

## Methionine can reduce the sublethal risk of Chlorantraniliprole to honeybees (*Apis mellifera* L.): Based on metabolomics analysis

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### ABSTRACT

Bees, essential for pollination in agriculture and global economic growth. However, the great wax moth (*Galleria mellonella*, GWM), a Lepidopteran insect, poses a substantial threat to bee colonies, contributing to a global decline in bee populations. Chlorantraniliprole (CH) is one of the primary insecticide used to control GWM due to its efficacy and low toxicity to bees. To improve beekeeping safety and reduce the risk of GWM developing resistance to prolonged use of CH, we investigated the potential of combining methionine (MET) which has been found to have insecticidal activity against certain Lepidoptera pests, with chlorantraniliprole for use in the apiculture industry. This study assessed the combined effects of MET and CH on GWM and honeybees by employing the maximum concentration of MET (1 %, w/w), previously reported as safe for honeybees, and the practical concentration of CH (1 mg/kg) for GWM control. The results revealed limited acute lethal toxicity of MET to GWM and honeybees, whereas the combined chronic exposure of MET and CH (MIX) led to significant synergistic lethal effects on GWM mortality. Nevertheless, the protective effect of MET on honeybees exposed to CH was significant under chronic exposure. Potential mechanisms underlying the synergistic actions of MET and CH may stem from MET-induced protection of the "Cysteine and methionine" and the "Glycine, serine, and threonine" metabolism pathways. Furthermore, immune stress mitigation was also observed in honeybee immune-related gene transcripts treated by the combination of MET and CH under both acute and chronic exposure. The effects of MET on CH activity in GWM and honeybees are likely due to metabolic regulation. This study suggests the potential of developing MET as a promising biopesticide or protective agent in the future.

### 1. Introduction

Honeybees are widely recognized as one of the primary pollinators worldwide (Garibaldi et al., 2013), providing invaluable pollination services to a diverse range of cultivated crops and wild flora (Calderone, 2012). *Galleria mellonella*, commonly known as the Great Wax moth (Lepidoptera: Pyralidae, GWM), is a prevalent parasitic pest found in honeybee colonies. The larvae of GWM can damage bee colonies by gnawing through wax cells, leading to galleriasis (Ellis et al., 2013) and subsequent absconding out of bee colonies (Kwadha et al., 2017). This pest is currently ubiquitous in apiculture practiced worldwide, with its sever impact on the tropical and sub-tropical areas rendering it a significant contributing factor to the decline in honeybee populations. (Chantawannakul et al., 2016; Kumar, 2018; Kwadha et al., 2017; Petreanu, 2001; Pirk et al., 2016; Shimanuki, 1980; Williams, 1997). Pesticide application is one of the effective means of controlling GWM

(Kwadha et al., 2017).

Chlorantraniliprole (CAS Number 500008-45-7, CH) is an anthranilic diamide insecticide developed by DuPont company. It exhibits remarkable efficacy in the eradication of lepidopteran pests such as GWM, diamondback moth, european grapevine moth and codling moth (Bassi et al., 2009; Han et al., 2012; Luo et al., 2020). Through the over-activation of insect ryanodine receptors (RyRs), CH induces the excessive release of calcium stores from the sarcoplasmic reticulum, resulting in insect mortality due to feeding cessation and contractile paralysis (Lahm et al., 2009). The global market value for CH was estimated to be USD 1886.8 million in 2022 (Business Research INSIGHTS, 2023). Due to its extensive usage, the cumulative toxicity of CH to honeybees has attracted considerable attention. According to reports, exposure to CH can lead to honeybees experiencing death, lethargy, apathy, and uncoordinated movements, resulting in severe damage to the colony (EPSA, 2008).

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Given the escalating risks of CH to honeybees and the imperative need for GWM control, there is an urgent requirement to identify compounds that can enhance the insecticidal efficacy of CH against pests while ensuring their safety or, at the very least, avoiding an exacerbation of toxicity to honeybees. Intriguingly, several studies have revealed the biocidal properties of methionine (MET), which exert an influence on ion flux and neurotransmitter transport, thereby controlling lepidopteran pests including *Principis (Papilio) demoleus* and *Manduca sexta* (L.) (Lewis et al., 2011; Long et al., 2003; Quick and Stevens, 2001). These findings suggest that MET might serve as a pivotal compound that not only augments the insecticidal activity of CH against lepidopteran pests but also ensures the safety of honeybees. However, to our knowledge, no studies have evaluated the impacts of combined applications of MET and CH on GWM and honeybees.

The essential amino acid MET is needed by both adult and larval honeybees. Bees cannot rear broods on MET-deficient pollen, and they exhibit a tendency to avoid collecting pollen or nectar from florets with MET deficiencies (Weeks et al., 2018). Consequently, the simultaneous application of MET and CH is unlikely to increase the risks of CH to honeybees; instead, it may potentially enhance CH's efficacy against lepidopteran pests. To test this hypothesis, we initially assessed the synergistic effects of MET and CH on the survival of both GWM and honeybees under acute and chronic conditions. Subsequently, we elucidated the potential protective mechanisms of MET in CH-treated bees through metabonomic analysis and transcription of target immune-related genes. This study is expected to offer new insights into bee protection and the development of pesticides suitable for the apicultural industry.

## 2. Methods and materials

### 2.1. Chemicals, solvents and devices

L-Methionine (99 % purity) was purchased from Macklin Biochemical Technology Co., Ltd (Shanghai, China) and stocked at room temperature. A commercial formulation of chlorantraniliprole (Coragen, 200 g/liter suspension concentrate, FMC Corporation, Jiangsu, China) and stored at 4 °C in the dark after opening. Artificial feed for the GWM was obtained from Keyun Biology Company (Henan, China). Electronic balance (METTLER TOLEDO, Swiss).

Carry Helix Total RNA Extraction Kit (Beijing, China), One-Step gDNA Removal and cDNA Synthesis SuperMix (AT311, TransScript), 2 × Master qPCR Mix SYBR Green I (TSE201, Tsingke), All the primers were synthesis by Beijing Tsingke Biotech Co., Ltd. qPCR instrument (A40425, Thermo Fisher), refrigerated centrifuge (5424R, Eppendorf), NanoDrop Nucleic acid content detection spectrophotometer (Nano-Drop 2000, Thermo Fisher).

Methanol and acetonitrile (Thermo Fisher), Tissue grinder (OSE-Y50, TIANGEN BIOTECH (BEIJING) CO., LTD), UPLC-qTOF-MS/MS (Agilent 6545, Agilent Technologies).

### 2.2. Test organisms and solution preparation

The GWM was purchased from Keyun Biology Company (Henan, China) and cultured in a controlled incubator under specific conditions (30 ± 1 °C, 65 ± 5 % RH, darkness) (MATSUMOTO and YANO, 1995; Sehnal, 1996). The third instar larvae were selected for the acute (48 h) and chronic (7d) exposure experiments.

Italian honeybee (*Apis mellifera* L.) colonies from the apiaries of the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, in Beijing, China, were utilized in this study. All experimental bees were sourced from these colonies. Each colony comprised eight frames of comb containing larvae, pupae, honey, and pollen. No miticides were applied to the colonies for a minimum of one month prior to the commencement of the study. Honeybees that flew around the hive entrance were collected for the acute toxicity test. They were transferred

into exposure container and kept in the incubator under conditions of 30 ± 1 °C, 50 ± 5 % relative humidity (RH), and darkness (Qi et al., 2022). In contrast to the acute test, newly emerged bees (< 24 h old) were employed for the chronic experiments. The acute and chronic oral exposure tests for GWM and honeybees involved four treatments: CK, MET, CH, and MIX. The final concentrations used were 1 mg/kg of CH, 1 % (w/w) of MET, and a combination of CH and MET (MIX). For the preparation, 100 g of artificial feed obtained from Keyun was ground to powder with a grinder (A11 basic, IKA) and then blended with 1 g MET (1 % MET), or 100 μL of 1000 mg/L CH solution (CH), or both (MIX) (Weeks et al., 2018). The original feed, without any additions, served as the blank control (CK). All powder was kneaded into tiny particles for easier application for GWM to consume. The same concentrations were used in the acute and chronic experiments for honeybees. The preparation method was similar to that used for GWM treatments, with the exception that the artificial feed was replaced by a 50 % sucrose solution.

### 2.3. Exposure, sample collection and parameter evaluation

The acute toxicity test for GWM treatments was performed with six biological replicates, each consisting of 10 GWM, whereas eight biological replicates, each consisting of 10 GWM, were used for the chronic toxicity test. All larvae were cultured in an incubator at 30 ± 1 °C, 65 ± 5 % RH, with darkness. The test feed was renewed daily and the mortality of GWM was calculated every 24 h.

In the acute toxicity test for honeybees, each treatment consisted of three biological replicates, with 20 bees in each replicate. The bees were cultured in an incubator under constant conditions of 30 ± 1 °C in darkness and 50 % ± 5% RH for a duration of 48 h. Prior to the test, all bees were starved for 2 h. At the start of the experiment, 200 μL of each test solution was provided as food to the bees in queen-rearing cups within each container. Once the solution was consumed, the cups were removed, and the bees were subsequently supplied with 1.5 mL of 50 % sucrose solution using a 2.5 mL injector. The mortality of honeybees was recorded on a daily basis.

For the chronic toxicity test, newly emerged bees were randomly assigned to transparent plastic containers. Each treatment group comprised three biological replicates, with 20 bees in each replicate. The bees underwent a consecutive 10-day incubation period in the same incubator as used in the acute toxicity test. Throughout the test period, the bees in each container were provided with 1.5 mL of the test solution as their daily food, with the solutions being renewed daily. The mortality, food consumption, and daily weight of the honeybees were recorded every 24 h. At the conclusion of the test, the midguts of honeybees within the same cage were collected and pooled to create a single sample. All samples were flash-frozen in liquid nitrogen and stored at -80 °C until further analysis.

### 2.4. Transcription of target genes in Honeybee midguts

The total RNA from the midgut tissue was extracted with the Carry Helix kit (Beijing, China) and then 1 ng of these qualified RNA were used to synthesize cDNA (AT311, Transgen Biotech). Applied Biosystems 7500 Real-Time PCR system was used to perform RT-qPCR with mixed reagent containing cDNA templates, primers, 2 × Master qPCR Mix SYBR Green I (TSE201, Tsingke) and DNase-free water. Two-step reaction program for qPCR setup are shown in Table S1.

Each treatment was tested with 3 biological replicates and each biological replicate contained three technical replicates. ddCT method was used to analyze the qPCR result data. *beta-actin* was used as a reference gene to normalize the expression level (Qi et al., 2020). The primers used in the RT-qPCR experiment are shown in Table S2.

## 2.5. Untargeted metabolomics analysis

### 2.5.1. Metabolite extraction

The metabolic actions in honeybees treated with MET, CH, and MIX were evaluated after a 10-day exposure period. Metabolites were extracted and analyzed following the procedures described in our previous investigation with some adjustments (Li et al., 2019). For each sample, 50 mg of bee midgut tissues were used, and three biological replicates were analyzed for each treatment. To initiate the extraction process, 400  $\mu$ L of a precooled methanol-acetonitrile (1:1, v/v) solution was added to each gut sample and homogenized with a TGrinder electronic tissue burnisher (Tiangen Biotech Co., LTD, Beijing, China). The mixtures were then ultrasonically extracted for 10 min and kept at  $-80^{\circ}\text{C}$  overnight. Later, the mixtures were centrifuged at 15,000 rpm for 15 min at  $4^{\circ}\text{C}$  (Centrifuge 5424 R, Eppendorf, Germany), placed the supernatant in a new 1.5 mL centrifuge tube and vacuum freeze dry it (Jiaimu CV200, Beijing, China). The concentrated extracts were then resuspended in 100  $\mu$ L of a mixture (1:1, v/v) of methanol and acetonitrile using an ultrasonic device for 10 min. In the end, the supernatant was transferred to brown injection bottles for metabolite analysis following a centrifugation stage at 15,000 rpm for 15 min.

### 2.5.2. UPLC-Q-TOF-MS/MS analysis

Metabolite analysis was carried out using an Agilent 6545 quadrupole time-of-flight (qTOF) tandem mass spectrometer (Agilent, USA) equipped with an Agilent 1290 Infinity II UPLC system. Following our previously reported procedure (Li et al., 2019), the separation of metabolites was executed using an Agilent Eclipse Plus C18 column (2.1 mm  $\times$  100 mm, 1.8  $\mu$ m) system. Mass spectrometric data were collected using both positive and negative ion modes employing an electrospray ionization (ESI) source. The identification of metabolites for each treatment was accomplished by referencing the METLIN database.

For data quality monitoring, an unsupervised dimensionality reduction method of Principal Component Analysis (PCA) and a supervised discriminant analysis method Orthogonal partial least-squares discriminant analysis (OPLS-DA) embedded Simca (14.1, Umetrics, Sweden) were conducted.

The quality of models was assessed using the fitting parameter ( $R^2$ ) and the predictive parameter ( $Q^2$ ).  $R^2$  and  $Q^2$  value greater than 0.5 and 0.4, respectively, indicate that the biological model is acceptable (Hrbek et al., 2018). Permutation analysis (200 times) was also employed for cross-validation of the model to check whether the model is over-fitted (Martin et al., 2008). Then, the Variable Influence on Projection (VIP) value and Welch's *t*-test was performed to filter the statistically differential metabolites between each treatment metabolites that both meet the  $\text{VIP} \geq 1$  and  $p \leq 0.05$  are identified as the statistically differential metabolites in each treatment (Wang et al., 2016). All differential metabolites between each treatment are used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway mapping using the MetaboAnalyst 5.0 program (<https://www.metaboanalyst.ca>).

## 2.6. Statistical analysis

The normality assumptions of data distribution for the survival rate of GWM and honeybees in acute toxicity test, the food consumption and body weight changes for honeybees in chronic toxicity test, and the transcription level of target genes in honeybee midguts were analyzed using the Kolmogorov-Smirnov test. Statistical differences between each treatment of the experiments described above were determined by one-way analysis of variance (ANOVA), followed by Turkey's post-hoc test, using SPSS 26.0 software. The survival curves for GWM and honeybees in chronic toxicity test were analyzed using the Log-Rank test by the R package 'Survminer' (V.4.2.2), and the *p* values were adjusted using the Benjamini-Hochberg method to account for a 5 % false-discovery rate (FDR).

The untargeted metabolomics analysis of the honeybee chronic

toxicity test was introduced in Section 2.5.2.

## 3. Results

### 3.1. The single and synergistic effect of CH and MET on GWM and honeybees

As shown in Fig. 1A, the survival rate of GWM exposed to MET at the first 24 h and 48 h was not greatly differed from  $93.33\% \pm 8.16\%$  and  $88.33\% \pm 11.69\%$ , respectively. GWM exposed to CH had a survival rate of  $90\% \pm 6.32\%$  at 24 h, which decreased to  $67\% \pm 7.53\%$  at 48 h. However, when CH and MET were mixed and exposed to GWM, a significant synergistic effect was observed, and the survival rate of GWM treated with MIX was  $85\% \pm 5.48\%$  at 24 h, while it plummeted to  $58.33\% \pm 11.69\%$  at 48 h. The synergistic effect was more pronounced in the chronic experiment (Fig. 1B), as the survival rate of the MIX group decreased to  $17.50\% \pm 8.86\%$  after 7 days of exposure.

Similar to the GWM experiment, the survival rates of bees were reaching 100 % and  $96.67\% \pm 5.77\%$  at 48 h in CK and MET treatment, respectively. Notably, the CH treatment exhibited the highest toxicity to honeybees, resulting in a survival rate of only  $85.00\% \pm 5.00\%$  at 48 h. However, the MIX treatment could significantly reduce the mortality of bees induced by CH, and its survival rate was merely reduced to  $98.33\% \pm 2.89\%$ , which was comparable to the CK level. This phenomenon was consistently observed in the chronic toxicity test of honeybees as well. After 10 days of chronic exposure, the final survival rate in the CH group was  $63.33\% \pm 7.64\%$ , significantly lower than the  $83.33\% \pm 5.77\%$  observed in the MIX treatment.

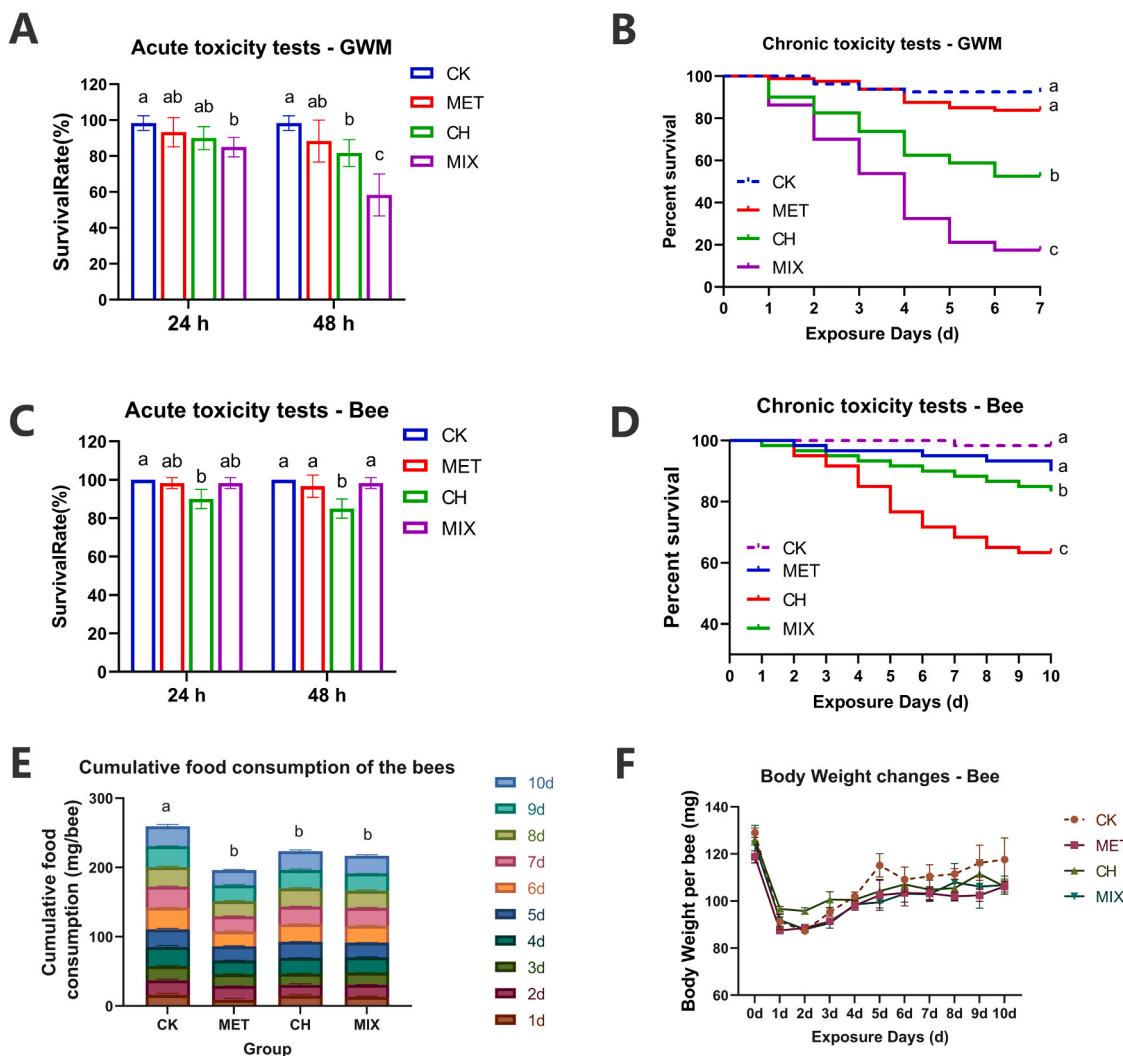
It takes a few days for newly emerged bees to fully develop. During the initial two days, the newly emerged bees ingested the least quantity of sucrose solution. Subsequently, their intake increased and ranged between 15 and 25 mg/bee/d (Fig. 1E). The average daily sugar ingestion per bee was  $21.23 \pm 1.12$  mg/bee for the CK group,  $16.23 \pm 0.70$  mg/bee for the MET group,  $18.31 \pm 0.97$  mg/bee for the CH group, and  $17.66 \pm 0.64$  mg/bee for the MIX group. Notably, bees exhibited an antifeedant effect towards MET, resulting in significantly lower sugar intake compared to the CK group ( $p < 0.001$ ). Consistent with food consumption of the newly emerged bees, the lowest average body weight of all treated newly emerged bees has happened on the initial two days of  $90.90 \pm 1.33$  mg per bee, then progressively climbed and stabilized at  $101.76 \pm 7.22$  mg per bee throughout the remaining exposure period (Fig. 1F). Overall, although the MIX treatment had a more pronounced mortality effect on GWM compared to the CK treatment, it mitigated the mortality caused by CH in bees. Moreover, none of the treatments had a noticeable impact on the weight change of bees ( $p > 0.05$ ).

### 3.2. The expression of immunity-related genes in honeybees

The treatment with MET, CH, and MIX led to changes in the expression of immunity-related genes. The transcription levels of five immunity-related genes in the midguts of honeybees were analyzed following both acute and chronic toxicity tests.

As shown in Fig. 2, In the acute feeding test of bees (AFB), MET treatment greatly up-regulated the transcription level of *Domeless* ( $2.80 \pm 0.75$  folds,  $p = 0.024$ ), but not significantly altered the expression level of *Hopscotch*, *Abaecin*, and *Apidaecin* compared to CK treatment, even significantly down-regulated the expression level of *Defensin* ( $0.47 \pm 0.03$  folds,  $p = 0.004$ ). CH treatment significantly up-regulated *Defensin* genes expression level ( $1.55 \pm 0.09$  folds,  $p = 0.003$ ), but had no significant effect on the remaining genes. Notably, except for *Apidaecin*, the transcription levels of various immune genes under MIX treatment were all lower than those under CH treatment.

As for the chronic feeding test of bees (CFB), MET treatment significantly up-regulated the transcription level of *Domeless* ( $3.20 \pm 0.34$  folds,  $p < 0.01$ ) which is similar to the result of AFB, however, there was



**Fig. 1.** The effects of MET and CH on GWM and Honeybees under different exposure conditions. (A) The survival rate of GWM fed by MET, CH and MIX at the indicated concentration in 48 h, N = 6 biological replicates, n = 6 \* 10 independent samples. (B) GWM survival curve during 7 days of exposure to MET, CH, and MIX (N = 8, n = 8 \* 10). (C) The survival rate of adult honeybees fed by MET, CH and MIX at the indicated concentration in 48 h (N = 3, n = 3 \* 20). Survival curve (D), cumulative food consumption per bee (E) and body weight changes (F) of newly emerged bees during 10 days of exposure to MET, CH, and MIX (N = 3, n = 3 \* 20). All the data were recorded daily. Statistical analyses were performed using the ANOVA followed by the post-hoc Turkey test, Survival statistical analyses were performed using the Log-Rank test, Benjamini - Hochberg was used as p adjustment method, Different letter marks indicate significant differences between two groups while the same letter does not ( $\alpha = 0.05$ ).

no significant alteration in the expression levels of the remaining genes. CH treatment significantly up-regulated the expression levels of each gene, including *Domeless* ( $2.33 \pm 0.11$  folds,  $p < 0.01$ ), *Hopscotch* ( $2.50 \pm 0.39$  folds,  $p = 0.002$ ), *Abaecin* ( $3.63 \pm 0.53$  folds,  $p < 0.01$ ), *Apidaecin* ( $3.31 \pm 0.41$  folds,  $p < 0.01$ ), and *Defensin* ( $2.09 \pm 0.07$ ,  $p < 0.01$ ). For MIX treatment, the expression levels of both *Hopscotch* and *Abaecin* were inferior to those of CH treatment, while the expression levels of *Domeless* ( $0.210 \pm 0.02$  folds,  $p < 0.01$ ), *Apidaecin* ( $0.60 \pm 0.20$  folds,  $p < 0.01$ ) and *Defensin* ( $0.66 \pm 0.06$  folds,  $p = 0.005$ ) were significantly lower than those of CH treatment.

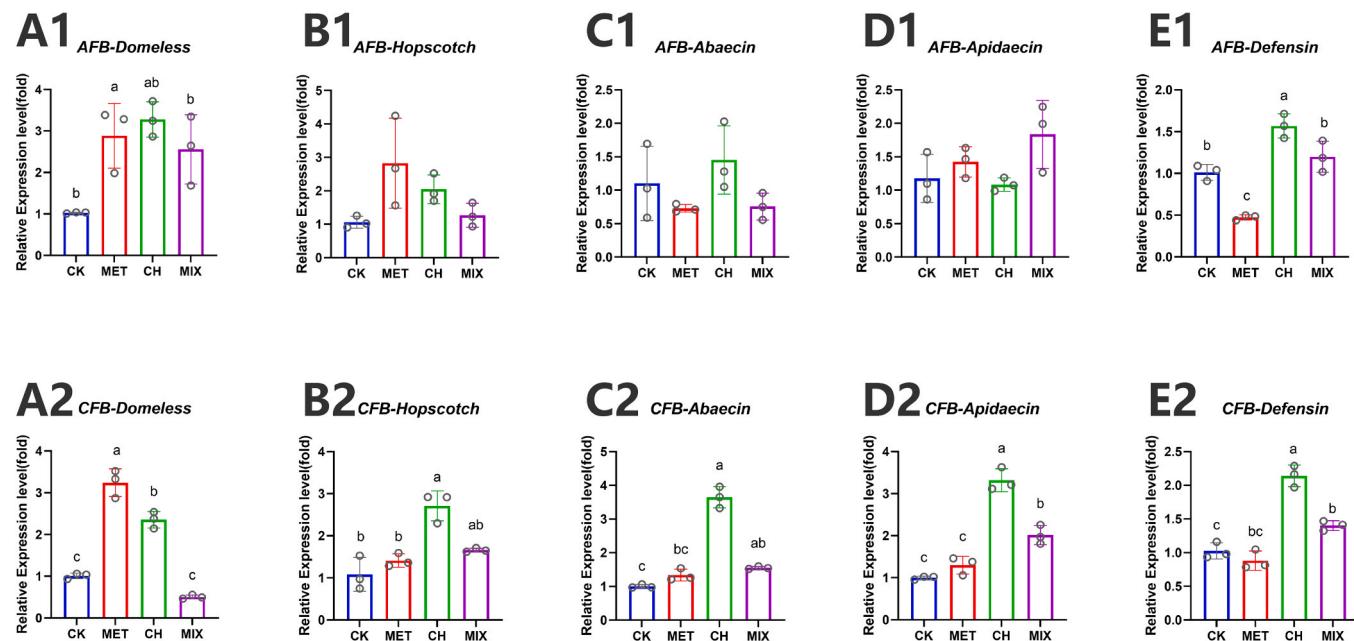
### 3.3. Metabolite profiles of the honeybee midgut

The functional and synergistic effects of MET and CH on the intestinal metabolism of bees have been investigated. As shown in Fig. 3A, all samples were clustered into four distinct groups in the PCA diagrams. These metabolic profile variations imply that 1 % MET, 1 mg/kg CH, and their mixtures caused significant biochemical alterations. Subsequently, the OPLS-DA model was established to find the differential metabolites among the treatment groups. The difference between each

treatment through OPLS-DA analysis which presents a good separation pattern has been shown in Fig. S1A. Additionally, the permutation plot displayed high values for the explained variation ( $R^2$ ) and predictive capability ( $Q^2$ ), as depicted in Figure. S1B, confirming the reliability and high predictability of the models. Detailed information regarding the fitting parameters  $R^2X$ ,  $R^2Y$ , and the predictive parameter  $Q^2$  for each OPLS-DA model can be found in Table S3.

Seen from Fig. 3B, a total of 1244, 1287, 1042, and 1227 metabolites were obtained from CK, MET, CH and MIX groups, respectively, of which 43, 38, 9 and 57 metabolites were specific to each group and 700 metabolites were shared among all four groups. Specifically, among the CK-MET, CK-CH, CK-MIX, MET-CH, MET-MIX, and CH-MIX comparisons, 288, 374, 437, 442, 397 and 226 different metabolites exhibited significant changes ( $VIP \geq 1$  and  $p \leq 0.05$ ), and the content variation of these metabolites whose VIP were rank in top 120 are also shown in Fig. 3C.

To further investigate the functional and synergistic effects of MET and CH on honeybee metabolism, the MetaboAnalyst (v5.0) platform was used to map these significantly altered metabolites to metabolic pathways. A total of seven metabolic pathways were identified as



**Fig. 2.** Effects of CK, MET, CH and MIX on immunity-related gene expression in midguts of honeybees after acute and chronic exposure (AFB represents Acute Feed Bees while CFB represents Chronic Feed Bees). Differential mRNA levels of immune system-related genes (A) *Domeless*, (B) *Hopscotch*, (C) *Abaecin*, (D) *Apidaecin*, (E) *Defensin*. The data shown are the results of three biological replicates per treatment. Statistical analyses were performed using the ANOVA followed by the post-hoc Turkey test, different letter marks indicate significant differences between the two groups while the same letter does not ( $\alpha = 0.05$ ). Error bars represent the SD of fold changes.

potential pathways in all intergroup comparisons, namely “Cysteine and methionine metabolism”, “Glycine, serine and threonine metabolism”, “Glycerophospholipid metabolism”, “Pantothenate and CoA biosynthesis”, “Pentose and glucuronate interconversions”, “Pyrimidine metabolism”, and “Alanine, aspartate and glutamate metabolism” (Fig. 4A). Among them, “Cysteine and methionine metabolism” and “Glycine, serine and threonine metabolism” were enriched in MET-MIX and CH-MIX comparison group, had a greater pathway-impact and involved more comparison groups than other metabolic pathways, demonstrated that these metabolic pathways were significantly altered in bees exposed to MIX compared to MET and CH alone. Further details, including the pathway-impact and *p*-value of these metabolic pathways, can be found in Table S4.

The significantly altered metabolites (selected based on VIP  $\geq 1$  and  $p \leq 0.05$  between each group) involved in the two pathways were matched in the KEGG, and shown as KEGG numbers, namely Methionine (MET, C00073), S-Adenosyl-L-methionine (SAM, C00019), S-Adenosyl-L-homocysteine (SAH, C00021) and Homocysteine (C00155) in the “Cysteine and methionine metabolism”; Choline (C00114), Betaine (C00719), Dimethylglycine (DMG, C01026), Creatine (Cr, C00213), Phosphocreatine (PCr, C02305) in the “Glycine, serine and threonine metabolism”.

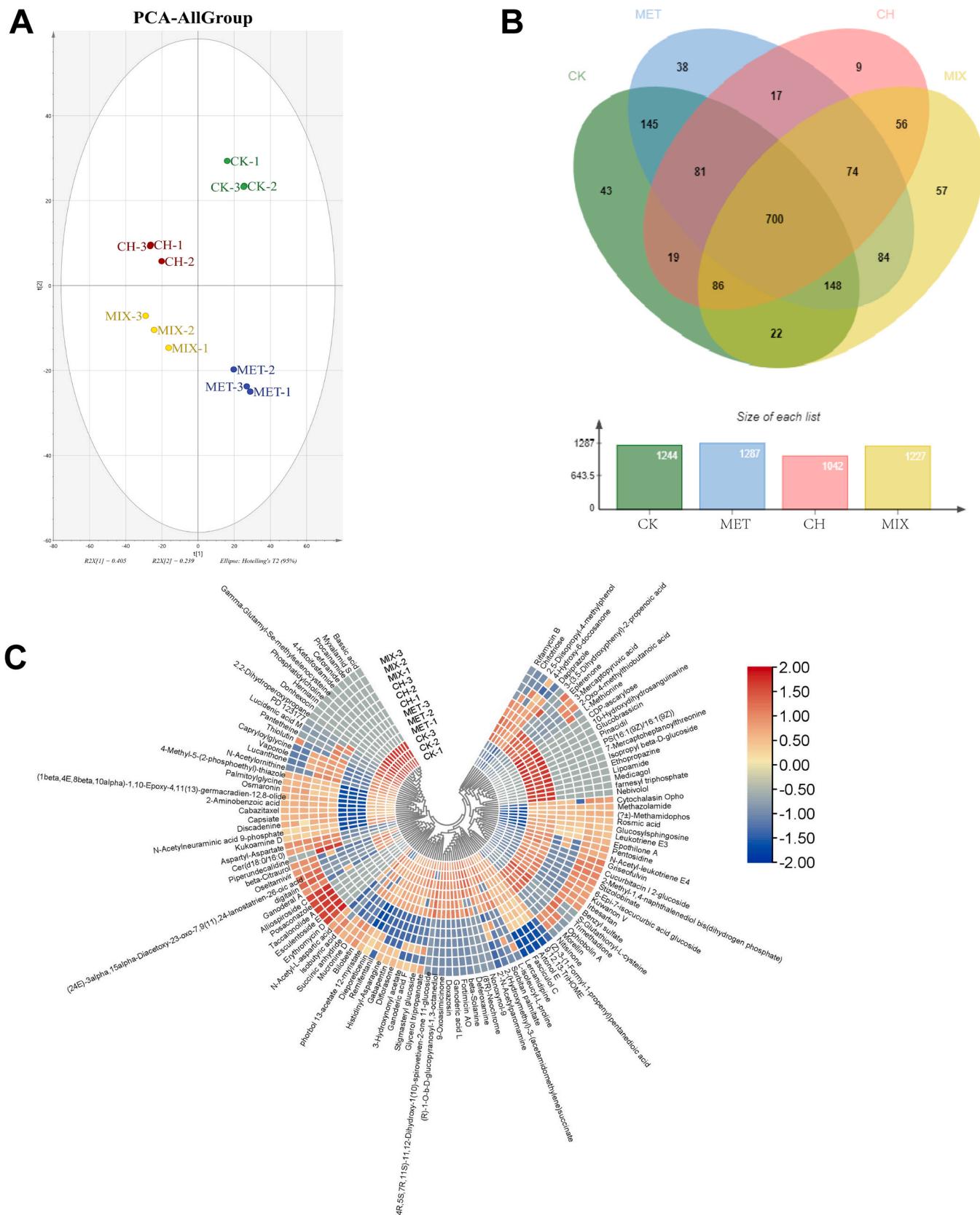
Among these metabolites, MET, Cr, and PCr have attracted our extensive attention due to their crucial biological functions. MET content was up-regulated by 15.060 and 4.480 fold in MET and MIX treatment, respectively. And the content of Cr, a metabolite involved in maintaining intracellular ATP levels, was up-regulated by 1.609 fold in the MET treatment and 1.461 fold in the MIX treatment. but the CH treatment led to a down-regulation of Cr with a fold change of 0.105. Furthermore, the content of PCr, a high-energy compound, was significantly up-regulated by 12.877 fold in the MET treatment and 9.101 fold in the MIX treatment (Fig. 4B).

#### 4. Discussion

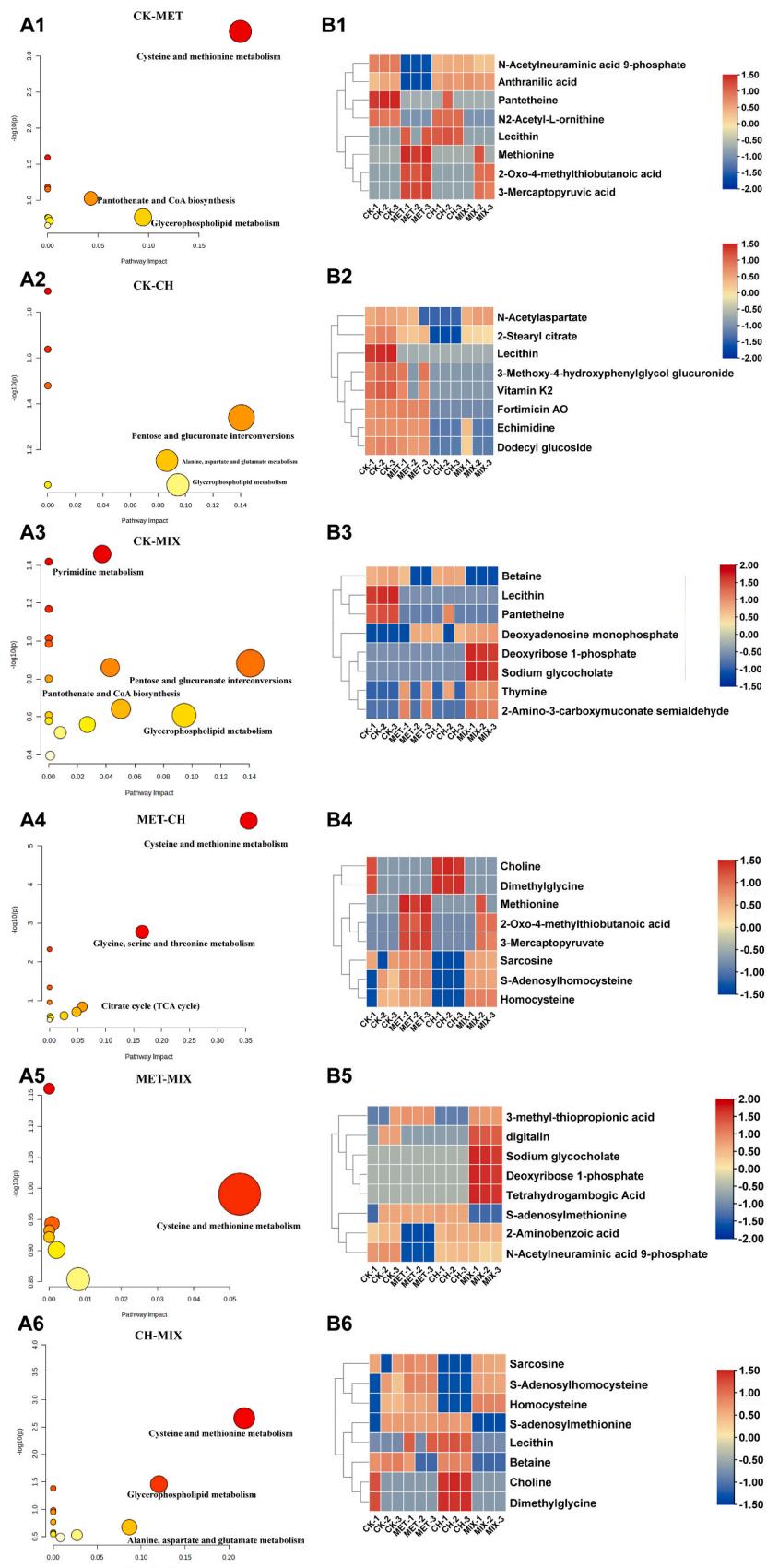
Honey bees play a crucial role as pollinators in agriculture and

provide valuable bee products to humans (Hung et al., 2018). However, honey bee colonies have been experiencing declines worldwide in recent years, with infestations of the GWM considered an important contributing factor. CH is widely employed to control the GWM due to its powerful insecticidal activity and relatively low toxicity to non-target organisms. However, some studies have shown that CH can induce bees lethargic, apathetic, uncoordinated move, and in severe cases can cause the mortality of bees (European Food Safety Authority (EFSA (EPSA (2015)). MET, as a nutritional amino acid, not only contributes to the development of bees but also has an insecticidal effect on kinds of lepidopteran pests, such as *Principis (Papilio) demoleus* and *Manduca sexta* (L.) (Lewis et al., 2011; Long et al., 2003; Quick and Stevens, 2001). The properties of MET offer the potential to mitigate the risk of CH to bees while preserving its efficacy against the GWM. In this study, we observed that exposure to MET alone exhibited limited toxicity against GWM. However, prolonged exposure of GWM to a MIX treatment (CH mixed with MET) demonstrated a significantly higher lethal effect compared to CH alone. Remarkably, the MIX treatment reduced bee mortality caused by CH exposure, as evidenced by the toxicity test. Nevertheless, it is important to emphasize that future exposure experiments with honeybee larvae could be conducted to provide a more comprehensive evaluation of the safety of the MIX treatment.

Antimicrobial peptides (AMPs) are widely acknowledged as essential elements of humoral immunity in honeybees (Danilhlik et al., 2015). Apidaecins, abaecin, hymenoptaecin, and defensins, four families of AMPs have been discovered in honeybees (Danilhlik et al., 2015). Pesticide exposure may have an impact on the transcription of AMPs coding genes in bees (Qi et al., 2022). In our study, we found that in AFB and CFB experiments, the treatment groups exposed to CH had up-regulated expression of *abaecin* and *defensin*, which were encoding antimicrobial peptides with the antibacterial effect produced by honeybee hemolymph (Saltykova et al., 2005), suggesting that CH may reduce the bacterial population in the honeybee gut thereby affecting the physiological health of honey bees. The JAK/STAT signaling pathway may contribute to innate immunity through the activation of complement-like proteins and the excessive proliferation of



**Fig. 3.** Metabolic profiling for CK, MET, CH and MIX treatments. (A) PCA models for classification of all treatments. (B) the number of different metabolites of CK, MET, CH and MIX treatments. (C) Heatmap of top 120 significant metabolites content in all treatment groups, Scale bars, Z-score log2 transformed content values. Metabolites were selected based on  $VIP \geq 1$  and  $p \leq 0.05$  between CK, MET, CH and MIX groups. Three biological replicates were tested.



**Fig. 4.** Metabolic changes by each treatment on newly emerged honeybees' midgut after 10 days of exposure. (A) Pathway enrichment analysis based on differential metabolites between each treatment. All the metabolites selected for the pathway enrichment analysis on account of VIP  $\geq 1$  and  $p \leq 0.05$ , the size of the bubble represents the amount of metabolites included. (B) The alteration in the content of identified differential metabolites involved in enriched metabolic pathways among each treatment. Scale bars, Z-score log<sub>2</sub> transformed content values. Number 1–6 represents CK-MET, CK-CH, CK-MIX, MET-CH, MET-MIX, and CH-MIX, respectively.

haemoglobin-producing cells (Evans et al., 2006). Domeless (related to cytokine receptors) and hopscotch (associated with tyrosine kinases) were crucial factors in the JAK/STAT signaling pathway (Dostert et al., 2005). In this study, we discovered that in AFB and CFB experiments, the transcription levels of *domeless* and *hopscotch* under CH treatment had opposing tendencies. Hopscotch was up-regulated in long-term exposure whereas domeless was up-regulated in short-term exposure, demonstrating how honeybee immunological stress varies over time in response to CH. Notably, when the bees are exposed to MIX, we could find that the expression levels of *domeless*, *hopscotch*, *abaecin*, *apidaecin*, and *defensin* were decreased compared to the CH treatment, which indicates that MET may lessen the impact of CH on intestinal microbes and the level of immunological stress.

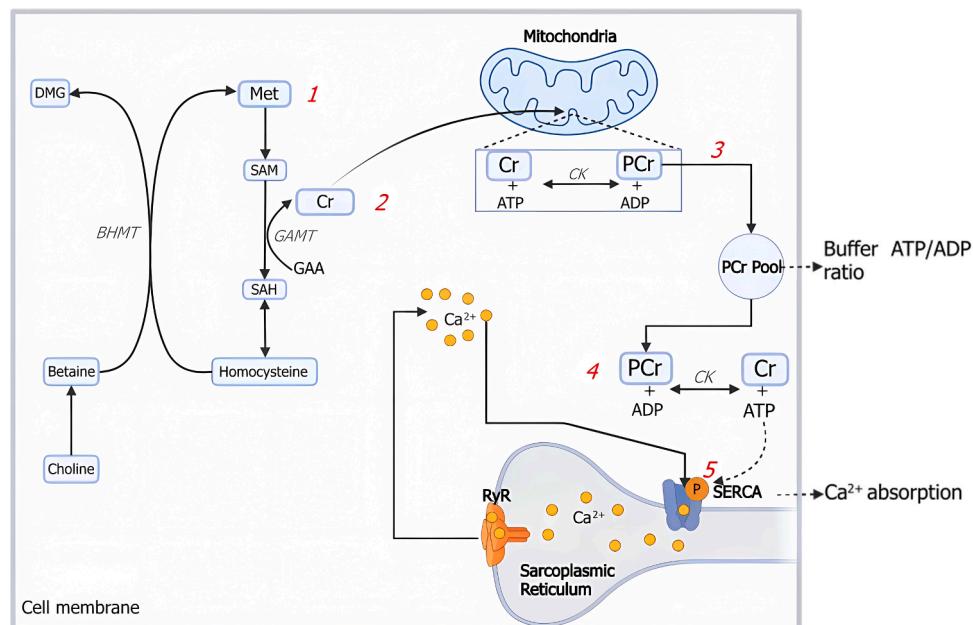
Metabolomics is effective for identifying the impacts of novel insecticides, fungicides, and acaricides on the biochemical pathways of the organism in insects. In our study, we discovered that a combination of 1 % MET and 1 mg/kg CH effectively eliminated the GWM while reducing the toxicity of CH to honey bees, which sparked our keen interest. Furthermore, our investigation revealed significant metabolic alterations in honey bees exposed to MET, CH, and the mixture (MIX) of both compounds. Through metabolic pathway enrichment analysis, we determined that MET may influence the effect of CH through two pathways: "Cysteine and methionine metabolism" and "Glycine, serine, and threonine metabolism".

The Cysteine and methionine metabolism pathway is known to play a crucial role in immunity and digestion in organisms (Martínez et al., 2017). Its key compound, MET, not only influences lipid metabolism but also activates endogenous antioxidant enzymes like methionine sulf oxide reductase A and participates in glutathione biosynthesis, thereby aiding in combating oxidative stress. MET is an essential amino acid that can be converted into S-adenosylmethionine (SAM) (He et al., 2019). SAM serves as a methyl donor for various methyltransferases involved in the methylation of DNA, RNA, proteins, lipids, and other molecules (He et al., 2019). In cases of MET abundance, guanidinoacetate N-methyltransferase (GAMT) plays a crucial regulatory role by catalyzing the

synthesis of Cr and S-adenosylhomocysteine from SAM and guanidinoacetate (Wyss and Kaddurah-Daouk, 2000). As for Cr, which was involved in the glycine, serine and threonine metabolism, reported effective in improving locomotion performance and strength (D'Antona et al., 2014). Cr within the intracellular environment undergoes transphosphorylation catalyzed by creatine kinase (CK), in the presence of ATP, resulting in the formation of phosphocreatine (PCr), a high-energy compound. Subsequently, the generated PCr enters the PCr pool, serving as a buffer to maintain cytosolic ATP/ADP ratios and balance local ATP consumption (Andres et al., 2008). External factor intrusion usually causes energy loss in the cell, i.e. ATP disorder, which leads to an increase in the  $\text{Ca}^{2+}$  levels. In such instances, if the PCr pool is sufficient, PCr can undergo dephosphorylation, generating ATP and activating the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) pumps. This enables the absorption of excess  $\text{Ca}^{2+}$  from the cytoplasm into the sarcoplasmic reticulum (Andres et al., 2008; De Groof et al., 2002; Pizzo et al., 2012; Salazar-Ramírez et al., 2021; Wuytack et al., 2002). In our study, cysteine and methionine metabolism pathway was significantly enriched in CK-MET, MET-CH, MET-MIX and CH-MIX groups, and its key compound MET content was significantly up-regulated in MET and MIX treatment, respectively. The content of creatine (Cr), an intermediate metabolite in this pathway, was up-regulated in MET and MIX treatments, while CH treatment markedly suppressed its levels. Similarly, the content of phosphocreatine (PCr), a high-energy compound, mirrored the changes observed in Cr, showing up-regulation in MET and MIX treatments. Considering that the mechanism of action of CH involves the excessive release of intracellular calcium stores from the sarcoplasmic reticulum, the alterations in MET, Cr, and PCr contents imply that the addition of MET may have increased the levels of Cr and PCr in honeybees, thereby mitigating the dysregulation of calcium release caused by CH, thus reducing its toxicity to bees. (Fig. 5).

## 5. Conclusion

The present study demonstrated that the concentration of CH used in



**Fig. 5.** Functional effects of methionine in honeybee "Cysteine and methionine metabolism" and "Glycine, serine and threonine metabolism" (1) MET ingested by the honeybees is initially converted into S-adenosylmethionine (SAM). (2) SAM combines with guanidinoacetate and is catalyzed by guanidinoacetate N-methyltransferase (GAMT) to generate creatine (Cr) and S-adenosylhomocysteine (SAH). (3) Within the mitochondria, Cr undergoes catalysis by creatine kinase (CK), utilizing ATP, resulting in the production of phosphocreatine (PCr) and ADP. (4) PCr is stored in the PCr pool, serving as a buffer for maintaining cytosolic ATP/ADP ratios and supporting localized ATP consumption (5) In response to excessive release of intracellular  $\text{Ca}^{2+}$ , PCr from the PCr pool is catalyzed by CK to regenerate Cr and ATP. The generated ATP then activates sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) located in the sarcoplasmic reticulum, facilitating the absorption of excess intracellular  $\text{Ca}^{2+}$ .

practice had a significant impact on the expression of certain immune genes and metabolic profiles in honey bees. However, MET supplementation was found to alleviate the toxicity of CH to honeybees. Our observations indicated that the addition of MET to CH led to a significant increase in the levels of creatine (Cr) and phosphocreatine (PCr) in bees. This increase in Cr and PCr content may help mitigate the dysregulation of intracellular calcium release and immune stress caused by CH in honeybees. These findings offer a new perspective for future studies on the risk assessment of bees exposed to CH and the potential development of MET as a novel biopesticide or protective agent.

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### CRediT authorship contribution statement

**Zhaoyong Liu:** Funding acquisition, Investigation, Methodology, Data curation, Validation, Visualization, Software, Formal analysis, Writing - Original draft. **Fangtong Wu:** Data curation, Validation. **FuQiang Li:** Investigation. **Yue Wei:** Funding acquisition, Conceptualization, Supervision, Writing – review & Editing, Resources, Validation. All authors reviewed the manuscript.

### Declaration of Competing Interest

I, Zhaoyong Liu, hereby declare that I have no conflict of interest to disclose in relation to the submitted manuscript titled "Methionine can reduce the sublethal risk of Chlorantraniliprole to honeybees (*Apis mellifera L.*): Based on metabolomics analysis" for publication in Ecotoxicology and Environmental Safety.

A conflict of interest arises when there are personal, financial, or professional relationships or affiliations that may influence or be perceived to influence the research and its outcomes. However, I affirm that no such conflicts exist for any of the authors involved in this study.

This research has been conducted with utmost integrity and impartiality, adhering to the ethical standards and guidelines set forth by the scientific community. Our findings and conclusions have been solely derived from the data and analysis presented in the manuscript, without any external influences that may compromise the objectivity or credibility of our work.

We are committed to transparency and scientific integrity and assure the editorial board and readers of Ecotoxicology and Environmental Safety that there are no competing financial, personal, or professional interests that could affect the interpretation or presentation of our research.

Should any conflicts of interest arise during the publication process or after its completion, we pledge to promptly disclose them to the Editor-in-Chief.

### Data Availability

Data will be made available on request.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115682.

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