCF influences the transcriptomic and metabolomic regulatory network in bees.

Fig. 2. Comparative analysis of the RNA-seq data from *Apis cerana cerana* and *Apis mellifera ligustica* under glyphosate commercial formulation (GCF) stress. A, Venn diagram showing the overlapping differentially expressed genes (DEGs) between *A. cerana cerana* and *A. mellifera ligustica* after GCF exposure for 3 days (left panel) and 5 days (right panel). AccGCF3d/AccUnt3d: 3-day GCF-treated *A. cerana cerana* compared with untreated *A. cerana cerana*. AccGCF5d/AccUnt5d: 5-day GCF-treated *A. cerana cerana* compared with untreated *A. cerana cerana*. AmGCF3d/AmUnt3d: 3-day GCF-treated *A. mellifera ligustica* compared with untreated *A. mellifera ligustica*. AmGCF5d/AmUnt5d: 5-day GCF-treated *A. mellifera ligustica* compared with untreated *A. mellifera ligustica*. B, Hierarchical clustering of transcripts co-regulated in AccGCF3d/AccUnt3d and AmGCF3d/AmUnt3d (upon panel) and in AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d (down panel). C, Correlation analysis of the log-fold change of DEGs between AccGCF3d/AccUnt3d and AmGCF3d/AmUnt3d (left panel) and between AccGCF5d/AmUnt5d and AmGCF5d/AmUnt5d (right panel). D, Gene Ontology (GO) analysis (biological process category) of co-regulated genes between AccGCF3d/AccUnt3d and AmGCF3d/AmUnt3d (blue column) and between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d (orange column). E, Expression levels of overlapping DEGs between AccGCF3d/AccUnt3d and AmGCF3d/AmUnt3d (upper panel) and between AccGCF5d/AmUnt5d and AmGCF5d/AmUnt5d (down panel) as determined by RT-qPCR, and *β-actin* was used as an internal control. The data are shown as the means ± SEs from three replicates of four individuals each. $^{**}P < 0.01$, as determined by Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

One of the main issues of concern worldwide is the impact of agrochemicals on the pollinator community, and thus, the adverse effects of different formulations or their active ingredients on nontarget pollinators, including bees, are being documented (Goulson et al., 2015; Potts et al., 2010). Despite the frequent use of herbicides, particularly glyphosate and GCF (Benbrook, 2016; Survey, 2012), the underlying molecular effects of glyphosate and GCF in bees remain largely unknown. Here, we showed that although GCF exerted adverse impacts on bee health, bees have developed several strategies to defend against GCF stress (Fig. 6). In addition, this study constitutes the first analysis of the differences and similarities between the responses of *A. cerana cerana* and *A. mellifera ligustica* to GCF at transcriptomics and metabolomics level.

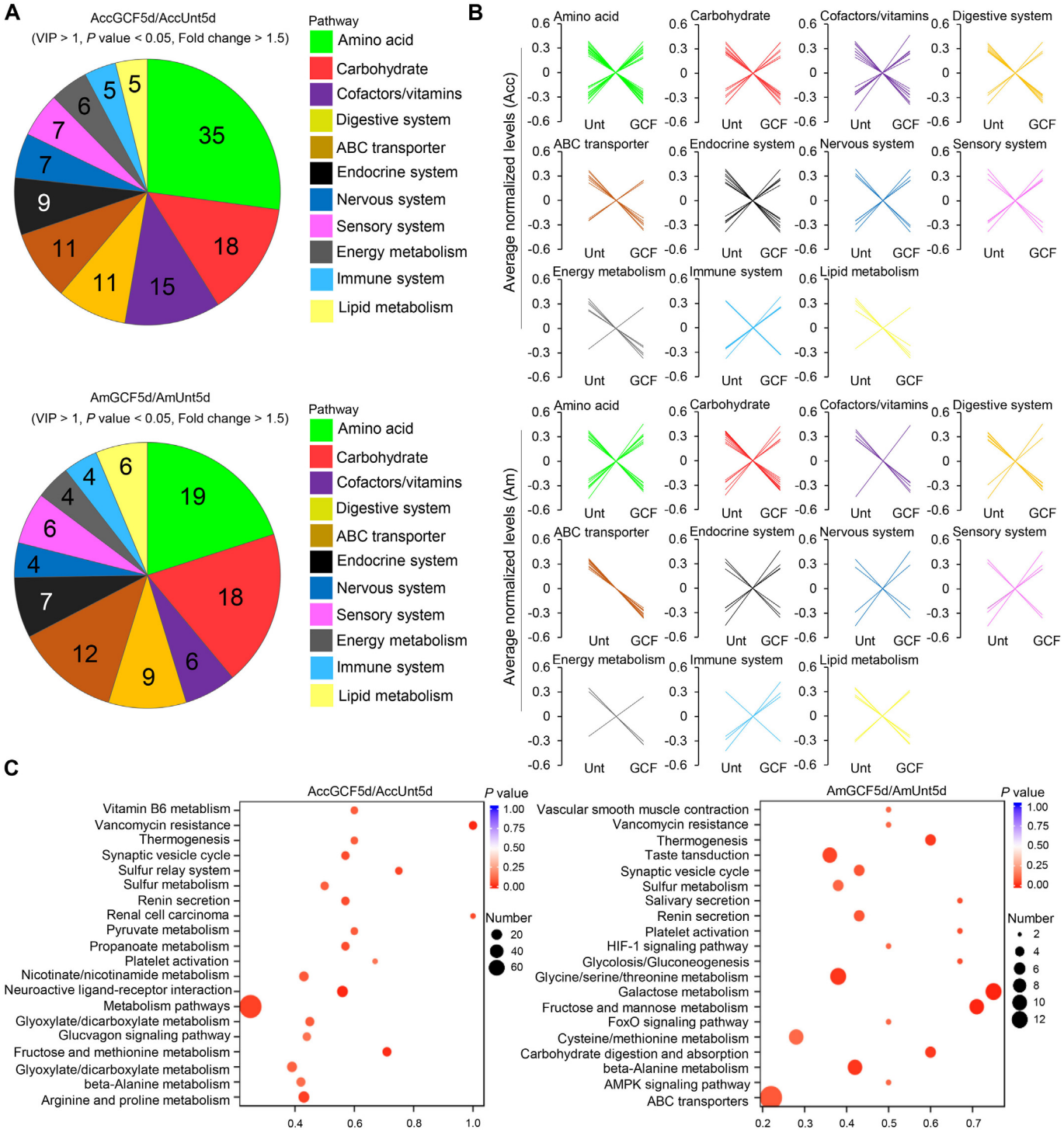


Fig. 3. Exposure to glyphosate commercial formulation (GCF) alters the abundance of metabolites involved in important pathways in bees. A, Effect of GCF exposure on differentially expressed metabolite classes (labelled by various colours) in *Apis cerana cerana* exposed to GCF for 5 days compared with untreated *A. cerana cerana* (AccGCF5d/AccUnt5d, upon panel) and in *Apis mellifera ligustica* exposed to GCF for 5 days compared with untreated *A. mellifera ligustica* (AmGCF5d/AmUnt5d, down panel). Metabolites were considered differentially expressed if VIP > 1, *P* value < 0.05 and fold change >1.5. B, Normalised profiles of the differentially expressed metabolites (grouped by class) in AccGCF5d/AccUnt5d and in AmGCF5d/AmUnt5d. The colour scheme defines the compound classes shown in panel A. C, Enrichment scatter diagram of the top 20 KEGG pathways of differentially expressed metabolites in *A. cerana cerana* and *A. mellifera ligustica* exposed to GCF stress for 5 days.

4.1. GCF disrupts the whole transcriptional and metabolic regulatory networks of bees and thus poses a challenge to bee health

Glyphosate has negative impacts on the associative learning processes and sensory and cognitive abilities of bees (Farina et al., 2019), but the underlying molecular mechanisms are poorly understood. *OBP* plays essential roles in the sensitivity of the olfactory system and cognitive abilities. *OBP69a* connects the modulation of social responsiveness to social interaction in *Drosophila* (Bentzur et al., 2018). *OBP7* plays a dual role in the recognition and perception of host-plant sex pheromones and volatiles in *Grapholita molesta* (Chen et al., 2018). The expression of *OBP4*, *OBP16*, *OBP18* and *OBP21* is obviously changed in *A. mellifera* to defend against sublethal doses of imidacloprid stress (Li et al., 2019). We found that the

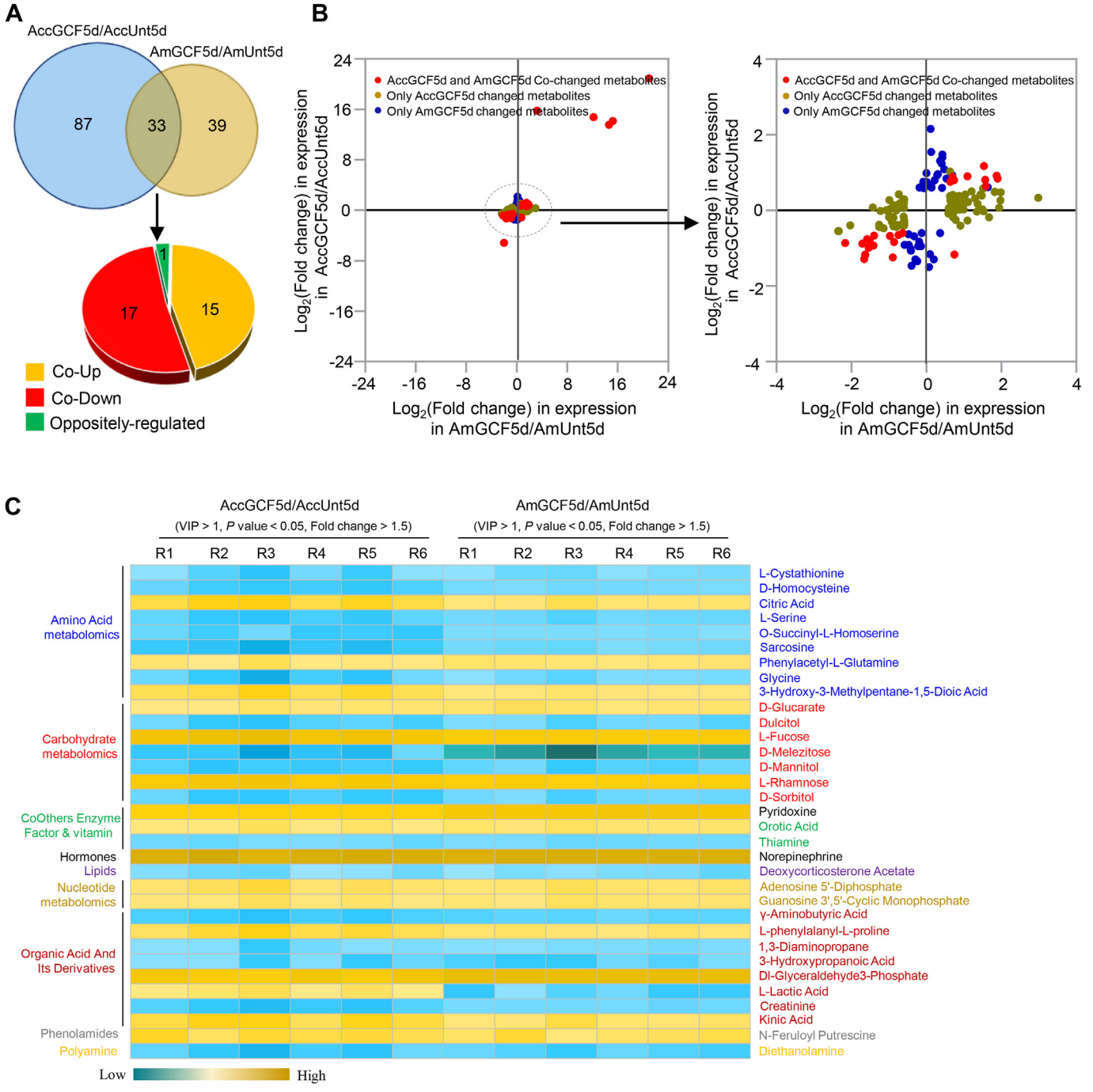
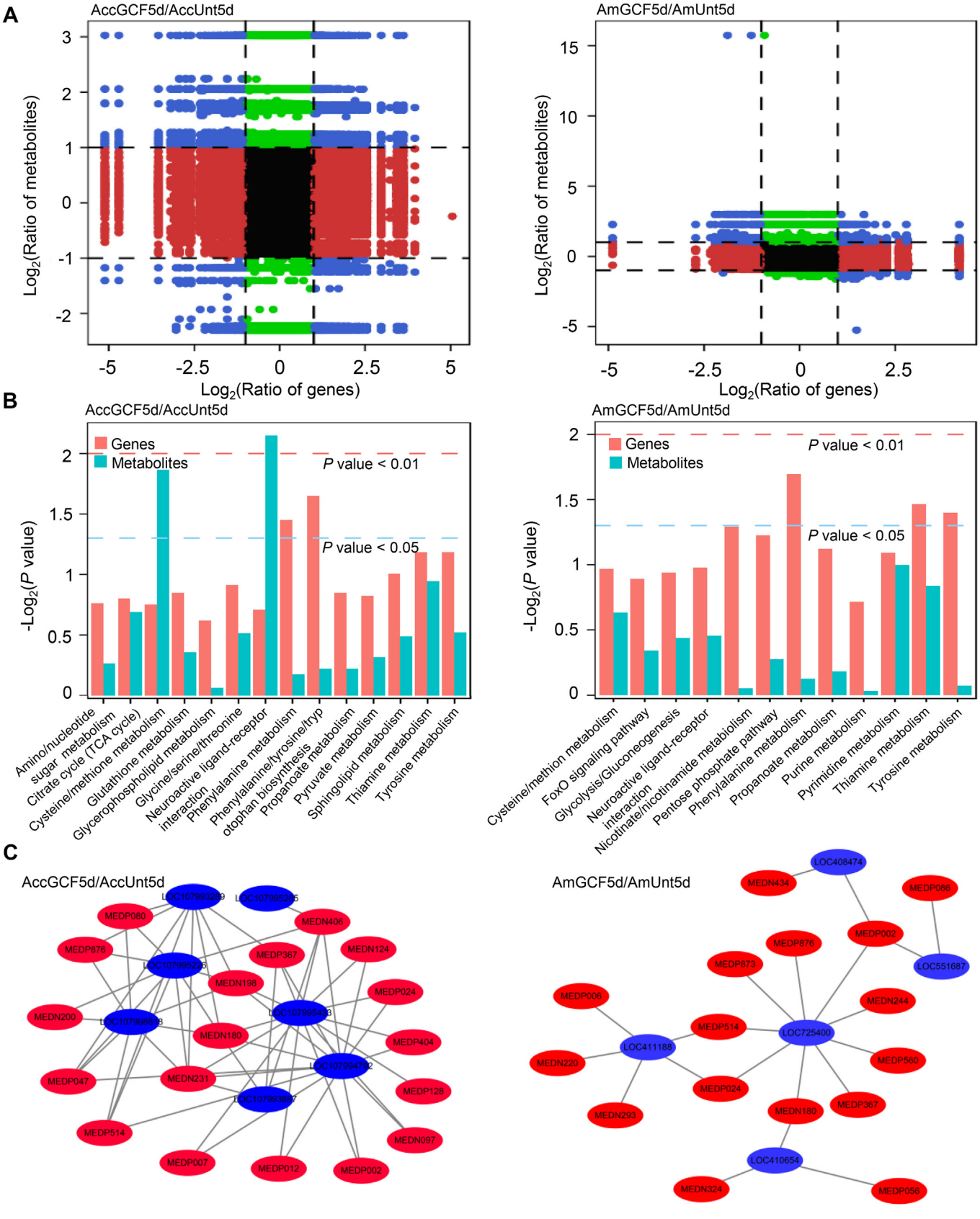


Fig. 4. Analysis of the overlapping differentially expressed metabolites between *Apis cerana cerana* and *Apis mellifera ligustica* under glyphosate commercial formulation (GCF) stress. A, Venn diagram (upper panel) showing the overlapping differentially expressed metabolites between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d. The pie chart (down panel) shows the co-upregulated compounds, co-downregulated compounds and oppositely regulated compounds between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d. AccGCF5d/AccUnt5d: *A. cerana cerana* exposed to GCF for 5 days compared with untreated *A. cerana cerana*. AmGCF5d/AmUnt5d: *A. mellifera ligustica* exposed to GCF for 5 days compared with untreated *A. mellifera ligustica*. B, Correlation analysis of the differentially expressed metabolites (left panel) between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d. Red dots: overlapping compounds between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d. Brown dots: differentially expressed metabolites only in AccGCF5d/AccUnt5d. Blue dots: differentially expressed metabolites only in AmGCF5d/AmUnt5d. Right panel in B: enlarged image of the black circle in the left panel in B. C, Hierarchical clustering of the log-fold change in the abundances of overlapping metabolites between AccGCF5d/AccUnt5d and AmGCF5d/AmUnt5d. R1–R6 represent biological replicates 1 to 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression levels of *OBP3* and *OBP15* in 3-day GCF-fed *A. cerana cerana*, *OBP7* and *OBP9* in 5-day GCF-fed *A. cerana cerana* and *OBP7* in 3-day GCF-fed *A. mellifera ligustica* were repressed compared with the control levels (Table S3). Metabolites related to the sensory system were altered in *A. cerana cerana* and *A. mellifera ligustica* under GCF stress (Fig. 3A–B). These results can help us better understand the damage caused by GCF to the sensory and cognitive abilities of bees at the molecular level.



For successful foraging, many insect pollinators depend on their capacity to learn and recall floral odours (Goulson, 1999). The change in the central nervous system induced by acute exposure to diesel exhaust has deleterious impacts on bee learning and memory (Reitmayer et al., 2019). It has been found that glyphosate-based herbicide has effects on the central nervous system of rat offspring possibly by changing neurotransmitter systems that regulate anxiety and locomotor (Gallegos et al., 2016). We found that several genes related to synapses (Fig. S2) and some metabolites involved in the nervous system, such as the synaptic vesicle cycle and neuroactive ligand-receptor interaction, were changed in *A. cerana cerana* and *A. mellifera ligustica* (Fig. 3). These results indicate that the nervous system of bees was disrupted by GCF, which might help explain the adverse effects of glyphosate in the short-term memory retention, elemental learning, foraging and navigation abilities of bees found in previous studies (Balbuena et al., 2015; Farina et al., 2019; Herbert et al., 2014). In addition, the expression of several genes and the abundance of some metabolites related to lipid metabolism were downregulated in *A. cerana cerana* and *A. mellifera ligustica* under GCF stress (Fig. 3 and Table S6). Energy from lipid metabolism is the preferred fuel for long-distance or sustained flight in insects (Haunerland, 1997; Van der Horst, 2003). Based on these and our results, we suggest that GCF exerts effects on the ability of bees to fly.

The availability of various micronutrients (minerals, vitamins and sterols) and macronutrients (carbohydrates, protein and fats) is vital for the health and longevity of adult bees (Brodschneider and Crailsheim, 2010; Negri et al., 2019; Wright et al., 2018). In addition, amino acids are essential for bee growth and development due to these compounds serve as building blocks for proteins and play multiple regulatory roles in immunity and gene expression (Hendriksma et al., 2019; Wu, 2010). We found that the abundance of many metabolites related to carbohydrate metabolism, vitamin digestion and absorption, mineral absorption, essential amino acid metabolism and digestion were downregulated in *A. cerana cerana* and *A. mellifera ligustica* under GCF stress (Fig. 3A–B). D-Glucose related to starch and sucrose metabolism was downregulated in *A. mellifera ligustica* exposed to GCF (Table S9_Sheet 2 and Table S10_Sheet 2). In addition, three genes connected with adult feeding behaviour, namely, the transcriptional regulator ovo (Gene symbol: LOC108000485), paired box pox-neuro protein (Gene symbol: LOC108001314) and protein takeout-like (Gene symbol: LOC108001880), were downregulated in *A. cerana cerana* exposed to GCF for 5 days (Fig. S3 and Table S3_Sheet 2). GCF at sub-lethal concentrations influences feed intake in Pacu (*Piaractus mesopotamicus*) and consequently restrains its growth (Giaquinto et al., 2017). Our results might contribute to the understanding that GCF treatment reduces adult food uptake and has an impact on the appetitive behaviour of bees (Herbert et al., 2014; Mengoni and Farina, 2018). Furthermore, the downregulation of various micronutrients and macronutrients may trigger malnourishment in bees, which further affects their growth and development.

The expression of ABC transporter G family member 20-like was downregulated in *A. cerana cerana* after exposure to GCF for 5 days (Table S3_Sheet 2), and eight and 12 metabolites related to the ABC transporter pathway were decreased in *A. cerana cerana* and *A. mellifera ligustica*, respectively, after GCF exposure (Fig. 3A–B and Tables S9–S10). In addition, eight compounds related to sulphur metabolism (energy metabolism) were reduced in both *A. cerana cerana* and *A. mellifera ligustica* under GCF stress (Fig. 3A–B and Tables S9–S10). ABC transporters play important roles in drug resistance and detoxification processes (Locher, 2016). Sulphur metabolism is a versatile platform for launching animal detoxification and defence operations (Gorlas et al., 2015; Nakajima, 2015). Based on these and our results, we suggest that the detoxification function of bees might be influenced by GCF exposure, which will increase the pernicious effects of GCF on bees. Because the specific functions of ABC transporters and sulphur metabolism remain unclear in bees, further study is needed to prove the above speculation.

4.2. Bees can cope with GCF stress by regulating key genes, metabolites and pathways

Pure glyphosate and Roundup® disrupt the expression of *CYP1A2*, *CYP1A4*, *CYP1B1*, *CYP3A5* and *CYP1C1* in chicken embryos (Fathi et al., 2020). RNA-seq and RT-qPCR assays have confirmed that the transcription of *CYP6AS2*, *CYP6AS3*, *CYP9Q3* and abaecin is upregulated in *A. mellifera* larvae exposed to glyphosate (Vazquez et al., 2018; Vazquez et al., 2020). We also found that *CYP15A1* (LOC107999579, LOC551179), *CYP9E2* (LOC108003159) and *CYP6A14* (LOC114576772, LOC727598) and abaecin were induced in *A. cerana cerana* and *A. mellifera ligustica* adults under GCF stress (Table S3). In addition, we revealed that genes encoding cuticular proteins, apidaecin, defensin and hymenoptaecin were also upregulated under GCF stress (Table S3). These results indicate that the defence mechanisms of larvae and adults differ under GCF stress. Insect cuticular proteins play critical roles in insecticide resistance (Huang et al., 2018). *Bombyx mori* with mutations in the cuticular protein CPH24 show reduced resistance to deltamethrin (Xiong et al., 2018). *CYP450* is well recognised for its vital function in pesticide and xenobiotic detoxification in animals (Chang et al., 2017; Manikandan and Nagini, 2018; Mao et al., 2011). *A. mellifera* can metabolise thiacloprid with high efficiency, and transgenic *Drosophila* strains expressing *A. mellifera CYP9Q3* show higher tolerance to thiacloprid compared with control flies without the transgene (Manjon et al., 2018). Apidaecin, abaecin, defensin and hymenoptaecin are antimicrobial peptides that play important roles in the immune response (Chan et al., 2009; Umnyakova et al., 2020). Based on these and our results, we hypothesize that the upregulation of cuticular proteins, *CYP450* and antimicrobial peptides can help bees defend against damage due to GCF stress.

Interestingly, the *Apis mellifera* gut microbiota upregulates the expression of genes related to antimicrobial peptides (apidaecin and hymenoptaecin), and one specific bacterial member of the gut microbiota, *Snodgrassella alvi*, contributes to the upregulation of apidaecin (Kwong et al., 2017). However, previous studies have also demonstrated that glyphosate treatment strongly affects the *S. alvi* abundance in the bee gut (Blot et al., 2019; Motta et al., 2018). Therefore, a decrease in the abundance of *S. alvi* after treatment with pure glyphosate is expected to downregulate the expression of some antimicrobial peptides, particularly apidaecin. We found that antimicrobial peptides, including apidaecin, abaecin, hymenoptaecin and defensin, were upregulated under GCF stress (Table S3). The agrochemical used in our study is a GCF rather than pure glyphosate, which might explain this divergent finding. It is possible that other components of the formulation and not glyphosate could upregulate the expression of genes related to

Fig. 5. Correlation analysis of the transcriptomic and metabolomic data of *Apis cerana cerana* and *Apis mellifera ligustica* under glyphosate commercial formulation (GCF) stress. A, Nine-quadrant diagram showing the correlation of genes (obtained from the RNA-seq analysis) and compounds (identified from the metabolomic analysis) in *A. cerana cerana* (left panel) and *A. mellifera ligustica* (right panel) exposed to GCF. AccGCF5d/AccUnt5d: *A. cerana cerana* exposed to GCF for 5 days compared with untreated *A. cerana cerana*. AmGCF5d/AmUnt5d: *A. mellifera ligustica* exposed to GCF for 5 days compared with untreated *A. mellifera ligustica*. The blue, red, green and black points indicate the differentially expressed gene-differentially expressed metabolite pairs, differentially expressed gene-non-differentially expressed metabolite pairs, non-differentially expressed gene-differentially expressed metabolite pairs, and non-differentially expressed gene-non-differentially expressed metabolite pairs, respectively. B, KEGG enrichment analysis of the differentially expressed genes (red column) and differentially expressed metabolites (dark cyan column) that were enriched in the same pathway in *A. cerana cerana* (left panel) and *A. mellifera ligustica* (right panel) upon GCF exposure. C, Connection network between differentially expressed genes (blue oval) and differentially expressed metabolites (red oval) with the absolute value of pearson correlation coefficient > 0.8 in *A. cerana cerana* (left panel) and *A. mellifera ligustica* (right panel) upon GCF exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

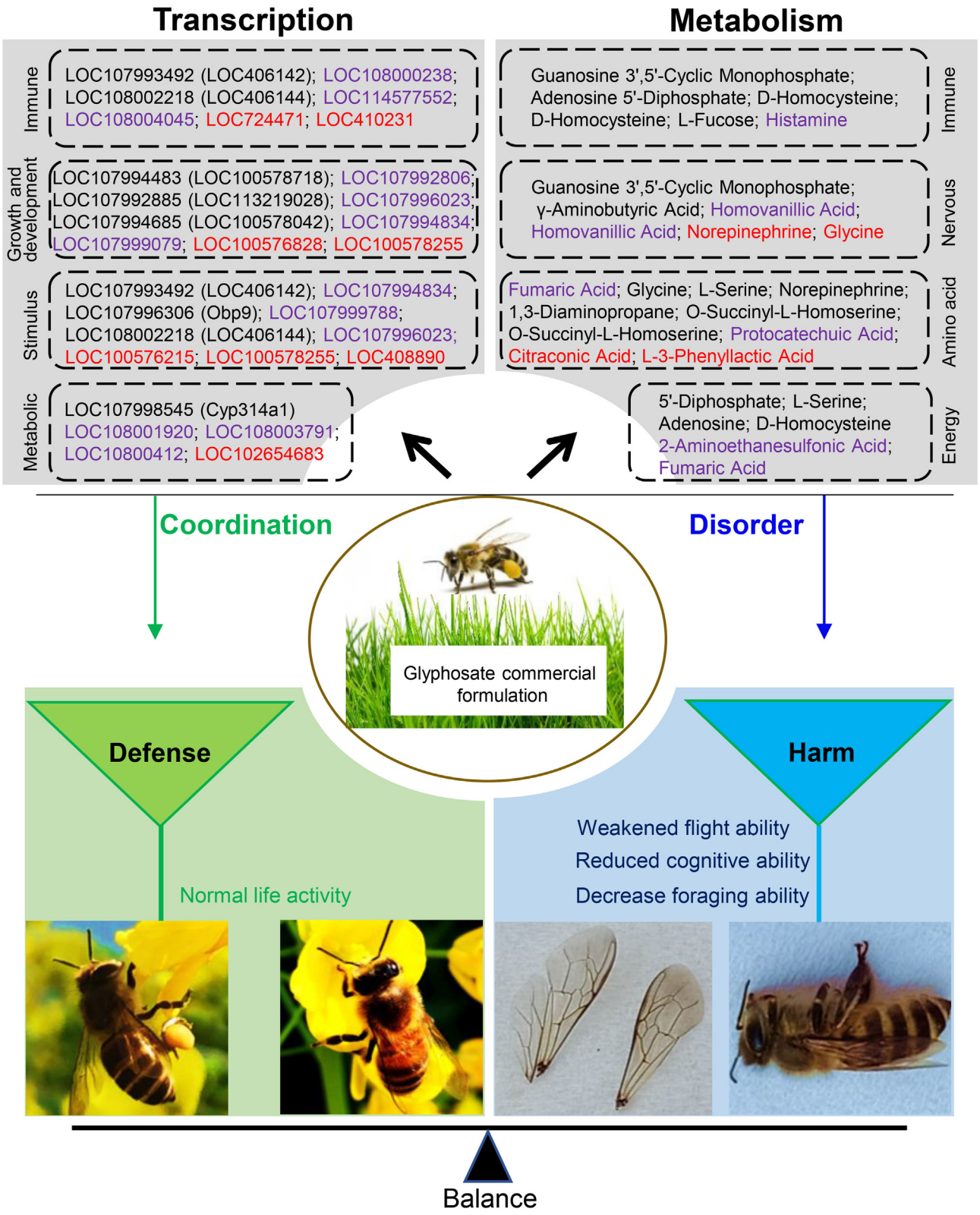


Fig. 6. Proposed working model of the molecular effects of glyphosate commercial formulation (GCF) on bees. When bees are subjected to GCF stress, they strive to defend against this stress by regulating many genes related to key pathways (such as immune system process, response to stimulus, metabolic process, and growth and development) and metabolites involved in important pathways (such as immune system, nervous system, amino acid metabolism and energy metabolism). Increases in the GCF exposure time decrease the defence ability of bees, which results in serious harm to bee health. Despite some differences, *Apis cerana cerana* and *Apis mellifera ligustica* express many co-regulated genes and metabolites in response to GCF stress. Genes and metabolites that are only regulated by *A. cerana cerana* and *A. mellifera ligustica* are shown in purple and red font, respectively. Coregulated genes and metabolites in *A. cerana cerana* and *A. mellifera ligustica* are shown in black font. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

these antimicrobial peptides. Although this is a speculation, it would be interesting to further study this speculation in the future.

Pesticides can affect key pathways and the metabolic capabilities of bees. For example, at environmentally relevant concentrations, the biopesticide spinosad triggers alterations in the expression of genes related to energy production, detoxification and neurons in *A. mellifera* (Christen et al., 2019). Metabolites enriched in amino acid metabolism, glycerophospholipid metabolism and glutathione metabolism are significantly affected by thiacloprid stress in *A. mellifera* (Shi et al., 2018). Glyphosate and its commercial agrochemical formulations influence the immune-related pathways of human skin cell (Lindberg et al., 2020). We found that the number of downregulated DEGs related to key pathways, such as response to stimulus, immune system, growth and developmental process, increased in both *A. cerana cerana* and *A. mellifera ligustica* as the GCF exposure time increased from 3 to 5 days (Fig. 1C). The abundance of compounds related to carbohydrate metabolism, energy metabolism, digestive system, nervous system, immune system and sensory system changed in *A. cerana cerana* and *A. mellifera ligustica* in response to exposure to GCF stress (Fig. 3A–B). Contamination of larval food with glyphosate (Roundup Original DI®) changes the development of the stingless bee *Melipona quadrifasciata*, and brings about sublethal or even lethal effects (Seide et al., 2018). Feed food containing Roundup® instead of glyphosate active ingredient to nurse *A. mellifera* triggers changes in its hypopharyngeal gland cellular ultrastructure (Faita et al., 2018). Based on these and our results, we suggest that bees attempt to coordinate key pathways to increase their growth and developmental processes under GCF stress. However, increases in the GCF exposure time will result in the exhaustion of this ability, which would culminate in detrimental effects on growth and development.

It is well known that the average body mass of *A. mellifera* (99.45 ± 0.70 mg/individual) is higher than that of *A. cerana cerana* (73.95 ± 0.55 mg/individual) (Yue et al., 2018). *A. mellifera* is more tolerant to cypermethrin, deltamethrin, malathion, fenvalerate and demeton-*s*-methyl than *A. cerana* (Sharma and Abrol, 2005), and despite its lower body mass, *A. cerana* is less sensitive to thiamethoxam than *A. mellifera* (Yue et al., 2018). However, the sensitivity of *A. mellifera* and *A. cerana* to imidacloprid observed in previous studies remains contradictory (Li et al., 2017; Yue et al., 2018). Although the defence mechanisms of *A. cerana cerana* and *A. mellifera ligustica* to GCF show some similarities, we also found some obvious differences between these two bees in their response to GCF stress. For example, the number of differentially expressed transcripts or metabolites was significantly higher in *A. cerana cerana* than in *A. mellifera ligustica* (Figs. 1A and 4A). Notably, the number of DEGs belonging to the OBP, CYP450, Hsp, antimicrobial peptide and cuticular protein families was clearly higher in *A. cerana cerana* than in *A. mellifera ligustica* under GCF stress (Table S3). The differentially expressed metabolites involved in the nervous system, sensory system, amino acid metabolism, energy metabolism, and cofactor and vitamin metabolism were more abundant in *A. cerana cerana* than in *A. mellifera ligustica* under GCF stress (Fig. 3). These results indicate that some specific metabolic pathways are more efficiently activated in *A. cerana cerana* than in *A. mellifera ligustica*, which could be reflected in the modulation of gene expression and might help *A. cerana cerana* cope better with the toxicity of GCF, and tolerate higher doses of GCF with low fitness costs. However, further phenotypic analysis is needed to prove this hypothesis and determine which bee species might be more sensitive to GCF.

Our experiment also has some limitations. First, the foraging bees had access to only one type of food source during the 3- and 5-day exposure sessions in the laboratory. The responses in the field are more complex than those observed in a laboratory, and thus, further studies are needed to investigate the mechanism underlying the molecular effects of GCF in bees under field-realistic scenarios. Second, using specific tissue to perform omics measurements might be better for investigating the effects of GCF in bees. However, to our knowledge, the tissues that are most highly affected by GCF have not been identified. We believe that GCF might affect more than one tissue in bees. We thus used the whole body of bees to explore the effect of GCF on bees during omics measurements. It will be fruitful to identify which tissue is most highly affected by GCF and then used it to explore the molecular effects of glyphosate in bees. Third, we used GCF rather than pure glyphosate as the treatment. GCF contain many adjuvants, such as surfactants, and these might exert many side effects on bees. The use of pure glyphosate will aid further investigation of the molecular effects of glyphosate in bees. Fourth, age, caste and behaviour have a significant impact on the transcriptome with thousands of genes changing of bees (Alaux et al., 2009; Chandrasekaran et al., 2011). Only a few genes were co-regulated in bees treated with GCF for 3 or 5 days (Fig. 1B). One explanation for this finding is that genes show differential expression based on the bee age, and we used foraging bees of mixed ages, which is a strong variable that could dictate the variations. For future work, foraging bees of the same age could be collected to further investigate the molecular effects of GCF in bees.

5. Conclusion

Our data clearly showed that GCF disturbed the transcriptional and metabolic regulatory networks of bees. The adverse impacts of GCF in bees were enhanced by increases in the GCF exposure time. However, bees strive to coordinate key defence pathways, such as metabolic processes, detoxification, the immune system and energy metabolism, to promote their survival under GCF stress (Fig. 6). *A. cerana cerana* might show a better ability to tolerate the toxicity of GCF than *A. mellifera ligustica*, and thus, the former is able to tolerate higher doses of GCF with lower fitness costs. Despite some differences, many genes and metabolites were found to be co-regulated in *A. cerana cerana* and *A. mellifera ligustica* to defend against GCF stress (Figs. 2A, 4A and 6). These results will improve our understanding of the molecular determinants underlying the physiological and behavioural damage observed in bees under GCF exposure. The adverse effects of GCF on bee populations and the pollination services that they provide in crop production should be given due consideration.

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CRediT authorship contribution statement

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Declaration of competing interest

There are no conflicts to declare.

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