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Thiacloprid exposure perturbs the gut microbiota and reduces the survival status in honeybees

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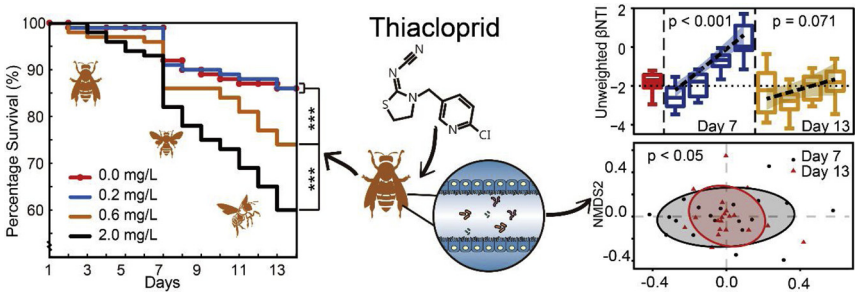
GRAPHICAL ABSTRACT



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ABSTRACT

Honeybees (*Apis mellifera*) offer ecosystem services such as pollination, conservation of biodiversity, and provision of food. However, in recent years, the number of honeybee colonies is diminishing rapidly, which is probably linked to the wide use of neonicotinoid insecticides. Middle-aged honeybees were fed with 50% (w/v) sucrose solution containing 0, 0.2, 0.6, and 2.0 mg/L thiacloprid (a neonicotinoid insecticide) for up to 13 days, and on each day of exposure experiment, percentage survival, sucrose consumption, and bodyweight of honeybees were measured. Further, changes in honeybee gut microbial community were examined using next-generation 16S rDNA amplicon sequencing on day 1, 7, and 13 of the exposure. When compared to control-treatment, continuous exposure to high (0.6 mg/L) and very high (2.0 mg/L) concentrations of thiacloprid significantly reduced percentage survival of honeybees ($p < 0.001$) and led to dysbiosis of their gut microbial community on day 7 of the exposure. However, during subsequent developmental stages of middle-aged honeybees (i.e. on day 13), their gut microbiome recovered from dysbiosis that occurred previously due to thiacloprid exposure. Taken together, improper application of thiacloprid can cause loss of honeybee colonies, while the microbial gut community of honeybee is an independent variable in this process.

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1. Introduction

Honeybees (*Apis mellifera*) support the global ecosystem by offering services such as pollination, conservation of biodiversity, and provision of food (Aizen et al., 2008). Honeybees pollinate approximately 90 percent of flowering plants and 75 percent of crop plants. However, in recent years, a number of causes, including destruction of habitat, widespread usage of pesticides (Doublet et al., 2015), over-intensive agriculture (Goulson, 2015), and specific parasites such as bacteria, and viruses (Fries, 2010; Philipp et al., 2013) have been linked to the decline in the number of honeybee colonies worldwide. Many of these factors, singly or in combination, can threaten survival and reproduction of honeybees (Brown et al., 2016).

On the other hand, the widespread use of neonicotinoid insecticides was considered as the main cause for the loss of honeybee colonies (Xiao et al., 2019; Farooqui, 2013). Neonicotinoids are broad-spectrum insecticides (Hladik et al., 2018) and are less toxic to mammalian systems when compared to previously employed insecticides such as carbamates, organophosphates, and pyrethroids (Jeschke et al., 2013). Further, these are amenable to an eco-friendly application (seed coating) instead of aerial spraying (Douglas and Tooker, 2015). These are systemic insecticides, which translocate from the seed-coat to different distal plant-parts such as leaves, flowers, and pollen; thus, protect the whole plant from insect pests (Simon-Delso et al., 2015). Because of these multiple advantages, usage of neonicotinoids has drastically increased during the past two decades (Hladik et al., 2018) and currently, these occupy 25 percent of global insecticide market (Bass et al., 2015). Neonicotinoids severely affect the central nervous system of insects by binding to the nicotinic acetylcholine receptors and blocking the neural signal transmission (Nauen et al., 2001). These indiscriminately affect both target and nontarget insects. Nontarget insects are honeybees and butterflies. Neonicotinoids were detected in nectar, pollen, and honey (Bonmatin et al., 2015). Honeybees through their collective behavior, get exposed to these insecticides and contaminate the whole colony (Laurent and Rathahao, 2003). 75% of honeybee colony samples contain at least one neonicotinoid (Tsvetkov et al., 2017). Neonicotinoids have been shown to reduce honeybee foraging (Mommaerts et al., 2010), life span, and immunity (Brandt et al., 2016; Dively et al., 2017).

After the availability of several lines of evidence about negative effects of neonicotinoids on honeybees, in 2013, European Union temporarily banned three of the most widely used neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) (Authority, 2013). Subsequently, from December 2018, these three neonicotinoids are banned from outdoor use (not limited to greenhouses and other countries) (Commission, 2018).

Other neonicotinoids, like thiacloprid, was not banned by the regulatory agencies because they are considered to be less-toxic under field conditions (Siede et al., 2017). However, thiacloprid affected honeybee immunity (Brandt et al., 2016) and behavior (Tison et al., 2016 and 2017). The lower median lethal dose (LD_{50}) of thiacloprid may have underestimated its toxicity to honeybees (Iwasa et al., 2004).

Interestingly, honeybees preferred to consume pollens contaminated with neonicotinoids (Kessler et al., 2015) and these compounds were thought to alter gut microbial communities in insects and vice versa. *Lactobacillus plantarum* of Drosophila gut mitigated neonicotinoid induced pathogen susceptibility in its host (Daisley et al., 2017). Honeybees, like mammals, acquire gut bacteria through sociality (Powell et al., 2014). Honeybees have a simple, yet specialized gut bacterial community; when compared to that of humans and other mammals, it has only eight core bacterial species, accounting for 95–99 % of gut bacteria (Sabree et al., 2012). The honeybee gut microbiome has been shown to play a role in its host metabolism, growth, development, and immunity (Philipp et al., 2013; Kwong et al., 2017a). For example, *Gilliamella apicola* and *Snodgrassella alvi* are required for nutrition and pathogens resistance (Lee et al., 2015). However, the invasion of opportunistic pathogens caused the dysbiosis of the honeybee gut microbiome (Brown et al., 2012). Further, the dysbiosis increased the mortality of worker honeybees (Raymann et al., 2017). Therefore, stable gut microbiome is essential for the health of honeybees.

Worker honeybees have a shorter life-span with distinct developmental stages such as 'nursing' and 'nectar-collection'. In the first three weeks (or 20 days), worker honeybees function as 'nursing honeybees', which build nests, clean nests and feed larvae. In the next developmental stage, 'nursing honeybees' become 'collecting honeybees', which fly out of the hive to collect nectar (Neukirch, 1982; Remolina and Hughes, 2008). The immunity of worker honeybees is optimum until they get transformed from'nursing honeybees' to 'collecting honeybees' (Munch et al., 2008). Honeybees obtain their gut microbiota within a few hours post-emergence from pupa and the colonization is complete within 6 days (Horner-Devine and Bohannan, 2006; Powell et al., 2014). The stability of honeybee gut microbial community decreases with age (Ellegaard and Engel, 2019). These unique characteristics of honeybee gut flora along with its strict social behavior make honeybee a model organism for intestinal flora research (Bonillarosso and Engel, 2018). However, the dynamics of the gut microbiota in different developmental stages of the honeybee has not been fully studied. Particularly, little is known about the relationship between honeybee gut microbiota and health under environmental stress such as insecticide exposure.

In this study, we investigated changes in the survival status of honeybees when exposed to thiacloprid at different developmental stages (or day 1 through 13) of middle-aged honeybees (see 'experimental design' under 'material and methods' of this manuscript). Further, we evaluated the relationship between the composition of honeybee microbiota and thiacloprid on day 1, 7, and 13. The results showed that continuous exposure to higher concentrations of thiacloprid (0.6 and 2.0 mg/L) caused significantly higher mortality in honeybees and dysbiosis of their gut microbiome on day 7 of middle-aged honeybees. The survival of honeybees was not affected when exposed to a low concentration of thiacloprid (0.2 mg/L). Meanwhile, on day 13 of middle-aged honeybees, we also observed the recovery of the gut microbial community from dysbiosis, which was independent of the monotonous decline of percent survival.

2. Materials and methods

2.1. Chemicals and solutions

Whenever required, 50% (w/v) sucrose solution was made just before use in the experiment. Thiacloprid stock solution (10 mg/L) was prepared by dissolving 1.0 mg thiacloprid powder (Sigma-Aldrich, St. Louis, MO, USA) in 100 mL 50% (w/v) sucrose solution in an amber laboratory bottle. The thiacloprid stock solution was divided into several aliquots as per the requirement of experiment and stored at −80 °C in dark. Two hours prior to the start of the daily-exposure during the 13-day experiment, the frozen thiacloprid stock solution was thawed and diluted to the working concentrations (0.2, 0.6 and 2.0 mg/L) using the freshly prepared 50% (w/v) sucrose solution and then filter-sterilized. The control group of honeybees was fed with freshly-prepared and filter-sterilized 50% (w/v) sucrose solution without thiacloprid (0 mg/L).

2.2. Experimental design

Colonization of honeybee-gut with microbes begins within a few hours and is completed by day 4 after the emergence of honeybee from its pupal stage (Ellegaard and Engel, 2019). Honeybees begin to age after three weeks of the emergence. We considered honeybees as 'young', 'middle-aged', and 'old', respectively, during the 1–7, 8–22 and after 22 days post-emergence (Fig. 1A).

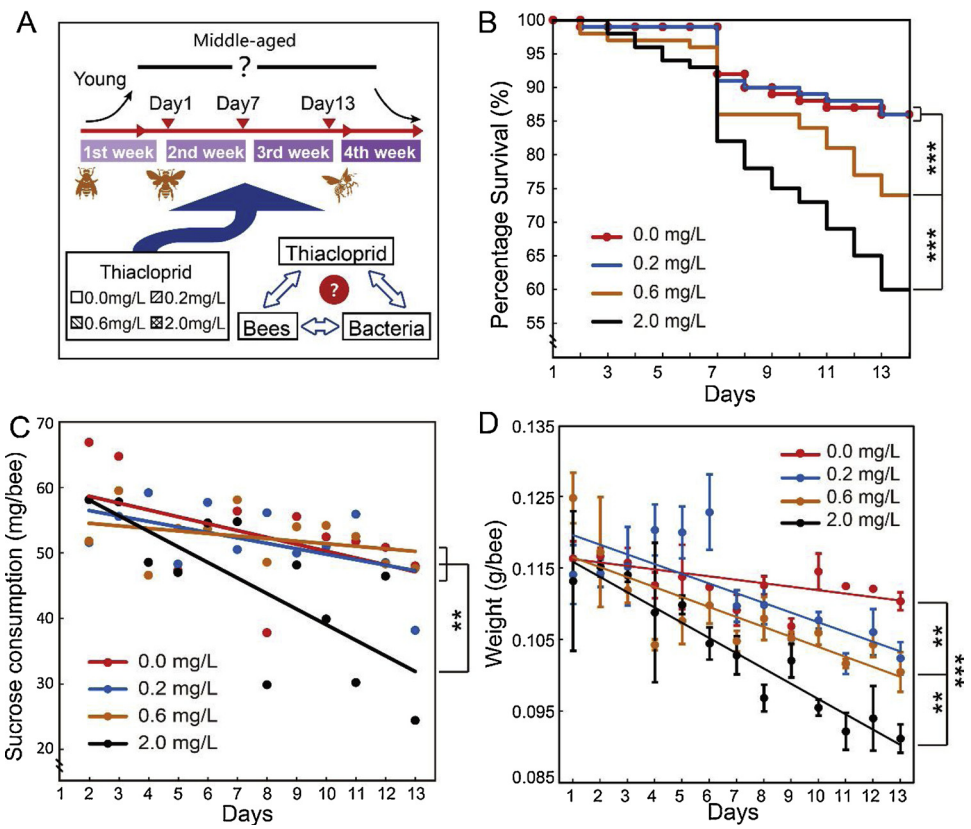

Fig. 1. Overview of the experimental design and the effect of thiacloprid on honeybees survival, sucrose consumption and bodyweight. A, the experimental design: Honeybees were considered as 'young', 'middle-aged' and 'old' during the 1–7, 8–22 and after 22 days post-emergence, respectively. Middle-aged honeybees were exposed to different concentrations of thiacloprid (0, 0.2, 0.6, and 2.0 mg/L) for 13 days. On each day of the exposure experiment, middle-aged honeybees were sampled in order to study the effects of thiacloprid on honeybees. Further, in order to study the effect of thiacloprid on middle-aged honeybee gut microbiome, samples were collected on day 1, 7, and 13; the same is indicated in the diagram using the inverted triangles. B: Kaplan–Meier survival curve depicting the effect of the four different concentrations of thiacloprid on the percentage survival of honeybees. Statistical analyses were performed using the Wilcoxon Rank-sum test (*** $p < 0.001$). C: Effect of the four different concentrations of thiacloprid on sucrose consumption by per honeybee. Statistical analyses were performed using the Wilcoxon Rank-sum test (** $p < 0.01$). D: The effect of the four different concentrations of thiacloprid on bodyweight of per honeybees. Statistical analyses were performed using the permutation test (** $p < 0.01$; *** $p < 0.001$).

'Young' honeybees, from day one to seven after their emergence, were fed with sterile 50% (w/v) sucrose solution without thiacloprid and pollen. On day 8 post-emergence (or on day 1 of middle-aged honeybees), a total of 485 honeybees were collected for the experiment. Out of 485 honeybees, 5 were used as initial samples for all the measurements and the remaining (i.e. 480) honeybees were divided into four groups containing 120 in each group. Subsequently, to study the effect of thiacloprid exposure on honeybee survival, sucrose consumption, and their gut microbiome, each group of the honeybees was fed with sterile 50% (w/v) sucrose solution containing a different concentration of thiacloprid. The four thiacloprid concentrations were: 0 (T0), 0.2 (T0.2), 0.6 (T0.6) and 2.0 (T2.0) mg/L. T0.2 and T0.6 represented the average (Brandt et al., 2016) and the highest (David et al., 2016) concentration of thiacloprid detected in the environment, respectively. Whereas T2.0 represented an acute toxic concentration i.e. 10 times the average concentration.

The exposure experiment was completed within two weeks to ensure that all honeybees were at their middle-age. Hereafter, day 1 is the first day of exposure experiment and it is also day 1 for middle-aged honeybees. On each day of 13-day exposure experiment with the four different concentrations of thiacloprid, percentage survival, sucrose consumption, and bodyweight of honeybees were monitored. These parameters represent the performance of honeybees under thiacloprid exposure stress. Further, changes in gut microbiomes of honeybees during exposure to different concentrations of thiacloprid were quantified using next generation 16S rDNA amplicon sequencing. Towards this, 5 honeybees from control and each thiacloprid treatment were collected on day 1, 7 and 13 of experiment. The changes in honeybee gut microbiome tease apart relationships among honeybees, the gut microbiome, and thiacloprid.

2.3. Sample and data collection

All the honeybees used in this study were collected from brood frames of a single hive at the Department of Honeybee Protection and Biosafety, Chinese Academy of Agricultural Sciences, Beijing, China, soon after their emergence from pupa (i.e. 1-day old 'young' honeybees). The honeybees were visually identified as healthy i.e. the honeybees were free from *Varroa* mites, bacterial diseases (American foulbrood and European foulbrood) and fungal diseases (Nosema, Chalkbrood and Stonebrood).

The honeybees were placed in a wooden box and incubated in dark at 30 °C and 50% relative humidity using an artificial climate incubator (RXZ - 380C, Ningbo Jiangnan, Ningbo, China) during August of the year 2017. During the 1–7 days prior to the exposure experiment, considering the demand for protein and other nutrients by the newly emerged (or 'young') honeybees in order to become healthy 'middle-aged' honeybees, sufficient quantity of 50% (w/v) sterile sucrose solution and pollen were provided. The pollen-feed helps the colonization of young honeybee gut by microbial communities. 50% (w/v) sucrose solution was fed to young honeybees using two microtiter plates (Corning Incorporated, Corning, NY, USA) (12 wells * 4 rows, 200 µl per well). The pollen was fed to young honeybees using two microtiter plates (12 wells * 2 rows, 200 µl per well). Both the pollen and the sucrose solution were considered sufficient supplies for honeybees. On each day, during the 1–7 days prior to the exposure experiment, at 10 a.m., new microtiter plates containing the sucrose and pollen that were prepared afresh were provided to the honeybees. By changing the microtiter plate with feed every day, sufficient food supply to the honeybees was ensured. We found that honeybees in the first three days were prefer to pollen, and later were more likely to eat sucrose solution.

On the day 8 post-eclosure i.e., the first day of the exposure experiment (or day 1 of middle aged honeybee), five bees from the wooden box in the artificial climate incubator, were collected, anaesthetised on ice and weighed. The remaining honeybees were assorted into four wooden boxes, each containing 120 honeybees. The wooden boxes were marked accordingly and returned to the climate incubator. Four concentrations of thiacloprid sucrose solution (0, 0.2, 0.6 and

2.0 mg/L) were provided to different boxes at 10 a.m. each day of exposure experiment. These thiacloprid sucrose solutions were prepared within two hours of the start of the daily exposure experiment. Five honeybees from each wooden box were collected on each day of 13-day exposure experiement for measuring their body weight. Similarly, on day 8 and day 13 of exposure experiment, five honeybees were sampled from each wooden box for gut microbiota studies, anaesthetised on ice and weighed. The sampled honeybees were placed in labeled centrifuge tubes (1.5 mL) and stored at −80 °C until use.

Honeybee whole gut was carefully collected from each honeybee using sterile forceps and transferred into a fresh centrifuge tube (1.5 mL). The tubes containing honeybee guts were stored at −80 °C until the DNA was extracted for further use. The percentage survival of honeybees was calculated from the counts of live and dead honeybees. For the determination of the mean bodyweight of honeybees, 5 live honeybees were anaesthetised on ice, placed in a sterile nylon bag and weighed using analytical balance (Mettler-Toledo, Shanghai, China). The daily sucrose consumption by honeybees was obtained by weighing the microtiter plate before and after feeding for 24 h and calculating the weight difference. Since the honeybees were raised in groups, the sucrose consumption by each honeybee was calculated by the formula: [each day sucrose consumption / (number survived the previous day - number died that day/2)]. The exposure concentration of thiacloprid per honeybee was obtained by multiplying the amount of sucrose solution consumed by per honeybee and the concentration of thiacloprid in the sucrose solution.

2.4. Nucleic acid extraction

Microbial DNA was extracted from each honeybee gut sample using the FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA, USA). The DNA was dissolved in Tris-EDTA (50 μL; pH 8.0) buffer, quantified with NanoDrop 2000 (ThermoFisher, Shanghai, China) and stored at −80 °C until use.

2.5. Quantitative polymerase chain reaction (qPCR)

The gut bacterial abundances of honeybees were estimated using qPCR of 16S rRNA genes with the forward primer 338 F (5′ –ACT CCT ACG GGA GGC AGC AG– 3′) and the reverse primer 806R (5′ –GGA CTA CHV GGG TWT CTA AT– 3′). Reactions (20 μL) were carried out in triplicate with 10 μL TB Green Premix Ex Taq (Takara Bio-Rad, Mountain View, CA, USA), 0.4 μL 10 μM primer (each), 0.4 μL ROX Reference Dye, 6.8 μL H_2O, and 2 μL template DNA. The PCR cycle was 95 °C (30 s) followed by 40 cycles of 95 °C (5 s) and 60 °C (30 s) and this reaction was performed on the 7500 Real-Time PCR System (ThermoFisher, Shanghai, China). The absolute gut bacterial copy number was determined using the standard curve that was generated from amplification of the cloned target sequence in a pGEM-T vector (Sangon Biotech, Shanghai, China).

2.6. Illumina sequencing

The V3-V4 hypervariable regions of 16S rRNA genes were amplified in triplicate using PCR with the primer set 338 F (5′ –ACT CCT ACG GGA GGC AGC AG– 3′) and 806R (5′ –GGA CTA CHV GGG TWT CTA AT– 3′) from each honeybee gut sample. The 5-bp bar-code was fused to each forward primer. PCR was conducted in 50 μL reaction mixtures under the following conditions: initial denaturation at 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The triplicate PCR products were pooled and purified using the QIAquick PCR Purification kit (Qiagen, Frankfurt, Germany), and then quantified based on the QuantiFluor™ dsDNA System (Promega, Madison, WI, USA). The purified amplicons from different samples, after normalization to yield equimolar amounts, were sequenced on an Illumina MiSeq platform (Illumina, Santiago, CA, USA).

2.7. Sequence preprocessing

Sequence reads were processed using Quantitative Insights Into Microbial Ecology (QIIME™). FASTQ files were filtered for quality with split_libraries_fastq.py allowing a minimum Phred quality score of Q20. Forward and reverse Illumina reads were joined using join_paired_ends.py with default settings. Chimeric sequences were removed using the Usearch 6.1 detection method implemented in the identify_chimeric_seqs.py script in QIIME. All sequences were clustered at 97% similarity level to obtain operational taxonomic units (OTUs), and the platform for clustering was Usearch (version http://drive5.com/uparse/ 7.0).

To obtain species classification information corresponding to each OTU, the RDP classifier Bayesian algorithm was used for the taxonomic analysis of OTUs representative sequences of 97% similar levels, and the database selected was Silva (Release128 http://www.arb-silva.de). To eliminate pyrosequencing errors, all OTUs with less than 0.1% abundance were removed.

2.8. Statistical analyses

Downstream analyses including alpha and beta diversity estimates were performed using the QIIME workflow core_diversity_analysis.py with a sampling depth of 30,530 reads and default parameters per sample. The absolute abundance of each bacterium was estimated by multiplying the total number of 16S rRNA genes (measured by qPCR and adjusting for rRNA operons per genome) by the relative abundance percentage of each bacterium (Motta et al., 2018).

Bray-Curtis distance matrices were constructed using rarefied OTUs abundance table and visualized by Nonmetric multidimensional scaling (NMDS) analysis and principal coordinate analysis (PCoA). Analysis of similarities (ANOSIM) was used to further examine the significance of the differences with 10,000 permutations. Both types of analysis can be used to compare the composition of microbial communities and can be used to visualize the differences of microbial communities between different samples (groups). The basic output of the comparison is a distance matrix representing the community difference between every two samples. Both analytical methods were calculated and plotted with the vegan package in R.

The explanatory power of factors, such as the concentration of thiacloprid, of the observed assembly of bacteria was determined by canonical correspondence analysis (CCA), which combined correspondence analysis with multiple regression analysis and conducted regression with environmental factors at every step of the calculation. The maximum Pearson correlation coefficient of environmental factor and sample community distribution difference was determined by bioenv function, and the subset of the environmental factor was obtained by the maximum correlation coefficient. The significance of CCA was determined by a permutest analysis similar to one-way analysis of variance (ANOVA). In the analysis of CCA, we adopted the R language vegan package to analyze and map CCA.

Nearest-taxon index (NTI) was used to evaluate the phylogenetic community composition within the community, with high or positive values indicated the clustering of taxa in the whole phylogenetic community, while low or negative values indicated the excessive dispersion of taxa across the phylogenetic community (Horner-Devine and Bohannan, 2006). The value of NTI was equal to the negative value of mean nearest-taxon distance (MNTD) standardization effect size, which was calculated by comparing the observed phylogenetic relatedness with the expected pattern, under the “taxa.lables” null model with 1000 randomizations in the “picante” R package. The unweighted β mean nearest-taxon distance (βMNTD) was calculated by “Phylocom” package to infer community phylogenetic turnover between communities in R. The value of β nearest-taxon index (βNTI) can be used to

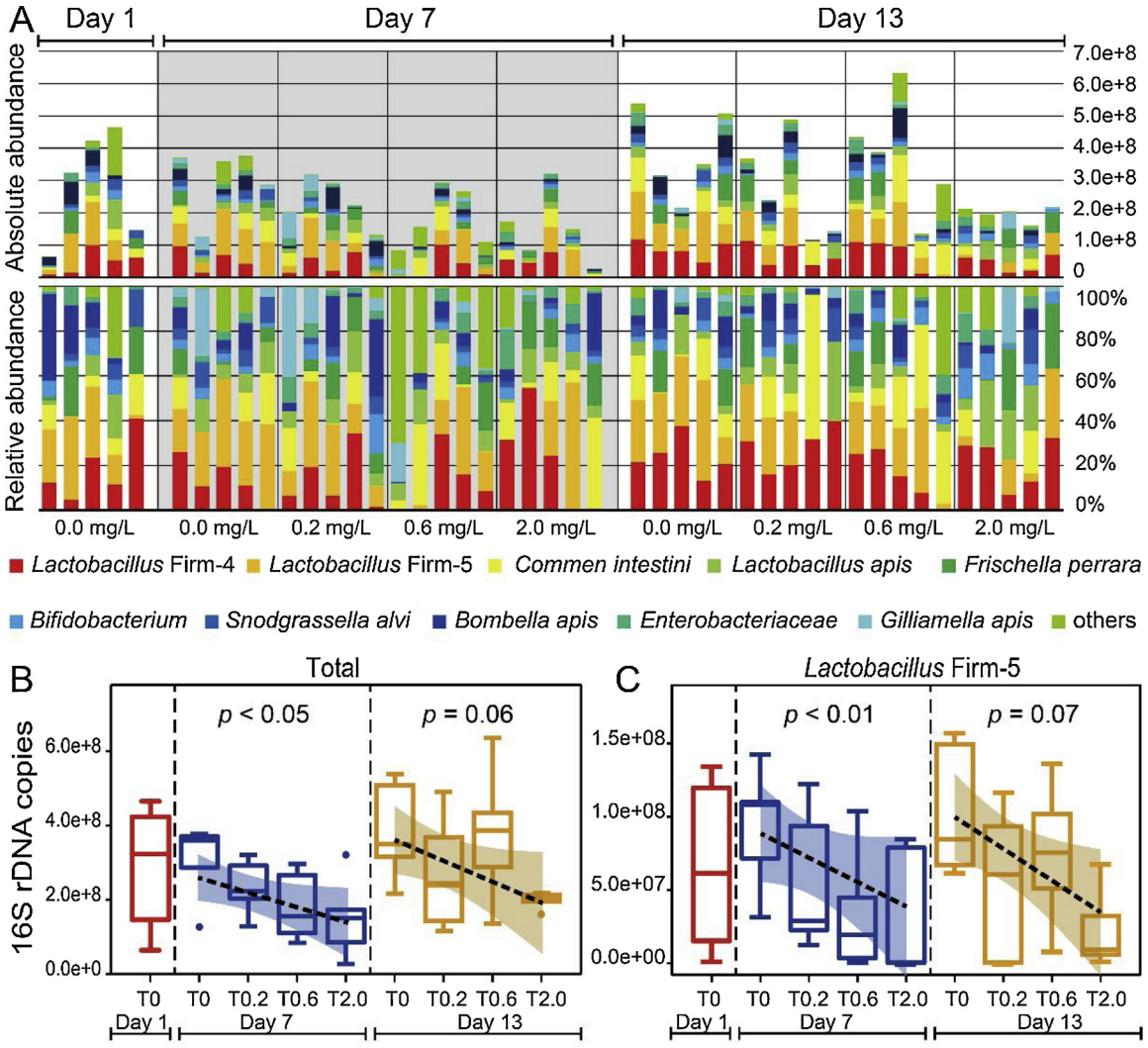

Fig. 2. Changes in gut microbiome composition of middle-aged honeybees after thiacloprid exposure. A: Stacked column graph showing the relative and absolute abundances of middle-aged honeybee gut core bacterial species in control (0 mg/L) and thiacloprid-treatments (0.2, 0.6, and 2.0 mg/L) on day 1, 7 and 13 of post-emergence from pupa. Each stacked column represents gut microbiome composition fromone of the 5 honeybees from every sample. B: Box-and-whisker plots of total number of 16S rDNA copies present in the gut microbiome of control (T0, 0 mg/L) and thiacloprid-treated (T0.2, 0.2; T0.6, 0.6 and T2.0, 2.0 mg/L) honeybees on day 1, 7 and 13 of post-emergence from pupa. The results (p-values) of the linear regression analysis are shown above each group. Each box-and-whisker plot shows the highest datum (top of the vertical line), lowest datum (bottom of the vertical line), median value (horizontal line in the box), first quartile (lower edge of the box) and third quartile (upper edge of the box). The black dotted line represents the linear line that represents the data set and the shades of different colours show the 95% confidence intervals. The actual concentration distance of thiacloprid was used in both line fitting and one-way analysis of variance. The same analysis was adopted for the box-and-whisker plots showed in the later parts of this manuscript (e.g. Fig. 2C, 3A and B etc.). C: Box-and-whisker plots of 16S rDNA copies belonging to a honeybee gut core bacterial species: *Lactobacillus* Firm-5 from the control and thiacloprid treated honeybees on the day 1, 7 and 13 of post-emergence from pupa.

infer the status of stochastic and deterministic processes in community assembly. βNTI is the deviation between the expected value of βMNTD and the observed value. Since the expected value of βMNTD represents the dominance of stochastic processes, the value of βNTI can be used to infer the dominance of stochastic and deterministic processes. βNTI comparisons falling into a null distribution ($-2 < \beta NTI < 2$) indicate the advantage of stochastic processes, while βNTI greater than or less than 2 indicates the advantage of deterministic processes (Dini-Andreote et al., 2015). Bray-Curtis distances were adopted for the community distances used here, and Mentel test with linear regression analysis was used to demonstrate the relationship between βNTI (NTI) and the treatment concentration and time of thiacloprid.

Analyses were executed using R (http://www.r-project.org/), SPSS (SPSS, Chicago, IL, USA) or SigmaPlot (Systat Software, San Jose, CA, USA).

3. Results

3.1. Thiacloprid affects the health of honeybees

During the course of 13 day exposure experiment, the percentage survival of middle-aged honeybees decreased in the three thiacloprid concentrations [0.2 (T0.2), 0.6 (T0.6), and 2.0 mg/L (T2.0)] and the control [0.0 mg/L (T0)] (Fig. 1B). The decline of percentage survival in control (T0) group represented the natural variation of the honeybee population under incubation conditions without external thiacloprid stress. There was no significant difference in the percentage survival of honeybees between the control group (T0) and the low thiacloprid concentration (T0.2) (Rank-sum test, $p = 0.210$). Whereas, high (T0.6) and very high (T2.0) concentration of thiacloprid, when compared to the control (T0), exacerbated the decline in the percentage survival (Rank-sum test, $p < 0.001$; Rank-sum test, $p < 0.001$). On the last day of exposure experiment (i.e. day 13), when compared to the control

group, 25% reduction in survival of middle-aged honeybees was observed with the very high thiacloprid concentration (T2.0).

When compared to the control group (T0), there was no significant difference in the sugar consumption by honeybees for the low-concentration group (T0.2) (Wilcoxon Rank-sum test, $p = 0.230$) and the high-concentration group (T0.6) (Wilcoxon Rank-sum test, $p = 0.175$) (Fig. 1C). However, there was a significant decrease in sugar consumption by honeybees between the control (T0) and the very high concentration group (T2.0) (Wilcoxon Rank-sum test, $p < 0.01$).

While weight loss was not observed for the honeybees in control (T0) over time during the experiment, it was observed for the honeybees from all of the three thiacloprid treatments (T0.2, T0.6 and T2.0) (Fig. 1D). When compared to the honeybees in the control group, a significant reduction in weight was observed for both the low (T0.2) and high (T0.6) concentrations of thiacloprid (permutation test, $p < 0.01$). There was no significant difference in the weight loss of honeybees between the low (T0.2) and high (T0.6) concentration treatments (permutation test, $p = 0.166$). The honeybees that were exposed tot he very high concentration of thiacloprid (T2.0) significantly lost their weight when compared to the control (T0) and two other thiacloprid concentrations (i.e. T0.2 and T0.6) (permutation test, $p < 0.001$).

Similarly, there was no change in the daily consumption of thiacloprid per honeybee at low (T0.2) or high (T0.6) concentrations. However, there was a decrease in the daily thiacloprid consumption per honeybee at the very high (T2.0) concentration (Fig. S1A). In addition, thiacloprid residues in honeybees were determined by liquid chromatography-tandem mass spectrometry (LC–MS/MS) at the end of the experiment (i.e. on day 13). High levels of thiacloprid exposure resulted in elevated levels of thiacloprid residue in honeybees (Fig. S1B).

3.2. Thiacloprid perturbs honeybee gut microbiome

Relative and absolute microbial abundances were used to describe honeybee gut microbial communities. When compared to the control group on the day 1 and the day 7, the absolute abundance of the bacterial community in the control group on the day 13 increased significantly (independent sample T-test, $p < 0.05$) (Fig. 2A and Fig. S2). On the day 7, when compared to the control group, the total bacterial abundances for the three thiacloprid treatment groups decreased significantly, and the thiacloprid treatment showed a dose-effect (linear regression analysis, $F = 4.4$, $p < 0.05$, Fig. 2B). Although a decrease in absolute abundance was observed on day 13, the decrease did not show the dose-effect, which was observed on day 7 (linear regression analysis, $F = 4.05$, $p = 0.059$, Fig. 2B).

The honeybee gut bacterial community is simple with 10 core bacterial species (Sabree et al., 2012 and this study), and the relative abundance of these core species when combined was more than 96% of the total bacterial species (Fig. 2A and Table S1). Of the ten core bacteria found in the gut of honeybees, two were significantly affected by thiacloprid treatment on day 7. The absolute abundance of *Lactobacillus* Firm-5, a gram-positive bacterium, was significantly decreased with the thiacloprid treatment (linear regression analysis, $p < 0.01$, Fig. 2C), but not on day 13. Similarly, the absolute abundance of *Bombella apis*, a gram-negative bacterium, was significantly reduced by thiacloprid (linear regression analysis, $p < 0.05$, Fig. S3F), but was not affected on day 13. None of the core bacterial species were significantly affected by thiacloprid on day 13 (Fig. S3).

3.3. Thiacloprid affects gut bacterial diversity on day 7

Alpha diversity represents the species diversity within a community and we used Shannon Index as a measure of alpha diversity. On the day 7, thiacloprid treatment had a dose-effect on alpha diversity of middle-aged honeybee gut microbiome (linear regression analysis, $F = 9.90$, $p < 0.01$, Fig. 3A); the alpha diversity decreased with increase in the thiacloprid concentration. The similar decrease in alpha diversity was not apparent on day 13 (linear regression analysis, $F = 0.04$, $p = 0.856$, Fig. 3A).

Beta diversity represents the extent of similarity between the compositions of the two communities. In our study, we estimated beta diversity from the Bray-Curtis distances, which were calculated by comparing the microbial communities from control (T0) and treatment groups (T0.2, T0.6, and T2.0). On the day 7, thiacloprid treatment had a dose-effect on Bray-Curtis distances of middle-aged honeybee gut microbiome (linear regression analysis, $F = 10.30$, $p < 0.01$, Fig. 3B); the Bray-Curtis distances increased with increase in thiacloprid concentration. The increase of Bray-Curtis distances with the increase in thiacloprid dose, however, did not appear on day 13 (linear regression analysis, $F = 0.04$, $p = 0.840$, Fig. 3B).

The beta diversity, which compares the composition of any two different microbial communities, can also be visualized using non-metric multidimensional scaling (NMDS) analysis. The results of NMDS analysis were similar to those of Bray-Curtis distance analysis (Fig. 3C-D). On day 7, the gut microbial community composition of control group and treatment groups differed significantly from each other (ANOSIM, $p = 0.001$). On the day 7, microbial communities of the control group (T0) were more clustered and the control group microbial community differed significantly from the three treatment groups (T0.2, T0.6, and T2.0) (ANOSIM, $p = 0.021$, $p = 0.020$, and $p = 0.015$). Further, the differences in microbial community composition increased with the thiacloprid concentration. When compared to the control group (T0), the low concentration treatment group (T0.2) had the smallest difference, and the very high concentration treatment group (T2.0) had the largest difference. On the day 13, the microbial community composition of control group (T0) and treatment groups (T0.2, T0.6, T2.0) did not differ significantly from each other (ANOSIM, $p = 0.175$).

3.4. Honeybee gut microbiome recovers from the dysbiosis on day 13

Distance decay analysis indicated that the similarity in the honeybee gut bacterial community composition varied significantly between the day 7 and the day 13 with different concentrations of thiacloprid treatment (permutation test, $p < 0.05$, Fig. 4A). On day 7, the Bray-Curtis distance increased significantly with thiacloprid treatment (mean slope values of 0.13 and slope 95% confidence intervals of (0.047, 0.207)). On day 13, this phenomenon did not happen, and the Bray-Curtis distance remained stable (mean slope values of -0.01 and slope 95% confidence intervals of (-0.064, 0.053)).

Also, we used canonical correspondence analysis (CCA) to determine the potential physical and chemical factors (such as age, sucrose-feeding, weight, accumulation of thiacloprid and concentration of thiacloprid) that affect honeybee gut microbial communities (Fig. 4B). Accumulation of thiacloprid (thiacloprid) significantly affected the honeybee gut microbial community composition ($p = 0.001$), and the concentration gradient of thiacloprid (thiacloprid level) was positively correlated with the accumulation of thiacloprid ($p < 0.01$). These two factors related to thiacloprid dominated the construction of the honeybee gut microbial communities (Table S2). Age phenotype (age) was also an important factor affecting microbial communities ($p < 0.05$). However, the arrow representing age phenotype pointed in the opposite direction from the arrow representing thiacloprid treatment, which suggests a negative correlation between the age phenotype and the thiacloprid treatment. Although it is not clear whether the age phenotype attempted to counteract the overwhelming role of thiacloprid in the construction of microbial communities, the aging phenotype was not as strong as that of the thiacloprid treatment.

NMDS analysis based on relative abundances of honeybee gut community members revealed that the microbiome compositions were different of all honeybees gut sample on day 7 and 13 (ANOSIM, $p < 0.05$, Fig. 4C). While microbial community composition of honeybees on day 7 was widely distributed, it was tightly clustered on day 13.

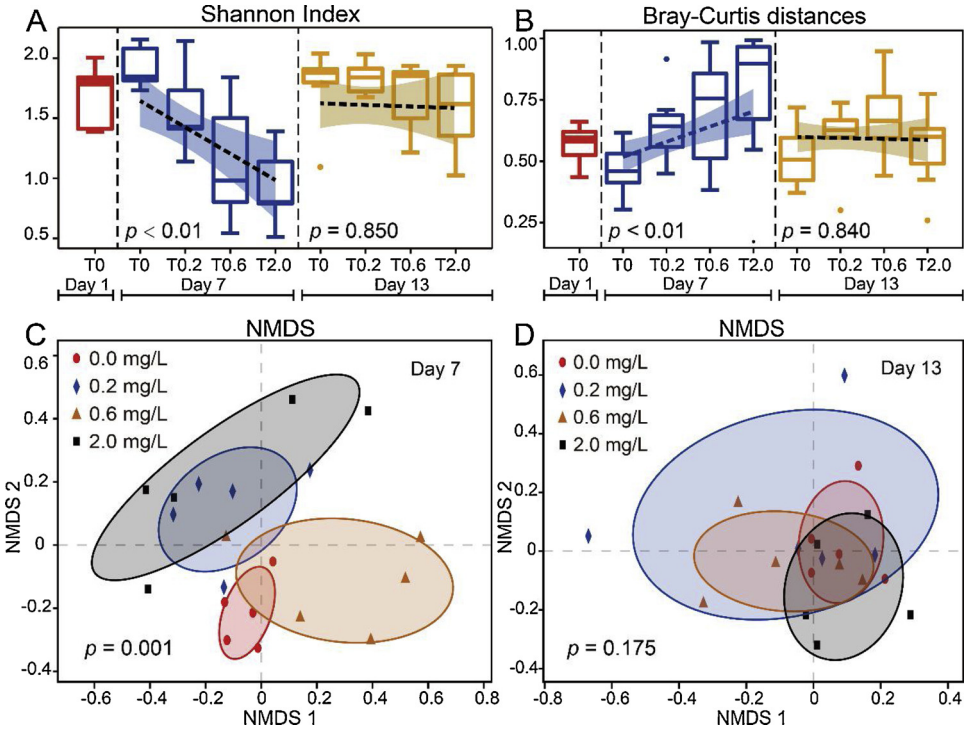

Fig. 3. Alpha and beta diversities of thiacloprid exposed and control honeybees. A: Box-and-whisker plots of alpha diversity (as determined by Shannon Index) of gut microbiomes of honeybees belonging to control (T0, 0 mg/L) and thiacloprid-treatments (T0.2, 0.2; T0.6, 0.6 and T2.0, 2.0 mg/L) on day 1, 7 and 13 of post-emergence from pupa. B: Box-and-whisker plots of beta diversity (as determined by Bray-Curtis distance) of gut communities of control and thiacloprid-exposed honeybees. C: Nonmetric multidimentional scaling (NMDS) analysis of gut microbial communities belonging to control and thiacloprid-exposed honeybees on the day 7 (ANOSIM with 999 permutations; p = 0.001, pseudo-F statistic = 2.677). D: NMDS analysis of gut microbial communities belonging to control and thiacloprid-exposed honeybees on the day 13 (ANOSIM with 999 permutations; p = 0.175, pseudo-F statistic = 1.305).

More detailed analyses showed that microbial community composition belonging to the control (T0), high-concentration (T0.6) and very high-concentration (T2.0) of thiacloprid were tightly clustered on day 13 than on day 7.

The effect of thiacloprid concentration on honeybee gut microbiome was further evaluated using inner unweighted nearest-taxon index (βNTI) values. On day 7, βNTI increased from -2 to 0 (linear regression analysis, F = 69.2, p < 0.001, Fig. 4D). With the increase of thiacloprid concentration, the process of bacterial community construction changed from a deterministic process to a stochastic process. This

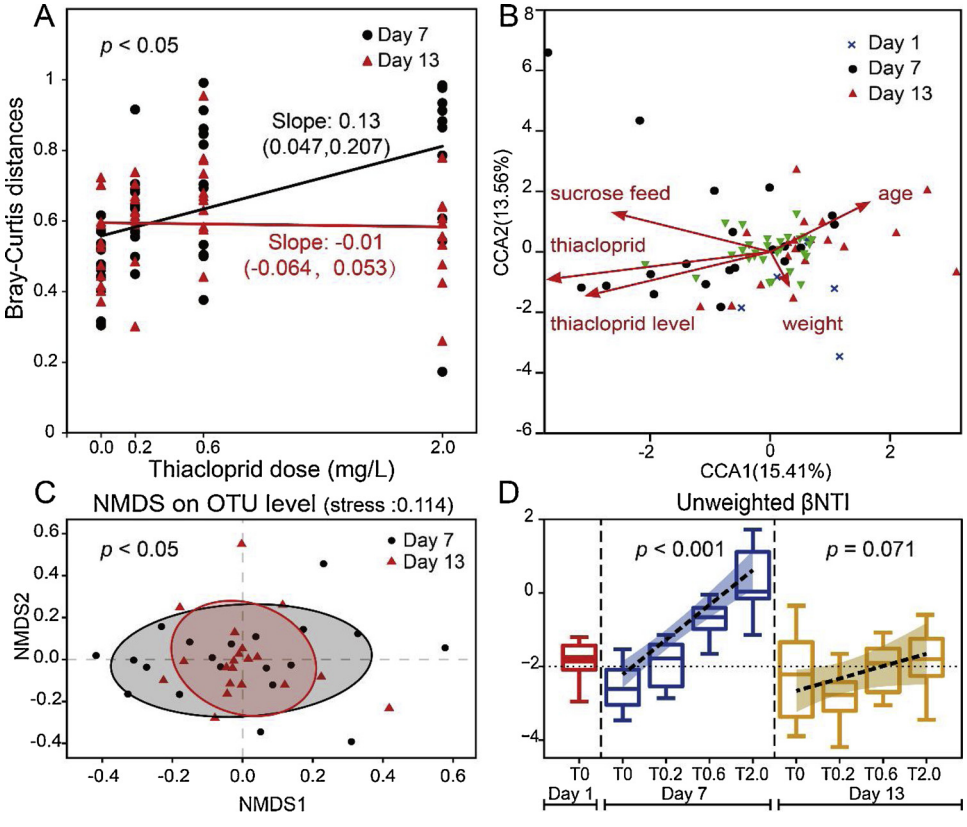

Fig. 4. Effect of exposure time and the role of environmental factors in shaping the structure of gut bacterial community in middle-aged honeybees. A: Distance decay analysis showing the trend of bacterial community similarity on day 7 and 13 for the four thiaclorpid concentrations; mean slope valuess are given and their 95 % confidence intervals were indicated in the parentheses. The X-axis represents concentration of thiacloprid, and the Y-axis represents the Bray-Curtis distance of the sample. Statistical analyses were performed using the permutation test (p < 0.05). B: Canonical correspondence analysis (CCA) examined the relationship between environmental factors, honeybee samples, and gut bacterial community, or between pairs. The dots with different colors or shapes represent the honeybees sample on different days (day 1, 7, and 13). The green triangle represents microbial species on OTU level; the red arrows indicate quantitative environmental factors and the statistical analyses were performed using the permutation test (Table S2). The angle between arrows of environmental factors represents positive and negative correlation (acute angle: positive correlation; obtuse angle: negative correlation; right angle: no correlation). C: NMDS analysis of honeybee gut community composition using Bray-Curtis at day 7 and 13 (ANOSIM with 999 permutations; p < 0.05, pseudo-F statistic = 2.097). D: Box-and-whisker plots withinner unweighted βNTI values for gut microbial communities in control (T0, 0 mg/L) and thiacloprid-treated (T0.2, 0.2; T0.6, 0.6 and T2.0, 2.0 mg/L) honeybees at day 1, 7 and 13 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

suggested that the community structure becomes more random due to the toxicity of thiacloprid. However, on day 13, βNTI did not change significantly and it remained at -2 (linear regression analysis, $F = 3.40$, $p = 0.072$, Fig. 4D). This indicated that the random effects caused by thiacloprid were not obvious at this time, and the construction of bacterial communities was mainly controlled by deterministic processes.

4. Discussion

In this study, we examined the effects of different concentrations of thiacloprid (T0, T0.2, T0.6, and T2.0) on the survival, sucrose consumption, and bodyweight of middle-aged honeybees. Further, during the exposure to different concentrations of thiacloprid, at three stages of middle-aged honeybees (i.e. on day 1, 7, and 13), we studied changes in their gut microbiomes.

4.1. Thiacloprid affects the health of honeybees

We found a reduction in the percentage survival, sugar consumption, and bodyweight of middle-aged honeybees during continuous exposure to thiacloprid. When compared to the control group, there was no significant reduction in honeybees percentage survival when they were exposed to a low concentration of thiacloprid (T0.2). However, exposure of middle-aged honeybees to high (T0.6) and very high (T2.0) concentrations of thiacloprid significantly increased honeybee mortality, suggesting that continuous exposure to thiacloprid can lead to a decline in honeybee colonies. A previous study has shown that, under field conditions, honeybee colonies were not affected by a long-lasting exposure to sublethal concentrations of thiacloprid (Siede et al., 2017). The apparent differences about the thiacloprid effects on honeybee mortlaity could be partially caused by differences between these two studies, such as experimental conditions (e.g., field versus laboratory), honeybee age, and food availability. Moreover, thiaclorprid is a neurotoxin and it interfered with learning and memory functions of honeybees (Tison et al., 2017; Shi et al., 2018). Further, honeybee blood cell density and capsulization were affected by thiacloprid (Brandt et al., 2016). Therefore, thiacloprid can have adverse effects on honeybee health.

Sucrose consumption by honeybees decreased significantly during the exposure to very high-concentration of thiacloprid (T2.0), when compared to all other treatments. Thiacloprid affected bodyweight of honeybees in a dose-dependent manner. While the honeybee weight of control group (T0) did not change significantly during the course of the experiment, thiacloprid treatments (T0.2, T0.6 and T2.0) markedly reduced the weight of honeybees. Honeybee weight is affected not only by their production activities but also by their sucrose consumption (Zheng et al., 2017). The decrease of sucrose consumption by each honeybee, at least during its exposure to very high-concentration of thiacloprid (T2.0), partly explains the observed weight loss in honeybees. However, the fresh sucrose solution was always available, so the honeybees may not have lost weight due to starvation. The dose effect exists in the residual thiacloprid in honeybees. High levels of thiacloprid, compared with the control group (T0), explain the decline in physiological status, including increased mortality, weight loss and reduced sucrose consumption. The thiacloprid-treated honeybees probably became anorexic, which might have indirectly disturbed the stability of honeybee gut microbial communities. Honeybee gut microbial community is an important factor that affects honeybee weight and its sucrose consumption. Similarly, by influencing the vitellogenin, insulin signal and taste response of the honeybee, the microbial communities promoted body-weight of the honeybee (Zheng et al., 2017; Bonillarosso and Engel, 2018).

4.2. Thiacloprid perturbs honeybee gut microbiome

The health of honeybees is affected by their environment and physiology. Honeybee physiology is regulated by the gut microbiota, which modulates its host metabolism, growth, development, and immunity (Kešnerová et al., 2017). The honeybees acquire their gut microbiota through sociality (Powell et al., 2014; Kwong et al., 2017b). The honeybee gut microbiome is simple and specific; it is consistently composed of eight to ten core bacterial species (Sabree et al., 2012; this study). The relative and absolute abundances of the core gut bacterial species as well as interactions among themselves are important for the health of their host (Philipp et al., 2013; Kwong and Moran, 2016). The normal and stable gut microbiome is beneficial to honeybees in the environment with or without hives (Shi et al., 2018).

We found that thiacloprid exposure significantly reduced absolute abundance (or total biomass) of honeybee gut microbiome in a dose-dependent manner on day 7 (but not on day 13). Specifically, on day 7 of thiacloprid exposure, two concentrations (T0.6 and T2.0) significantly reduced the absolute abundance of gut microbiota, when compared to the control group (T0). On day 13 of thiacloprid exposure, T2.0 concentration significantly reduced the absolute abundance (or total biomass) of gut microbiota, when compared to the control group (T0). In addition, on day 7, the absolute abundances of core honeybee gut bacterial species such as *Lactobacillus* Firm-5 and *Bombella apis* were significantly reduced in response to thiacloprid exposure in a dose-dependent manner. *Lactobacillus* Firm-5 is important for processing food and resisting pathogenic bacteria such as *Crithidia* (Schmidt and Engel, 2016). These results strongly suggest that thiacloprid not only affected the absolute abundance of honeybee gut microbiota but also significantly reduced the abundance of two of ten honeybee gut core bacterial species.

Also, an increase in the Bray-Curtis distance (inner and inter-group) of the gut microbial community on day 7 confirmed that the thiacloprid caused a disorder in the honeybee gut microbiome. Interactions between members of honeybee gut microbiome such as mutualism and antagonism, the responses of different species and genotypes depend on environmental conditions (Ellegaard and Engel, 2019). The order in which symbiotic bacteria colonized honeybee gut affected the susceptibility of their host towards infection by *Lotpanosomatid passim*, suggesting that disorder of gut microbiome promotes pathogen invasion (Regan et al., 2018). Environmental conditions such as exposure to antibiotics and pesticides can either damage honeybee gut microbiota or alter their immune system, which makes honeybees vulnerable to opportunistic pathogens (Brandt et al., 2016). Similarly, we found a strong correlation between thiacloprid exposure and reduction in honeybee percentage survival under laboratory conditions. Further, the reduction in percentage survival was accompanied by changes in honeybee gut microbiome i.e. the symbiotic core microbiome was replaced by the dysbiotic microbiome. Overall, while stable and undisturbed gut microbiome is important for honeybee health, thiacloprid disturbed the normal honeybee gut microbiome. It is possible that thiacloprid induced changes in honeybee gut microbiome affected the honeybee health. The relationship between gut microbes, hosts, and environmental pressures is complex and secretive, and further research is needed to reveal their interactions.

4.3. Honeybee gut microbiome recovers from the dysbiosis on day 13

Exposure of honeybees to thiacloprid perturbed gut microbial communities on day 7; however, on day 13, the gut communities recovered from the dysbiosis that was observed on day 7. CCA analyses were used to reveal the relationship between environmental factors and microbial communities. The time variable was negatively correlated with insecticide treatment (thiacloprid and thiacloprid level). The time variable (age) attempts to modify or has corrected the effects of thiacloprid on honeybee gut microbial communities, suggesting that the gut

microbial communities recovered from thiacloprid damage, although percentage survival was reduced by thiacloprid. NMDS analysis visualized the effects of time variable on the gut microbial community of honeybees. Sample spatial loci of gut microbiota of honeybees describe the degree of the community diversity in different time-periods. The tight clustering of the community was observed on day 13, whereas the microbial community on day 7 was more widely dispersed. The higher clustering level of the microbial community indicates more convergent and stable community structure (Raymann et al., 2017). More specific analyses revealed that the samples of the control group (T0) were more clustered on the day 13 when compared to the samples on day 7, indicating that the similarity of honeybee gut microbial communities was higher on day 13, and the microbiome became more stable. Higher microbial community stability contributes to the role of intestinal microbes in the host (Ge et al., 2012). Moreover, this phenomenon also occurred with the high (T0.6) and very high (T2.0) concentrations of thiacloprid treatments, which indicated that the construction of honeybee gut microbial community, same as the control group, also occurred in the environment affected by thiacloprid (Ge et al., 2016). Time effect corrected the damage of some environmental pressure on honeybee gut microbial community.

The alpha diversity of honeybee gut microbiome was higher on day 13 when compared to that of day 7, and beta diversity was lower, representing the recovery of the gut microbiome from the dysbiosis that was caused by the thiacloprid exposure. Bray-Curtis distance decay analysis showing the similarity of communities also strongly supports the phenomenon of recovery from dysbiosis. The recovery from dysbiosis is evident in live honeybees; however, dead honeybees were not examined for changes in their honeybee gut microbiome.

4.4. The reasons of honeybee gut microbiome recovers

Honeybees are very special and strictly social insects, and it is more convenient and practical to compare them as a whole rather than as a single individual (Osterman et al., 2019). Worker honeybees generally survive for more than a month in its natural state. According to the social discipline, they get transformed from nursing honeybees to collecting honeybees around day 20 of post-emergence from pupa (Neukirch, 1982). The physiological indices of honeybees reach to the highest level before they are converted into collecting honeybees. Once converted into collecting honeybees, their mortality rate increases significantly (Munch et al., 2008; Ellegaard and Engel, 2019). In this study, the middle-aged honeybees are nursing honeybees. Thus, a strong and stable gut microbial community builds up during their period of middle-age, and the process is likely to exist even under stressful environmental conditions (Doublet et al., 2015). As environmental stress, thiacloprid tries to hurt the physiological status of honeybees and the symbiotic gut microbiota.

The effect of thiacloprid exposure on honeybee gut microbiome was assessed from the perspective of microbial community assembly. The results presented in the NTI and βNTI null models represent the destructive effects of thiacloprid, demonstrated that the degree of damage to the honeybee gut microbiome was alleviated on the day 13, when compared to the damage on the day 7. The results also indicated that the gut microbial community of the middle-aged honeybees treated with thiacloprid was recovered to the normal microbiome. The positive correlation between thiacloprid and βNTI only appeared on the day 7, and its value changed from -2 to a positive value, indicating that the construction process of the sample changed from a deterministic process to stochastic processes (Feng et al., 2018). This indicates that the toxicity effect of thiacloprid caused the microbial communities to become more random and uncertain, which might have detrimental effects on honeybee digestion and immunity (Kwong et al., 2017a). This situation did not appear on the day 13, and the βNTI value remained near -2, indicating that the randomness effect caused by the thiacloprid was reduced, and the community was constructed as a deterministic process, which was conducive to the recovery of microbial functions in honeybees. The recovery of the microbial community on day 13 may represent the adjustment of honeybees. Even when the percentage survival of honeybees decreases, the microbial community is adjusted against the trend, indicating that the gut microbial communities of honeybees exist as a system closely related to honeybee individuals but is relatively independent.

The monotonous decline in the survival status (percentage survival, sucrose consumption, and body weight) of middle-aged bees that were exposed to thiacloprid and the recovery of gut microbial communities from dysbiosis are likey two different phenomena. The recovery from dysbiosis was not observed in a previous study (Kwong and Moran, 2016). The recovery of honeybee gut microbial community depends on the degree of damage to the microbial community. The composition of gut microbial communities in healthy honeybees over time did not change significantly in the control honeybees. Although, gut microbiome of honeybees was shown to vary with their age (or developmental stage) and the season (Ellegaard and Engel, 2019), the middle-aged honeybees have the most stable gut microbiome and the optimum immunity, when compared to other stages of honeybees; this makes the middle-aged honeybees resist the effect of thiacloprid on their gut microbiota on day 13 (Christen et al., 2019). Also, bacteria rapidly adapt to changes in their external environment. The thiacloprid may have retained most of the symbiotic core bacterial species through its selection on honeybees or on the honeybee gut microbiota to improve the stability of the microbial community (Ge et al., 2014; Sousa et al., 2018). Moreover, honeybee sociality is a powerful additive homogeneous mechanism that compensates for the loss of honeybee communities or its gut microbial communities caused by environmental stresses (Kwong and Moran, 2016). The honeybees or their microbial community should be more adept at adopting a comprehensive approach to counteract environmental stresses, so the superposition of the mentioned above possibilities may be the cause of community recovery.

5. Conclusion

In general, our research showed that continuous exposure to high concentrations of thiacloprid significantly reduced the survival of honeybees under laboratory conditions. Furthermore, thiacloprid disturbed the gut microbiome of honeybees, meanwhile, we also observed the recovery of the microbiome from dysbiosis during the later stages of exposure. The recovery process is independent of the honeybee survival status i.e. there was no causal relationship between the recovery of honeybee gut microbiome from dysbiosis and the increase of the honeybee mortality. The toxicity of thiacloprid to honeybees may include different mechanisms. The role of the toxic effects of the pesticide was greater than the changes in gut microbiota, since there was some recovery in the microbial community, but it did not alleviate the mortality rate. Therefore, we believe that the cause of increased mortality in honeybee community was mainly the influence of thiacloprid, including indirect effects on food consumption. Although the application of insecticides is the most economical way to control insect-pests and increase crop yields (Siede et al., 2017), one should pay attention to the possible negative impact of thiacloprid on honeybee colonies. The relationship between the dysbiosis of honeybee gut microbiome and its health-status must be explored. Although our results suggest that utilization of thiacloprid in agriculture is harmful to honeybees, one should consider other factors that contribute to the decline of the honeybee colonies. Overall, a comprehensive understanding of honeybee environment and its physiology will solve the worldwide problem of the decline in the honeybee colonies.

CRediT authorship contribution statement

Yong-Jun Liu: Conceptualization, Methodology, Investigation, Data curation, Writing - review & editing. **Neng-Hu Qiao:** Data

curation, Visualization, Writing - original draft. **Qing-Yun Diao:** Methodology, Investigation. **Zhongwang Jing:** Investigation, Data curation. **Raja Vukanti:** Writing - review & editing. **Ping-Li Dai:** Investigation. **Yuan Ge:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.121818.

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