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Metabolomic Analysis of Honey Bee (*Apis mellifera* L.) Response to Glyphosate Exposure

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Metabolomics, honey bees, glyphosate, metabolic profiling, essential amino acids

Abstract

Glyphosate is among the world most commonly used herbicides in agriculture and weed control. The agrochemical use could have unintended consequences for non-target organisms, such as honey bee (*Apis mellifera L.*), the earth most prominent insect pollinator. However, the understanding of the detailed biological effects in response to sublethal 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) of glyphosate affects the regulation of honey bee metabolites in 2, 5, and 10 days. The glyphosate exposure on Day 2

honey bees showed significant downregulation of several essential amino acids including leucine, lysine, valine, and isoleucine. The phenomenon indicates that the glyphosate gave an obvious metabolic perturbation when the honey bees were at 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) study. The separation is the result of the combinational changes of the essential amino acids such as threonine, histidine, methionine, and the non-essential amino acids glutamine, proline as well as carbohydrate sucrose were all downregulated. In summary, our study demonstrated that though no significant behavior observation differences were observed on honey bees under the sublethal doses of glyphosate, the metabolomic level perturbation can be observed under short-term exposure when meet with the other environmental stressors or long-term exposure.

1. Introduction

Honey bees (*Apis mellifera* L.), the earth most prominent insect pollinator, have become an ambassador for pollinators globally, due to their wide distribution and popularity among humans. Honey bees and other pollinators account for approximately 87.5% of the world 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, drastic declines in survival have 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 the potential causes of

the sharp decline of beehives. Among all the factors, agrochemicals are considered the main reason affecting healthy beehives which is due to the direct contact between workers and the polluted flowers (14).

Glyphosate is a broadly used herbicide introduced to the US in 1974 and is "the most used herbicide in the United States crop production in terms of acres treated" according to US EPA (15). While extensive research has indicated that glyphosate has negligible threats to humans without direct exposure (16), glyphosate's short and long-term effects on honey bees have not been well studied. Most studies have been focused on the behavior effect of glyphosate. For example, Herbert et al. tested the effects of sub-lethal glyphosate doses on honey bee appetitive behavior and discovered that environmental levels of glyphosate can reduce the bees effectiveness of foraging activities and impair associative learning (17). Balbuena et al. tested the effects of a sub-lethal dose of glyphosate at 10 mg/L and discovered that glyphosate exposure impairs the honey bees cognitive capacity to retrieve and store spatial information for successful return flights to the hive (18). On the biology 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). The delayed brood development of 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 the negative effects of honey bee health under sub-lethal exposure to glyphosate such as decreased memory retention, lowered navigational abilities, impaired learning, and decreased gustatory responsiveness to disrupted sleep cycles have been reported, the more detailed molecular and cellular mechanism 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 after interaction with an environmental stressor (21-23). The metabolomics approach in ecotoxicology can help identify the unique metabolite profiles or 'fingerprints' in an organism after toxin exposure, which could serve as biomarkers for future exposures 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 the mammalian systems since the 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 serve as evidence of metabolic disorders along periods (29). In this study, the hemolymph of honey bees was collected after different time points of glyphosate exposure, and the hemolymph metabolomic profiling was analyzed using high resolution nuclear magnetic resonance (NMR). Hemolymph is the only biofluid in the honey bee that circulates in the insect body, which makes it critical for metabolite biomarkers discovery. Additionally, 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 honey bees in carbohydrates and amino acids on whole-body studies (32). Hemolymph is critical for insect immune defense and primary energy storage in addition to molecular transport (33, 34). The metabolites in hemolymph will provide critical results for honey bee responses to glyphosate. However, the metabolomics studies in honey bee hemolymph are rare. In this study, hemolymph metabolomics has been applied to investigate the effects of sub-lethal doses of glyphosate herbicides on honey bees in both short and long terms.

2. Experiment

2.1 Honey bee sampling and experimental design

The experimental setup for this study required six mesh insect cages with dimensions (15.7in x 15.7in x 24.0in) including 3 experimental groups and 3 control groups. A desk lamp was placed on an outlet timer that cycled in 12-hour light and dark increments 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 solution of glyphosate-sucrose that had been diluted from its original concentration using a 30% sucrose solution. The glyphosate used for this study was sourced from the New College of Florida landscape department in the form of RangerPro concentrate. The original concentration of glyphosate in the sample was 356 g/L, which was diluted to 7.12mg/L using the sucrose solution to bring it into a plausible range for environmental levels found in plant nectar and pollen (35).

Honey bees were collected from the New College of Florida apiary located on the south Caples campus. Using the measure of ½cup = roughly 300 bees (UMN Bee Lab), bees were scooped from a bucket after being sprayed with 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 bees ± 3 bees. After the bees were divided between the cages, they were brought into the lab space and fed. Feedings occurred once daily by pipetting the appropriate solution (plain sucrose or glyphosate-sucrose) onto the cotton balls that sat within the 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 re-sealed. The relative behavior and mortality levels of each cage were recorded each day after feeding occurred. 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 cage process may affect the glyphosate results, at each data point, the control and glyphosate treatment were under the same conditions.

2.2 Honey bee hemolymph collection

Sample collections were carried out on days 2, 5, and 10 after the treatment. The hemolymph was collected using the previously reported method with slight revisions (36). Briefly, the honey bees were placed in a -20 ^{o}C freezer for 3 minutes while in the cages to slow down the activities of honeybees. 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 water-soluble glue to prevent backflow. Capillary tubes were used to collect the hemolymph droplets and then deposit them into centrifuge tubes. A total amount of 25 µL hemolymph was collected for each vial from approximately 3-6 bees and all the samples were stored in a -80 ℃ 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 the 25 µL hemolymph, and the final samples contained 10% of 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 being centrifuged for further NMR acquisition. A Bruker Ascend 400 MHz high-resolution NMR with a sampleXpress autosampler was applied in this study and all the experiments were carried out using ICON-NMR software (Bruker Biospin) and controlled by ICON-NMR. A 1D NOESY experiment with water suppression (noesygppr1d) was carried out with 32k increments, 64 transients. All the spectra were carefully phased and calibrated to TSP in Bruker Topspin 4.06 (Bruker Biospin).

135 2.4 Data interpretation

136 All the NMR processing was carried out in Amix 4.0 (Bruker BioSpin) and the NMR spectra
137 were bucketed using a previously reported automatic method (37) to minimize peak overlap and
138 splitting. The processed data were normalized to the total peak intensity exported to Excel
139 (Microsoft) for further data analysis. Metabolite identification was carried out using Chenomx
140 8.4 (Chenomx Inc). The Student t-tests (two tails) were calculated in Excel (Microsoft). The
141 principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA)
142 were carried out in PLS-toolbox (Eigenvector Research). Venetian Blinds cross-validation was
143 applied for PLS-DA, and the Matthews correlation coefficient (MCC) was used to evaluate the
144 confusion matrix categories (38).

145 **3. Results**

146 3.1 Physical behavior observations of the honey bees

147 The behavior and mortality of the bees were recorded daily after treatment until the sample date
148 (**Table 1)**. The honey bees were lethargic with low levels of mortality for the first 2 days of the
149 experiment, and the mortality was negligible after Day 4. The initial lethargic phenomenon and
150 low mortality are likely due to the caging process since the active behavior was consistently
151 observed after Day 5. Exhibiting behaviors such as clustering, waggle dancing, and flying (**Table**
152 **1**) were observed in most experimental and control groups after Day 5. Though at Day 8, the
153 control group showed a louder buzzing than the glyphosate group, the Day 9 data showed a
154 reversed result which indicated the behavior difference was not significant. In summary,
155 behavior differences were observed among the different experimental days, and the honey bees
156 were less active on Day 2 which is likely due to caging. However, the behavior difference
157 between control and the glyphosate treatment group at each stage is very limited.

158 3.2 Overall metabolites change after glyphosate treatment

159 For metabolic profiling, hemolymph samples from both experimental groups and control groups
160 were collected at three time points considered as early (Day 2), middle (Day 5), and late (Day
161 10) stages of treatment, respectively. A number of 36 samples (12 for each experimental day)
162 were analyzed by ^{1}H NMR and 33 total metabolites were identified from the honeybee
163 hemolymph samples. The PCA study (**Figure 1**) showed that the early stage of treatment and
164 caging (Day 2) metabolites were distinctly different from the middle and late stages in both
165 control and glyphosate treatment groups. The Day 5 and Day 10 data showed a similar
166 distribution in the PCA score plot which indicated that the caging influence to Day 5 and 10 is
167 relatively low. Since the caging process generated a weak condition for the honey bee, the
168 glyphosate treatment analysis was analyzed at each stage separately.

169 The PCA score plot for Day 2 group (**Figure 2A**) showed a clear separation between the control
170 and experimental groups which indicates the glyphosate effect on the honey bee global
171 metabolites level, and the PCA loading plot indicates that the upregulation of glucose and
172 downregulation of amino acids such as alanine, isoleucine, leucine, lysine, and valine are the
173 main factors for the separation. The PLS-DA study (**Figure S1**) showed a relatively reliable
174 model (error rate is 0.25) after cross-validation which indicates the high metabolic level
175 perturbation after the glyphosate exposure.

176 However, the separation pattern was not observed in the Day 5 group. The control and
177 glyphosate data showed a very similar distribution in the PCA score plot (**Figure 2B**). The PLS-
178 DA study (**Figure S2**), though showed separation, did not have reliable cross-validation values
179 with a high error rate (0.583). The results indicate that glyphosate has a much weaker influence
180 on Day 5.

On Day 10, the separation between the control and glyphosate groups in the PCA score plot (**Figure 2C**) became clear which is different than Day 5 but is similar to Day 2. The loading plot showed the main loading contributes to the separation of amino acids such as histidine, glutamine, glutamate, and threonine which are down-regulated in the glyphosate group. The PLS-DA study (**Figure S3**) showed high confidence in the cross-validation with an error rate as low as 0.008 which indicates the metabolites could be used to distinguish the control and glyphosate groups in the PLS-DA model.

3.3 The detailed metabolites profiling changes after glyphosate treatment

While PCA and PLS-DA showed the metabolites changes as a group, the details in metabolites changes can show more information about the potential glyphosate effect on honeybee health. Day 2 showed a clear downregulation of essential amino acids 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), all of which were significantly downregulated in the treatment group ($p < 0.05$) (**Table 2**). On Day 5, similar to the results of the PCA (**Figure 2B**) and PLS-DA (**Figure S2**) study, no metabolites showed high significance ($p < 0.05$) at this stage except AMP. The upregulating of the expression of AMP is potentially related to the immune system (39), however, the discovery was not supported in the combinational models (PCA and PLS-DA). The cause of the sole metabolite upregulation is unclear by the current data and needs further studies.

Though both PCA and PLS-DA showed evidence of difference after glyphosate treatment on Day 10 groups (**Figure 2C**), the individual metabolites significance level is not very high, and only sucrose showed statistical significance ($p < 0.05$). However, the downregulation of the non-essential amino acids glutamine (FC = 0.47) and proline (FC = 0.54) also showed high significance ($p < 0.1$) (**Table 2**).

204 3.4 The initial caging process effect to honey bees

205 The initial caging process has a significant effect on the honey bee activities which directly leads
206 to lethargic and low mortality that is a weak condition of honey bees. The PCA study showed
207 distinctly that the metabolomic level changes compared to Day 5 and Day 10 regardless of the
208 control or treatment groups. The loadings (**Figure 1**) indicate that metabolites choline, acetate,
209 and essential amino acids such as lysine, leucine, and valine are positively contributed to the Day
210 2 samples, and the sucrose and fructose are positively contributed to the Day 5 and 10 groups.

211 **4. Discussion**

212 4.1 Metabolomic profiling changes by glyphosate at different stages

213 Both the PCA and PLS-DA studies showed that the metabolomic profiling of honey bees has
214 been highly perturbated by the low concentrations of glyphosate at the early stage of caging (Day
215 2). The activities of honey bees became normal around Day 5 and metabolomic level
216 perturbation also turned to negligible. However, after relatively long-term exposure (Day 10), the
217 glyphosate influence on honey bee metabolomic profiling was observed again in the PCA and
218 PLS-DA study though the amino acids changes are weaker compared to Day 2 data. The honey
219 bees were highly active on Day 10, the metabolomic level perturbation indicated that glyphosate
220 has a long-term effect on the honey bee metabolism which is a potential concern for the health of
221 honey bees.

222 In addition, though the caging process is not the focus of this study, the PCA study showed that
223 the day 2 bees had lower concentrations in fructose and sucrose, but higher concentrations in the
224 amino acids compared with day 5 and day 10 bees. The results may indicate that the honey bees

tend to consume more sugar to produce amino acids under caging process, and potentially for somatic maintenance (40).

4.2 Honey bees essential amino acids

The early-stage (Day 2) samples showed high perturbation in honey bee's essential amino acids. At this stage, the honey bee activities were not fully recovered from the caging process and glyphosate has a high impact on the honey bee metabolism according to the PCA and PLS-DA results. For example, the essential amino acids such as methionine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine, and valine had a downregulation trend after glyphosate exposure at Day 2. Leucine, isoleucine, lysine, threonine, and valine showed low *p*-value ($p < 0.05$) and high contribution in both PCA and PLS-DA model 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 stages of the bees' lives (41). The essential amino acids have been reported to have reduced concentration when the bees are older but are still required for regular somatic maintenance and during reproductive periods (41).

Disturbance of the essential amino acids is a common metabolic response to stress and the stressed organisms must balance intracellular osmolality. In this study, the high perturbation of the essential amino acids is a potential sign of honey bees' stress response to the 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, they are also important for neurotransmission and overall brain function. Many honey bee amino acids act as precursors to enzymes, neurohormones, and neuropeptides, with some even acting themselves as neurotransmitters (40). Previous research has suggested that the essential amino acid lysine has direct involvement in nitric oxide synthesis, a known neurotransmitter that relates to memory in

bees (42). 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 the Day 2 honey bees were also struggling with the new caging environment for both groups, the high glyphosate effect to honey bee 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 at Day 2 were potentially for regular somatic maintenance (**Figure 1**, loadings), but the glyphosate exposure weakened the process. This can also be observed on the PCA score plot (**Figure 1**) where the Day 2 glyphosate treated experimental group showed a separation direction to the Day 5 data (normal activity data). In conclusion, glyphosate tends to slow down the protection process of honey bees during the caging process.

The glyphosate effect became negligible on Day 5 when the activity become normal (**Table 1)** which indicated the short-term glyphosate exposure has limited influence on the health and active bees. However, while the Day 10 honey bees were still active, the essential amino acids showed perturbations though not as significant as Day 2. The essential amino acids also showed downregulation at Day 10 but the significant level is 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 of the score plot separation (**Figure 2C**) which indicates a potential combinational perturbation in long term glyphosate exposure (Day 10).

4.3 Honey bees non-essential amino acids

Non-essential amino acids also have effects on the functioning and development of the bee brain, sometimes serving as “neuro-protectants” against oxidative stress (43). The non-essential amino

acids detected such as glutamate, glutamine, and proline showed downregulation in terms of fold change in 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 are the important metabolites in the same direction of the model separation (**Figure S3**). The t-test showed that Day 10 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 potentially increase the mortality rates of bees infected with parasites and pathogens and put them at 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), and the proline downregulation could also be a sign of potential health problem.

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 caste, the honey bees' diets shift towards an increased need for carbohydrates, such as sugars found in honey, to allow them to expend mass amounts of energy during foraging flights. The reduced levels of both proline ($p = 0.07$) and sucrose ($p < 0.05$) in the day 10 treatment group suggest that metabolic priority was shifted away from flight and redirected to more vital processes that could impact the health of the honey bees. The significant sucrose downregulation was also observed on Day 2 without a significant proline change which is likely due to the less activity of the honey bees on Day 2.

In summary, the findings of this study indicate that honey bees exposed to environmentally-consistent 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 that may be exposed to glyphosate.

5. Conclusion

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

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

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Declarations

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

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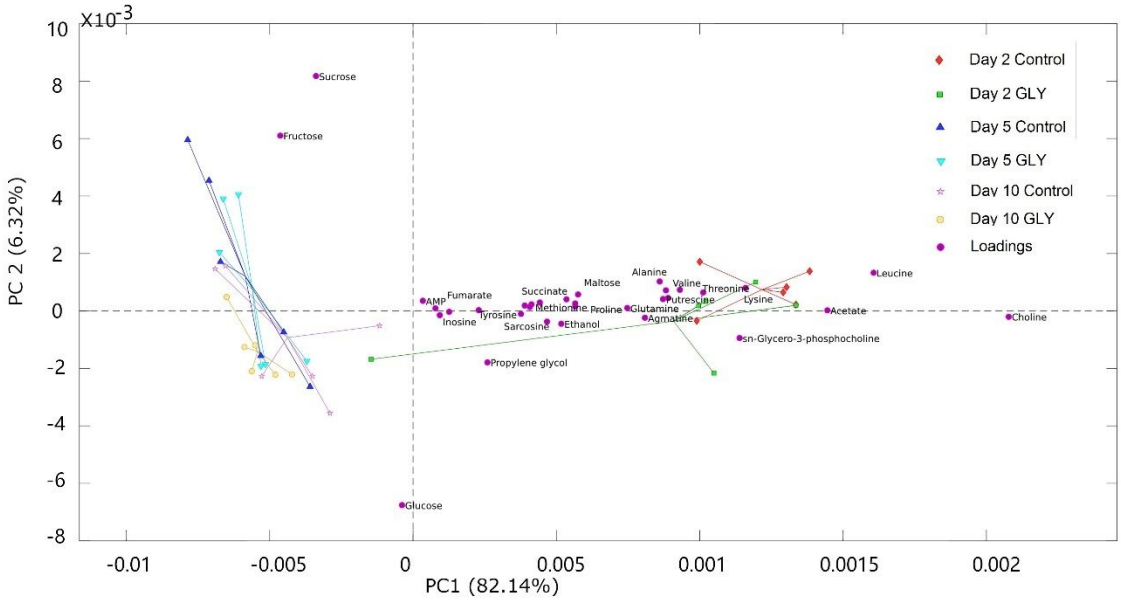
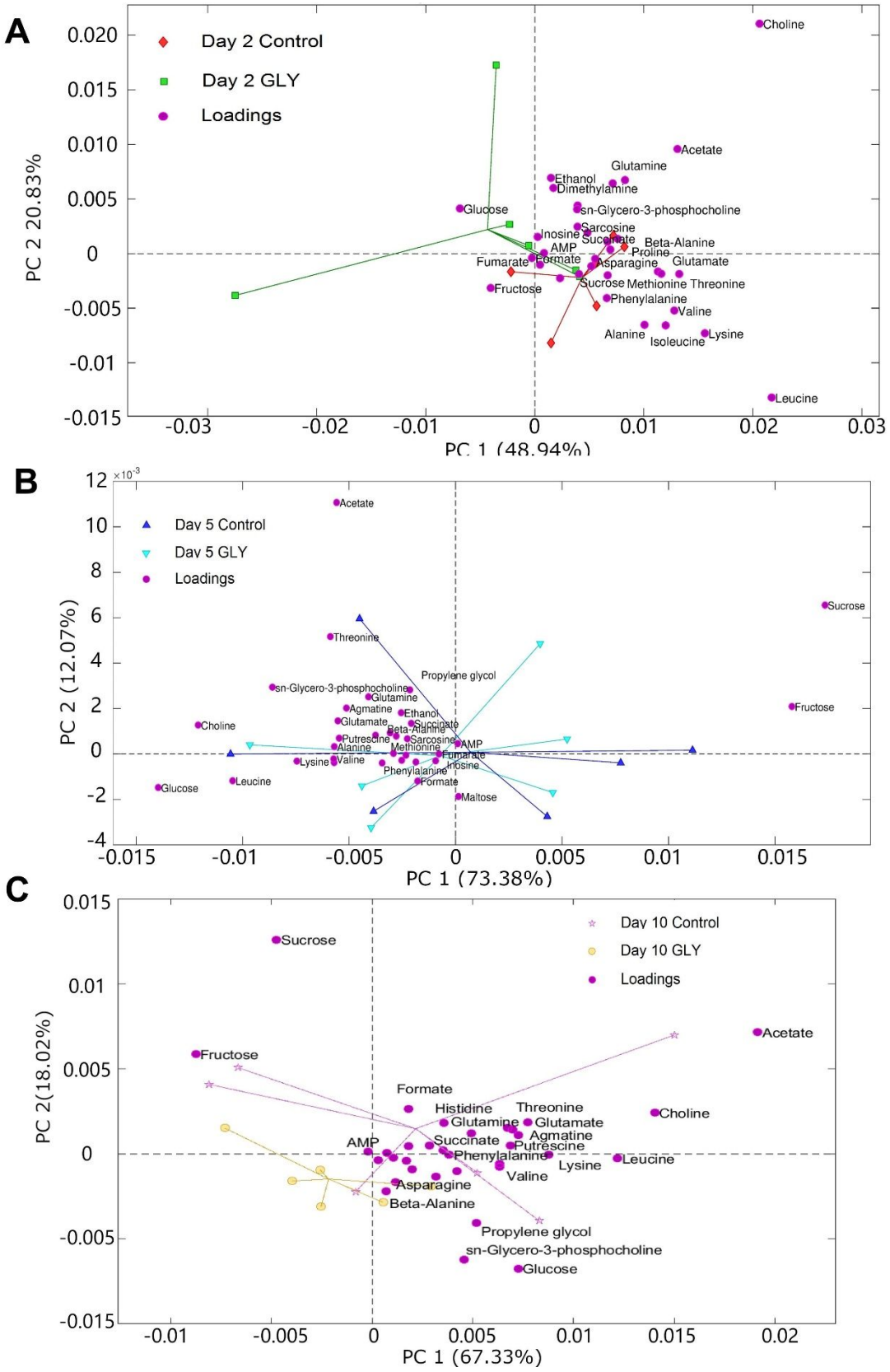
441 **Figures**



442

443 **Figure 1.** The overall PCA for all study groups.


A
Day 2 Control
Day 2 GLY
Loadings
PC 2 20.83%
PC 1 (48.94%)
Choline
Acetate
Glutamine
Ethanol
Dimethylamine
Glucose
sn-Glycero-3-phosphocholine
Sarcosine
Inosine
Succinate
Beta-Alanine
AMP
Proline
Fumarate
Formate
Asparagine
Glutamate
Sucrose
Methionine
Threonine
Fructose
Phenylalanine
Valine
Alanine
Isoleucine
Lysine
Leucine
B
Day 5 Control
Day 5 GLY
Loadings
PC 2 (12.07%)
PC 1 (73.38%)
Acetate
Sucrose
Threonine
Propylene glycol
sn-Glycero-3-phosphocholine
Glutamine
Fructose
Agmatine
Ethanol
Choline
Glutamate
Succinate
Beta-Alanine
Putrescine
Sarcosine
AMP
Alanine
Methionine
Lysine
Valine
Fumarate
Inosine
Phenylalanine
Leucine
Formate
Glucose
Maltose
C
Day 10 Control
Day 10 GLY
Loadings
PC 2(18.02%)
PC 1 (67.33%)
Sucrose
Acetate
Fructose
Formate
Histidine
Threonine
Choline
Glutamine
Glutamate
Agmatine
AMP
Succinate
Putrescine
Phenylalanine
Lysine
Leucine
Valine
Asparagine
Beta-Alanine
Propylene glycol
sn-Glycero-3-phosphocholine
Glucose

445 **Figure 2 A.** The Principal Component Analysis for day 2 groups. **B.** The Principal Component
446 Analysis for day 5 groups. **C.** The Principal Component Analysis for day 10 groups.

447 **Table 1** Honey Bee Behavior Observations. Note: L = Lethargic; M = Mortality; AAF = Active
448 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

449

450 **Table 2** Metabolites fold change (FC) and *p* values via a Student t-test. The FC was calculated
451 using the experimental group over the control group.

		Day 2	Day 2	Day 5	Day 5	Day 10	Day 10
Metabolites	ppm	FC	*p*-values	FC	*p*-values	FC	*p*-values
Acetate	1.92	0.96	8.84E-01	0.73	6.50E-01	0.32	1.11E-01
Agmatine	3.05	0.96	7.74E-01	1.39	3.51E-01	0.70	3.57E-01
Alanine	1.49	0.76	2.71E-01	0.91	8.36E-01	0.62	3.95E-01
AMP	8.27	0.64	1.28E-02	3.04	9.86E-03	0.48	2.79E-01
Asparagine	2.93	0.80	2.69E-01	0.96	9.58E-01	0.67	5.61E-01
Choline	3.20	0.96	7.97E-01	1.38	5.35E-01	0.74	4.32E-01
Dimethylamine	2.76	1.15	3.96E-01	1.30	4.31E-01	0.81	4.65E-01
Ethanol	1.17	1.69	2.40E-02	1.08	8.44E-01	0.53	2.06E-01
Formate	8.46	1.82	1.56E-01	0.94	9.10E-01	0.38	3.81E-01
Fructose	4.03	1.09	2.73E-01	0.88	5.34E-01	1.20	1.73E-01
Fumarate	6.53	0.69	3.45E-01	1.54	5.58E-01	0.99	9.91E-01
Glucose	3.27	1.06	3.41E-01	1.06	7.39E-01	0.99	8.53E-01
Glutamate	2.33	0.79	1.15E-01	1.37	4.06E-01	0.64	2.99E-01
Glutamine	2.41	0.73	1.62E-01	1.33	4.57E-01	**0.47**	5.96E-02
Histidine	7.09	0.81	2.35E-01	1.50	3.34E-01	0.37	2.70E-01
Inosine	8.24	1.16	5.72E-01	1.18	7.82E-01	0.69	5.40E-01
Isoleucine	1.02	**0.67**	3.95E-02	1.01	9.79E-01	0.69	4.47E-01
Leucine	0.96	**0.66**	3.30E-02	1.01	9.85E-01	0.67	4.34E-01

Lysine	1.73	**0.70**	4.36E-02	1.07	8.84E-01	0.68	4.18E-01
Maltose	3.29	0.81	7.69E-02	0.95	6.66E-01	1.15	4.35E-01
Proline	3.36	0.88	3.32E-01	1.10	5.21E-01	**0.54**	7.24E-02
Methionine	2.64	0.69	9.00E-02	1.00	9.96E-01	0.38	1.11E-01
Phenylalanine	7.31	0.74	1.08E-01	1.11	8.30E-01	0.59	3.91E-01
Propylene glycol	1.14	0.95	7.83E-01	0.66	5.44E-01	0.64	4.93E-01
Putrescine	1.75	0.77	7.60E-02	1.17	6.97E-01	0.67	3.82E-01
Sarcosine	2.74	0.94	7.10E-01	1.21	7.13E-01	0.66	3.48E-01
sn-Glycero-3-phosphocholine	3.24	1.16	6.82E-01	1.41	1.69E-01	1.05	7.55E-01
Succinate	2.40	0.77	8.42E-02	1.49	2.94E-01	0.67	2.27E-01
Sucrose	4.24	**0.75**	3.41E-02	1.04	9.45E-01	**0.27**	4.86E-02
Threonine	1.32	0.73	5.20E-02	0.74	5.98E-01	0.59	2.90E-01
Tyrosine	7.21	0.83	3.90E-01	0.75	5.85E-01	1.19	7.68E-01
Valine	1.04	**0.70**	4.90E-02	1.06	9.01E-01	0.67	4.00E-01
β-Alanine	3.18	0.83	2.79E-01	0.87	8.05E-01	0.96	9.30E-01

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