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Exposure of honey bee larvae to thiamethoxam and its interaction with *Nosema ceranae* infection in adult honey bees☆

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ABSTRACT

During their lifetime honey bees (*Apis mellifera*) rarely experience optimal conditions. Sometimes, a simultaneous action of multiple stressors, natural and chemical, results in even greater effect than of any stressor alone. Therefore, integrative investigations of different factors affecting honey bees have to be carried out. In this study, adult honey bees exposed to thiamethoxam in larval and/or adult stage and infected with *Nosema ceranae* were examined. Newly emerged bees from colonies, non-treated or treated with thiamethoxam, were organized in six groups and kept in cages. Thiamethoxam treated bees were further exposed to either thiamethoxam or *Nosema* (groups TT and TN), or simultaneously to both (group TTN). Newly emerged bees from non-treated colonies were exposed to *Nosema* (group CN). From both, treated and non-treated colonies two groups were organized and further fed only with sugar solution (groups C and TC). Here, we present the expression profile of 19 genes in adult worker honey bees comprising those involved in immune, detoxification, development and apoptosis response. Results showed that gene expression patterns changed with time and depended on the treatment. In group TC at the time of emergence the majority of tested genes were downregulated, among which nine were significantly altered. The same gene pattern was observed on day six, where the only significantly upregulated gene was *defensin-1*. On day nine most of analyzed genes in all experimental groups showed upregulation compared to control group, where upregulation of antimicrobial peptide genes *abaecin*, *defensin-1* and *defensin-2* was significant in groups TT and TTN. On day 15 we observed a similar pattern of expression in groups TC and TT exposed to thiamethoxam only, where most of the detoxification genes were downregulated. Additionally RNA loads of *Nosema* and honey bee viruses were recorded. We detected a synergistic interaction of thiamethoxam and *Nosema,* reflected in lowest honey bee survival.

1. Introduction

In recent years many factors (physical, biological, chemical and nutritional) were identified to have a negative effect on honey bee health. However, there is still no definitive explanation for their multiple losses (Goulson et al., 2015; Stanimirovic et al., 2019; vanEngelsdorp et al., 2009). Two most important factors that contribute to honey bee colony losses are definitely pesticides and pathogens (Aufauvre et al., 2012; Doublet et al., 2015). These factors co-occur in colonies most of the time, as honey bees infected with different pathogens come in contact with pesticides when collecting pollen and nectar (Goulson et al., 2015; Neumann and Carreck, 2010). Therefore, integrative investigation of different factors on honey bee colonies have to be carried out. In this study we investigated the effects of *Nosema ceranae* infection and thiamethoxam treatment on expression of immune, detoxification, apoptosis and developmental genes in adult honey bees.

Neonicotinoid insecticides are most in-depth investigated cause of honey bee losses, as they are liable to cause changes in honey bee physiology, such as hypopharyngeal gland development (Hatjina et al., 2013; Renzi et al., 2016), and honey bee behavior (Suchail et al., 2001; Williamson et al., 2013; Williamson and Wright, 2013), colony development (Wu et al., 2011), foraging (Schneider et al., 2012; Yang et al., 2008), memory and learning (Desneux et al., 2007; Frost et al., 2013; Williamson and Wright, 2013). Most worldwide used is thiamethoxam, one of systemic insecticide for seed coating or direct use on crops (Nauen et al., 2003).

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Thiamethoxam is known to effectively transform into its metabolite chlothianidin when present in plants or insects (Benzidane et al., 2010; Coulon et al., 2018; Nauen et al., 2003). Metabolite clothianidin accumulates in the honey bee rectum after metabolisation and is thereafter excreted during cleansing flights (Coulon et al., 2018; du Rand et al., 2015). Previous studies demonstrated that thiamethoxam induce deleterious effects in adult honey bees (Badiou-Beneteau et al., 2012; Coulon et al., 2018; Henry et al., 2012; Tosi et al., 2017), honey bee pupae (Tesovnik et al., 2017), and larvae (Friol et al., 2017; Grillone et al., 2017; Tavares et al., 2015; Tavares et al., 2017). At the same time, microsporidians from the genus *Nosema*, are one of most common intracellular spore-forming fungal gut parasites that infect adult honey bees. *N. ceranae* is ubiquitous honey bee parasite that infects their midgut after ingestion of mature spores through the activities of cleaning and trophallaxis (Higes et al., 2010). It is known to cause several negative effects (degeneration of gut epithelial cells, immune suppression, and energetic stress) on health of individual bees which has consequently an impact on whole colony (Antunez et al., 2009; Badaoui et al., 2017; Dussaubat et al., 2012; Glavinic et al., 2017; Higes et al., 2013). Moreover, higher sugar solution consumption of bees infected with *Nosema* causes energetic stress (Badaoui et al., 2017; Mayack and Naug 2009; Stanimirovic et al., 2019). Higher food consumption would lead to higher exposure to pesticides if their food in natural habitat contains one or more pesticides.

Most data about *Nosema* and pesticide interactions were obtained by investigated exposure of honey bees to pesticides and *Nosema* only after emergence (Alaux et al., 2010; Aufauvre et al., 2014; Pettis et al., 2013; Vidau et al., 2011). Other studies were based on exposure of young brood to pesticides and later infection with *Nosema* spores (Glavinic et al., 2019; Pettis et al., 2012; Wu et al., 2012). In this study, we designed experiment where honey bees were exposed to pesticide first in larval and later in adult stage together with *Nosema* infection. In this experiment we wanted to explore how exposure to different combination of stressors affects newly emerged honey bees, by monitoring their survival and the expression of genes for immunity, detoxification, apoptosis, and development.

2. Material and methods

Experiment was conducted at the Faculty of Veterinary Medicine (FVM), University of Belgrade, Serbia. The honey bees used in this study were from *Apis mellifera* colonies kept in Langstroth-Root hives at the FVM apiary. Subspecies of honey bees used in this study was not determined, as our previous investigations revealed that honey bees in Serbia are mostly hybrids of *Apis mellifera carnica* and *Apis mellifera macedonica* (Muñoz et al., 2012). Before the experiment hives where left to feed naturally on the available bee forage and were maintained in accordance to good beekeeping practice. The last treatment on hives was performed against *Varroa destructor* with coumaphos stripes according to CheckMite+® instructions (CheckMite+®, Bayer) in November of the previous year (six months before experiment; November 2016). Honey bee colonies did not present visible symptoms of any known brood disease. All samples were also tested for nine most common honey bee pathogens using RT-qPCR method (Table S1).

2.1. Experimental setup

2.1.1. Field experiment

Four healthy colonies headed by sister queens without clinical signs of brood and adult bee disease were chosen for the experiment. Two colonies were treated with thiamethoxam and were placed away from the apiary to prevent chance for drifting during the experiment. Another two colonies served as control, and remained untreated throughout experiment. Honey reserves were completely removed from colonies. Once a day colonies were fed with 200 mL of sugar syrup. Experimental colonies received sugar syrup containing 10 μg/kg thiamethoxam. Used concentrations of thiamethoxam were within the range detected in contaminated hives (Pilling et al., 2013). After one month of feeding, frames with sealed brood (a day or two prior to emergence) from treated and control colonies were transferred to the laboratory at FVM, where they were kept in separated incubators at 34 ± 1 °C until emergence. The laboratory experiment started immediately after emergence of honey bees.

2.1.2. Laboratory experiment

For establishing six experimental groups (C, CN, TC, TN, TT and TTN; Fig. 1 and Table 1), newly emerged worker bees were removed from frames and divided into 12 cages (two cages per group) designed by Glavinic et al. (2017). Each cage consisted of 30 newly emerged honey bees. The bees were fed *ad libitum* with 50% w/v sucrose solution. Four cages were created using newly emerged bees originating from control colony, two of them were fed only with sugar syrup and represent control group (group C), and other two cages contained honey bees infected with *N. ceranae* spores on day three after emergence as later described (group CN). Newly emerged honey bees obtained from thiamethoxam treated colonies, were divided into eight cages. First two cages were given sugar syrup (group TC), second two received sugar syrup with addition of thiamethoxam in concentration of 10 μg/kg (group TT). Third two cages were infected with *Nosema* spores (group TN), and last two cages were infected with *Nosema* spores and received sugar syrup with thiamethoxam (group TTN). To obtain *N. ceranae* inoculum, fresh spore suspension was mixed with sucrose solution in final concentration of 10^6 spores per mL according to the protocol described by Glavinic et al. (2017). Bees in cages from groups CN, TN and TTN were infected with inoculum on the 3rd day after emergence as described by Fries et al. (2013).

Samples for gene expression analysis were collected on day of honey bee emergence (day 0) from frames obtained in treated and control colonies and later from each cage on day 6th, 9th and 15th after emergence (Fig. 1). Each day, we removed dead bees from cages and recorded mortality.

2.2. RNA extraction and cDNA synthesis

Each individual honey bee was placed in sterile 1.5 mL polypropylene microtube (Eppendorf) containing 1 mL of TRIZOL® (Invitrogen™, Thermo Fisher Scientific, USA) and homogenized with sterile disposable micro tube pestles (VWR, San Francisco, CA). Extracted RNA was then purified with Quick-RNA™ MiniPrep columns (Zymo Research, USA). Residual DNA was removed following Quick-RNA™ MiniPrep instructions. RNA concentrations were quantified using Biospec-nano (Shimadzu biotech, Japan). Extracted RNA was immediately transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

2.3. Real-time qPCR

The primers used to amplify 19 genes (12 immune, five detoxification, one developmental, one apoptosis and three reference genes) were those reported by Gregorc et al. (2012), Cizelj et al. (2016) and Evans (2006) and synthesized by IDT (Integrated DNA Technologies, US) (Table S2). For quantitative real-time PCR, 10 μL reactions were prepared, containing 5 μL of Fast Start Universal

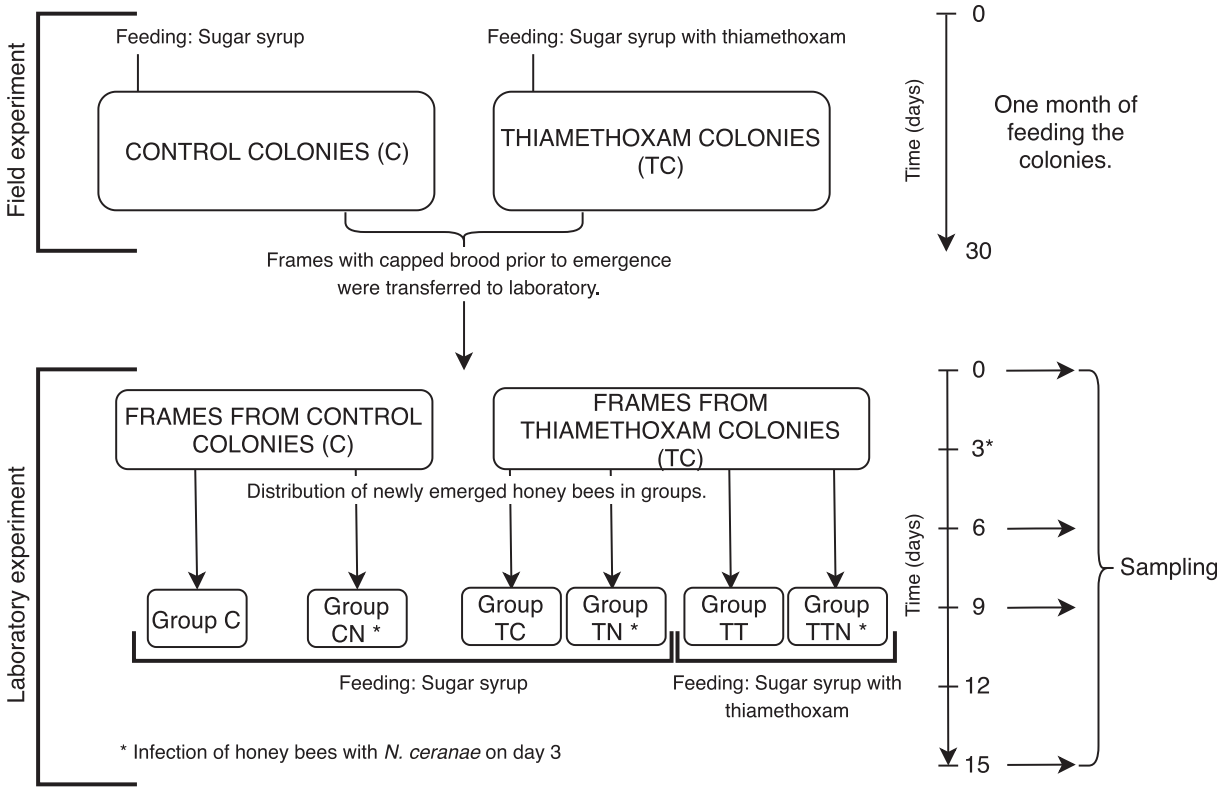

Fig. 1. Schematic diagram of experimental design.

Table 1
Experimental design. Boxes marked with the symbol (N) in the table represent groups inoculated with *N. ceranae* spores and the symbol (/) represent groups that were not inoculated with spores. The boxes with the plus sign (+) show which experimental group belongs to which treatment.

Groups	Feeding					
	Sugar syrup (larval stage)		Sugar syrup with thiamethoxam (larval stage)			
	Sugar syrup (adult stage)		Sugar syrup (adult stage)		Sugar syrup with thiamethoxam (adult stage)	
N. ceranae infection	/	N	/	N	/	N
C	+					
CN		+				
TC			+			
TN				+		
TT					+	
TTN						+

SYBR Green Master (ROX) (Roche Diagnostics GmbH, Germany), 250 nM of forward and reverse primer, DEPC treated water and 1 μL of cDNA (6.25 ng per reaction). Amplification of targeted molecules were performed with ViiA7 (Applied Biosystems, USA) and analyzed with QuantStudio™ Real-Time PCR Software. For the experimental run the following cycle profile was used: denaturation step at 95 °C for 10 min and 40 cycles of amplification at 95 °C for 20 s, 20 s at Tm of each primer pair and 72 °C for 20 s, followed by dissociation curve step at 95 °C for 15 s, 60 s at Tm and 15 s at 95 °C, where in each cycle temperature is gradually rising from Tm to 95 °C by 0.5 °C increments per cycle. Reactions for quantitative real-time PCR were carried out in 384-well plates (MicroAmp®, Life Technologies). Each experiment contained three no-template controls and test samples preformed in duplicates. Gene expression was analyzed using Rp49, Tbp-af and *RPS5* as reference genes. Gene expression values of non-treated groups were used for gene expression calibration. For each gene the level of gene expression was calculated using the method described by Pfaffl (2001) where the normalized relative expression ratio between treated and non-treated group is based on PCR efficiency. These results were then graphically represented on a heatmap illustrating differential expression of genes as a consequence of different treatments. The significance of the treatment effect was indicated according to statistical analysis described below.

All collected samples were also tested for nine most common honey bee-pathogen targets (Table S1) using qRT-PCR, also performed as described above. The expression of pathogen genes of all the experimental samples were evaluated by comparing threshold cycle (Cq) values between treatment groups.

2.4. Statistical analysis

Gene expression levels were measured on the individual level; experimental unit was individual honeybee. The effects of

N. ceranae infection, thiamethoxam treatment during different honey bee developmental stages (larvae, adult) and their interactions on gene expression were analyzed using linear model for fixed effects (lm) according to the following model:

$$y_{ijkl} = \mu + N_i + L_j + LA_{jk} + NLA_{ijk} + e_{ijkl}$$

where y_{ijkl} is gene expression (Cq), μ is overall mean, μ is fixed effect of *Nosema* infection, N_i is fixed effect of thiamethoxam treatment in larval stage, A_k is the effect of thiamethoxam treatment in adult stage and e_{ijkl} is residual error. The estimation of least squares means followed by Dunnett's post hoc test was used for pairwise comparisons among the treatment groups. The assumption of normal distribution was tested and met via examination of the residuals (coefficients of skewness and kurtosis).

Mortality among different treatments was analyzed using Cox proportional hazard model. In modeling of survival data of caged bees under exposure to pesticides (Dechaume et al., 2003) and pathogen, the treatment, the effect of cage and interaction between them were considered. Bees taken for the gene expression analysis were censored. Statistical significance was declared at $P < 0.05$. All statistical analyses and plotting were performed using R software version 3.5.0 (Team R 2017) with relevant libraries (lsmeans, moments, ggplot2, survminer and survival) (Komsta and Novomestky, 2015; Lenth, 2016; Wickham, 2009).

3. Results

3.1. Effect of thiamethoxam treatment and Nosema infection on survival rate of bees in cages

Analysis of survival revealed that each treatment led to significantly decreased survival of honey bees compared to control (Fig. 2). Mortality rates in groups C (10%) and TC (15%) were the lowest. For groups CN (35%), TN (40%) and TT (53%) mortality rates were significantly higher (using Cox proportional hazard model; $P < 0.05$). Honey bees exposed to both stressors (group TTN) reached 68% mortality rate which was significantly higher from control and all other treated groups.

3.2. Time- and treatment-dependent gene expression

3.2.1. Day 0

On the day of honey bee emergence (day 0), samples were collected from frames obtained in treated and control colonies to compare their gene expression. In this time point, we recorded significant downregulation in nine out of 19 of tested genes (Fig. 3; Table 2). There was significant downregulation of four detoxification genes (*PKA-R1*, *CYP6AS14*, *CYP4G11* and *CYP306A1*) in honey bees from group TC. Among significantly dysregulated immune related genes, *PGRP SC 4300* and *lysozyme-2*, had the lowest gene expression following by *relish*, *domeless*, and *iap-2*.

3.2.2. Day 6

On the day six after the beginning of cage experiment, the majority of genes were downregulated in all treated groups (Fig. 3). In the group CN, ten genes were significantly downregulated three days after *Nosema* infection. The most downregulated genes were two immune related and two detoxification genes, *abaecin*, *defensin-1*, *CYP4G11* and *CYP6AS14*. Group TC showed increased number of significantly dysregulated genes (12 out of 19) compared to day 0. Most downregulated were immune related genes (*toll*, *domeless* and *lysozyme-2*), developmental gene *VGMC* and detoxification genes (*CYP4G11*, *CYP6AS14* and *catalase*). In the group TT only three genes, *defensin-1*, *domeless* and *PGRP SC 4300*, were significantly dysregulated. Group TN had two upregulated immune related genes, *defensin-1* and *dorsal*, while other immune genes were downregulated. Downregulated expression was also observed for all detoxification genes. Another group that had seven immune and antimicrobial peptide (AMP) genes significantly downregulated was TTN, where *abaecin* had the lowest expression (Table 2).

3.2.3. Day 9

Most of analyzed genes in experimental groups on the day nine showed upregulated expression compared to control (Fig. 3; Table 2). At this time point, the lowest number of significantly

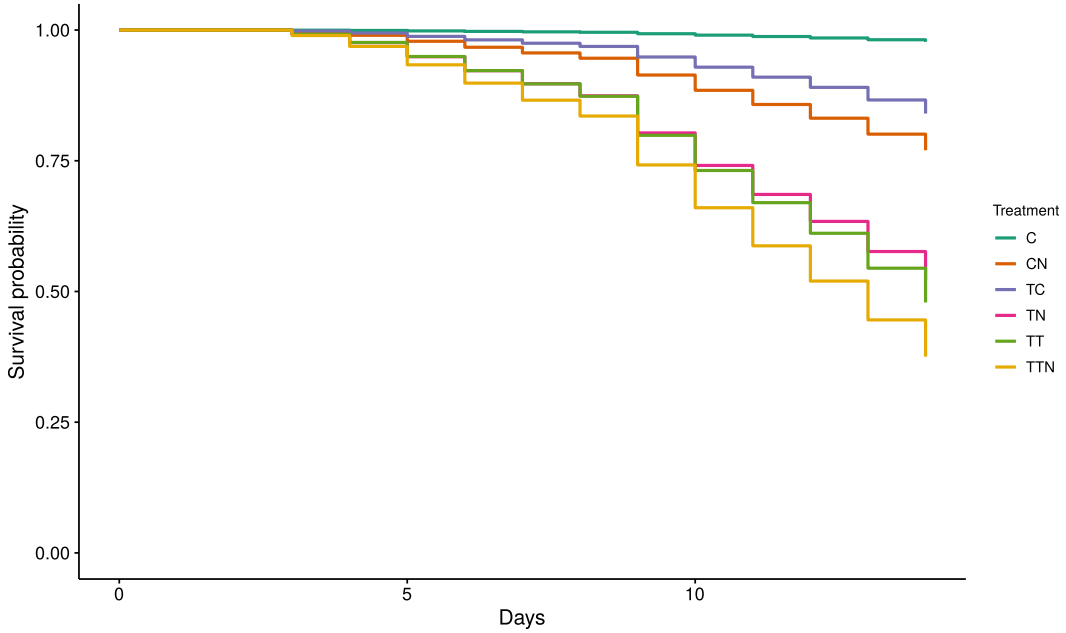

Fig. 2. Effects of *N. ceranae* infection and exposure to thiamethoxam on survival of honey bees. Data show the survival probability over 15 days treatment period observed in cage experiments of newly emerged honey bees. Survival probability among different treatments was analyzed using Cox proportional hazard model.

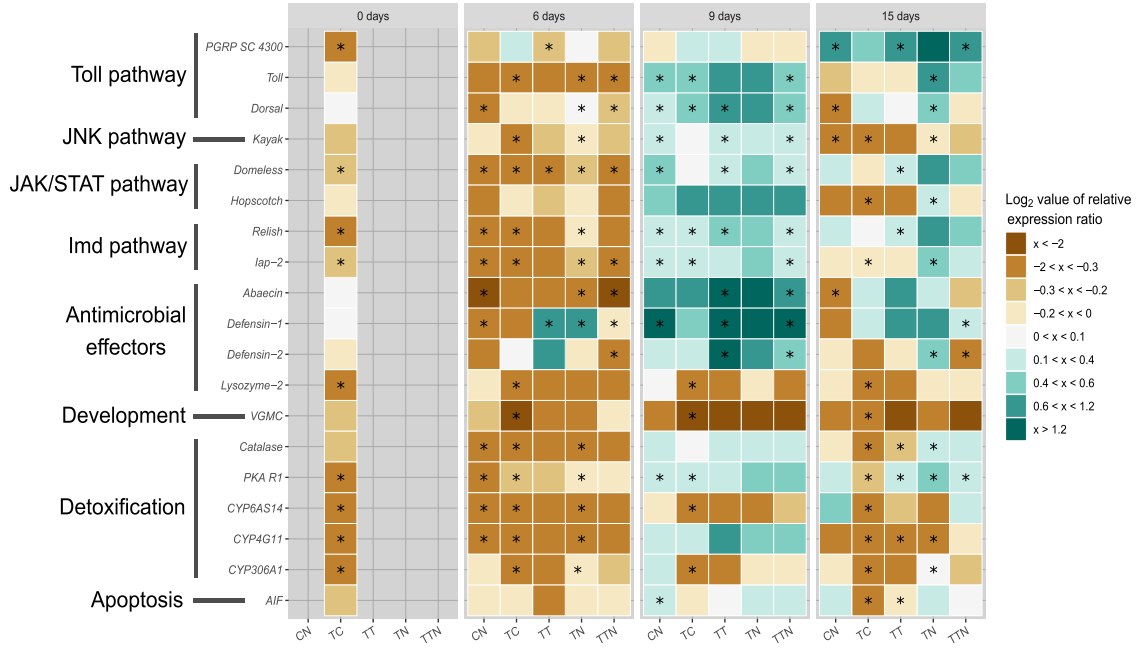

Fig. 3. Gene expression heatmap of adult honey bees in different time points and treatments. The relative expression ratio of analyzed genes between treated and non-treated groups is demonstrated in heatmap graph. Four boxes represents four time points in our experiment (day zero, six, nine, and 15). In each box columns correspond to the expression profile of treatments (CN, TC, TT, TN and TTN), and each row presents the expression level of the gene whose name is indicated on the left. Range $\log_2$ value of relative expression ratio is indicated in the legend on the right. Boxes marked with asterisk shows statistically significant effect of treatment on gene expression, when p-value was equal or less than 0.05. The effects of *N. ceranae* infection and thiamethoxam treatment during different honey bee developmental stages (larvae, adults) and their interactions on gene expression were analyzed using linear model for fixed effects (lm).

Table 2
Relative expression ratios for studied genes at different time points for each treatment group. Numbers with an asterisk sign printed in bold type indicates significantly elevated or decreased transcript abundances; *** indicate P < 0.001, ** indicate P < 0.01 and * is for P < 0.05. The effects of *N. ceranae* infection and thiamethoxam treatment during different honey bee developmental stages (larvae, adults) and their interactions on gene expression were analyzed using linear model for fixed effects.

	0 day	6 days					9 days					15 days				
	TC	TC	TT	CN	TN	TTN	TC	TT	CN	TN	TTN	TC	TT	CN	TN	TTN
PGRP SC 4300	**−0,66** **	0,15	**−0,21** *	−0,28	0,01	−0,24	0,14	0,13	−0,07	−0,04	−0,19	0,59	**1,1** **	**0,68** *	1,26	**0,7** **
Toll	−0,07	**−0,82** ***	−0,71	−0,35	**−0,43** *	**−1,1** *	**0,54** **	0,75	**0,45** **	0,79	**0,49** *	−0,18	−0,01	−0,22	**0,66** **	0,46
Dorsal	0,05	−0,17	−0,13	**−0,37** **	**0,01** **	**−0,29** *	**0,6** ***	**0,81** *	**0,32** ***	0,69	**0,46** ***	0,12	0,09	**−0,54** **	**0,46** **	0,00
Kayak	−0,25	**−0,37** ***	−0,21	−0,14	**−0,12** *	−0,24	0,00	**0,26** *	**0,33** **	0,31	**0,19** *	**−0,69** ***	−0,78	**−0,52** **	**−0,03** ***	−0,26
Domeless	**−0,29** *	**−0,56** ***	**−0,35** *	**−0,47** ***	**−0,23** ***	**−0,42** **	0,09	**0,28** *	**0,48** ***	0,46	**0,3** **	−0,09	**0,19** **	0,32	0,64	0,41
Hopscotch	−0,09	−0,16	−0,28	−0,46	−0,15	−0,68	0,64	1,02	0,47	0,78	0,62	**−0,56** *	−0,54	−0,64	**0,22** **	−0,05
Relish	**−0,32** *	**−0,46** ***	−0,48	**−0,51** ***	**−0,19** ***	−0,51	**0,2** *	**0,42** *	**0,37** ***	0,55	**0,37** **	0,09	**0,32** *	0,15	0,63	0,51
Iap 2	**−0,29** *	**−0,37** ***	−0,38	**−0,39** ***	**−0,21** ***	**−0,62** **	**0,28** **	0,37	**0,36** ***	0,51	**0,36** *	**−0,14** *	−0,02	−0,01	**0,46** *	0,31
Abaecin	0,03	−1,27	−0,50	**−2,38** ***	**−1,32** *	**−2,67** *	1,00	**2,45** *	0,97	2,76	**1,14** ***	0,31	1,05	**−1,18** *	0,28	−0,25
Defensin 1	0,03	−0,49	**0,64** *	**−1,2** *	**0,86** **	**−0,18** **	0,53	**1,81** **	**1,38** **	2,49	**1,63** ***	0,21	0,97	−0,39	0,87	**0,25** *
Defensin 2	−0,06	0,10	0,66	−0,39	−0,01	**−0,55** *	0,23	**1,56** ***	0,30	0,89	**0,57** ***	−0,37	−0,01	−0,14	**0,55** *	**−0,38** **
Lysozyme 2	**−0,5** **	**−0,56** **	−0,68	−0,18	−0,52	−0,43	**−0,51** *	−0,83	0,07	−0,13	−0,68	**−0,51** **	−0,49	−0,07	−0,11	−0,18
VGMC	−0,29	**−2,04** **	−1,81	−0,22	−1,08	−0,02	**−2,94** ***	−3,78	−0,74	−2,13	−2,06	**−1,93** ***	−2,46	−0,75	−1,91	−2,04
Catalase	−0,22	**−0,58** ***	−0,66	**−0,44** **	**−0,55** *	−0,55	0,07	0,22	0,13	0,35	0,36	**−0,85** ***	**−0,27** *	−0,19	**0,36** **	0,25
PKA-R1	**−0,39** **	**−0,22** *	−0,21	**−0,32** ***	**−0,03** ***	−0,04	**0,25** **	0,30	**0,39** ***	0,51	0,50	**−0,27** **	**0,12** ***	0,15	**0,55** *	**0,37** *
CYP6AS14	**−0,34** *	**−1,21** ***	−1,33	**−0,76** **	**−0,97** **	−0,47	**−0,71** **	−0,87	−0,04	−0,39	−0,23	**−0,73** *	−0,25	0,60	−0,41	0,18
CYP4G11	**−0,37** *	**−1,23** ***	−1,30	**−1,27** ***	**−1,05** ***	−0,71	0,38	0,73	0,24	0,43	0,42	**−0,97** ***	**−0,6** *	−0,41	**−0,69** *	−0,18
CYP306A1	**−0,39** **	**−0,44** **	−0,40	−0,16	**−0,14** *	−0,25	**−0,4** **	−0,37	0,21	−0,14	−0,07	**−0,63** ***	−0,50	−0,14	**0,08** **	−0,24
AIF	−0,24	−0,13	−0,30	−0,19	−0,16	−0,20	−0,09	0,04	**0,26** **	0,15	0,16	**−0,45** **	**−0,07** *	0,20	0,34	0,03

dysregulated gene expressions were observed. There were nine significantly upregulated genes in the group CN where immune related *defensin*-1 had the highest upregulation. Expression of four immune related genes (*toll*, *relish*, *dorsal* and *iap-2*) and detoxification gene *PKA-R1* in the group TC was upregulated. Other analyzed genes were downregulated (*VGMC*, *CYP6AS14*, *CYP306A1* and *lysozyme-2*). In the group TT only immune related genes (*dorsal*, *relish*, *domeless* and *kayak*) and genes of AMPs (*abaecin*, *defensin-1* and *defensin-2*) were significantly upregulated. There was no significant dysregulation in gene expression among tested genes in the group TN. Apart from AMPs genes (*defensin-1*, *abaecin* and *defensin-2*) other immune related genes (*toll*, *dorsal*, *domeless*, *relish*, *iap-2* and *kayak*) in the group TTN were also upregulated.

3.2.4. Day 15

On the day 15 most significantly dysregulated genes were observed in the groups TC and TN (Fig. 3). Decrease in expression of genes related to immunity (*kayak*, *hopscotch*, *lysozyme-2* and *iap-2*),

development (*VGMC*), detoxification (*CYP4G11*, *catalase*, *CYP6AS14*, *CYP306A1* and *PKA R1*) and apoptosis (*AIF*) were recorded in the group TC. In group TN only detoxification gene *CYP4G11* and immune gene *kayak* were downregulated. In the same group, other immune related genes *toll*, *defensin-2*, *iap-2*, *dorsal* and *hopscotch* and detoxification genes *PKA-R1*, *catalase* and *CYP306A1* were upregulated. In the group CN the expression of four immune related genes was changed, *abaecin*, *PGRP SC 4300*, *dorsal* and *kayak*. The genes involved in immunity, *domeless*, *relish* and *PGRP SC 4300*, along with detoxification gene *PKA R1* were upregulated in the group TT. In the same group, two detoxification genes (*CYP4G11* and *catalase*) and one related to apoptosis, were significantly downregulated. Upregulation of genes *PKA-R1*, *defensin-1* and *PGRP SC 4300*, and downregulation of *defensin-2* gene were recorded in the group TTN (Table 2).

3.3. Effect of thiamethoxam and Nosema infection on pathogen RNA loads

3.3.1. Deformed wing virus

On days six, nine, and 15 we observed the effect of *Nosema* infection on DWV load (Fig. 4A). In the group CN, *Nosema* infection significantly increased DWV load in each sampling time (day six, nine and 15). In the group TTN significant gene expression dysregulation was observed only on day nine. The only significant effect of thiamethoxam alone on lower DWV load was recorded in group TT on day six.

3.3.2. Nosema ceranae

The level of *Nosema* RNA was low but significantly differed at day 0 between control bees and bees fed with thiamethoxam in larval stage, even though at this time point bees were not infected with *Nosema* spores (infection was performed on day three, see Material and methods). On days six, nine and 15, two groups of bees exposed to *Nosema,* CN and TTN, had significantly higher *Nosema* levels than other groups. Interestingly, the third group which was exposed to *Nosema* spores (TN) had no significant changes in *Nosema* levels (Fig. 4B).

3.3.3. Black queen cell virus

The only stress factor which significantly affected Black queen cell virus (BQCV) load was thiamethoxam. In the group TC it was the lowest on the day 0 and later increased on day 9, and on day 6 the only change was seen in the group TT (Fig. S1).

4. Discussion

This study provides new insights into how honey bee exposure to a pesticide first in larval and later in adult stage in combination with *Nosema* infection have an influence on their survival and expression of immune, detoxification, developmental and apoptosis genes.

In the study we showed that exposure of honey bees in larval stage to thiamethoxam had significant effect on the survival of adult honey bees (TC group). In groups CN, TN, TT and TTN the mortality was significantly higher than in control group. In honey bee larvae exposed to thiamethoxam and then in adult stage to both stressors (group TTN), combined effect of thiamethoxam and *Nosema* was found to cause the highest mortality rate (Fig. 2). Such interaction between *Nosema* and pesticides has also been reported in other studies before (Alaux et al., 2010; Aufauvre et al., 2012; Glavinic et al., 2019; Gregorc et al., 2016; Vidau et al., 2011).

Two groups of bees exposed to *Nosema*, CN and TTN, had significantly higher *Nosema* levels than other groups on days six, nine, and 15 (Fig. 4B). This results coincide with highest mortality rate of group TTN (Fig. 2), where we can clearly see combined effect of thiamethoxam and *Nosema* infection on honey bee survival (Fig. 4B). Our results suggest that exposure to thiamethoxam could intensify *Nosema* infection, which in long term could cause possible synergistic effect of both stressors on honey bees life span. Similar negative effects of pesticides and *Nosema* infection on honey bee health were reported in previous studies (Alaux et al., 2010; Pettis et al., 2012; Pettis et al., 2013). The *Nosema* load slightly increased with time even in control groups, which could mean that control bees were in contact with *Nosema* at the beginning of the experiment similarly as in the study of Alaux et al. (2010). In study of Malone and Gatehouse (1998) they suggest possibility of spore ingestion by chewing the wax capping at emergence or it could happen accidently by contaminated *Varroa* mites (Glavinic et al., 2014), which explain low detection of *Nosema* RNA in control bees and those treated with thiamethoxam (group TC) on day 0 (Malone and Gatehouse, 1998). However, the mortality in the group C and TC was not significantly different and low (10–15% respectively) (Fig. 2).

It was previously observed that exposure to high dose of thiamethoxam may not increase viral loads of CBPV in honey bees (Coulon et al., 2018). In our study thiamethoxam alone was also not associated with increase in any tested viral loads at different time points in groups TC and TT. Whereas, in group TTN we observed that combination of thiamethoxam and *Nosema* infection could have induced significantly higher DWV viral loads in nine-day-old honey bees. Honey bees have often been reported to harbor two pathogens simultaneously. Pathogens that co-infect honey bees may interact positively, negatively or independently of each other (Cox, 2001). On days six, nine, and 15 we observed correlation between *Nosema* infection and DWV load in groups CN, TN and TTN (Fig. 4). It was previously reported that *N. ceranae* have synergistic effect not only with DWV (Glavinic et al., 2019; Zheng et al., 2015) but also with other viruses like CBPV (Toplak et al., 2013). With *Nosema* infection, increased DWV levels may have resulted from damages to midgut epithelial cells done by *Nosema*, which created suitable conditions for DWV amplification, or its transmission through the midgut protective barrier (Chen and Siede, 2007; Zheng et al., 2015). Furthermore, it was shown that *Nosema* affects the immune response in honey bees (Antunez et al., 2009; Glavinic et al., 2017; Li et al., 2017; Sinpoo et al., 2018), which could make honey bees more susceptible for viral infections.

As in bees originating from colonies treated with thiamethoxam, group TC, exclusively downregulation was registered on day 0, we could propose that thiamethoxam exposure acts as a challenge that suppress immune, detoxification and developmental systems in newly emerged honey bees. The same gene pattern was observed on day six in cages, where the majority of genes were also downregulated in all groups (Fig. 3). Pesticides can exert a suppressive effect on the immune (Aufauvre et al., 2014; Boncristiani et al., 2012; Cizelj et al., 2016; Desneux et al., 2007; Garrido et al., 2013; Glavinic et al., 2019; Gregorc et al., 2012; Tesovnik et al., 2017; Wu et al., 2017) and detoxification system (Aufauvre et al., 2014; Boncristiani et al., 2012; Johnson et al., 2006; Wu et al., 2017) which was also shown in previous studies. Downregulation of genes in groups infected with *Nosema* (CN, TN and TTN) might be a result of energy loss, as it is known that *Nosema* have high dependency on host ATP, especially at the time of germination which is energy intensive process (Badaoui et al., 2017; Keeling and Fast, 2002). In previous studies (Higes et al., 2007; Martin-Hernandez et al., 2009) researchers observed that gut epithelia cells were already parasitized in two to three days post infection with *Nosema*, which pairs with our sampling on day six (day three after *Nosema* infection). The only gene that was significantly upregulated was that for *defensin-1* (group TT and TN, Fig. 3), an AMP that plays a

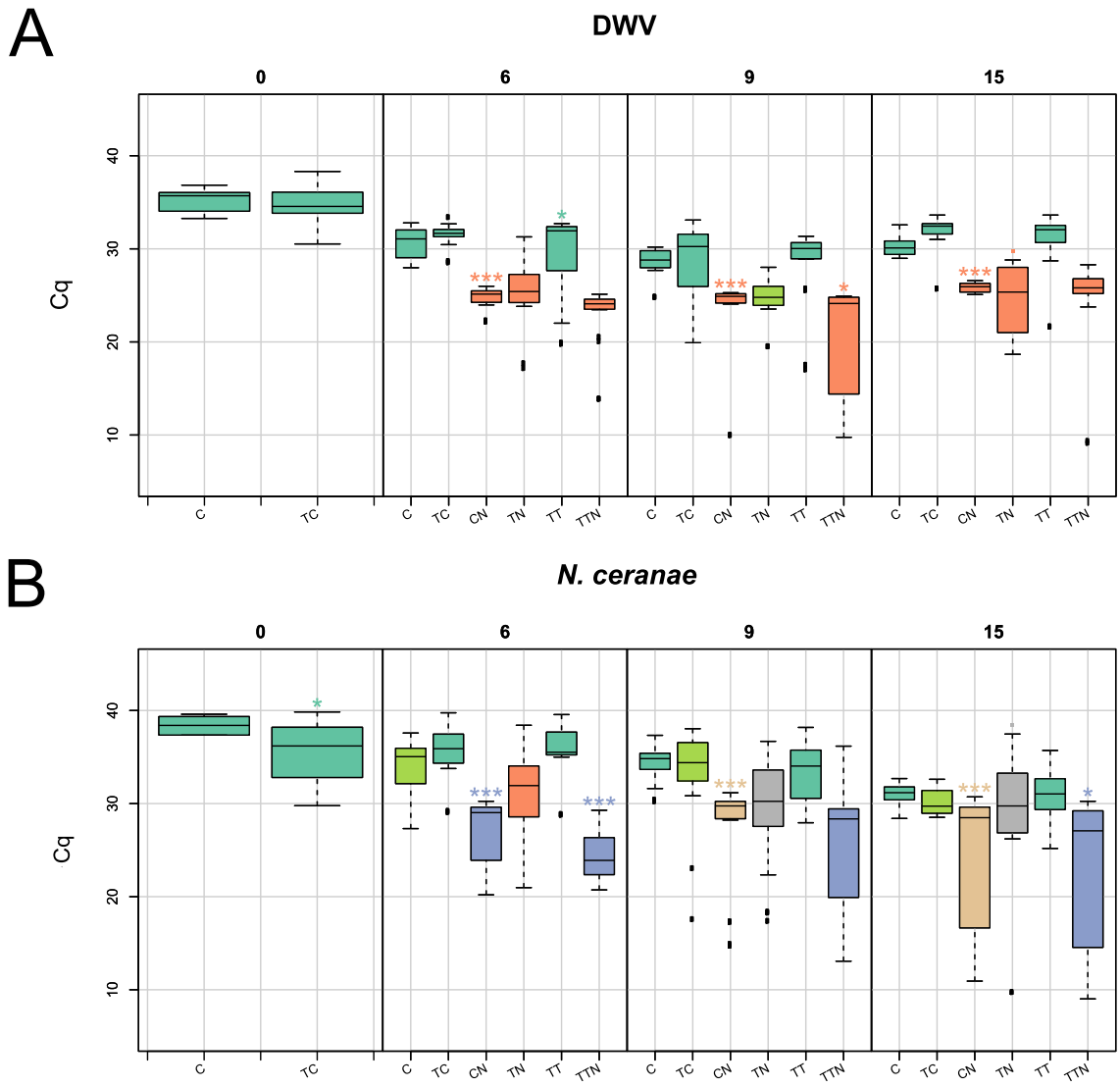

Fig. 4. DWV and *N. ceranae* RNA loads of adult honey bees in different time points. Cq: qPCR signal for (A) DWV and (B) *N. ceranae* pathogen load. Treatments are indicated at the bottom of the plots. Boxes marked with asterisk symbol shows statistically significant effect of treatment on gene expression, when p-value was equal or less than 0.05. The effects of *N. ceranae* infection, thiamethoxam treatment during different honey bee developmental stages (larvae, adults) and their interactions on pathogen RNA loads were analyzed using linear model for fixed effects (lm).

crucial role in insects immune systems, where it can act as part of individual or social immunity (Evans, 2004; Evans et al., 2006; Ilyasov et al., 2012).

Most of analyzed genes in all experimental groups on day nine show upregulation compared to control group (Fig. 3). Immune genes which are involved in Toll, Imd, JAK/STAT and JNK pathways were upregulated in all experimental groups, which is in line with the significant upregulation of AMPs genes for *abaecin*, *defensin-1* and *defensin-2*. Only in group TC we observed significant downregulation of two detoxification genes *CYP6AS14* and *CYP306A1*, and similar, although non-significant pattern can also be seen in other experimental groups. In insects, cytochrome P450s are involved in multiple functions (biosynthesis and oxidative metabolism of endogenous substances, and detoxification of exogenous substances) (Malka et al., 2009; Mao et al., 2015). Changes in the expression of P450 enzymes in honey bees exposed to different pesticides has been demonstrated before (Aufauvre et al., 2014; Boncristiani et al., 2012; Dussaubat et al., 2012; Mao et al., 2015; Wu et al., 2017). Boncristiani et al. (2012) previously observed downregulation of *CYP306A1* gene and upregulation of *PKA-R1* gene in bees exposed to pesticides, thymol and coumaphos. They suspected that changing regulations of genes, related to hormone synthesis, by indirect effect of pesticides may cause reduction in lifespan and could also induce unpredictable consequences among honey bees (Boncristiani et al., 2012; Chaimanee et al., 2016; Wu et al., 2017).

Nosema infection dysregulated the gene expression throughout the experiment in the group CN. On the day 15 of the experiment it was observed that again (similarly to day six) the majority of genes were downregulated, especially those immune related, which could explain the downregulation of AMPs among which only *abaecin* was significantly changed. Interestingly on the day 15, almost all immune related genes were upregulated in the group TN where honey bees were as larvae exposed to thiamethoxam and later infected with *Nosema* spores. Also three out of five detoxification genes were upregulated. The survival between groups CN and TN was not significantly different even though their gene expression pattern at day 15 is changed. Results from groups TC and

TT on 15th day also show similar downregulated gene expression pattern, however we observed major difference in survival with group TC at 85% and TT at 47%.

Vitellogenin, a product of a pleiotropic gene (*VGMC*), has a versatile regulatory functions in different physiological processes in honey bees. It is known to be involved in regulation of early forager behavior and in immune system pathways (Amdam et al., 2005; Nelson et al., 2007). In our study *vitellogenin* was downregulated in all treatment groups in each sampling time point, but its significant change was recorded only in group TC (Fig. 3). In previous studies shorter lifespan of honey bees with downregulated *vitellogenin* was observed (Antunez et al., 2009; Badaoui et al., 2017; Boncristiani et al., 2012; Nelson et al., 2007; Remolina et al., 2007), therefore the induced downregulation of *vitellogenin* with thiamethoxam and/or *Nosema* in our experiment could contribute to reducing of lifespan of honey bee (Fig. 2).

An important outcome of this study is the combined effect of *Nosema*, DWV and thiamethoxam on hosts, decreasing their survival. The observed higher mortality rates could result from multiple treatments, which caused the activation and maintenance of different reactions in honey bees and produced an extra burden. In our study, data showed that gene expression patterns change with time in each treatment group. Insecticide treatment in larval stage led to a significant downregulation of almost all tested detoxification and immune related genes in different time points (day zero, six and 15) of adult honey bees, suggesting a possible immunetoxicity. Honey bees infected with *Nosema* and those exposed to thiamethoxam at the same time (groups CN, TN and TTN) showed the strong alternations of immune genes expression six days after infection. But on day 15, group infested with *Nosema* (CN) showed downregulation of most tested genes, whilst in groups TN and TTN the majority of tested genes were upregulated. The impact of treatments increased with time, leading to the imbalance of transcriptome which may have a great reflection on recovery from stress and, subsequently, on mortality rates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113443.

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