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Metabolomic analysis of honey bee (*Apis mellifera* L.) response to glyphosate exposure†

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Glyphosate is among the world's most commonly used herbicides in agriculture and weed control. The use of this agrochemical has unintended consequences on non-target organisms, such as honey bees (*Apis mellifera L.*), the Earth's most prominent insect pollinator. However, detailed understanding of the biological effects in bees in response to sub-lethal glyphosate exposure is still limited. In this study, ^{1}H NMR-based metabolomics was performed to investigate whether oral exposure to an environmentally realistic concentration (7.12 mg L^{-1}) of glyphosate affects the regulation of honey bee metabolites in 2, 5, and 10 days. On Day 2 of glyphosate exposure, the honey bees showed significant downregulation of several essential amino acids, including leucine, lysine, valine, and isoleucine. This phenomenon indicates that glyphosate causes an obvious metabolic perturbation when the honey bees are subjected to the initial caging process. The mid-term (Day 5) results showed negligible metabolite-level perturbation, which indicated the low glyphosate impact on active honeybees. However, the long-term (Day 10) data showed evident separation between the control and experimental groups in the principal component analysis (PCA). This separation is the result of the combinatorial changes of essential amino acids such as threonine, histidine, and methionine, while the non-essential amino acids glutamine and proline as well as the carbohydrate sucrose were all downregulated. In summary, our study demonstrates that although no significant behavioral differences were observed in honey bees under sub-lethal doses of glyphosate, metabolomic level perturbation can be observed under short-term exposure when met with other environmental stressors or long-term exposure.

1. Introduction

Honey bees (*Apis mellifera* L.) are the most prominent insect pollinators globally due to their wide distribution and popularity among humans. Honey bees and other pollinators account for approximately 87.5% of the pollination of flowering plants.[1] Therefore, various ecosystems are dependent on bee pollination to remain stable, which includes 35% of global crops.[2] While the number of managed beehives has seen a steady increase since the 1960s, a drastic decline in bee survival has been reported in the United States and Europe.[3] Since 2006, the average reported overwintering mortality has doubled from 15% to 30% in the United States.[4] Factors such as malnutrition,[5,6] pests and parasites,[7,8] viruses,[9] and agrochemicals[10–13] and their combinations are potential causes of the sharp decline in beehives. Among all these factors, agrochemicals are considered the main reason affecting healthy beehives due to the direct contact between workers and polluted flowers.[14]

Glyphosate is a broadly used herbicide introduced to the US in 1974 and is the most used herbicide for crop production in the United States in terms of acres treated, according to the US EPA.[15] While extensive research has indicated that glyphosate is a negligible threat to humans without direct exposure,[16] the short- and long-term effects of glyphosate on honey bees have not been well studied. Most studies have been focused on the behavioral effect of glyphosate. For example, Herbert *et al.* tested the effects of sub-lethal glyphosate doses on honey bee appetite and discovered that environmental levels of glyphosate can reduce the effectiveness of foraging activities and impair associative learning in bees.[17] Balbuena *et al.* tested the effects of a sub-lethal dose of glyphosate at 10 mg L^{-1} and discovered that glyphosate exposure impairs the honey bees' cognitive capacity to retrieve and store spatial information necessary for successful return flights to the hive.[18] On the biological side, Motta *et al.* developed a novel study to investigate the effects of glyphosate exposure on the gut microbiota of honey bees and discovered that glyphosate exposure to honey bees can perturb their beneficial gut microbiota, possibly having consequences on their overall health and pollination efficiency.[13]

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Delayed brood development in workers and reduced hatching weight of adults after long-term exposure to environmental traces of glyphosate[19,20] have also been discovered. In summary, though negative health effects under sub-lethal exposure to glyphosate, such as decreased memory retention, lowered navigational ability, impaired learning, and decreased gustatory responsiveness to disrupted sleep cycles have been reported in honey bees, the detailed molecular and cellular mechanisms of these negative effects remain poorly analyzed.

Metabolomics is an analytical tool that can quickly and quantitatively measure the changes in a range of metabolites in response to an external stressor and provide an evaluation of the overall biological functioning of an individual at the molecular level.[21–23] In ecotoxicology, the metabolomics approach can help identify the unique metabolite profiles or 'fingerprints' in an organism after toxin exposure, which can serve as biomarkers for future exposure to the same compound.[24–26] The most established protocol in metabolomics is the metabolic profiling of biological fluids, such as plasma and urine, in mammalian systems since these fluids are easy to collect and store.[27,28] Besides this, these biological fluids contain a broad range of metabolites that can be used as biomarkers for the early diagnosis of infectious diseases and as evidence of metabolic disorders.[29] In this study, the hemolymph of honey bees was collected after different time points of glyphosate exposure, and the hemolymph metabolomic profile was analyzed using high-resolution nuclear magnetic resonance (NMR). Hemolymph is the only biofluid that circulates in the honey bee body and hence is critical for metabolite biomarker discovery. Additionally, this metabolomic analysis is expected to provide insights into bee development, behavior, and physiology.[8,30,31] Glyphosate has been reported to have an adverse effect on carbohydrates and amino acids in honey bees based on whole-body studies.[32] Hemolymph is critical for immune defense and primary energy storage in addition to molecular transport in insects.[33,34] Thus, metabolites in the hemolymph can provide critical results regarding honey bee responses to glyphosate. However, metabolomics studies using honey bee hemolymph are rare. In this study, hemolymph metabolomics has been applied to investigate the effects of sub-lethal doses of glyphosate on honey bees both in the short and long term.

2. Experimental methods

2.1 Honey bee sampling and experimental design

The experimental setup for this study required six mesh insect cages with dimensions (15.7 in × 15.7 in × 24.0 in) for 3 experimental groups and 3 control groups. A desk lamp was placed with an outlet timer that cycled between 12 hour light and dark phases in order to maintain the circadian rhythm of honey bees. The control group received a 30% sucrose solution made from table sugar and distilled water. The experimental group received a 7.12 mg L^{-1} solution of glyphosate–sucrose. The glyphosate used in this study was sourced from the landscape department of the New College of Florida in the form of RangerPro concentrate. The original concentration of glyphosate in the sample was 356 g L^{-1}, which was diluted to 7.12 mg L^{-1} using the sucrose solution to achieve a plausible range matching the environmental levels found in plant nectar and pollen.[35]

Honey bees were collected from the apiary located at the south Caples campus of the New College of Florida. Using the measure of $\frac{1}{2}$ cup = roughly 300 bees (UMN Bee Lab), the bees were scooped from a bucket after being sprayed with the sucrose solution to prevent escaping. Smaller scoops were used to divide the bees among the six mesh insect cages used for the experimental study. Each cage received around 50 ± 3 bees. After the bees were divided between the cages, they were brought into the lab space and fed once daily by pipetting the appropriate solution (plain sucrose or glyphosate–sucrose) onto cotton balls placed in Petri dishes inside the cages. Each cage door was opened just enough to fit the plastic pipette in, and the cotton balls were soaked thoroughly. After feeding, the pipette was quickly removed, and the cage door was re-sealed. The relative behavior and mortality levels of each cage were recorded each day after feeding. In summary, three sample collection time points were designed in this study, and each time point has two groups: the control group and the glyphosate group. Day 2, 5, and 10 represent the second, fifth and tenth day after caging and glyphosate treatment. Though the caging process may affect the glyphosate results, at each data point, the control and glyphosate-treated bees were maintained under the same conditions.

2.2 Honey bee hemolymph collection

Sample collection was carried out on days 2, 5, and 10 after the treatment. The hemolymph was collected using a previously reported method with slight modifications.[36] Briefly, the honey bees were placed in a −20 °C freezer for 3 minutes while still in the cages to slow down their activity. The honey bees were then removed from the cages and terminated in entomology jars with ethyl acetate for 20 minutes. Each bee had its anus sealed using a water-soluble glue to prevent backflow. Capillary tubes were used to collect hemolymph droplets, which were then deposited in centrifuge tubes. A total of 25 μL hemolymph was collected in each vial from approximately 3–6 bees, and all the samples were stored in a −80 °C freezer until further analysis.

2.3 Sample preparation and ^{1}H NMR analysis

A phosphate buffer of D_2O (180 μL) was then added to 25 μL hemolymph, and the final samples contained 10% D_2O with 0.1 M phosphate buffer (pH = 7.4) and 0.5 mM trimethylsilylpropanoic acid (TSP). The samples were then transferred to 3 mm NMR tubes after centrifugation for further NMR acquisition. A Bruker Ascend 400 MHz high-resolution NMR with a sampleXpress autosampler was employed in this study, and all the experiments were carried out using the ICON-NMR software (Bruker Biospin) and controlled by ICON-NMR. A 1D NOESY experiment with water suppression (noesygppr1d) was carried out with 32k increments and 64 transients. All the spectra were carefully phased and calibrated to TSP in Bruker Topspin 4.06 (Bruker Biospin).

2.4 Data interpretation

All NMR processing was carried out in Amix 4.0 (Bruker BioSpin), and the NMR spectra were obtained using a previously reported automatic method[37] to minimize peak overlap and splitting. The processed data were normalized to the total peak intensity and exported to Excel (Microsoft) for further data analysis. Metabolite identification was carried out using Chenomx 8.4 (Chenomx Inc.). Student *t*-tests (two tails) were carried out in Excel (Microsoft). The principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were carried out in PLS toolbox (Eigenvector Research). Venetian Blinds cross-validation was applied for PLS-DA, and the Matthews correlation coefficient (MCC) was used to evaluate the confusion matrix categories.[38]

3. Results

3.1 Physical behavior observations in honey bees

The behavior and mortality of the bees were recorded daily after treatment until the sampling day (Table 1). The honey bees were lethargic with low levels of mortality for the first 2 days of the experiment, and mortality was negligible after Day 4. The initial lethargic phenomenon and low mortality are likely due to the caging process since active behavior was consistently observed after Day 5. Behaviors, such as clustering, waggle dancing, and flying, (Table 1) were observed in most experimental and control groups after Day 5. Though the control group showed louder buzzing than the glyphosate group on Day 8, Day 9 data showed a reversed result, which indicated that the behavioral difference was not significant. In summary, behavioral differences were observed between the different experimental days, and the honey bees were less active on Day 2 likely due to caging. However, the behavioral difference between the control and the glyphosate treatment groups at each stage was very limited.

3.2 Overall metabolite profile change after glyphosate treatment

For metabolic profiling, the hemolymph samples from both experimental groups and control groups were collected at three time points denoted as early (Day 2), middle (Day 5), and late (Day 10) stages of treatment, respectively. A total of 36 samples (12 for each experimental day) were analyzed by ^{1}H NMR, and 33 metabolites were identified from the honeybee hemolymph samples. The PCA study (Fig. 1) showed that the metabolites found during the early stage of treatment and caging (Day 2) were distinctly different from those in the middle and late stages in both control and glyphosate treatment groups. The Day 5 and Day 10 data showed a similar distribution in the PCA score plot, which indicated that the influence of caging on Day 5 and 10 was relatively low. Since the caging process generated a weak condition for the honey bees, the glyphosate treatment was analyzed at each stage separately.

The PCA score plot for Day 2 (Fig. 2A) showed a clear separation between the control and experimental groups, which indicated the effect of glyphosate on the global honey bee metabolite level, and the PCA loading plot indicated that the upregulation of glucose and downregulation of amino acids, such as alanine, isoleucine, leucine, lysine, and valine, were the main reasons for the separation. The PLS-DA study (Fig. S1, ESI†) showed a relatively reliable model (error rate is 0.25) after cross-validation, which indicates high metabolic-level perturbation after glyphosate exposure.

However, the separation pattern was not observed in the Day 5 groups. The control and glyphosate data showed a very similar distribution in the PCA score plot (Fig. 2B). Though the PLS-DA study (Fig. S2, ESI†) showed separation, it did not have reliable cross-validation values and had a high error rate (0.583). These results indicate that glyphosate had a much weaker influence on Day 5.

On Day 10, the separation between the control and glyphosate groups in the PCA score plot (Fig. 2C) became clear, which is different from the Day 5 data but similar to those of Day 2. The loading plot showed the main loading contributors to the separation of amino acids, such as histidine, glutamine, glutamate, and threonine, which were down-regulated in the

Table 1 Honey bee behavioral observations. Note: L = Lethargic; M = Mortality; AAF = Active after feeding; A = Active. Low M was classified as <5 deaths. E = Experiment day

Study group	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
C2	E	—	—	—	—	—	—	—	—
E2	E	—	—	—	—	—	—	—	—
C5	L low M	L low M	L, AAF	E	—	—	—	—	—
E5	L low M	L low M	L, AAF low M	E	—	—	—	—	—
C10	L low M	L low M	A	L, AAF	Very A, buzzing	Very A, loud buzzing	A, loud buzzing, flying, clustering	Very A, loud buzzing, clustering	E
E10	L low M	L low M	A	A	Very A, buzzing	Very A, loud buzzing	A, buzzing, clustering	Very A, waggle dancing, loud buzzing, clustering	E

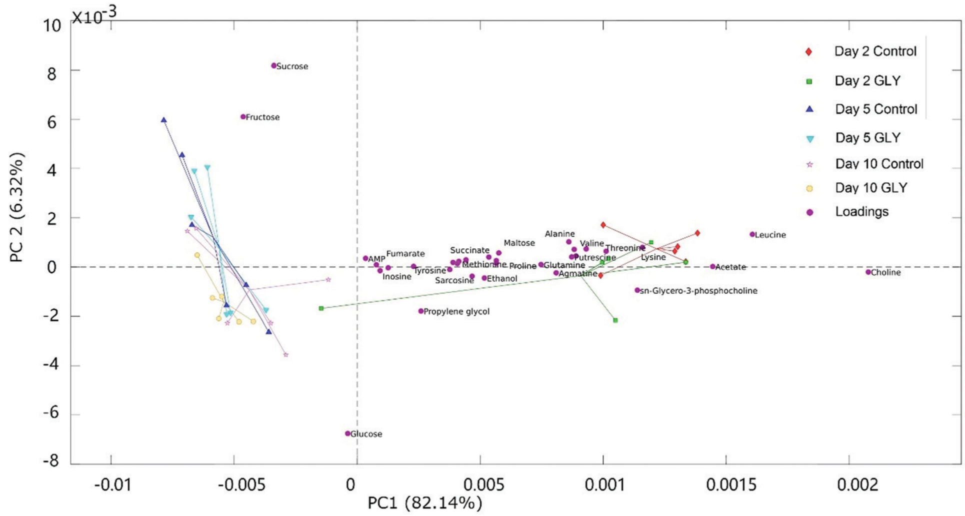

Fig. 1 The overall PCA for all study groups.

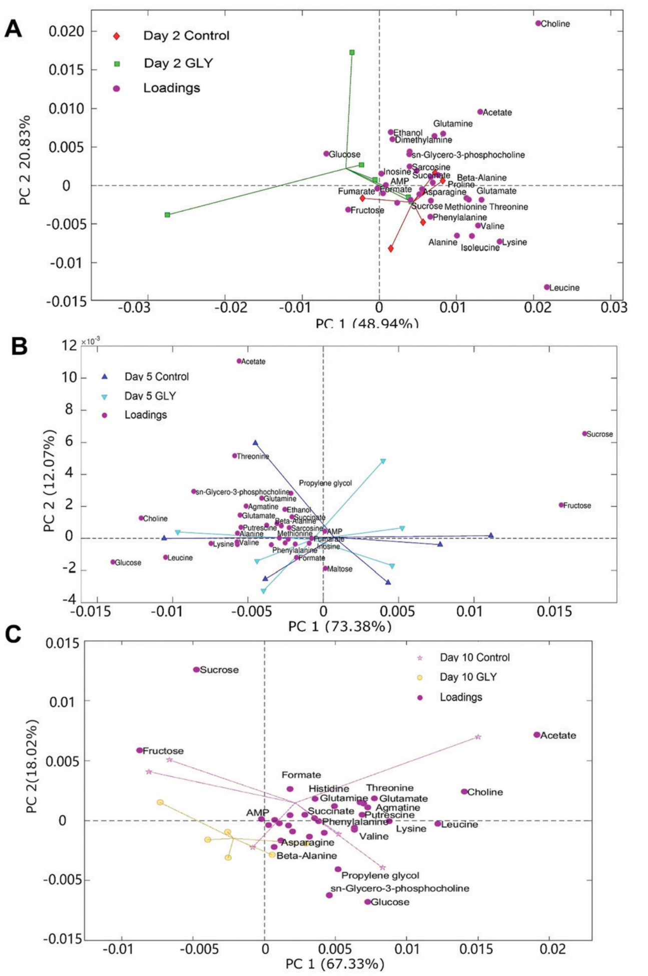

Fig. 2 A. Principal component analysis for the day 2 groups. B. Principal component analysis for the day 5 groups. C. Principal component analysis for the day 10 groups.

glyphosate group. The PLS-DA study (Fig. S3, ESI†) showed high confidence in the cross-validation, with an error rate as low as 0.008, which indicated that the metabolites could be used to distinguish the control and glyphosate groups in the PLS-DA model.

3.3 The detailed metabolite profile changes after glyphosate treatment

While PCA and PLS-DA showed the metabolite changes as a group, the details of these changes can provide more information about the potential glyphosate effect on honeybee health. Day 2 showed a clear downregulation of essential amino acids, including leucine (FC = 0.66), lysine (FC = 0.70), valine (FC = 0.70), and isoleucine (FC = 0.67), as well as carbohydrate sucrose (FC = 0.75), in the treatment group ($p < 0.05$) (Table 2). On Day 5, similar to the results of the PCA (Fig. 2B) and PLS-DA (Fig. S2, ESI†) study, no metabolites showed high significance ($p < 0.05$) except AMP. The upregulation of the expression of AMP is potentially related to the immune system;[39] however, this was not supported by our combinatorial models (PCA and PLS-DA). The cause for the upregulation of this sole metabolite is unclear and needs further studies.

Though both PCA and PLS-DA showed evidence for metabolic differences after glyphosate treatment in the Day 10 groups (Fig. 2C), the significance level of the individual metabolite changes was not very high, and only sucrose showed statistical significance ($p < 0.05$). However, the downregulation of the non-essential amino acids, namely glutamine (FC = 0.47) and proline (FC = 0.54), also showed high significance ($p < 0.1$) (Table 2).

3.4 The effect of the initial caging process on honey bees

The initial caging process had a significant effect on honey bee activities, directly leading to lethargy and low mortality but a weak condition in honey bees. The PCA study distinctly showed that the metabolomic level changed between Day 5 and Day 10 in the control and treatment groups. The loadings (Fig. 1) indicate that certain metabolites, including choline and

Table 2 Metabolite fold change (FC) and p values based on the student's t-test. FC was calculated using the experimental group with respect to the control group

Metabolites	ppm	Day 2 FC	Day 2 p-values	Day 5 FC	Day 5 p-values	Day 10 FC	Day 10 p-values
Acetate	1.92	0.96	8.84×10^{-1}	0.73	6.50×10^{-1}	0.32	1.11×10^{-1}
Agmatine	3.05	0.96	7.74×10^{-1}	1.39	3.51×10^{-1}	0.70	3.57×10^{-1}
Alanine	1.49	0.76	2.71×10^{-1}	0.91	8.36×10^{-1}	0.62	3.95×10^{-1}
AMP	8.27	0.64	1.28×10^{-2}	3.04	9.86×10^{-3}	0.48	2.79×10^{-1}
Asparagine	2.93	0.80	2.69×10^{-1}	0.96	9.58×10^{-1}	0.67	5.61×10^{-1}
Choline	3.20	0.96	7.97×10^{-1}	1.38	5.35×10^{-1}	0.74	4.32×10^{-1}
Dimethylamine	2.76	1.15	3.96×10^{-1}	1.30	4.31×10^{-1}	0.81	4.65×10^{-1}
Ethanol	1.17	1.69	2.40×10^{-2}	1.08	8.44×10^{-1}	0.53	2.06×10^{-1}
Formate	8.46	1.82	1.56×10^{-1}	0.94	9.10×10^{-1}	0.38	3.81×10^{-1}
Fructose	4.03	1.09	2.73×10^{-1}	0.88	5.34×10^{-1}	1.20	1.73×10^{-1}
Fumarate	6.53	0.69	3.45×10^{-1}	1.54	5.58×10^{-1}	0.99	9.91×10^{-1}
Glucose	3.27	1.06	3.41×10^{-1}	1.06	7.39×10^{-1}	0.99	8.53×10^{-1}
Glutamate	2.33	0.79	1.15×10^{-1}	1.37	4.06×10^{-1}	0.64	2.99×10^{-1}
Glutamine	2.41	0.73	1.62×10^{-1}	1.33	4.57×10^{-1}	0.47	5.96×10^{-2}
Histidine	7.09	0.81	2.35×10^{-1}	1.50	3.34×10^{-1}	0.37	2.70×10^{-1}
Inosine	8.24	1.16	5.72×10^{-1}	1.18	7.82×10^{-1}	0.69	5.40×10^{-1}
Isoleucine	1.02	0.67	3.95×10^{-2}	1.01	9.79×10^{-1}	0.69	4.47×10^{-1}
Leucine	0.96	0.66	3.30×10^{-2}	1.01	9.85×10^{-1}	0.67	4.34×10^{-1}
Lysine	1.73	0.70	4.36×10^{-2}	1.07	8.84×10^{-1}	0.68	4.18×10^{-1}
Maltose	3.29	0.81	7.69×10^{-2}	0.95	6.66×10^{-1}	1.15	4.35×10^{-1}
Proline	3.36	0.88	3.32×10^{-1}	1.10	5.21×10^{-1}	0.54	7.24×10^{-2}
Methionine	2.64	0.69	9.00×10^{-2}	1.00	9.96×10^{-1}	0.38	1.11×10^{-1}
Phenylalanine	7.31	0.74	1.08×10^{-1}	1.11	8.30×10^{-1}	0.59	3.91×10^{-1}
Propylene glycol	1.14	0.95	7.83×10^{-1}	0.66	5.44×10^{-1}	0.64	4.93×10^{-1}
Putrescine	1.75	0.77	7.60×10^{-2}	1.17	6.97×10^{-1}	0.67	3.82×10^{-1}
Sarcosine	2.74	0.94	7.10×10^{-1}	1.21	7.13×10^{-1}	0.66	3.48×10^{-1}
sn-*Glycero*-3-phosphocholine	3.24	1.16	6.82×10^{-1}	1.41	1.69×10^{-1}	1.05	7.55×10^{-1}
Succinate	2.40	0.77	8.42×10^{-2}	1.49	2.94×10^{-1}	0.67	2.27×10^{-1}
Sucrose	4.24	0.75	3.41×10^{-2}	1.04	9.45×10^{-1}	0.27	4.86×10^{-2}
Threonine	1.32	0.73	5.20×10^{-2}	0.74	5.98×10^{-1}	0.59	2.90×10^{-1}
Tyrosine	7.21	0.83	3.90×10^{-1}	0.75	5.85×10^{-1}	1.19	7.68×10^{-1}
Valine	1.04	0.70	4.90×10^{-2}	1.06	9.01×10^{-1}	0.67	4.00×10^{-1}
β-Alanine	3.18	0.83	2.79×10^{-1}	0.87	8.05×10^{-1}	0.96	9.30×10^{-1}

acetate, and essential amino acids, such as lysine, leucine, and valine, positively contributed to the changes in the Day 2 samples, and sucrose and fructose positively contributed to the changes in the Day 5 and 10 groups.

4. Discussion

4.1 Metabolomic profile changes caused by glyphosate at different stages

Both the PCA and PLS-DA studies showed that the metabolomic profile of honey bees was highly perturbed by low concentrations of glyphosate at the early stage of caging (Day 2). The activities of honey bees became normal around Day 5, and the metabolomic level perturbation also turned out to be negligible. However, after relatively long-term exposure (Day 10), the glyphosate influence on the honey bee metabolomic profile was observed again in the PCA and PLS-DA studies though the amino acid changes, which are weaker compared to the Day 2 data. The honey bees were highly active on Day 10; the metabolomic level perturbation indicated that glyphosate had a long-term effect on honey bee metabolism, which is a potential concern for the health of honey bees.

In addition, though the caging process was not the focus of this study, the PCA study showed that the bees had lower concentrations of fructose and sucrose but higher concentrations of amino acids on Day 2 compared with Day 5 and day 10 bees. These results may indicate that the honey bees tended to consume more sugar during the caging process to produce amino acids and potentially for somatic maintenance.[40]

4.2 Honey bee essential amino acids

The early-stage (Day 2) samples showed high perturbation in essential amino acids. At this stage, the honey bee activities had not fully recovered from the caging process, and glyphosate had a high impact on honey bee metabolism according to the PCA and PLS-DA results. For example, essential amino acids, such as methionine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine, and valine, had a downward trend on Day 2 after glyphosate exposure. Leucine, isoleucine, lysine, threonine, and valine showed low p-values ($p < 0.05$) and high contribution in both PCA and PLS-DA models, which indicated their importance in classifying the control and glyphosate groups. The essential amino acids are used for somatic maintenance, growth, and reproduction in the early life stages of bees.[41] These essential amino acids have been reported to exist in reduced concentrations when the bees are older but are still required for regular somatic maintenance and during reproductive periods.[41]

Disturbance to essential amino acids is a common metabolic response to stress as stressed organisms must balance intracellular osmolality. In this study, the high perturbation of the essential amino acids is a potential sign of stress response to glyphosate by balancing the cell osmolytes. Amino acids do not only play a critical role in the production of essential proteins and polypeptides during honeybee development but are also important for neurotransmission and the overall brain function. Many honey bee amino acids act as precursors to enzymes, neurohormones, and neuropeptides, while some even act as neurotransmitters themselves.[40] Previous research has suggested that the essential amino acid lysine has direct involvement in nitric oxide synthesis, a known neurotransmitter related to memory in bees.[42] The downregulation of lysine (as seen in the Day 2 treatment group) is a potentially important contributor to memory impairment, as seen in previous behavioral studies using bees exposed to glyphosate.[17,18] Since both groups of Day 2 honey bees were also struggling with the new caging environment, the high glyphosate effect on essential amino acids is more likely due to the weak state of the honey bees. Our results indicated that the honey bees produced a higher concentration of essential amino acids on Day 2 potentially for regular somatic maintenance (Fig. 1, loadings), but glyphosate exposure weakened this process. This was also observed in the PCA score plot (Fig. 1), wherein the Day 2 glyphosate-treated experimental group showed a separation similar to the Day 5 data (normal activity data). In conclusion, glyphosate tends to slow down the protective processes of honey bees during the caging process.

The glyphosate effect became negligible on Day 5 when the activity became normal (Table 1), which indicated that short-term glyphosate exposure had a limited influence on the health of active bees. However, while the Day 10 honey bees were still active, the essential amino acid levels showed perturbations though not as significant as on Day 2. The essential amino acids also showed downregulation on Day 10 but the statistical significance was relatively low with p values higher than 0.05 in most cases. However, the essential amino acids, such as leucine, lysine, and threonine, showed relatively high contributions in the PCA loading plots in the same direction as the score plot separation (Fig. 2C), which indicates a potential combinatorial perturbation under long-term glyphosate exposure (Day 10).

4.3 Honey bee non-essential amino acids

Non-essential amino acids also influence the functioning and development of the bee brain and sometimes even serve as "neuro-protectants" against oxidative stress.[43] The non-essential amino acids detected (glutamate, glutamine, and proline) showed downregulation in terms of fold change on both Day 2 and Day 10; however, the significance levels were generally low. On Day 10, the PLS-DA loading results indicated that proline and glutamine were the important metabolites in the same direction as model separation (Fig. S3, ESI†). The t-test showed that on Day 10, the honey bee metabolite glutamine was dramatically downregulated in the experimental group (p = 0.06, FC = 0.47). Glutamine is crucial to protein expression in insects, specifically in infected cells.[44] Therefore, low levels of glutamine may potentially increase the mortality rates in bees infected with parasites and pathogens and put them at a greater risk of colony collapse. The non-essential amino acid proline (p = 0.07, FC = 0.54) was also notably downregulated in the Day 10 treatment group. Proline has been linked to flight metabolism in honey bees, along with sucrose, which is the primary metabolic source for flight;[45] moreover, proline downregulation could also be a sign of potential health problems.

4.4 Carbohydrates

Honey bees acquire essential amino acids from pollen collected from a diverse array of flora. This pollen is then used to make bee bread and royal jelly, the primary food source for young bees. As they age into the forager stage, the honey bee diet shifts towards the increased need for carbohydrates, such as sugars found in honey, to allow them to expend high amounts of energy during the foraging flights. The reduced levels of both proline (p = 0.07) and sucrose ($p < 0.05$) in the day 10 treatment group suggest that the metabolic priority had shifted from flight and was redirected to more vital processes that impact the health of the honey bees. A significant sucrose downregulation was also observed on Day 2 without a significant change in proline, which is likely due to the low activity of honey bees on Day 2.

In summary, the findings of this study indicate that honey bees exposed to environmental levels of the herbicide glyphosate experience adverse metabolic effects. The downregulation of key metabolites in the treated bees has many implications for the overall health of hives exposed to glyphosate.

5. Conclusion

Glyphosate exposure consistent with realistic field conditions negatively impacts the development and nutritional health of honey bees. These impacts potentially stem from a disruption in the maintenance of metabolites used for the development and somatic maintenance of individual bees due to the stress response to glyphosate ingestion. Our results indicate that even exposure to a low concentration of glyphosate is a mild threat to regular healthy honey bees, and its influence on honey bee health is not negligible. On the one hand, when the honey bees were under other stress conditions (in this case, the caging process), glyphosate exposure showed a significant effect on essential amino acids, such as isoleucine, leucine, and lysine. While the influence of mid-term exposure (Day 5) on honey bees is limited, the relatively long-term exposure to glyphosate showed highly combinatorial metabolic profile perturbation in honey bees in both PCA and PLS-DA studies, and the metabolites proline, glutamine, and sucrose were highly downregulated. In summary, our study indicates that metabolomic perturbation can be observed under long-term exposure or short exposure when honey bees struggle with other stress-inducing stimuli. Long-term glyphosate application in areas with other environmental issues can potentially influence the health of honey bees, which will be investigated in our future studies.

Data availability

Data for this paper, including the NMR raw data are available at Mendeley Data at https://data.mendeley.com/datasets/r7ptfzsm97/1.

Author contributions

Lin Jiang: conceptualization, methodology, supervision, software, writing – original draft preparation, writing – review & editing. Calypso Habermehl: investigation, visualization. Bo Wang: investigation, data curation, software, writing – review & editing, validation, visualization.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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