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Enhancement of chronic bee paralysis virus levels in honeybees acute exposed to imidacloprid: A Chinese case study

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HIGHLIGHTS

- Reported cases of honeybee colony decline or large number of losses incidents were investigated.
- Acute imidacloprid ingestion can elevate the level of chronic bee paralysis virus.
- Environmental factors might be accelerator for pathogens rapid replication and transmission.
- Acute toxicity of pesticides should be incorporated in bee risk assessments.

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ABSTRACT

Though honeybee populations have not yet been reported to be largely lost in China, many stressors that affect the health of honeybees have been confirmed. Honeybees inevitably come into contact with environmental stressors that are not intended to target honeybees, such as pesticides. Although large-scale losses of honeybee colonies are thought to be associated with viruses, these viruses usually lead to covert infections and to not cause acute damage if the bees do not encounter outside stressors. To reveal the potential relationship between acute pesticides and viruses, we applied different doses of imidacloprid to adult bees that were primarily infected with low levels (4.3×10^5 genome copies) of chronic bee paralysis virus (CBPV) to observe whether the acute oral toxicity of imidacloprid was able to elevate the level of CBPV. Here, we found that the titer of CBPV was significantly elevated in adult bees after 96 h of acute treatment with imidacloprid at the highest dose 66.9 ng/bee compared with other treatments and controls. Our study provides clear evidence that exposure to acute high doses of imidacloprid in honeybees persistently infected by CBPV can exert a remarkably negative effect on honeybee survival. These results imply that acute environmental stressors might be one of the major accelerators causing rapid viral replication, which may progress to cause mass proliferation and dissemination and lead to colony decline. The present study will be useful for better understanding the harm caused by this pesticide, especially regarding how honeybee tolerance to the viral infection might be altered by acute pesticide exposure.

1. Introduction

A variety of food crops and wild flowers in China require bee pollination, especially in wild areas and villages (Su and Chen, 2009). Nevertheless, honeybee health is being damaged by a variety of stressors, such as bacteria, fungi and viruses, as well as environmental stressors, such as pesticides (Goulson et al., 2015). Despite considerable effort (Di Prisco et al., 2013; Coulon et al., 2017), the exact causes and mechanisms underlying the interactions between viruses and pesticides have yet to be fully understood. Many potential causes of declining honeybee health have been proposed, including Israeli acute paralysis virus (IAPV) (Cox-Foster et al., 2007), neonicotinoid pesticides (Doublet et al., 2015), malnutrition (Brodschneider et al., 2010), loss of habitat and lack of nectar floral diversity (Winfree et al., 2009), and these causes may act singly or synergistically. Among the great number of threats, viruses and

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pesticides are thought to play vital roles in honeybee population depletion and include chronic bee paralysis virus (CBPV) and imidacloprid (Coulon et al., 2017; Goulson et al., 2015).

CBPV, generally called paralysis virus, is an infectious disease of adult bees. It is also called hairless black syndrome or bloated abdomen due to the two obvious symptoms it induces (Ribière et al., 2010). CBPV was first characterized by Bailey as part of another work in 1963, and the two distinct symptoms were described as type I and type II (Bailey and Ball, 1991). Type I is represented by abnormal trembling of the wings and bodies of infected bees and leads to crawling in the front of hive as well as becoming flightless, while type II is described as a hairless black bright in diseased bee and a broader abdomen (Ribière et al., 2010). Both types of CBPV symptoms render adult bees flightless, trembling and crawling in the front of their hives and can lead to death. In addition, CBPV can also induce similar symptoms in queens, and leads to overt infection six days after exposure (Amiri et al., 2014). CBPV has been shown to have serious adverse effects on honeybee colony health and survival (Ribière et al., 2010). Colony losses have frequently been associated with high infection levels of viruses in the absence of other stressors, especially covert infections, such as CBPV (Mcmenamin and Genersch, 2015). CBPV is especially prevalent in spring, and >75% of dead adult bees were found to be CBPV positive in a study in the Canary Islands (Chauzat et al., 2016). In other regions of Europe, the infection rate of CBPV remains at lower levels, usually between 4% and 18.3% (Berényi et al., 2006; Forgách et al., 2008; Nielsen et al., 2008; Toplak et al., 2012), with a maximum prevalence of 28% in France (Toplak et al., 2013). Recently, many beekeepers in China have reported a large number of adult bees crawling in the front of beehives as well as seriously affected colony health and production (Hou et al., 2016; Hou et al., 2017). We found a high prevalence of CBPV in these samples and analyzed its full genome sequence (Li et al., 2017). The results indicated that despite CBPV presenting as a low-level infection under natural conditions, it has the potential to cause overt infection or increase its proliferation when it encounters certain stressors, such as *Nosema ceranae* (Toplak et al., 2013).

Neonicotinoids are an important environmental stressor that is not targeted at the honeybee. Among neonicotinoids, imidacloprid is widely used to control agricultural and home insect pests, but it might present a potential problem for honeybee survival because it can persist for a long period of time in nectar and pollen (Blacquière et al., 2012; Chauzat et al., 2011). Generally, the imidacloprid concentrations contacted by honeybees are low, resulting in sublethal effects, such as damage to learning and homing abilities (Blanken et al., 2017), as well as the learning ability of *Apis cerana* (Tan et al., 2015). Experimental evidence has shown that the immune defense of the honeybee is decreased by imidacloprid (Gregorc et al., 2012). In addition, imidacloprid can reduce colony growth and queen production in bumblebees (Whitehorn et al., 2012). Recently, pesticide residues, including fungicides, insecticides, herbicides and miticides, have also been detected in pollen and wax combs (Mullin et al., 2010; Wu et al., 2011). In addition, several reports have identified the acute toxicity (LD50 value) of a high dose imidacloprid to honeybees. For example, Iwasa et al. (2004) found that the acute LD50 dose of imidacloprid reached 21.8 ng/bee and was as high as 152.2 ng/bee (Gong et al., 1999). Moreover, pesticide-exposed honeybees are more susceptible to infection by the microsporidian parasite *N. ceranae* (Aufauvre et al., 2012; Pettis et al., 2012). Given that imidacloprid has been widely used and is highly variable in amount and potential impact on honeybees and because it is highly probable that exposure to this pesticide will occur in the field (Chen et al., 2015), we investigated the effects of an acute dose of imidacloprid on honeybees infected with CBPV under natural conditions.

Interaction studies have reported that exposure of honeybees to imidacloprid and *Varroa destructor* leads to significantly negative effects on honeybee immunity and increases bees' energy usage (Abbo et al., 2016), suggesting that a causal link exists between external stressors and pathogens. Di Prisco et al. (2013) demonstrated that the neonicotinoid clothianidin adversely affects a member of the gene family NF-κB and promotes replication of deformed wing virus (DWV) in honeybees harboring a covert infection. Moreover, interactions between *V. destructor* and viruses have obvious effects on the health and survival of individual honeybees (Di Prisco et al., 2013). The black queen cell virus (BQCV) infection levels were significantly higher in bees treated with imidacloprid compared to controls (Doublet et al., 2015). Furthermore, a large-scale correlation study over an 11-year period revealed a significant correlation between honeybee colony losses and national imidacloprid usage patterns across England and Wales (Budge et al., 2015). However, the effect of the acute oral toxicity of imidacloprid on CBPV levels has not yet been investigated, although synergistic effects between imidacloprid and DWV, CBPV and *N. ceranae* were reported some years ago (Di Prisco et al., 2013; Toplak et al., 2013).

The aim of this study was to investigate whether a high dose of imidacloprid can enhance the infection level and induce rapid replication when the virus at a low level. This will help us to understand why the titer of virus varies with the season. Here, using imidacloprid as an acute environmental stressor, we examined the hypothesis that honeybees infected with low levels of CBPV, a virus that is completely different from other common bee viruses, such as DWV, and exposed to acute imidacloprid will show reduced survival. Our results provide clear evidence that acute exposure of honeybees to imidacloprid can accelerate bee mortality by enhancing the level of CBPV.

2. Material and methods

2.1. Honeybee sample collection

All colonies were raised using standard beekeeping practices and treated for mites (*V. destructor*) (only for *A. mellifera*) with acaricide twice each year, in spring and autumn. No typical symptoms of viral infection or other pathogens were observed in these colonies, but some of the bees lost the ability to fly and were found crawling in the front of their hives. Three colonies were randomly selected from each of three apiaries per province. Seemingly healthy samples were obtained from *A. mellifera* and *A. cerana* colonies in ten provinces, namely, Henan, Liaoning, Beijing, Gansu, Fujian, Hainan, Guangdong, Zhejiang, Sichuan and Jiangsu. Honeybee samples were collected from inside each colony. The samples were immediately transported to the laboratory on dry ice to avoid degradation of the active substances therein.

Half of the bee samples were used to detect the presence of eight common viruses, *Israeli acute paralysis virus* (IAPV), *Sacbrood virus* (SBV), *Deformed wing virus* (DWV), *Black queen cell virus* (BQCV), *Chronic bee paralysis virus* (CBPV), *Acute bee paralysis virus* (ABPV), *Varroa destructor virus-1* (VDV-1) and *Chinese sacbrood virus* (CSBV), and the remaining samples were used to calculate the amount of imidacloprid residue.

To detect imidacloprid residue, live adult worker honeybee samples were immediately collected from colonies maintained in apiaries in five provinces, Beijing, Jiangsu, Henan, Sichuan and Gansu, where it was reported that colonies had large numbers of crawling bees or losses during the spring of 2015 or 2016.

2.2. RNA extraction and PCR amplification

Total RNA was extracted from approximately 50 pooled honeybee samples (per colony) using the TRIzol Kit (Invitrogen, CA, USA) according to the manufacturer's protocol in a TissuePrep homogenizer (Gening Scientific, Beijing, China). The obtained RNA was dissolved in 20 μL of sterile water and stored at −80 °C prior to analysis. The quantity and purity of the RNA were measured using a NanoDrop spectrophotometer (Thermo Scientific, Beijing, China).

The primer sequences, orientation and references are provided in Table S1. For the eight RNA viruses, the initial cycle for reverse

transcription was 50 °C for 30 min and 85 °C for 5 min, followed by PCR. PCR consisted of a total volume of 20 μL with 2 × GoTaq reaction buffer (Promega, WI, USA), 1 μM of the sense and antisense primers, 1 μL of cDNA, and nuclease-free water. The cycling conditions were as follows: 1 min at 95 °C; 33 cycles of 30 s at 94 °C, 30 s at 55 °C and 72 °C for 1 min; a final extension of 10 min at 72 °C; and cooling to 4 °C. The PCR amplification products were separated on a 1.5% agarose gel stained with GV II (Biomec, BJ, China) and photographed with a FR-200A luminescent and fluorescent biological image analysis system (Furi, China). The product sizes were determined using a 100-bp molecular size ladder.

2.3. Determination of imidacloprid residues in honeybee samples

Honeybees were sampled from six regions of five provinces, Beijing, Gansu, Jiangsu, Sichuan, Henan (Pingyu) and Henan (Hebi), which experienced a large number of losses during the spring in 2015 and 2016. To identify whether imidacloprid was present in seemingly healthy samples and to determine the amount of imidacloprid residues in seemingly healthy honeybee samples from six regions of five provinces, we used enzyme-linked immunosorbent assay (ELISA), which is a convenient and effective tool for monitoring imidacloprid residues (Lee et al., 2001). We selected three colonies from each of the six regions.

Honeybee samples (~5 g per colony) were ground to a fine powder and dissolved in 5 mL of methanol (70% v/v). Then, the mixture was strongly vortexed in a 10 mL tube for three minutes and filtered through filter paper. The supernatant was added to a tube containing antibody enzyme conjugates of imidacloprid (50 μL) and moved to an incubator at 37 °C for 30 min, and then, the concentration was detected at a wavelength of 450 nm. Imidacloprid was used to make standard solutions following the manufacturer's instructions (Glory Science, Hangzhou, China): 0 μg/L,0.1 μg/L,0.3 μg/L,0.9 μg/L,2.7 μg/L,and 8.1 μg/L. A standard curve with imidacloprid as the standard sample was made with the concentration of imidacloprid and absorbance as the X and Y axes, respectively. In addition, the linear regression equation was $y = -0.5017x + 0.4963$ with a linear range of 0–8.1 μg/L ($R^2 = 0.9931$). Briefly, rapid determination of the imidacloprid levels in honeybee samples was performed with an ELISA Kit (Glory Science, Hangzhou, China) using a microplate reader (Eppendorf, Biospectrometer, Germany). The concentration was calculated as ng/bee according to the consumption of syrup, which was estimated to be 33 μL/bee (Decourtye et al., 2003).

2.4. Purification of CBPV

Honeybee samples collected from Beijing colonies showed clinical symptoms of viral infection. Paralyzed bees were homogenized in 50 mL sterile tubes in extraction buffer using a Tissueprep® homogenizer (Getting Scientific Instrument Ltd., Beijing, China). The mixture was centrifuged at 3000 rpm for 10 min and then recentrifuged at 4000 rpm for 30 min after filtration through net gauze. The supernatant was centrifuged at 24,000 rpm for 4 h in a BY 30 rotor. The precipitated virus was resuspended in 1 mL of Brij buffer, layered onto a 30% or 50% (w/v) sucrose gradient and centrifuged at 20,000 rpm for 4 h in a BY 30 rotor. The viral fraction was recovered, layered onto a 30–60% (w/v) CsCl gradient and centrifuged at 27,000 rpm for 24 h in a BY 30 rotor. Finally, the purified virus was dissolved in sucrose at −80 °C and aliquoted until use. Briefly, CBPV particles were purified through differential centrifugations as described by Olivier et al. (2008).

2.5. Interaction experiments between CBPV and imidacloprid

For the interaction experiment between imidacloprid and CBPV, honeybee samples were collected from *A. mellifera* colonies in the spring of 2016 and maintained in the experimental apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences. Newly emerged bees were obtained from brood frames taken from the experimental honeybee hives and kept in an incubator at 30 °C and 60% relative humidity (RH) for approximately 12 h. Then, we divided the samples into 9 groups, each group containing 30 emerged bees and three replicates. In each experiment, three cages of 30 bees were used for each dose of treatment for oral acute studies. We confirmed the absence of eight common viruses (Fig. S1) before the interaction experiment was performed.

Newly emerged worker bees were collected from *A. mellifera* colonies at the experimental apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences during the spring of 2016 and screened for the presence of the common honeybee viruses IAPV, CBPV, ABPV, DWV, VDV-1, KBV, SBV, and BQCV as well as *Nosema apis* and *N. ceranae* under a microscope. These seemingly healthy bees were transferred to standard cages (8 cm × 6 cm × 12 cm, 30 bees per cage) containing water and a sucrose solution (1:1 v/v) under no consideration of nutrition condition. Cages were kept in an incubator at 30 °C with 65% humidity. The cages had several ventilation holes in the side and two holes in the lid to allow the insertion of feeding tubes (500 μL in a 1.5 mL Eppendorf tube). The solution in each feeding tube was replenished with 500 μL of sucrose solution daily.

Certified imidacloprid standards were purchased from Sigma-Aldrich (Seelze, Germany). These standards were dissolved in acetone, and the final concentrations were obtained by successive dilutions. Solutions of imidacloprid were made to achieve concentrations of 26.9 ng/bee, 44.6 ng/bee and 66.9 ng/bee as experimental doses according to the preliminary experiment and related references (Di Prisco et al., 2013; Kasiotis et al., 2014; Suchail et al., 2001), which reported that the lowest effective concentration value for honeybee larvae was ≥40 ng/bee. Newly emerged honeybees were anesthetized in a 4 °C refrigerator for 30 min, transferred into a small cage for 1 h, and then used to perform the infection experiment.

It is well known that CBPV is often detected in asymptomatic honeybee samples and builds persistent infections under natural conditions (Ribière et al., 2010). Therefore, we first infected honeybees with CBPV at a low level (4.3×10^5 genomic copies) in a volume of 500 μL of a sucrose solution, which was similar to the level found in asymptomatic bees. Then, after 24 h, we fed honeybees with each of three concentrations of imidacloprid for two successive days to observe whether imidacloprid could elevate the level of CBPV. Previous studies on the effects of imidacloprid on DWV titers have confirmed that imidacloprid at amounts from 10 to 20 ng/bee can rapidly enhance the levels of DWV (Di Prisco et al., 2013). Therefore, an equivalent amount was used to test CBPV, but we did not achieve acute mortality. Then, we applied imidacloprid in a 500 μL sugar solution at concentrations of 26.9 ng/bee, 44.6 ng/bee and 66.9 ng/bee based on our preliminary experiment. Both control and treatment group bees, including the DMSO treatment group, received sugar syrup (50%). Each group consisted of three replicates. Dead bees were recorded and removed daily.

2.6. qRT-PCR assay

Total RNA was extracted from dead samples of the different treatment groups with the TRIzol Kit (Invitrogen, CA, USA). cDNA was subjected to real-time quantitative RT-PCR (qRT-PCR) using a LineGene9600 instrument (Bioer, Hangzhou, China). The primers used for CBPV and housekeeping genes were as follows: CBPV Forward 5′-CAACCTGCCTCAACACAG-3′ and Reverse 5′-AATCTGGCAAGGTTGACTGG-3′ were used to amplify a 296-bp fragment (Ribière et al., 2007); the housekeeping gene primers β-actin forward 5′-TTGTATGCCAACACTGTCCTTT-3′ and β-actin reverse 5′-TGGCGCGATGATCTTAATTT-3′ were used to amplify a reference gene fragment (Ai et al., 2012). Quantitative PCR was performed using the SYBR-Green-based KapaSybr^R Fast qPCR kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. The cycling protocol was 95 °C for 10 min followed by 40 cycles of 95 °C for10 s, 60 °C for 15 s, and 72 °C for 20 s. Standard curves were prepared by performing real-time qPCR

with serial 10-fold dilutions of known concentrations of the CBPV-specific amplicons, which were approximately 296 bp in length. The melt curve dissociation was analyzed to confirm each amplicon. Linear detection of CBPV over a 7-log range, 10^1 to 10^7 genome equivalents, was obtained by plotting the C_T values vs. the logarithm of the concentration of genome equivalent copies, $R^2 = 0.99795$. The concentration of nonspecific primer amplification was measured by performing a RT reaction in the absence of primers followed by specific-primer quantitative real-time PCR and was found to be negligible under our working conditions. The results were analyzed using 9600 Plus Software. For the detection of negative strand RNA of CBPV, we used the TRIzol Kit (Invitrogen, CA, USA) to separately extract RNA from the head, thorax, abdomen, wing and leg of samples from Henan (Hebi) province, and reverse transcription was performed using M-MLV Reverse transcriptase (Invitrogen, CA, USA) at 55 °C for 50 min followed by 70 °C for 10 min for inactivation. The tagged primer (agcctgcgcaccgtgCAACCTGCCTCAACACAG) was used for reverse transcription. qPCR amplifications were performed under the same conditions and using the SYBR Fast qPCR kit (Sigma-Aldrich, MI, USA) described above with the tag sequence (agcct gcgcaccgtg) and CBPV primer sequence (AATCTGGCAAGGTTGACTGG).

2.7. Statistical analysis

The prevalence of viruses in 10 provinces and potential associations between pathogens within samples were examined by multiple comparison analysis, and we considered a virus to be present if samples from one colony in each province were positive for one virus. The cumulative survival rate was calculated by summing live bees at each day of the survival assessment and then dividing this number by the total number of bees introduced into each cage (Aufauvre et al., 2012). The viral levels of different tissues (head, thorax, abdomen, wing and leg) were analyzed using one-way ANOVA followed by post-hoc *t*-tests or Tukey HSD tests. The viral level data were log-transformed to approximate normality as described previously (Hou et al., 2014). A nonparametric Mann-Whitney test was used to compare the CBPV expression levels of samples from different groups at different concentrations of imidacloprid. The variance components are expressed as standard deviations. The software Origin 8.0 was used for statistical analysis.

3. Results

3.1. Occurrence and prevalence of honeybee viruses

To better understand the occurrence and distribution of honeybee viruses in particular regions, we screened for eight RNA viruses in bee samples from ten provinces. Different types of viruses were detected in all provinces, with the exception of ABPV, which was not found in any province (Fig. 1). CBPV, BQCV and IAPV were the most prevalent viruses and were present in 80% of the *A. mellifera* apiaries in the 10 provinces. CSBV, IAPV and BQCV were detected in 70% of the *A. cerana* apiaries in the 10 provinces. In addition, the prevalence of the rest of the viruses was equal to or <50%, regardless of whether the apiaries contained *A. mellifera* or *A. cerana*. Although IAPV and BQCV were both highly prevalent, the characterization of IAPV has been reported in relatively greater detail, while BQCV has been less studied and poses a relatively lower threat compared to CBPV (Chen and Siede, 2007). Therefore, we focused on whether CBPV could replicate in infected bee samples and possibly pose a serious threat to bee health. As shown in Fig. 2, CBPV can replicate in the major body regions of the honeybee, such as the head, thorax and abdomen. However, the virus loads were significantly different in different tissues; head and thorax replication were extremely significantly higher than abdomen and leg replication ($p < 0.01$) and significantly higher than wing replication ($p < 0.05$).

The co-occurrence of more than one virus in one region was fairly common in both *A. mellifera* and *A. cerana* apiaries (Table S2). In *A. mellifera*, viral co-infections varied from 2 to 5 viruses and usually involved CBPV, IAPV and BQCV. Viral co-infections in *A. cerana* primarily involved 2 to 4 viruses, and CSBV was the major virus. These results indicate that the co-infection of multiple viruses included CBPV as one of the major viruses in *A. mellifera*.

3.2. Detection of imidacloprid residues

Initially, beekeepers from Beijing, Gansu, Jiangsu, Sichuan, Henan (Hebi) and Henan (Pingyu) reported that bee colonies (*A. mellifera*) from these six regions suffered from large losses, and we therefore hypothesized they might be affected by imidacloprid, which is a commonly used pesticide in these regions. To determine whether honeybee samples from colonies that had undergone incidents of

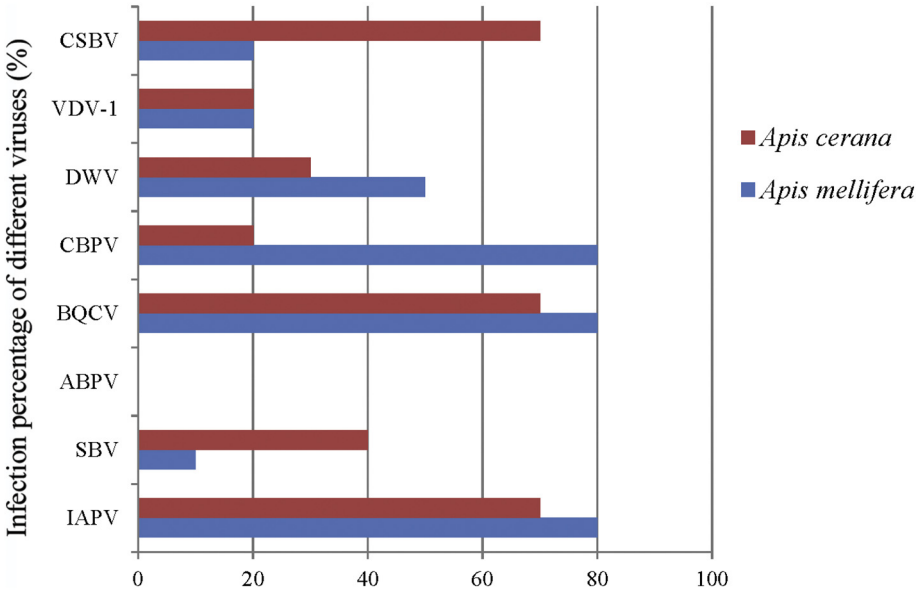

Fig. 1. Prevalence of honeybee viruses. The infection percentage of eight honeybee viruses (CSBV, VDV-1, DWV, CBPV, BQCV, ABPV, SBV, IAPV) was assessed in ten provinces. The red and blue bars indicate bee samples from *Apis cerana* and *Apis mellifera*, respectively. The X-axis represents the infection percentage of each virus, and the y-axis represents different viruses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

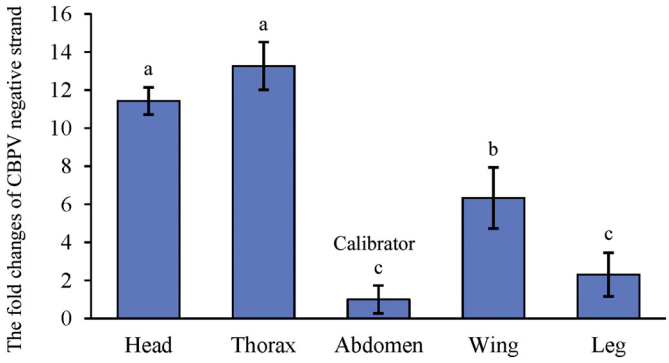

Fig. 2. Replication of CBPV in different tissues of adult bees. The abdomen harbored the minimal level of CBPV and was chosen as a calibrator. The means and standard deviations are shown.

serious loss contained more pesticide residues than those from other regions, samples from six regions in five provinces were collected from the field during the spring of 2015 and 2016 (Fig. 3). The analyses confirmed the presence of imidacloprid residues in all of the samples collected, and the results showed that the range of the imidacloprid content was from 18.86 (Sichuan) to 27.89 ng/bee (Henan, Pingyu) (Fig. 4). The level of imidacloprid residues in the samples from Gansu and Henan provinces was significantly higher than those from the other three provinces ($p < 0.05$).

To confirm whether seemingly healthy bees can carry imidacloprid, we took samples from the six regions of the five provinces mentioned above. As shown in Fig. 4, seemingly healthy bees also carried imidacloprid residues, but at lower amounts than those from colonies that had experienced a large number of losses. The amount of imidacloprid was between 13.80 and 22.97 ng/bee. The amounts from the Gansu and Henan province bee samples were higher than those from the other three provinces.

3.3. Effect of interaction on survival

These results showed that bees in the control and CBPV groups had the highest survival rates (>80%) compared to the co-exposure to CBPV and imidacloprid groups at 96 h (Fig. 5A). Generally, there was not a large difference in survival rate among all groups within 48 h. However, a significant synergistic effect on mortality was found at 72 h, even in the CBPV and imidacloprid group (22.6 ng/bee), and reached to the lowest survival rate at 96 h ($p < 0.05$). After 96 h, the survival rate was only 20% when bees were treated with imidacloprid at 66.9 ng/bee in combination with CBPV. The mortality in the co-exposure group exceeded what expectations from an additive effect between CBPV and 66.9 ng/bee of imidacloprid. Although the survival rates of the treatment groups of IMI and IMI with CBPV were 48.3% and 47.8% at 96 h, respectively, they changed to 35% and 21.6% in the IMI III and IMI III groups with CBPV at 96 h. The survival rate varied from 48.3% and 42% to 35% in the IMI, IMI II and IMI III treatment groups, while it changed to 47.8%, 40% and 21.6% in the IMI with CBPV, IMI II with CBPV and IMI III with CBPV treatment groups, respectively (Fig. 5A). These results demonstrate that there was a synergistic effect between the CBPV and imidacloprid at the highest dose of 66.9 ng/bee.

We tested whether imidacloprid could elevate the level of CBPV, which is significantly different from other honeybee viruses. Indeed, the results showed that imidacloprid accelerated individual bee death with increasing amounts of imidacloprid (Fig. 5A). The quantitative CBPV levels confirmed this result, and a positive correlation between the imidacloprid dose and CBPV titer ($r = 0.79$, $p < 0.05$) was observed (Fig. 5B). The titer of CBPV in honeybees treated with CBPV and imidacloprid reached its highest level at 4.3×10^8 genome copies at 96 h after treatment. However, no difference in CBPV level was observed

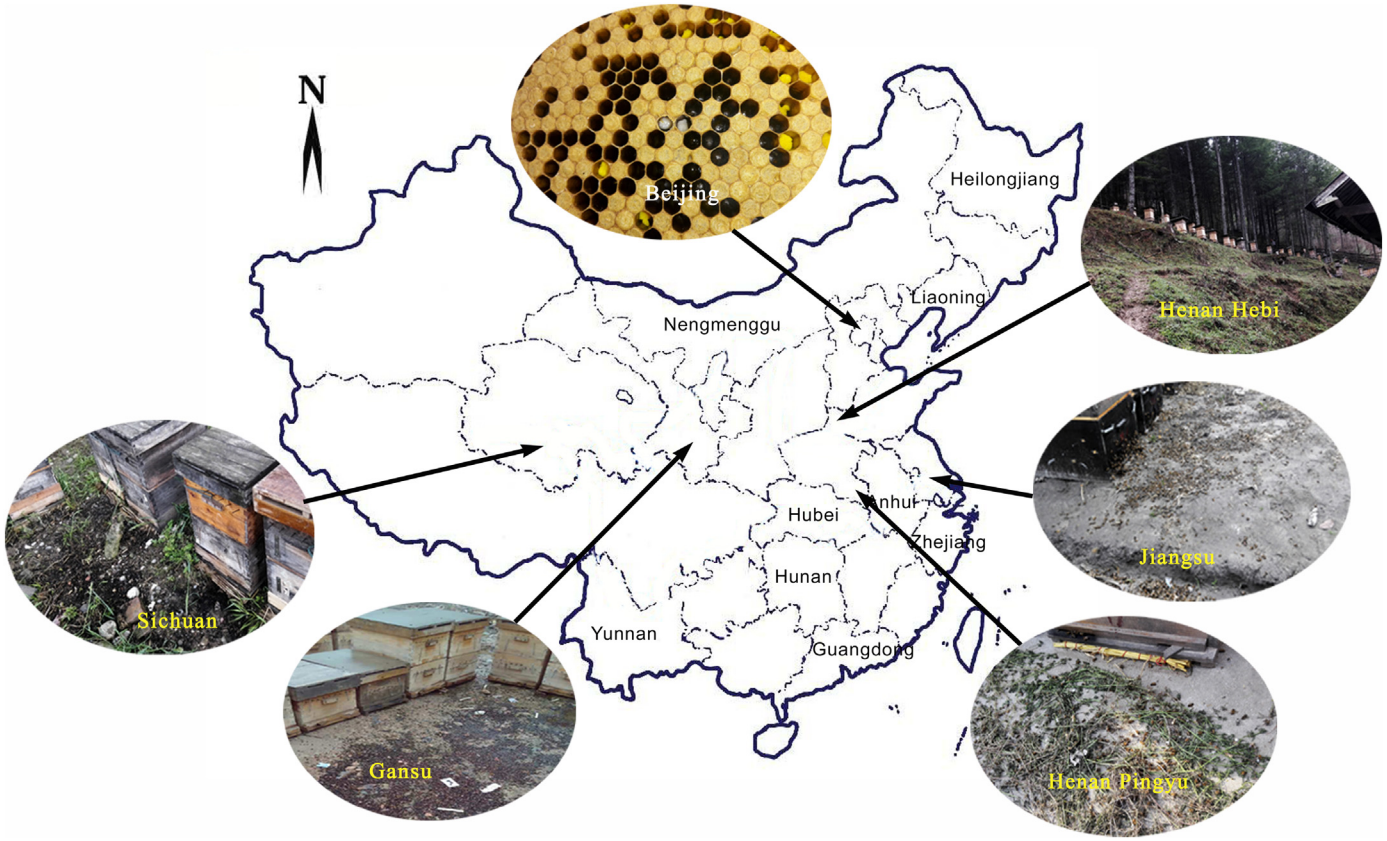

Fig. 3. Distribution of major loss incidents reported from six sampling sites.

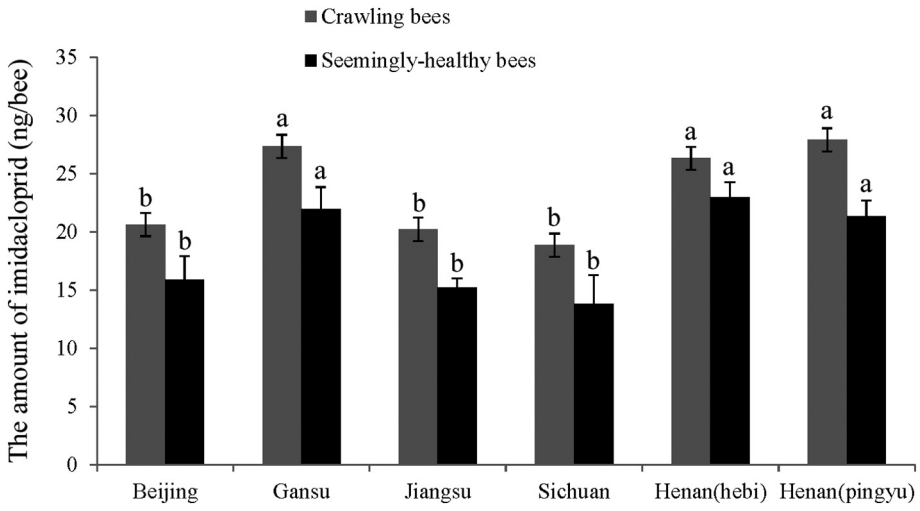

Fig. 4. Amount of imidacloprid residue in the honeybee samples from sites that went through losses or had many crawling bees compared with those from seemingly healthy colonies in six regions of five provinces. The gray and black bars represent bees that went through losses or crawling and seemingly healthy bees, respectively. Letters indicate significant differences. The means and standard deviations are shown.

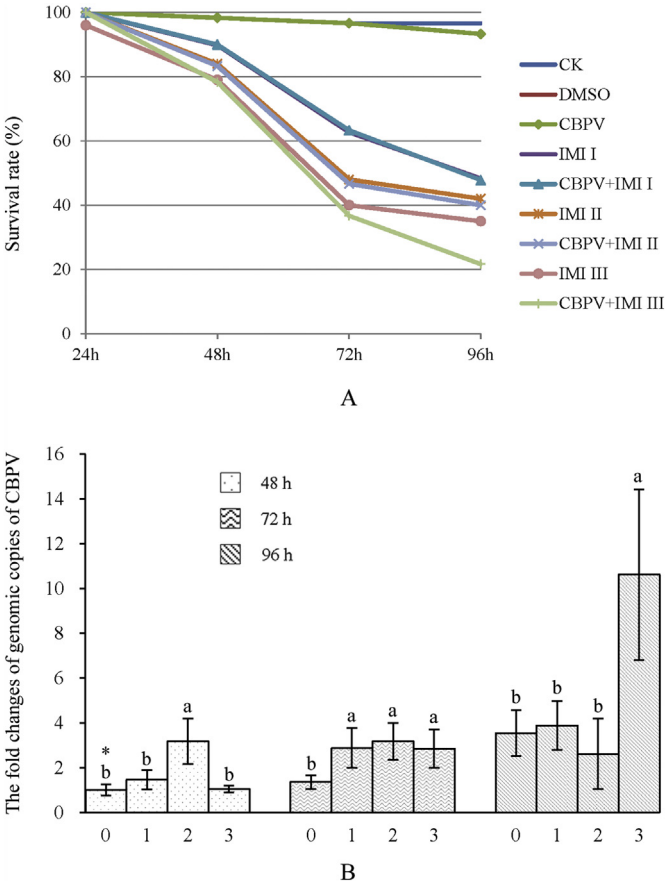

Fig. 5. Effects of imidacloprid on the survival rate of bees and the level of CBPV replication. The survival rates of honeybees in the control groups and those treated with imidacloprid and CBPV are shown (A). The dotted, waved, and diagonally shaded columns represent time points at 48, 72 and 96 h, respectively. The CBPV titers were transformed into the fold change of genomic copies at 48, 72 and 96 h. The numbers 0, 1, 2 and 3 indicate the groups treated with CBPV, CBPV + imidacloprid (26.9 ng/bee), CBPV + imidacloprid (44.6 ng/bee) and CBPV + imidacloprid (66.9 ng/bee), respectively (B). The level of CBPV from the group treated with CBPV at 48 h harbored the minimal level of CBPV and therefore was chosen as a calibrator (indicated with an asterisk). IMI, IMI II, IMI III and CK indicate the different doses of imidacloprid, 26.9 ng/bee, 44.6 ng/bee, 66.9 ng/bee and blank control, respectively. The means and standard deviations are shown.

between controls and honeybees fed a low level of CBPV alone (4.7×10^5, 5.1×10^5, 1.7×10^6 at 48, 72 and 96 h). It is worth noting that the imidacloprid doses (44.6 and 66.9 ng/bee) used here caused a large number of deaths in experimental honeybees after fewer than 72 h, and the survival rates were 40% and 20% at 44.6 and 66.9 ng/bee after 96 h, respectively. Workers infected with CBPV and exposed to imidacloprid showed significantly higher mortality compared with control individuals and with those treated with CBPV alone.

4. Discussion

Imidacloprid and CBPV have been found to impact bee health (Di Prisco et al., 2013; Toplak et al., 2013). We found that one environmental stressor, imidacloprid, and one common virus, CBPV, act synergistically on adult honeybees and lead to rapid mortality by exacerbating the CBPV titer under laboratory conditions, although we used the lower number replicates for testing this effects.

We found unprecedented levels of imidacloprid in honeybees from several provinces that went through large losses or an increase in bee crawling. In the present study, the acute oral toxicity of the imidacloprid LD50 value obtained in *A. mellifera* was 46.65 ng/bee at 72 h, while Suchail et al. (2001) reported that the acute oral toxicity of imidacloprid in honeybees was 57 ± 28 ng/bee at 48 h and 37 ± 10 ng/bee at 72 h (Suchail et al., 2001). Subsequently, Decourtye et al. (2003) found that the acute oral toxicity of imidacloprid in honeybees was 30.6 ng/bee at 48 h. Thus, the acute toxicity studies show that there is great flexibility of imidacloprid sensitivity as it varies from 37 to 73.9 ng/bee (Kasiotis et al., 2014). By contrast, however, the amount of imidacloprid in dead bee samples from Greece in 2011 ranged from 0.3–5.74 ng/bee (Chauzat and Faucon, 2007), but the acute toxicity LD50 for imidacloprid was 18 ng/bee at some points (Kamel, 2010). Although the amount of our samples was also relatively higher than those from other regions and recent reports (Kiljanek et al., 2017), there was a large fluctuation in amount for some reason. Previous studies have found that the acute oral LD50 values of imidacloprid on honeybees were from 3.7–81 ng/bee (Schmuck et al., 2001) or 41–81 ng/bee at 48 h (Nauen et al., 2001), could exceed 170 ng/g (Kasiotis et al., 2014), and occasionally reached as high as 200 ng/bee (Suchail et al., 2001). The difference between our results and those of previous studies might be due to a variation in the detoxification capacity in honeybee colonies from colony to colony (Suchail et al., 2001), fewer detoxifying enzymes (Claudianos et al., 2006), and inhibition of xenobiotic transporters of honeybee (Hawthorne and Dively, 2011; Guseman et al.,

2016). Second, bee samples were collected from different time periods, and imidacloprid is lower in summer (12 μg/kg) than winter bees (48 μg/kg), which means winter bees are less sensitive than summer bees (Decourtye et al., 2003). Likewise, samples in our investigation were collected in spring when sudden losses or bee crawling were observed. Our experimental colonies were frequently impacted by pesticides, which were sprayed to control all types of pests because our Institute is located in Beijing Botanical Garden. Another reason might be sampling differences from different regions. For example, imidacloprid was detected in 26.2% bee samples in Central and Southern France from 2002 to 2005 (Chauzat et al., 2009), but in Western France, several neonicotinoids, such as imidacloprid, clothianidin and thiamethoxam, were not detected in samples of live honeybees during 2008 to 2009 (Olivier et al., 2013). Beekeeping management practice can also have an impact on pesticide residues. Calatayud-vernich et al. (2017) described that more pesticides were found in old comb beeswax. Last, the interaction between pathogens and pesticides was able to increase the pathogens level or reduce the ability of the host to degenerate the pesticide, as reported by Bacandritsos et al. (2010), who found that the concentrations of imidacloprid ranged from 14 to 39 ng/kg following virus infection with the accumulation of imidacloprid.

Combinations of sublethal doses of modern pesticides often produce additive or even synergistic effects on animal mortality and behavior (Laetz et al., 2009; Di Prisco et al., 2013; Martinello et al., 2017; Fine et al., 2017). In our experiments, we combined a neonicotinoid pesticide, imidacloprid, with a segmental virus, CBPV, to determine whether honeybees infected with CBPV and exposed to pesticides would exhibit accelerated colony mortality. We found that high doses of imidacloprid after 96 h of treatment caused significantly higher mortality by accelerating the replication and increasing the level of CBPV. We also assessed the level of CBPV after 120 h, but the level was not increased. This result is similar to the report by Di Prisco et al. (2013), in which the level of DWV remained unchanged after treatment with imidacloprid at 72 h. Generally, the level of CBPV in the group treated with CBPV and imidacloprid (66.9 ng/bee) steadily increased between the 48 h and 96 h time points and reached an increase of approximately four-fold that of the 48 h level at 96 h. Similarly, imidacloprid has been shown to elevate the level of DWV by four-fold between 24 h and 72 h (Di Prisco et al., 2013). These results demonstrate that the effects of imidacloprid on DWV and CBPV might be similar. The increase in pathogen growth within individual bees reared in colonies exposed to acute doses of imidacloprid is dose-dependent (Pettis et al., 2012). A recent study found a significant correlation between the presence of fungicide residues in hives and honeybee colony viruses (Simon-Delso et al., 2014).

There are two potential reasons that imidacloprid could lead to an increase in the level of CBPV. One is that imidacloprid increases the energetic cost of detoxification during pesticide exposure by reducing the Vg titer in honeybees (Abbo et al., 2016). Sublethal imidacloprid doses reduce waggle dancing 24 h after ingestion (Eiri and Nieh, 2012). Likewise, the level of acetylcholinesterase (Ache) was shown to be elevated in a neonicotinoid-treated honeybee group (Alburaki et al., 2014). The other reason is that imidacloprid might decrease the immune response of the host by reducing its enzyme activity. A synergistic interaction between *N. ceranae* and sublethal doses of fipronil (a phenylpyrazole that disrupts GABA-regulated chloride channels) or the neonicotinoid thiacloprid resulted in higher bee mortality than in non-exposed bees by changing the enzyme (glutathione-S-transferase) activity in both the mid-gut and fat body (Aufauvre et al., 2012; Vidau et al., 2011). Although the present study did not detect changes in immunity genes, the results showed that the treatment used in this study might also reduce the expression of immunity genes based on similar responses to a case in which NF-κB activation was impaired by clothianidin exposure (Di Prisco et al., 2013). This report demonstrated that sublethal doses of clothianidin negatively modulate NF-κB immune signaling in insects and that both clothianidin and imidacloprid adversely affect the honeybee antiviral defenses controlled by this transcription factor (Di Prisco et al., 2013). Therefore, neonicotinoids can modulate the virulence of bee pathogens and may more generally modulate interactions between insects and their natural antagonists, as indicated by the increased virulence of entomopathogenic fungi against *Aedes aegypti* induced by exposure to imidacloprid (Paula et al., 2011). Colonies infected with *V. destructor* and exposed to imidacloprid showed significantly reduced populations compared with non-exposed colonies (Dively et al., 2015). As demonstrated by Rundlöf et al. (2015), however, neonicotinoids can not only impact the domestic honeybee but also reduce the density, colony growth and reproduction of wild bees, such as bumblebees.

Further research is now underway to investigate the molecular mechanism of the synergistic effects of imidacloprid and CBPV on honeybee physiology and immunity, with the hope of gaining deeper insight into the problems caused by their interaction and finding solutions to reverse the steep decline in populations of honeybees. Even if a lethal chronic toxicity of imidacloprid on individual bees can be demonstrated (Rondeau et al., 2013), the implications this has for the survival of colonies cannot be easily assessed in field trials in the long term unless the data are analyzed using fully crossed factorial designs and mathematical models that consider the effect of multiple stressors (Wehling et al., 2010). Although our results show that the mortality of honeybees is related to acute imidacloprid toxicity, we cannot be sure whether imidacloprid, its metabolites or both induce the synergistic interaction on honeybee mortality. In the future, it would be interesting to follow imidacloprid metabolism in different parts of *A. mellifera* to observe the possible accumulation of imidacloprid or its metabolites in specific compartments of the honeybee.

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Author contributions

HCS and DQY conceived the study; DQY, LBB and ZHX carried out the experiments; HCS, WYY, WQ and DPL analyzed the data; HCS, GR and CDF prepared and edited the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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