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ORIGINAL RESEARCH ARTICLE

Interactions between sublethal doses of thiamethoxam *and Nosema ceranae* in the honey bee, *Apis mellifera*†

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Both *Nosema ceranae* and insecticides are adversely affecting honey bees. How both factors affect gene expression and honey bee survival are well studied. However, only a few studies dealt with the interactions of the two factors. Here we studied the effects of both, when alone and in combination, on honey bee survival and expression changes of immune and detoxification genes. Newly emerged bees were randomly assigned four different treatments: control bees, bees exposed to thiamethoxam, bees exposed to thiamethoxam and then infected with *Nosema*, and bees infected with *N.ceranae* only. When combined with *Nosema,* thiamethoxam caused a significant reduction in survival from the control, but this reduction is not significantly different from *N. ceranae* alone. Exposure to thiamethoxam caused significantly increased expressions compared to the control for the genes of abaecin, apidaecin and hymenoptacin, but significantly decreased expression for the defensin gene. When the two factors were combined, the expression patterns were similar to *N.ceranae* infection alone (apidaecin, defencin and hymenoptaecin), except abaecin, which was increased compared to *Nosema* infected bees. For detoxification genesCCE8 and CYP315A1, thiamethoxam caused significantly increased gene expression but *Nosema* caused significantly decreased expression. When combined, the two factors did not show a further increase in Mortality compared to *Nosema infection* alone. Our study shows a clear combined effect of thiamethoxam and *Nosema*, but mainly in worker mortality and very little effect on gene expression. This study highlights the importance of observing mortality when studying the effect of insecticide on gene expression.

Keywords: Beekeeping; neonicotinoid; microsporidian; immunity gene; detoxifying gene

Introduction

Honey bees (*Apis mellifera*) are regarded as one of the most important insects to humans due to their pollination service (Calderone, 2012), hive products (Boukraâ, 2015) and as a model species for research (Giurfa, 2003; Ihle et al., 2015). However, managed honey bee colonies are experiencing high mortality worldwide, which could be caused by interactions of multiple factors (Cox-Foster et al., 2007), including parasites, pathogens, nutrition, pesticides, transportation, and interactions among them (Goulson et al., 2015). Pesticides are indispensable in agriculture because each agricultural crop will have its own pests, diseases, and weeds. However, honey bees will forage for pollen and nectar on most fruit and vegetable flowers. This can be a problem because both pollen (Mullin et al., 2010) and nectar (Dively & Kamel, 2012) have been found to contain high amounts of many types of pesticides. Among the pesticides, neonicotinoid insecticides are the most widely used. In recent years, neonicotinoids including the first generation of them, such as imidacloprid (Mengoni Gonalons & Farina, 2015; Nicodemo et al., 2014; Yang et al., 2012), acetamiprid (El Hassani et al., 2008), and thiacloprid (Tison et al., 2016), and the second generation of neonicotinoids, such as thiamethoxam (Oliveira et al., 2014) and clothianidin (Brandt et al., 2017), all had a huge impact on honey bees. Honey bees exposed chronically to thiacloprid in the field for several weeks at a sublethal concentration can receive impacts such as reduced foraging effort, homing success, navigation performance, and impaired social communication (Tison et al., 2016). Therefore, three neonicotinoid insecticides (clothianidin, imidacloprid, and thiamethoxam) are currently subjected to a moratorium in the EU (Wood & Goulson, 2017).

Parasites and pathogens may also affect honey bees by inducing changes in their development, behavior, reproduction, and parasite tolerance (Vidau et al., 2011). Two microsporidian species, *Nosema apis* and *Nosema ceranae*, are the agents of two major diseases known as nosemoses A and C (Hges et al., 2010). Both species are obligate intracellular parasites of adult honey bees. *N. ceranae* increases energetic demand in honey bees and decreases hemolymph sugar levels (Mayack & Naug, 2010). *N. ceranae*-infected honey bees have shorter life-spans than uninfected ones (Alaux et al., 2010).

Many recent studies have been conducted to study the interactions of nicotinic insecticides and *Nosema*.

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†Zhiyong Liu and Shouming Li contributed equally to this paper.

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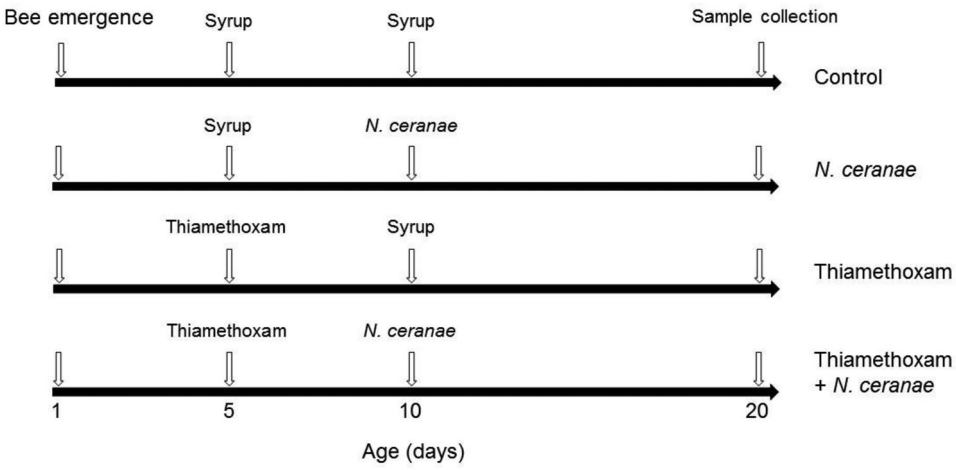

Figure 1. Experimental arrangement.

Recent studies suggest that *N. ceranae* can sensitize the honey bees to pesticides and vice versa (Alaux et al., 2010; Aufauvre et al., 2012; 2014; Pettis et al., 2012; Retschnig et al., 2014). The combined stress of neonicotinoid insecticides and *N. ceranae* can increase honey bee mortality (Vidau et al., 2011) and weaken their population (Alaux et al., 2010). Although *N. ceranae*-insecticide combinations cause a significant increase in honey bee mortality, the molecular mechanism remains unclear. In this study, we evaluate changes in the gene expression of *A.mellifera* contaminated with thiamethoxam and/or microsporid *N.cerana.*

Materials and methods

Experimental bees, treatments and artificial rearing

Experiments were conducted in the fall of 2017 at Michigan State University Bee Biology Building, East Lansing (42°40′44″N, 84°28′38″W), according to standard bee-keeping practices. *Nosema*-free colonies were used as source colonies for providing experimental bees. These colonies were confirmed to be free of *Nosema* by dissecting midgut and checking for *Nosema* spores (N = 10 bees per colony). Two frames of sealed brood were taken from a source colony and placed in an incubator in the dark at 35 °C with 65% relative humidity. Newly emerged honey bees were collected in 24 h, ~90 bees from each colony were separated into 4 groups in wooden cages (14 × 12 × 16 cm), and maintained in the incubator for 5 days. During this time, the caged honey bees were fed with sugar and water *ad labium;* pollen was provided through a glass tube(12 × 75 mm)through a hole in the cage (Huang, 2012). On day 5 (day 1 = day of emergence), we exposed half of the bees (randomly selected) to thiamethoxam. On day 10, half of two groups (insecticide treated or not) were exposed to *N.ceranae* (Figure 1). Thus we have four different treatments: control bees (no *N.ceranae* and no pesticide), bees exposed to thiamethoxam, bees exposed to thiamethoxam and then infected with *Nosema*, and bees infected with *N. ceranae* only. The experiment was replicated with bees from three different colonies.

Exposure to thiamethoxam

On day 5, honey bees were exposed to thiamethoxam (Sigma Aldrich, purity > 99%) by feeding insecticide-containing sugar syrup. LD_{50} for thiamethoxam was estimated as29.9 ng/bee (Iwasa et al., 2004). We used1% of the $LD_{50,}$ which was 0.299 ng/bee. A stock solution of thiamethoxam (500 mg/L) was prepared in acetone. One μl of stock solution was added to 1 ml of acetone, mixed well, then 14.9 μl was removed and left in a fume hood to air-dry. After the acetone was evaporated, 50 μl50% sugar water was added to the tube to obtain a solution of thiamethoxam at 0.149 ng/μl. Each bee in the pesticide group was fed 2 μl. Control bees were fed with 50% sugar water.

Honey bee infection of N. ceranae

Spores of *N. ceranae* were obtained from caged bees that were inoculated 8-14 days earlier to provide a reliable source of *Nosema*. *Nosema* spores were purified similarly to (Milbrath et al., 2015) and (Solter et al., 2012) methods. To obtain *Nosema* spores for our infections, we homogenized the midgut tissues of infected bees in distilled water using a plastic pestle. The spore suspension was centrifuged to pellet spores and the supernatant with insect cells was discarded. *Nosema* species was confirmed by PCR (Milbrath et al., 2015).

Five days after thiamethoxam exposure, bees to be infected with *Nosema ceranae* only, and bees exposed to thiamethoxam and *Nosema* were anesthetized on ice. Each bee was then fed 2 μl of syrup containing 100,000 *N. ceranae* spores. Non-infected bees were fed syrup without spores.

Table 1. Sequences of real-time PCR primers.

Gene	Sequence 5′ to 3′	Reference
Immune genes		
Abaecin-F	CAGCATTCGCATACGTACCA	Evans (2006)
Abaecin-R	GACCAGGAAACGTTGGAAAC	
Apidaecin-F	CCAACCTAGATCCGCCTACTCGACCT	Van Vaerenbergh et al. (2013)
Apidaecin-R	TATTTCACGTGCTTCATATTCTTC	
Defensin-F	TGCGCTGCTAACTGTCTCAG	Evans (2006)
Defensin-R	AATGGCACTTAACCGAAACG	
Hymenopt-F	CTCTTCTGTGCCGTTGCATA	Evans (2006)
Hymenopt-R	GCGTCTCCTGTCATTCCATT	
Detoxification Genes		
CCE8-F	TCTGCTTGCGCATTCTATTG	Alptekin et al. (2016)
CCE8-R	CTTTACGCGCTTCTTTGTCC	
CYP315A1-*F*	CTGGGTCCCGTTTACAAAGA	Alptekin et al. (2016)
CYP315A1-*R*	GGTGTTGACCCTTCAAGTCG	
Control Genes		
Actin-F	TTGTATGCCAACACTGTCCTTT	Simone et al. (2009)
Actin-R	TGGCGCGATGATCTTAATTT	
GAPDH-F	GATGCACCCATGTTTGTTTG	Scharlaken et al. (2008)
GAPDH-R	TTTGCAGAAGGTGCATCAAC	

Daily check and observation

Throughout the experiment, each cage was checked daily and dead honey bees were removed. Each dead bee was dissected for its midgut (rectum was not included) and the *N. ceranae* spore concentration was determined by optical microscopy and spore count using a hemocytometer. R 3.3.2 (R foundation for statistical computing, 2016) was used for survival analysis (Kaplan Meier analysis using Log-Rank tests) and plotting survival curves. At age 20, 10 days after infection of *N. ceranae*, all remaining honey bees in each group were sampled for *N. ceranae* spores.

RNA isolation and cDNA synthesis

Bees were anesthetized on ice and their midgut dissected. One midgut in each sample (about 50% of the midgut) was placed in a 1.5 ml Eppendorf tube and added 300 µl of Trizol. After being homogenized using a motorized pestle, we washed the pestle with 500 µl of Trizol. The rest of the RNA extraction was done we used according to the manufacturer's instructions (Trizol kit, Invitrogen, Carlsbad, CA). Nanodrop 2000 spectrophotometer (Thermo scientific, Wilmington, DE, USA) was used to check the RNA quality. Values between $1.8-2.0$ were considered acceptable as pure RNA.

First strand cDNA synthesis was performed by QuantiTect Reverse Transcription Kit (QIAGEN, USA) in accordance with the manufacturer's instructions. The cDNA was kept at $-20\,^{\circ}C$ until use. The quality of cDNA was measured by a Nanodrop 2000 spectrophotometer (Thermo scientific, Wilmington, DE, USA).

Quantitative PCR

Primers for four immune genes (Evans, 2006; Van Vaerenbergh et al., 2013), two detoxification genes (Alptekin et al., 2016) and two reference genes Actin (Simone et al., 2009) and *GAPDH* (Scharlaken et al., 2008) were synthesized by Integrated DNA Technologies, Inc. (Skokie, IL, USA) (Table 1). Two reference q-PCR was then performed with the SYBR Green master mix kit (Qiagen) and an ABI 7900 Real Time PCR system (Applied Bio systems, Foster City, CA, USA). Gene-specific primers were used in each well on a 96-well plate, in which every qPCR reaction contained 7.5 µl SYBR mix, 0.15 µl each of F and R primers (Table 2), 1 µl cDNA, and 6.2 µl RNase-free water (15 µl total volume). All samples were measured in duplicates and the reaction cycle consisted of a melting step of 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. If the coefficient of variation of the duplicate exceeded 8%, we reran that sample again using duplicates.

$$\text{Relative gene expression} = \Delta CT_1\text{-}\Delta CT_2$$

Here: ΔCT_1=CT(Samples)-[CT(actin)+CT(GAPDH)]/2

ΔCT_2=average CT of 10 control samples

Statistical analysis

Statview5.0.1 (SAS in statute Inc., USA) was used for the gene expression statistical analysis. The data were examined of normal distribution using the homogeneity test of variance and were used ANOVA to analyze the data with homogeneity of variance. Gene expression data were found to be not normally distributed after K-S Normality Tests, they were therefore transformed by taking log (gene expression level) to satisfy requirement of ANOVA. Fisher's Protected Least Significant Difference tests were used for multiple comparisons after a significant effect was found by ANOVA first.

The effect of thiamethoxam and *N. ceranae* on honey bee mortality was analyzed with Log rank tests, of the Kaplan-Meier survival analysis by using the survival package of R 3.3.0.

Results

Sublethal doses of thiamethoxam increased mortality of N. ceranae-infected honey bees

In all three colonies, thiamethoxam did not show significantly reduced survival compared to the control, nor did *N. ceranae* except in colony A (Figure 2 A to C). However, when both stresses were combined, thiamethoxam and *Nosema* showed significantly reduced survival in all three colonies. When all the data were combined (Figure 2D), thiamethoxam alone did not cause reduced survival compared to the control, but *N. ceranae* caused a significant reduction in survival. Combining both factors caused further are reduction in survival but this reduction is not significantly different from *N. ceranae* alone.

Pre-exposure to thiamethoxam did not increase the spores of N. ceranae

The number of spores in the midgut of live bees (20 days old) from *Nosema*-infected bees was (1,223,500 ± 224,200) and thiamethoxam treated and *Nosema* infected bees (1,220,500 ± 149,500) were not significantly different ($t = 0.04$, $P > 0.05$).

Effect of thiamethoxam and N. ceranaeon immune gene expression

There were no differences in immune gene expression for bees from different colonies (ANOVA for colony effect: abaecin $p = 0.1741$, apidaecin $p = 0.2155$, defensin $p = 0.2238$, hymenoptacin $p = 0.4741$). However, significant differences were found for bees from different treatments. Exposure to thiamethoxam caused significantly increased gene expression compared to the control for abaecin, apidaecin and hymenoptacin, but significantly decreased expression for defensin (Figure 3). *Nosema* infection followed the same pattern, with the exception of apidaecin, which did not show a change of expression compared to the control. When the two factors were combined, the expression patterns were similar to *N. ceranae* infection alone for apidaecin, defencin and hymenoptaecin, except abaecin, which showed no change compared to the control, i.e. the increased levels are seen in thiamethoxam exposed and *Nosema* infected bees disappeared.

Effect of thiamethoxam exposure on detoxification gene expression

There were no significant differences in detoxification gene expression for bees from different colonies (ANOVA for colony effect: cce8, $p = 0.1128$;cyp315SA, $p = 0.2402$). For both CCE8 and CYP315A1, thiamethoxam caused significantly increased gene expression but *Nosema* caused significantly lower expression (Figure 4). Combining the two stresses did not show a further increase compared to *Nosema* infection alone.

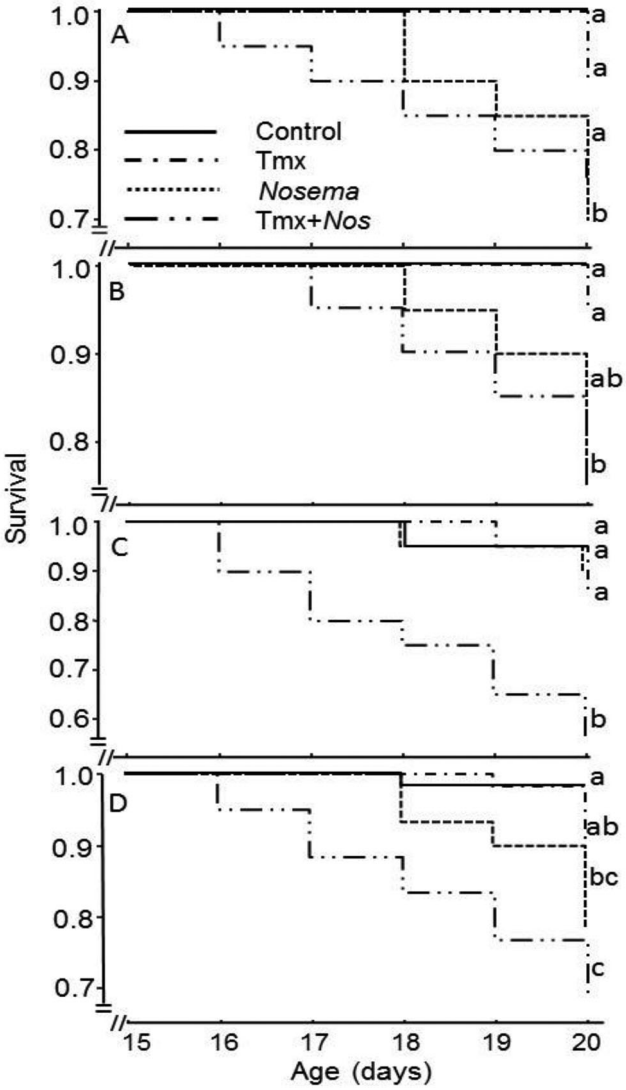

Figure 2. Sublethal doses of thiamethoxam increased mortality of *Nosemaceranae*-infected honey bees.Different letters in the column of average lifespan indicate significantdifference ($P < 0.05$).

Discussion

N. ceranae is a widespread microsporidium that seems to have recently jumped from its host, the Asian honey bee *Apis ceranae*, to the worldwide honey producer, *A. mellifera* (Higes et al., 2006). Meanwhile, pesticides especially neonicotinoid have produced serious harm to the health of bees as well (Mainz, 2016; Wessler et al., 2016). These pesticides were generally not targeting bees, but because of their widespread and non-standard use, they have already threatened the safety of bee individuals and even influenced the bee population. With the arising public awareness, highly toxic pesticides were gradually eliminated, while insecticides such as nicotine insecticides (developed in the 1990s as an alternative pesticide) are increasingly used. The researcher enthusiasm towards these insecticides saw an annually increasing trend as well, whose third generation production already used in world, because they are safety

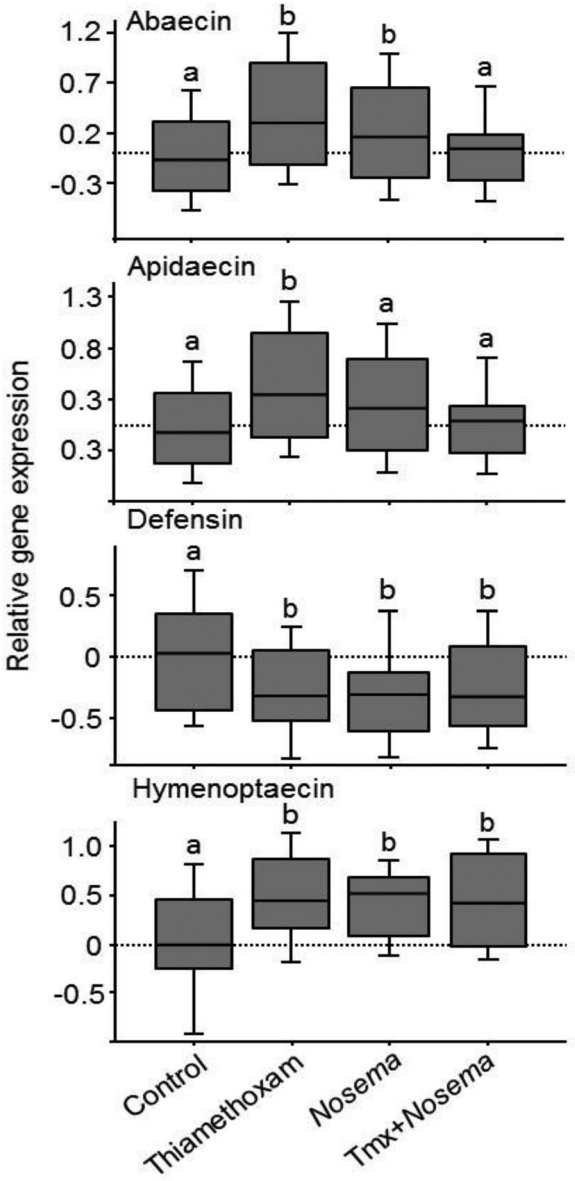

Figure 3. Effect of thiamethoxam exposure on detoxification gene expression. Different letters in the column of average lifespan indicate significant difference ($P < 0.05$).

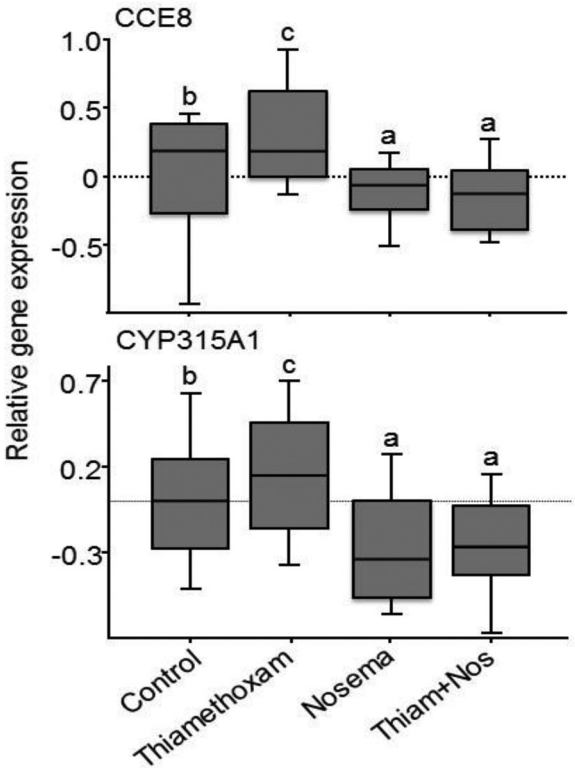

Figure 4. Effect of thiamethoxam exposure on detoxification gene expression. Different letters in the column of average lifespan indicate significant difference ($P < 0.05$).

to mankind and environment. They have high toxicity in sucking insects such as beetles and some Lepidoptera, particularly cutworms. Due to the fact that their targets are nicotinic acetylcholine receptors (nAChRS) in insect nervous system, these insecticides also have high toxicity towards honey bees since they can block bees' receptors as well, causing paralysis and death (Matsuda et al., 2005). Even though the honey bees are never meant to be the target, they widely experience potential exposure from the whole plant, including pollen, nectar, and guttation. Acute high dose exposure can directly lead to the death of bees (Shoumin et al., 2015), and nicotine insecticides can also affect adult worker bees under sublethal doses of behavior (Aliouane et al., 2009; El Hassani et al., 2008; Mengoni Gonalons & Farina, 2015), affecting the larvae (Yang et al., 2012) and even the drone (Kairo, Biron et al. 2017) and queen's health (Brandt et al., 2017).

As a social organism, the honey bee colony can be considered as a complex living system. Therefore, sometimes an individual's illness may affect the whole group, such as the case of infectious diseases. This disease can spread quickly between honey bees because there have to feed each other behavior. In addition to causing these changes, *Nosema* infection also causes the death of bees. Higes et al. (2007) reported that in their experiment, the infected groups' mortalities were not observed until day 6 p.i. (66.7%+/-5.6). Total mortality on day 7 p.i. was 94.1% in the three infected replicates and by day 8 p.i. no infected bee was alive (Higes et al., 2007). In our results, *Nosema* infected honey bees started to die from post infection day 8. Our findings were different from Higes' but similar to other researchers (Vidau et al., 2011). The risk of colony depopulation is six times higher in colonies infected with *N. ceranae* than in uninfected ones (Martín-Hernández et al., 2007) .

Insects have a robust immune system to defend themselves against the attack of microbial and eukaryotic pathogens. Insects' humoral immunity involves the synthesis of a battery of antimicrobial peptides in response to infection by bacteria, fungi, or parasites. In honey bees (*A. mellifera*), the immune repertoire consists of at least four peptides, including apidaecin,

abaecin, hymenoptacin, and defensin. A localized immune response in the midgut can be implemented by the production of reactive oxygen species (ROS) which are toxic to pathogens (Broderick et al., 2009, Nathan & Cunningham-Bussel, 2013). But ROS can also have cytotoxic effects on host tissues, a balance between the generation and elimination of ROS must be maintained. Kairo, Biron et al. (2017) reported that drones infected with *Nosema* alone can induce moderate and localized effects in the midgut (Kairo, Biron et al. 2017). Hymenoptacin is a key gene of congenital immunity gene. Antúnez et al. (2009) reported that infection of honey bees with *N. ceranae* caused significant down-regulation ofabaecin and hymenoptacin expression. Other researchers, Chaimanee et al., (2012) and Aufauvre et al.,(2014) had another result that results showed that the mRNA levels of four antimicrobial peptides, defensin, abaecin, apidaecin and hymenoptacin were down-regulated in *N. ceranae*-infected workers (Aufauvre et al., 2014; Chaimanee et al., 2012). However, after *N. ceranae* infection, honey bees also had unanimous changes in these genes. Antúnez et al. (2009) had found infection of honey bee with *N. ceranae* for 4 days without change, but after day 7, the expression of abaecin diminished significantly when compared with control. Defensin expression increased in the first 4 days but became stable until day 7. The expression level of hymenoptacin mRNA level first increased but then decreased after day 7 (Antúnez et al., 2009). We also found these four genes thought to be involved in immunity were the inconsistent change. The mRNA level of defensive gene had decreased and reached significant difference ($p < 0.05$). The abaecinand hymenoptacin experienced upregulation ($p < 0.05$). Why is there such a difference in expression? This may be related to the amount of *Nosema* infection and the time of the sampling. There was no significant reduction in survival because pesticides were used at a sublethal level. Pesticides might also act on insect immune systems (Aufauvre et al., 2014), and fungicides and acaricides have been shown to down regulate immune-related genes in honey bees (Boncristiani et al., 2012; Garrido et al., 2013; Gregorc et al., 2012). However, Li, Li et al. (2017) reported in their paper that with neonicotinoid insecticide exposure in *A. mellifera* and *A. ceranae*, all four immune-related gene expression levels were significantly increased (Li, Li et al., 2017). Gene ofdetoxification for the phase I detoxification enzymes used by organisms, including insects, to metabolize xenobiotic, including phytochemicals and insecticides. Neonicotinoids can be metabolized by insecticide-resistant insect pests as a result of enhanced expression of specific cytochrome P450s. Cytochrome P450 monooxygenases (P450s) are among the principal component. However, honey bees have a relatively low number of CYP genes encoding P450s compared to other insect species (Claudianos et al., 2006). A particular insecticides xenobiotic, might work as an inducing factor which may potentially increase metabolic activity and allow identification of specific metabolic enzymes from honey bees that are involved in chemical defense (Alptekin et al., 2016). Mao, Schuler et al. (2013) determined that constituents found in honey, including p-coumarou acid, pinocembrin, and pinobanksin 5-methyl ether, can specifically induce detoxification and change immunity genes. Vidau et al. (2011) failed to demonstrate a change in the pesticide detoxification system in adult bees.

Although the above two reasons alone may lead to the disease and even result in the death of bees, and these reasons for bee's CCD seems hard to convince. A lot of long-term studies performed through many years suggested that CCD may include both of up reason. We found the joint action of pathogens and chemicals and observed these stressors in honey bee. It is also reported that sublethal doses of neonicotinoid pesticide and infected *N. ceranae* can significantly increase honey bee mortality (Aufauvre et al., 2012; 2014; Retschnig et al., 2014; Vidau et al., 2011). All generations of nicotine insecticides have shown the same effect. Therefore, these two factors can be superimposed to affect the honey bee health. Although that combination of *Nosema* and insecticide causes significant mortality in bees, do they have different mechanisms of action? *N. ceranae* can sensitize honey bees to pesticides and vice versa. Nevertheless, the mechanism of this action is not fully understood yet. First, it is known that the parasite development and transmission success can be modified in intoxicated organisms, so insect detoxification system may be changed on parasite development, either by disrupting or enhancing. *Nosema* infection induced a higher cell death rate in comparison with uninfected bees by the 5th day post-infection (Gregorc et al., 2016). In the first stages of the parasite infection, the bee's midgut experienced accelerating levels of apoptosis. This defense mechanism could prevent the spread of infection to neighboring cells (James & Green, 2002). Nicotine insecticides can also be entirely killed in midfield cells (Oliveira et al., 2014). It is clear that *Nosema* and nicotine insecticides have a common target organ. But compared with other researchers, such as Aufauvre et al. (2014), they found no effect on these four immunity genes was caused by *Nosema* and fipronil(Aufauvre et al., 2014). According to gene expression profiles, chronic exposure to insecticides had repressed the expression of immunity-related genes.

In our study, it was shown that the first exposure to sublethal doses of thiamethoxam followed by *N. ceranae* infection also resulted in an increase of honey bee mortality. During our experiments, for the 3 colonies of bees only received the sublethal doses of thiamethoxam by oral, no mortality was observed on 5^{th}day until after the 14^{th} day (age19), while the group infected by

N. ceranae only died from day 8 (age18), the group first exposed to thiamethoxam than infected with *N. ceranae*died from 6 days (age 16).In all three colonies, thiamethoxam did not show significantly reduced survival compared to the control, nor did *N. ceranae* except in colony A in our study. However, when both stresses were combined, thiamethoxam+*Nosema* showed significantly reduced survival in all three colonies. When all the data were combined, thiamethoxam alone did not cause reduced survival but *N. ceranae* caused a significant reduction in survival, combining both factors caused further reduced survival but this reduction is not significantly different from *N. ceranae* alone. A decrease in lifespan is commonly observed in *Nosema*-infected *A. mellifera*. The life span in *Nosema*-infected honey bee is 14th day after infection (Dussaubat et al., 2012), as well as the 20th day post-infection (Huang & Solter, 2013).

In our study, we found that single administration thiamethoxam significantly enhanced the detoxifying gene expression but lowered the detoxifying gene in *Nosema* infected honey bee. While the detoxifying gene expression is low, the experiment first administrated thiamethoxam, then infected *Nosema*. Chronic exposure to insecticides had no significant impact on detoxifying genes but repressed the expression of immunity-related genes. Honey bees treated with *N. ceranae*, alone or in combination with an insecticide, showed a strong alteration of midgut immunity together with modifications affecting cuticle coatings and trehalose metabolism.

Conclusions

This paper shows a clear combined effect of thiamethoxam and *Nosema* in adult worker honey bees which result in immunity gene and detoxifying gene expression and mortality changed.

Disclosure statement

No potential conflict of interest was reported by the authors.

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