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## ORIGINAL RESEARCH ARTICLE

# Immune gene expression in developing honey bees (*Apis mellifera* L.) simultaneously exposed to imidacloprid and *Varroa destructor* in laboratory conditions

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The impact of widespread pesticide use in agriculture has been a major concern to the beekeeping industry. Accumulating evidence suggests that pesticides have a negative impact on honey bees. Additionally, honey bees exposed to different stressors, such as parasites and the pathogens they vector, may further affect their health. The aim of this study was to investigate the combined or sole effect of two stressors on developing honey bees under laboratory conditions where larvae were *per os* treated with realistic field doses of imidacloprid and later infested with *Varroa destructor*. Here, we present a gene expression profile of 15 immune-related genes in different honey bee development stages (white-eyed pupae, brown-eyed pupae, and emerged honey bees). Our results show that pesticide consumed in the larval stage lead to a decrease in immune response of bees in early development stages (white- and brown-eyed pupae) and later to an increase in honey bee immune response, and thus honey bee health may be challenged. The gene expression patterns of Varroa infested honey bees changed during development with the number of significantly differentially expressed genes increasing from white-eyed pupae to newly emerged honey bees. The effect of both stressors has a significant effect on antimicrobial peptides and genes involved in defense (*apidaecin*, *hymenoptaecin*, *defensin-1*, *lysozyme-2*, and *PPO*) in different developmental stages. All tested honey bees exposed to Varroa had significantly elevated DWV loads. Overall, our data showed changes in immune-related gene expression of three honey bee development stages, exposed to each of two stressors alone or combined, which varies depending on the developmental stage.

**Keywords:** *Apis mellifera*; gene expression; imidacloprid; immune response; pesticides; Varroa; DWV

## Introduction

Apiculture is facing a worldwide habitat loss, increased use of pesticides, and exposure to a large number of parasites and pathogens. With time, honey bee losses could have drastic consequences as demand for pollination of crops increases (Aizen, Garibaldi, Cunningham, & Klein, 2008; Goulson, Nicholls, Botias, & Rotheray, 2015; Ratnieks & Carreck, 2010). Despite extensive research, the exact cause of recent, unexplained colony losses is not completely understood. Therefore, a detailed analysis of molecular mechanisms in honey bees and other standard survey methods should be implemented in the research of colony collapses.

One of the most important factors that are thought to play key roles in honey bee decline are the ectoparasitic mites *Varroa destructor* (Anderson and Trueman; Varroa) (Abbo et al., 2017; Blacquiere, Smagghe, Van Gestel, & Mommaerts, 2012; Neumann & Carreck, 2010; Pettis, vanEngelsdorp, Johnson, & Dively, 2012; Rosenkranz, Aumeier, & Ziegelmann, 2010). These mites are obligate ectoparasites that feed on honey bee hemolymph and have been relatively rapidly expanding from the primary host *Apis cerana* Fabricius, originating from Asia, to their new host, *Apis mellifera* (Francis, Nielsen, & Kryger, 2013). Varroa mites have been responsible in recent decades for the largest losses of honey bee colonies worldwide. According to some studies, Varroa has a significant impact on honey bee health and is also known as a vector of viral diseases and other pathogens that affect honey bees. Several studies reveal that Varroa parasitism induces immune suppression (Gregory, Evans, Rinderer, & de Guzman, 2005; Koleoglu, Goodwin, Reyes-Quintana, Hamiduzzaman, & Guzman-Novoa, 2017; Navajas et al., 2008; Yang & Cox-Foster, 2005), while others also showed increased expression of immune related genes (Gregorc, Evans, Scharf, & Ellis, 2012; Khongphinitbunjong et al., 2015; Kuster, Boncristiani, & Rueppell, 2014; Ryabov, Fannon, Moore, Wood, & Evans, 2016; Tesovnik et al., 2017).

Pesticides can also contribute to colony mortality, especially in combination with pathogens (Boncristiani et al., 2012; Kevan, 1999). There are studies supporting the assumption that pesticide residues present in honey bee colonies may have contributed to the weakening of honey bee health (Aufauvre et al., 2012; Gregorc et al., 2012). Neonicotinoid pesticides are a class of systemic insecticides that act on the insect nervous system as analogs of nicotine but with much greater affinity for

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nicotine acetylcholine receptors (Aliouane et al., 2009; Johnson, 2015). Imidacloprid, even though its half-life is short, is still present in the whole honey bee body up to 48 hours (Suchail, De Sousa, Rahmani, & Belzunces, 2004). Because of its neurotoxic activity, the neonicotinoid imidacloprid has been of particular concern for sub lethal effects on honey bees and other wild pollinators. A specific concern is the use of imidacloprid as a seed coating that results in distribution of the insecticide throughout plant tissues, being excreted in nectar, guttation fluids, and pollen which are all collected by foraging bees (Fairbrother, Purdy, Anderson, & Fell, 2014).

Previous studies investigated gene expression in adult honey bees exposed individually to imidacloprid or Varroa (Abbo et al., 2017; Di Prisco et al., 2016; Khongphinitbunjong et al., 2015; Koleoglu et al., 2017; Kuster et al., 2014; Yang & Cox-Foster, 2005). Other studies focused on only the immature stages of honey bees exposed to imidacloprid and/or Varroa (Gregorc et al., 2012; Koleoglu et al., 2017; Kuster et al., 2014; Zaobidna, Zoltowska, & Lopienska-Biernat, 2015, 2017). Understanding the effects of imidacloprid and Varroa by themselves, as well as their combined interaction on developing honey bees is crucial to improve the knowledge of honey bee health and survival. To the best of our knowledge this is the first study of immune gene expression in developing honey bees (white- and brown-eyed pupae, and newly emerged bees) simultaneously exposed to imidacloprid in larval stage and after defecation infested with Varroa in laboratory conditions.

## Materials and methods

The honey bees used in this study were from *A. mellifera* colonies, and did not present visible symptoms of any known brood disease. All collected samples were also tested for the ten most common honey bee pathogens using RT-qPCR (Supplemental Table S1). Experiments were conducted at USDA, ARS, Thad Cochran Southern Horticultural Laboratory, Poplarville, MS.

### *Experimental setup*

This experiment consisted of four groups of 48 larvae: the imidacloprid group (group 1), the Varroa group (group 2), the combination group (group 3; treated with both imidacloprid and Varroa), and the control group. Each larva was fed or/and infested individually according to its treatment group. The larvae in the pesticide groups received the imidacloprid diet as detailed below. Varroa mites were introduced to larvae in the Varroa groups, after larval feeding and defecation, on the surface of each well of the tissue culture plates.

Using a standard 10-frame Langstroth-style hive, queens in three honey bee colonies were confined to a newly-drawn comb using a metal queen excluder cage covering the comb side at time $t = -24$ h. The caged queen and frame were returned to the center of the brood nest where worker bees could access and tend to the queen. After 24 h of queen confinement, we removed the queen from the cage and placed the cage back on the comb as before but this time for 72 h (from $t = 0$) to allow the eggs to hatch and larvae to reach an appropriate age for grafting. During this time, worker bees were able to access the comb to feed the developing larvae. At 72 h, we removed the test frames containing 0 ± 24 h old larvae from the colonies and took them to the laboratory (Gregorc & Ellis, 2011).

Larvae were grafted using a Chinese grafting tool to sterile 48-well tissue culture plates (Celltreat Scientific Products, Shirley, MA, US) with previously pipetted 10 μL larval diet on the bottom of each well. The diet consisted of 50% fresh organic royal jelly (GloryBee, OR, US) and 50% sugar solution. Sugar solution was prepared with 12% D-glucose (CAS Number 50-99-7, Sigma-Aldrich, US), 12% D-fructose (CAS Number 57-48-7, Sigma-Aldrich, US) and 2% yeast extract (EC Number 232-387-9, Sigma-Aldrich, US) dissolved in distilled water. Prior to grafting the plates, the diet was pre-warmed to 34.5 ± 0.5 °C in the incubator (Nor-Lake Scientific) (Aupinel, 2005).

Each day the larvae were given fresh diet dependent on their age. On the first feeding day (day one) the larvae received 10 μL of diet, on day two, 20 μL on day three, 30 μL on day four, 40 μL and on day five 50 μL. Plates with larvae were kept in the incubator at 34.5 ± 0.5 °C and 96 ± 1% relative humidity.

To test the effect of the pesticide on different stages of developing honey bees, imidacloprid (Pestanal, CAS#138261-41-3) was mixed into the larval diet stated above to a concentration of 20 ppb to assess sub-lethal effects (Dively, Embrey, Kamel, Hawthorne, & Pettis, 2015; Pettis et al., 2012; Schmuck, Schoning, Stork, & Schramel, 2001). Prior to administration to the larval diet, dry imidacloprid was diluted in water. The diet/imidacloprid mixtures were prepared and stored in 1.5 mL snap-top plastic vials (Fisher Scientific, Pittsburgh, PA, USA). The larvae received this pesticide diet daily, beginning on the second feeding day and continuing through day five.

On the sixth day, when larvae began to defecate, they were transferred into new 48-well tissue culture plates. On this day larvae in treatment groups 2 and 3 received one Varroa mite in phoretic phase collected from three highly infested colonies using the sugar-shake method (Dietemann et al., 2013). All plastic wells were sealed with transparent wax foundation. The plates with the transferred larvae were then vertically set in the incubator at 34.5 ± 0.5 °C and 80 ± 1% relative humidity, until adult honey bees emerged.

In order to obtain three developmental stages for further molecular analyses, developing honey bees in each group were sampled on day 10 as pupae with white eyes, on day 13 as pupae with brown eyes, and finally on day 17 as developed bees. We only sampled

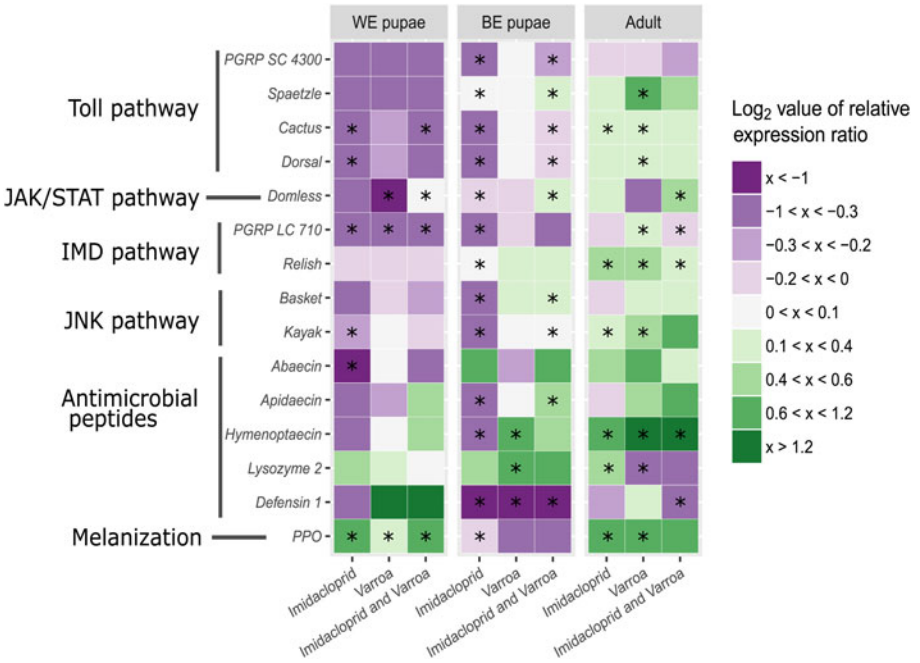


Figure 1. Gene expression heatmap at three honey bee development stages. Columns correspond to the expression profile of treatments (Imidacloprid; Varroa; Imidacloprid +Varroa), and each row presents one gene transcript. The names of genes and corresponding immune pathways are indicated on the left. Colors indicate the average mRNA levels compared to average mRNA levels of control groups. Range log2 value of relative expression ratio is indicated in the legend on the right. Boxes marked with an asterisk show statistically significant effects of the treatment on gene expression, when *p*-value was equal or less than .05.

alive bees with an alive mite in the cell. Bees with a dead mite in their cell were excluded from any analysis.

### *RNA isolation and cDNA synthesis*

For preservation individual specimens were stored in tubes with RNAlater® (Thermo Scientific, USA) at –20 °C until RNA isolation. Each individual white-eyed pupae, brown-eyed pupae, and emerged bee were then grounded using BioPulverizer (Biospec products, USA) in liquid nitrogen. The BioPulverizer quickly disintegrated hard frozen insect samples into powder, from which total RNA was extracted using TRIZOL® (Ambion®, Life Technologies). Extracted RNA was then purified with PureLink® RNA Mini Kit columns (Ambion®, Life Technologies). RNA concentrations were determined using NanoDrop One (Thermo Scientific, USA). Residual DNA was removed by incubating 1 μg of RNA with 1 U of RNase-free DNase I (Fermentas, Germany) for 30 min at 37 °C. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to synthesize complementary DNA (cDNA), according to the manufacturer's instructions.

### *Real-time qPCR*

The primers used to amplify 15 immune related genes (PGRP SC 4300, Spaetzle, Cactus, Dorsal, Domeless, PGRP LC 710, Relish, Basket, Kayak, Abaecin, Apidaecin, Hymenoptaecin, Lysozyme 2, Defensin 1, PPO), and two reference genes (RPS5, Tbp-af) were those reported in Gregorc et al. (2012) and Tesovnik et al. (2017) (Supplemental Table S2). For quantitative real-time PCR, 10 μL reactions were prepared, containing 5 μL of PowerUp™ SYBR® Green Master Mix (Applied Biosystems, USA), 500 nM of forward and reverse primer, DEPC treated water and 5 ng of cDNA. Amplification of targets were performed with CFX96 Deep Well Real-Time system (BioRad, UK) and analyzed with Bio-Rad CFX Manager software. For the experimental run the following cycle profile was used: denaturation step at 95 °C for 10 min, and 40 cycles 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 of each primer pair and 95 °C for 15 s, where 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 96-well plates (Multiplate® PCR Plates™ 96-well, Bio Rad). Each experiment contained three no-template controls and test samples performed in duplicates. Gene expression was analyzed for 15 immune related genes. We ranked the set of candidate normalization genes (Rp49, RPS5 and Tbp-af) according to their expression stability in a given sample set using NormFinder software (Andersen, Jensen, & Orntoft, 2004). Based on software analysis and according to its stable expression in our and previous studies we used RPS5 and Tbp-af as reference genes (Cizelj et al., 2016; Lourenco, Mackert, Cristino, & Simoes, 2008). Gene expression values of non-treated group were used for

Table 1. Relative expression ratios for studied immune related genes in three developmental stages (white-, brown-eyed pupae and newly emerged bees) for each treatment group. Bold indicates significantly elevated or decreased transcript abundances. Numbers marked with an asterisk show statistical significance: *** $p < .001$, ** $p < .01$ and * $p < .05$.

| | Imidacloprid | | | Varroa | | | Imidacloprid and Varroa | | |
|---|---|---|---|---|---|---|---|---|---|
| | WE | BE | Adult | WE | BE | Adult | WE | BE | Adult |
| **PGRP SC 4300** | –0.38 | **–0.53**$^{***}$ | –0.09 | –0.3 | 0.02 | –0.18 | –0.36 | **–0.21**$^{**}$ | –0.29 |
| **Spaetzle** | –0.36 | **0.0002**$^{*}$ | 0.12 | –0.37 | 0.02 | **0.65**$^{***}$ | –0.42 | **0.12**$^{*}$ | 0.53 |
| **Cactus** | **–0.73**$^{***}$ | **–0.43**$^{***}$ | **0.29**$^{**}$ | –0.3 | 0.08 | **0.26**$^{*}$ | **–0.38**$^{**}$ | **–0.15**$^{*}$ | 0.37 |
| **Dorsal** | **–0.42**$^{*}$ | **–0.41**$^{***}$ | 0.13 | –0.22 | 0.02 | **0.28**$^{*}$ | –0.61 | **–0.09**$^{***}$ | 0.33 |
| **Domeless** | –0.35 | **–0.04**$^{*}$ | 0.31 | **–1.57**$^{***}$ | –0.03 | –0.42 | **0.08**$^{***}$ | **0.19**$^{*}$ | **0.56**$^{*}$ |
| **PGRP LC 710** | **–0.60**$^{***}$ | **–0.45**$^{***}$ | –0.02 | **–0.55**$^{***}$ | –0.15 | **0.35**$^{***}$ | **–0.62**$^{*}$ | –0.74 | **–0.18**$^{***}$ |
| **Relish** | –0.18 | **0.02**$^{**}$ | **0.44**$^{***}$ | –0.11 | 0.21 | **0.58**$^{***}$ | –0.06 | 0.12 | **0.26**$^{***}$ |
| **Basket** | –0.36 | **–0.42**$^{***}$ | –0.08 | –0.04 | 0.27 | 0.18 | –0.2 | **0.15**$^{**}$ | 0.28 |
| **Kayak** | **–0.27**$^{*}$ | **–0.44**$^{***}$ | **0.35**$^{**}$ | 0.04 | 0.05 | **0.47**$^{***}$ | –0.11 | **0.08**$^{***}$ | 0.73 |
| **Abaecin** | **–5.04**$^{*}$ | 1.04 | 0.44 | 0.01 | –0.24 | 1.11 | –0.61 | 0.74 | 0.34 |
| **Apidaecin** | –0.33 | **–0.34**$^{***}$ | –0.05 | –0.27 | 0.09 | 0.51 | 0.49 | **0.45**$^{***}$ | 0.86 |
| **Hymenoptaecin** | –0.36 | **–0.56**$^{*}$ | **1.07**$^{*}$ | 0.07 | **1.11**$^{*}$ | **2.46**$^{***}$ | 0.56 | 0.58 | **2.19**$^{*}$ |
| **Lysozyme 2** | 0.54 | 0.44 | **0.59**$^{*}$ | 0.28 | **0.79**$^{*}$ | **–0.77**$^{**}$ | 0.08 | 1.05 | –0.52 |
| **Defensin 1** | –0.48 | **–2.31**$^{***}$ | –0.23 | 1.55 | **–2.26**$^{***}$ | 0.23 | 2.46 | **–2.01**$^{***}$ | **–0.93**$^{*}$ |
| **PPO** | **1.08**$^{***}$ | **–0.15**$^{*}$ | **0.62**$^{***}$ | **0.34**$^{*}$ | –0.37 | **0.85**$^{***}$ | **0.84**$^{*}$ | –0.74 | 1.06 |

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 (Pfaffl, 2001). These results were then visualized on a heatmap illustrating differential expression of immune-related genes as a consequence of different treatments (Figure 1). The significance levels of the treatment effects were indicated according to the statistics described below. The results demonstrating the level of gene expression are shown in log2 scale.

All collected samples were also tested for the ten most common honey bee-pathogen targets (*Ascosphaera apis*, *Acarapis woodi*, ABPV, BQCV, DWV, IAPV, KBV, *Nosema apis, Nosema ceranae*, *Paenibacillus larvae;* Supplemental Table S1) using RT-qPCR, performed as described before (Cizelj et al., 2016).

### *Statistical analysis*

Relative expression levels of studied genes were normalized with two reference genes (*RPS5* and *Tbp-af*). Delta Cq ($\Delta$Cq = Cq(ref) – Cq(target)) between the mean of reference genes Cq values and target genes Cq values was calculated. To analyze the effects of Varroa infestation, imidacloprid treatment and interaction of both treatments (imidacloprid treatment + Varroa infestation) on gene expression, we used a linear model for fixed effects (lm function in R) for each of the 15 genes and each sampling group (white-eyed pupae, brown-eyed pupae, and adult bees) according to the following model (1):

$$y_{ijk} = \mu + V_i + I_j + V_i I_j + e_{ijk} \quad (1)$$

where $y_{ijkl}$ is $\Delta$Cq value, $\mu$ is overall mean, $V_i$ is fixed effect of Varroa infestation ($i$ = yes, no), $I_j$ is fixed effect of imidacloprid treatment ($j$ = yes, no) and $eijk$ 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). The gene expression data ($\Delta$Cq values) and the results of statistical analysis were then graphically summarized using boxplots. All statistical analyses and plotting were carried out using R software version 3.5.1 (R Core Team, 2013) with relevant libraries (lsmeans, moments, ggplot2) (Komsta & Novomestky, 2015; Lenth, 2016; Wickham, 2009).

## Results

### *Effects of imidacloprid or/and Varroa infestation on immune related gene expression in three developmental stages (white-eyed, brown-eyed pupae, and newly emerged bees)*

The majority of immune related genes in the white-eyed pupae of group 1 were downregulated when compared to control white-eyed pupae, while the only upregulated gene was *PPO* involved in melanization (Figure 1). Changes in gene expression between white-eyed pupae of group 2 included the downregulation of *domeless* and *PGRP LC 710*, whereas only *PPO* was significantly upregulated. In white-eyed pupae of group 3, an upregulation of two genes (*PPO* and *domeless*) and downregulation of two genes (*PGRP LC 710* and *cactus*) were recorded (Figure 1 and Supplemental Figure S1; Table 1).

In group 1, brown-eyed pupae had significant upregulation for genes encoding Relish and Spaetzle, while the majority of genes were downregulated. Alternations in gene expression between group 2 and the control group were significant for downregulation of *defensin-1*. Significant upregulation was observed in genes acting as antimicrobial peptides, *lysozyme-2* and *hymenoptaecin*.

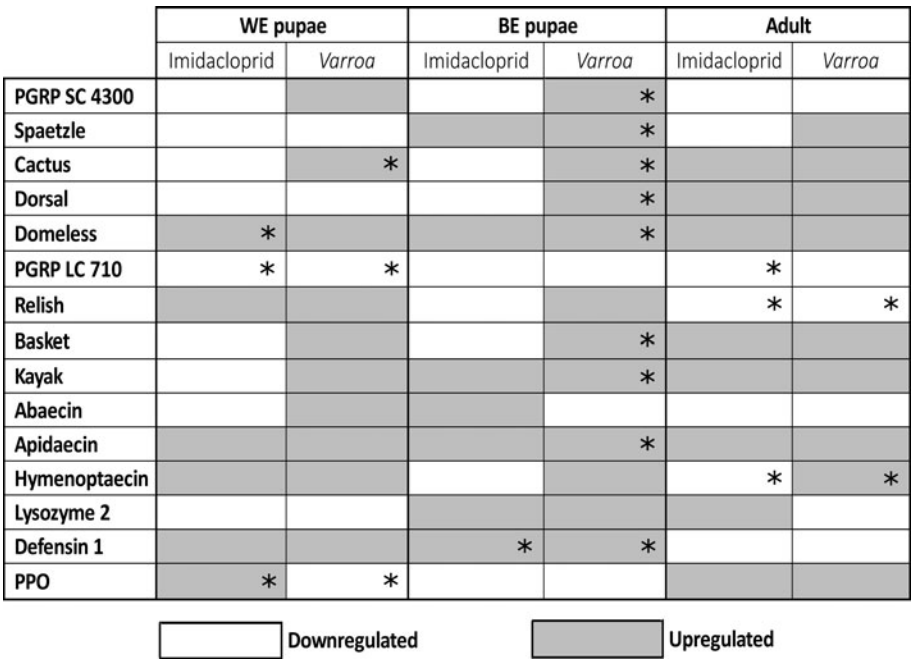


Figure 2. Analysis of differences in the mean expression of target genes between brood that was Varroa infested and received no imidacloprid, imidacloprid treated group without Varroa infestation and imidacloprid treated + Varroa infested group. WE pupae, white-eyed pupae; BE pupae, brown-eyed pupae; Adult, newly emerged bees. The downregulation or upregulation indicate the average mRNA levels in Varroa infested - imidacloprid treated group compared to average levels of mRNA in Varroa infested group or imidacloprid treated. Boxes marked with asterisk shows statistically significant effect of treatment on gene expression, when p-value was equal or less than 0.05.

Brown-eyed pupae of group 3 had significant changes in the immune related genes *apidaecin*, *kayak*, *basket*, *domeless* and *spaetzle*. The only significantly downregulated genes was AMP Defensin-1 and genes involved in Toll pathway (*PGRP SC 4300*, *cactus* and *dorsal*) (Figure 1; Table 1).

In newly emerged honey bees of group 1, transcript levels of genes *cactus*, *relish*, *kayak*, *hymenoptaecin*, *lysozyme-2* and *PPO* were significantly higher than transcript levels in control group. In response to Varroa infestation in group 2, the majority of immune genes were upregulated and the only significant downregulated gene encoded AMP Lysozyme-2. Comparing newly emerged bees from group 3 to the control group showed that the majority of genes involved in immune response were upregulated. The most significant upregulated changes were in *hymenoptaecin*, *domeless*, and *relish*. Conversely, two immune-related genes, *defensin-1* and *PGRP LC 710* were significantly downregulated (Figure 1, Supplemental Figures S1 and S2; Table 1).

### *Impact of second stressor on honey bees*

In this section, we present the impact of imidacloprid or Varroa infestation as a second stressor on honey bees. To estimate the impact of Varroa in imidacloprid treated bees, we compared the gene expression between group 1 and imidacloprid + Varroa treated bees (group 3). In white-eyed pupae from group 3, the gene *cactus* involved in the Toll immune pathway was expressed significantly higher in comparison to group 1. Whereas, transcript levels of *PGRP LC 710* and *PPO* were significantly lower. In brown-eyed pupae, AMP genes (*defensin-1* and *apidaecin*), genes involved in Toll (*PGRP SC 4300*, *spaetzle*, *cactus* and *dorsal*), JAK/STAT (*domeless*) and JNK pathway (*basket* and *kayak*) had higher transcript levels in group 3 than group 1. In newly emerged bees from group 3, the *relish* gene had a lower expression pattern, while gene *hymenoptaecin* had higher expression than bees in group 1 (Figure 2; Table 1).

To see the impact of imidacloprid in Varroa infested honey bees, we compared gene expression between two treatment groups: Varroa (group 2) and imidacloprid + Varroa infested (group 3). In white-eyed pupae, the expression of *PGRP LC 710* was significantly lower in group 3 when compared to group 2. Conversely, transcript levels of two genes *domeless* and *PPO* were significantly higher. In brown-eyed pupae alternations in gene expression were significant only for the gene *defensin-1*. In newly emerged bees of group 3, gene expression was lower for genes *hymenoptaecin*, *relish* and *PGRP LC 710* compared to group 2 (Figure 2; Table 1).

### *Effects of Varroa and imidacloprid treatment on DWV loads*

In Varroa infested groups, DWV RNA loads significantly increased in white-eyed and brown-eyed pupae, and in newly emerged bees. Imidacloprid alone had no effect on DWV replication in white-eyed pupae and emerged

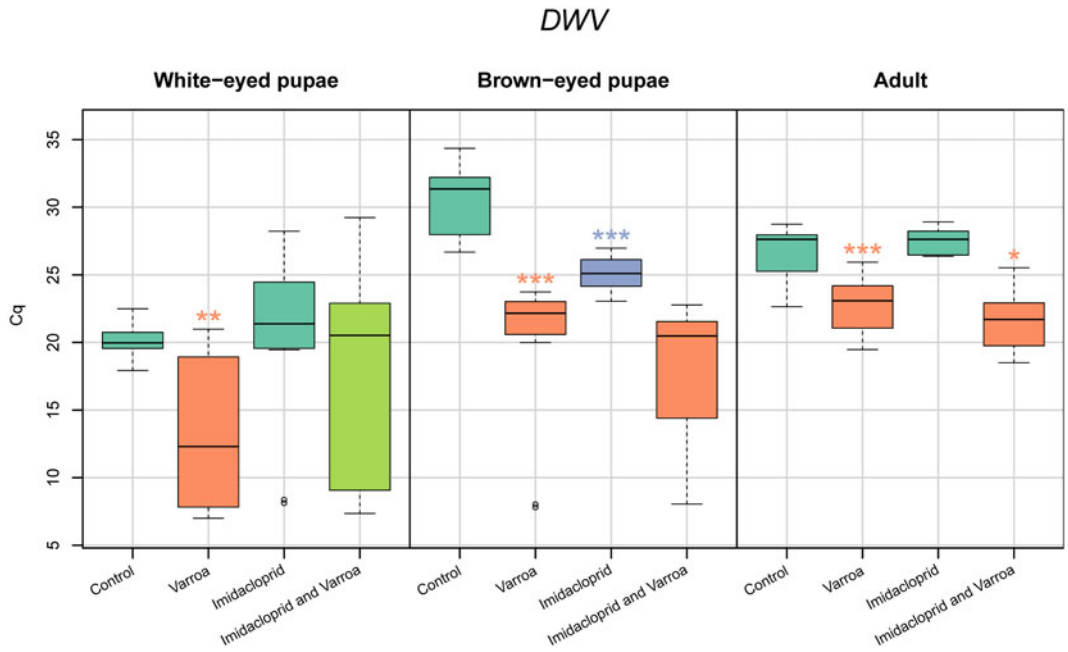


Figure 3. Deformed Wing Virus RNA loads in different development stages of honey bees. Cq: qPCR signal for pathogen load. Treatments are indicated at the bottom of the plots. Boxes marked with an asterisk show statistically significant effect of the treatment on gene expression when the *p*-value was equal or less than .05.

bees, whereas in brown-eyed pupae replication of DWV was significantly higher in imidacloprid treated bees in comparison to untreated bees. We also observed significantly increased DWV RNA loads in newly emerged bees for group treated with imidacloprid + Varroa infested (Figure 3). Beside DWV virus there were no other tested pathogens detected in our samples.

## Discussion

Imidacloprid and Varroa have an important impact on honey bee health. Consequently, an understanding of interactive effects of these two stressors and their individual effect on honey bees is crucial to improve honey bee health and survival. This study represents the first experiment in which individual and combined effect of imidacloprid and Varroa on immune related gene expression in developmental stages of *A. mellifera* were examined.

In our study, the exposure of imidacloprid showed that most immune related genes are downregulated in white- and brown-eyed pupae. A number of studies demonstrated that detoxifying enzymes significantly contribute to detoxification when insects are exposed to pesticides. The detoxification process demands a lot of metabolic energy and is therefore energetically expensive (Abbo et al., 2017; du Rand et al., 2015; Johnson et al., 2012; Mao, Schuler, & Berenbaum, 2011). As du Rand et al. (2015) suggested, exposure of larvae to imidacloprid increased the energetic cost for enhanced detoxification that could result in an altered immune status. Also Abbo et al. (2017) observed a significant decline of vitellogenin titer in bees exposed to imidacloprid, and suggest that exposure could result in altered nutritional status. Our present study coincides with other studies where they showed that insecticides influence the immune system of insects which could be a negative factor for honey bee immunity (Abbo et al., 2017; Aufauvre et al., 2014; Hoffmann, 1995; Nicolas, Reichhart, Hoffmann, & Lemaitre, 1998). However, we also observed that two immune related genes, *lysozyme-2* and *PPO*, were still significantly upregulated. *Lysozyme-2*, one of the antimicrobial peptide (AMP), is known to play an important role in immune response to bacterial infections (Chan, Melathopoulos, Pernal, & Foster, 2009) and in promotion of other antimicrobial peptide synthesis in honey bees (Imler & Bulet, 2005). When bees were exposed to synthetic acaricides, no statistically significant changes were detected in gene expression of *lysozyme-2* (Garrido et al., 2013). In studies by Chan et al. (2009) and Gatschenberger, Gimple, Tautz, and Beier (2012), upregulation of *lysozyme-2* was shown as a response to bacterial infections. In contrast, in our study upregulation of *lysozyme-2* was shown as a response to pesticide challenge in white- and brown-eyed pupae.

Prophenoloxidase (PPO) is important for the melanization cascade, which leads to the encapsulation of infectious agents and is one of the most important mechanism of innate immunity. Our results are in correspondence with the expression trend of other studies where elevated expression of *PPO* is shown due to immune challenge by pesticide or pathogen infection (Chan et al., 2009; Gregorc et al., 2012; Hu et al., 2017). These results suggest that imidacloprid affects

mechanisms and pathways in charge of honey bee self-defense. During brood development the impact of imidacloprid on immune related gene expression is decreasing in line with earlier studies showing that imidacloprid has a half-life of five hours, but it can be still present in honey bees up to 48 hours after treatment (Iwasa, Motoyama, Ambrose, & Roe, 2004; Johnson, 2015; Suchail et al., 2004). Two of five metabolites, 5-dihydroxy-imidacloprid and olefin, might also be involved in imidacloprid toxicity as they showed long persistence in significant amounts even 30 hours after treatment. Our results in this respect are in accordance with findings about prolonged action of imidacloprid due to accumulation of metabolites (Suchail et al., 2004).

Infestation with the parasitic Varroa, and most likely viruses and other pathogens carried by mites, is well known to affect expression pattern of the immune system in honey bees (Di Prisco et al., 2016; Gregorc et al., 2012; Khongphinitbunjong et al., 2015; Koleoglu et al., 2017; Kuster et al., 2014; Tesovnik et al., 2017; Zaobidna et al., 2015, 2017). The gene expression pattern of Varroa infested honey bees changes during development. With time, the number of differentially expressed genes increased from the white-eyed stage to enclosure. It seems that the honey bee immune system is slowly activated after Varroa parasitism, and is shown in upregulation of most tested immune genes. *Lysozyme-2* expression was higher in Varroa infested white-eyed pupae, but only significant in brown-eyed pupae. During development *lysozyme-2* expression decreased, and became significantly lower in Varroa infested newly emerged bees, which supports findings by Zaobidna et al. (2017).

Zhang, Liu, Zhang, and Han (2010) tried to determine why Varroa has a lower degree of pathogenicity for Asian bees. They noted differences in gene expression; an increase of *PPO* gene in *A. cerana*, but no significant changes in *A. mellifera* (Zhang et al., 2010). On the contrary, our results showed significantly increased expression of *PPO* gene in infested white-eyed pupae, which is consistent with results reported by Zaobidna et al. (2015). Similar to other studies (Khongphinitbunjong et al., 2015; Kuster et al., 2014; Zaobidna et al., 2015), we noticed decreased gene expression of *PPO* in brown-eyed pupae. As already mentioned, a phenoloxidase cascade is involved in two processes, melanogenesis and cuticle sclerotisation, that are important in barrier development, formation of muscle and other organs attachments, and other substances secreted by cuticle and subcuticular glands, such as proteolytic enzymes (Strachecka et al., 2014). Decrease in gene expression of important defense mechanisms could contribute to the risk of other infections and the spread of different pathogens in the long term. Our data showed little indication of immunosuppression of honey bees by Varroa (Figure 1; Supplemental Table S3) especially in newly emerged bees, contrary to earlier studies (Gregory et al., 2005; Koleoglu et al., 2017; Yang & Cox-Foster, 2005).

In our study, we also explored DWV dynamics in Varroa infested honey bees, and host immune response of challenged bees with both mites and pesticides. With this we attempt to expand on information on host-pesticide-parasite-virus interactions. Studies related to Varroa infestation support the role of Varroa mites as a vector of virulent viruses, like DWV (Abbo et al., 2017; Di Prisco et al., 2016; Gregorc et al., 2012; Koleoglu et al., 2017; Ryabov et al., 2014; Yue & Genersch, 2005). In this study, all three tested developmental stages (white-, brown-eyed pupae, and newly emerged bees) exposed to Varroa had significantly elevated DWV loads in relation to the control group or the imidacloprid- treated group. The only significant effect imidacloprid had, was in brown-eyed pupae where virus levels of DWV were higher than in the control group. DWV viruses cause covert infections and are often responsible for latent infections that can appear after a stress situation. To our knowledge, there is only one study (Di Prisco et al., 2013) where researchers have seen clear statistically significant impact of imidacloprid on DWV replication in adult honey bees. In the imidacloprid + Varroa group, we observed high levels of DWV virus in white-, brown-eyed pupae and newly emerged bees, but the only significance was seen in the latter (Figure 3).

In several studies researchers noticed suppressed expression of immune related genes, especially AMPs, when honey bees were infested with Varroa (Koleoglu et al., 2017; Navajas et al., 2008; Yang & Cox-Foster, 2005). As co-effect of both stressors, imidacloprid and Varroa, we observed only suppression of the AMP gene *defensin-1* in both brown-eyed pupae and newly emerged bees. Whereas expression levels of other defense genes and AMPs, *PPO* in white-eyed pupae, *apidaecin* in brown-eyed pupae and *hymenoptaecin* in emerged bees, were significantly elevated when honey bees were exposed to two stressors. With development of honey bees from white-eyed pupae to newly emerged bees we also noticed elevated expression in genes *domeless*, *relish*, and *kayak*. Each gene, *relish* in Imd pathway and *kayak* in JNK pathway, transcriptionally regulate expression of AMPs and other genes involved in defense (Brutscher, Daughenbaugh, & Flenniken, 2015), which correlates with our findings of AMPs upregulated expression.

The first contact young honey bees have with environmental stressors is usually the residues of pesticides in their food and wax. Later in their life cycle they come in contact with other stressors, like Varroa. Therefore, we compared gene expression of the group treated with imidacloprid and the group in which larvae were treated and then infested with Varroa. Even though the expression of *PPO*, is upregulated in white-

eyed pupae, if we compare the mean expression level of the same gene between these two groups, we can see that Varroa infestation leads to weaker upregulation. At the same time, when we compared gene *cactus* that was significantly downregulated, we noticed that Varroa infestation caused slightly higher transcript levels. The same pattern was noticed in brown-eyed pupae for two AMP genes, *defensin-1* and *apidaecin*. Whereas in newly emerged bees Varroa infestation of bees treated with imidacloprid caused even lower expression of genes *relish* and *PGRP LC 710*. To conclude, we noticed that earlier exposure of larvae to imidacloprid and later infestation with Varroa has positive and negative synergistic effects on some immune related genes.

Overall, our data showed changes in immune related gene expression of three honey bee development stages, exposed to each of two stressors alone or combined. We observed that responses to stress vary depending on the developmental stage. Results from previously published studies correspond with ours, and we confirm that pesticide residues at the tested concentration may lead to decreased or increased honey bee immune response and, thus, honey bee health may be challenged. In contrast, the gene expression pattern of Varroa infested honey bees changed during development with increasing number of significantly expressed genes from white-eyed pupae to newly emerged honey bees. We further observed that exposure of larvae to imidacloprid and additional infestation with Varroa have significant synergistic effects on AMPs and other genes involved in defense of immune system (*apidaecin*, *hymenoptaecin*, *defensin-1*, *lysozyme-2*, and *PPO*) in different developmental stages. We therefore found an extensive diversity of studied stress factors that can interfere with the immune system of developing honey bees. Further studies should be performed in order to understand the studied effects of gene expression in laboratory conditions and exposure to the same stressors under real environmental conditions.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

## Supplementary material

Supplementary (Figure 1/Table 1/content) is available via the 'Supplementary' tab on the article's online page (https://doi.org/10.1080/00218839.2019.1634463).

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## References

Abbo, P. M., Kawasaki, J. K., Hamilton, M., Cook, S. C., DeGrandi-Hoffman, G., Li, W. F., … Chen, Y. P. (2017). Effects of Imidacloprid and Varroa destructor on survival and health of European honey bees, *Apis mellifera*. *Insect Science*, *24*(3), 467–477. doi:10.1111/1744-7917.12335

Aizen, M. A., Garibaldi, L. A., Cunningham, S. A., & Klein, A. M. (2008). Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. *Current Biology*, *18*(20), 1572–1575. doi:10.1016/j.cub.2008.08.066

Aliouane, Y., el Hassani, A. K., Gary, V., Armengaud, C., Lambin, M., & Gauthier, M. (2009). Subchronic exposure of honeybees to sublethal doses of pesticides: Effects on behavior. *Environmental Toxicology and Chemistry*, *28*(1), 113–122. doi:10.1897/08-110.1

Andersen, C. L., Jensen, J. L., & Orntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research*, *64*(15), 5245–5250. doi:10.1158/0008-5472.CAN-04-0496

Aufauvre, J., Biron, D. G., Vidau, C., Fontbonne, R., Roudel, M., Diogon, M., … Blot, N. (2012). Parasite-insecticide interactions: A case study of *Nosema ceranae* and fipronil synergy on honeybee. *Scientific Reports*, *2*(1), 1–7. doi:10.1038/srep00326

Aufauvre, J., Misme-Aucouturier, B., Vigues, B., Texier, C., Delbac, F., & Blot, N. (2014). Transcriptome analyses of the Honeybee response to *Nosema ceranae* and insecticides. *Plos One*, *9*(3), e91686. http://www.plosone.org/article/fetchObject.action?uri=info doi:10.1371/journal.pone.0091686

Aupinel, P. (2005). Improvment of artificial feeding in standard *in vitro* method for rearing *Apis mellifera* larvae. *Bulletin of Insectology*, *58*(2), 107–111. doi:10.3896/IBRA.1.52.1.05

Blacquiere, T., Smagghe, G., Van Gestel, C. A. M., & Mommaerts, V. (2012). Neonicotinoids in bees: A review on concentrations, side-effects and risk assessment (vol 21, pg 973, 2012). *Ecotoxicology*, *21*(5), 1581–1581. doi:10.1007/s10646-012-0890-7

Boncristiani, H., Underwood, R., Schwarz, R., Evans, J. D., Pettis, J., & vanEngelsdorp, D. (2012). Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. *Journal of Insect Physiology*, *58*(5), 613–620. doi:10.1016/j.jinsphys.2011.12.011

Brutscher, L. M., Daughenbaugh, K. F., & Flenniken, M. L. (2015). Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science*, *10*, 71–82. doi:10.1016/j.cois.2015.04.016

Chan, Q. W. T., Melathopoulos, A. P., Pernal, S. F., & Foster, L. J. (2009). The innate immune and systemic response in honey bees to a bacterial pathogen, *Paenibacillus larvae*. *BMC Genomics*, *10*(1), 387. doi:10.1186/1471-2164-10

Cizelj, I., Glavan, G., Bozic, J., Oven, I., Mrak, V., & Narat, M. (2016). Prochloraz and coumaphos induce different gene expression patterns in three developmental stages of the Carniolan honey bee (*Apis mellifera* carnica Pollmann). *Pesticide Biochemistry and Physiology*, *128*, 68–75. http://www.ncbi.nlm.nih.gov/pubmed/26969442. doi:10.1016/j.pestbp.2015.09.015

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., … Pennacchio, F. (2013). Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees.

[Research Support, Non-U.S. Gov't]. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(46), 18466–18471. http://www.ncbi.nlm.nih.gov/pubmed/24145453. doi:10.1073/pnas.1314923110

Di Prisco, G., Annoscia, D., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., … Pennacchio, F. (2016). A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. [Research Support, Non-U.S. Gov't]. *Proceedings of the National Academy of Sciences*, *113*(12), 3203–3208. http://www.ncbi.nlm.nih.gov/pubmed/26951652. doi:10.1073/pnas.1523515113

Dietemann, V., Nazzi, F., Martin, S. J., Anderson, D. L., Locke, B., Delaplane, K. S., … Ellis, J. D. (2013). Standard methods for varroa research. *Journal of Apicultural Research*, *52*(1), 1. In V. Dietemann, J. D. Evans, & P. Neumann (Eds.), The Coloss Beebook: Vol. 2. Standard methods for *Apis mellifera* pest and pathogen research. doi:10.3896/IBRA.1.52.1.09

Dively, G. P., Embrey, M. S., Kamel, A., Hawthorne, D. J., & Pettis, J. S. (2015). Assessment of chronic sublethal effects of imidacloprid on Honey Bee Colony Health. *Plos One*, *10*(4), e0118748. doi:10.1371/journal.pone.0126043

Du Rand, E. E., Smit, S., Beukes, M., Apostolides, Z., Pirk, C. W., & Nicolson, S. W. (2015). Detoxification mechanisms of honey bees (*Apis mellifera*) resulting in tolerance of dietary nicotine. [Research Support, Non-U.S. Gov't]. *Scientific Reports*, *5*(1), 11779. http://www.ncbi.nlm.nih.gov/pubmed/26134631. doi:10.1038/srep11779

Fairbrother, A., Purdy, J., Anderson, T., & Fell, R. (2014). Risks of neonicotinoid insecticides to honeybees. *Environmental Toxicology and Chemistry*, *33*(4), 719–731. doi:10.1002/etc.2527

Francis, R. M., Nielsen, S. L., & Kryger, P. (2013). Varroa-virus interaction in collapsing Honey Bee colonies. *Plos One*, *8*(3), e57540. doi:10.1371/journal.pone.0057540

Garrido, P. M., Antunez, K., Martin, M., Porrini, M. P., Zunino, P., & Eguaras, M. J. (2013). Immune-related gene expression in nurse honey bees (*Apis mellifera*) exposed to synthetic acaricides. *Journal of Insect Physiology*, *59*(1), 113–119. http://www.ncbi.nlm.nih.gov/pubmed/23147024. doi:10.1016/j.jinsphys.2012.10.019

Gatschenberger, H., Gimple, O., Tautz, J., & Beier, H. (2012). Honey bee drones maintain humoral immune competence throughout all life stages in the absence of vitellogenin production. [Research Support, Non-U.S. Gov't]. *Journal of Experimental Biology*, *215*(8), 1313–1322. http://www.ncbi.nlm.nih.gov/pubmed/22442369. doi:10.1242/jeb.065276

Goulson, D., Nicholls, E., Botias, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957. doi:10.1126/science.1255957

Gregorc, A., & Ellis, J. D. (2011). Cell death localization in situ in laboratory reared honey bee (*Apis mellifera* L.) larvae treated with pesticides. *Pesticide Biochemistry and Physiology*, *99*(2), 200–207. doi:10.1016/j.pestbp.2010.12.005

Gregorc, A., Evans, J. D., Scharf, M., & Ellis, J. D. (2012). Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and Varroa mites (*Varroa destructor*). *Journal of Insect Physiology*, *58*(8), 1042–1049. http://ac.els-cdn.com/S0022191012000790/1-s2.0-S0022191012000790-main.pdf?_tid=8b5851bc-c8db-11e5-8794-00000aab0f27&acdnat=1454328276_db730f116116b1273e4cef466542f128. doi:10.1016/j.jinsphys.2012.03.015

Gregory, P. G., Evans, J. D., Rinderer, T., & de Guzman, L. (2005). Conditional immune-gene suppression of honeybees parasitized by Varroa mites. *Journal of Insect Science*, *5*(1), 1–5. insectscience.org/5.7

Hoffmann, J. A. (1995). Innate immunity of insects. [Review]. *Current Opinion in Immunology*, *7*(1), 4–10. http://www.ncbi.nlm.nih.gov/pubmed/7772280. doi:10.1016/0952-7915(95)80022-0

Hu, Y. T., Wu, T. C., Yang, E. C., Wu, P. C., Lin, P. T., & Wu, Y. L. (2017). Regulation of genes related to immune signaling and detoxification in *Apis mellifera* by an inhibitor of histone deacetylation. *Scientific Reports*, *7*(1), 41255. http://www.ncbi.nlm.nih.gov/pubmed/28112264. doi:10.1038/srep41255

Imler, J. L., & Bulet, P. (2005). Antimicrobial peptides in Drosophila: Structures, activities and gene regulation [Review]. *Chemical Immunology and Allergy*, *86*, 1–21. http://www.ncbi.nlm.nih.gov/pubmed/15976485. doi:10.1159/000086648

Iwasa, T., Motoyama, N., Ambrose, J. T., & Roe, R. M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection*, *23*(5), 371–378. doi:10.1016/j.cropro.2003.08.018

Johnson, R. M. (2015). Honey Bee toxicology. *Annual Review of Entomology*, *60*(1), 415–434. doi:10.1146/annurev-ento-011613-162005

Johnson, R. M., Mao, W. F., Pollock, H. S., Niu, G. D., Schuler, M. A., & Berenbaum, M. R. (2012). Ecologically appropriate Xenobiotics induce cytochrome p450s in *Apis mellifera*. *Plos One*, *7*(2), e31051. doi:10.1371/journal.pone.0031051

Kevan, P. G. (1999). Pollinators as bioindicators of the state of the environment: Species, activity and diversity. *Agriculture Ecosystems & Environment*, *74*(1–3), 373–393. doi:10.1016/S0167-8809(99)00044-4

Khongphinitbunjong, K., de Guzman, L. I., Tarver, M. R., Rinderer, T. E., Chen, Y. P., & Chantawannakul, P. (2015). Differential viral levels and immune gene expression in three stocks of *Apis mellifera* induced by different numbers of *Varroa destructor*. *Journal of Insect Physiology*, *72*, 28–34. doi:10.1016/j.jinsphys.2014.11.005

Koleoglu, G., Goodwin, P. H., Reyes-Quintana, M., Hamiduzzaman, M. M., & Guzman-Novoa, E. (2017). Effect of *Varroa destructor*, Wounding and Varroa Homogenate on Gene Expression in Brood and Adult Honey Bees. *Plos One*, *12*(1), e0169669. http://www.ncbi.nlm.nih.gov/pubmed/28081188. doi:10.1371/journal.pone.0169669

Komsta, L., & Novomestky, F. (2015). Moments, cumulants, skewness, kurtosis and related tests [Package]. https://cran.r-project.org/web/packages/moments/moments.pdf

Kuster, R. D., Boncristiani, H. F., & Rueppell, O. (2014). Immunogene and viral transcript dynamics during parasitic *Varroa destructor* mite infection of developing honey bee (*Apis mellifera*) pupae. *Journal of Experimental Biology*, *217*(10), 1710–1718. http://jeb.biologists.org/content/jexbio/217/10/1710.full.pdf. doi:10.1242/jeb.097766

Lenth, R. V. (2016). Least-squares means: The R Package lsmeans. *Journal of Statistical Software*, *69*(1), 1–33. doi:10.18637/jss.v069.i01

Lourenco, A. P., Mackert, A., Cristino, A. D., & Simoes, Z. L. P. (2008). Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. *Apidologie*, *39*(3), 372. U333. doi:10.1051/apido:2008015

Mao, W. F., Schuler, M. A., & Berenbaum, M. R. (2011). CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). *Proceedings of the National Academy of Sciences of the United States of America*, *108*(31), 12657–12662. doi:10.1073/pnas.1109535108

Navajas, M., Migeon, A., Alaux, C., Martin-Magniette, M. L., Robinson, G. E., Evans, J. D., … Le Conte, Y. (2008). Differential gene expression of the honey bee *Apis mellifera* associated with *Varroa destructor* infection. *BMC Genomics*, *9*(1), 301. doi:10.1186/1471-2164-9

Neumann, P., & Carreck, N. L. (2010). Honey bee colony losses. *Journal of Apicultural Research*, *49*(1), 1–6. doi:10.3896/IBRA.1.49.1.01

Nicolas, E., Reichhart, J. M., Hoffmann, J. A., & Lemaitre, B. (1998). In vivo regulation of the IkappaB homologue cactus during the immune response of Drosophila. [Research Support, Non-U.S. *Journal of Biological Chemistry*, *273*(17), 10463–10469. http://www.ncbi.nlm.nih.gov/pubmed/9553105. doi:10.1074/jbc.273.17.10463

Pettis, J. S., vanEngelsdorp, D., Johnson, J., & Dively, G. (2012). Pesticide exposure in honey bees results in increased levels of the gut pathogen Nosema. *Naturwissenschaften*, *99*(2), 153–158. doi:10.1007/s00114-011-0881-1

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), 45e. http://nar.oxfordjournals.org/content/29/9/e45.full.pdf. doi:10.1093/nar/29.9.e45

Ratnieks, F. L. W., & Carreck, N. L. (2010). Clarity on Honey Bee collapse? *Science*, *327*(5962), 152–153. doi:10.1126/science.1185563

Core Team (Producer), R. (2013). R: A language and environment for statistical computing. *Foundation for Statistical Computing*. Vienna, Austria. http://www.R-project.org/

Rosenkranz, P., Aumeier, P., & Ziegelmann, B. (2010). Biology and control of *Varroa destructor*. *Journal of Invertebrate Pathology*, *103*, S96–S119. doi:10.1016/j.jip.2009.07.016

Ryabov, E. V., Fannon, J. M., Moore, J. D., Wood, G. R., & Evans, D. J. (2016). The Iflaviruses Sacbrood virus and deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate their horizontal or vertical transmission. *PeerJ*, *4*, e1591. doi:10.7717/peerj.1591

Ryabov, E. V., Wood, G. R., Fannon, J. M., Moore, J. D., Bull, J. C., Chandler, D., … Evans, D. J. (2014). A virulent strain of deformed wing virus (DWV) of Honeybees (*Apis mellifera*) prevails after Varroa destructor-mediated, or in vitro, transmission. *Plos Pathogens*, *10*(6), e1004230. doi:10.1371/journal.ppat.1004230

Schmuck, R., Schoning, R., Stork, A., & Schramel, O. (2001). Risk posed to honeybees (*Apis mellifera* L. Hymenoptera) by an imidacloprid seed dressing of sunflowers. *Pest Management Science*, *57*(3), 225–238. doi:10.1002/ps.270

Strachecka, A., Borsuk, G., Paleolog, J., Olszewski, K., Bajda, M., & Chobotow, J. (2014). Body-surface compounds in buckfast and Caucasian Honey Bee Workers (*Apis mellifera*). *Journal of Apicultural Science*, *58*(1), 5–15. doi:10.2478/jas-2014-0001

Suchail, S., De Sousa, G., Rahmani, R., & Belzunces, L. P. (2004). In vivo distribution and metabolisation of C-14-imidacloprid in different compartments of *Apis mellifera* L. *Pest Management Science*, *60*(11), 1056–1062. doi:10.1002/ps.895

Tesovnik, T., Cizelj, I., Zorc, M., Čitar, M., Božič, J., Glavan, G., & Narat, M. (2017). Immune related gene expression in worker honey bee (*Apis mellifera* carnica) pupae exposed to neonicotinoid thiamethoxam and Varroa mites (*Varroa destructor*). *Plos One*, *12*(10), e0187079. doi:10.1371/journal.pone.0187079

Wickham, H. (2009). *Ggplot2: Elegant graphics for data analysis*. New York, NY: Springer.

Yang, X., & Cox-Foster, D. L. (2005). Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. [Comparative Study]. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(21), 7470–7475. http://www.ncbi.nlm.nih.gov/pubmed/15897457. doi:10.1073/pnas.0501860102

Yue, C., & Genersch, E. (2005). RT-PCR analysis of Deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *Journal of General Virology*, *86*(12), 3419–3424. doi:10.1099/vir.0.81401-0

Zaobidna, E. A., Żółtowska, K., & Łopieńska-Biernat, E. (2015). Expression of the prophenoloxidase gene and phenoloxidase activity, during the development of *Apis mellifera* brood infected with *Varroa destructor*. *Journal of Apicultural Science*, *59*(2), 85–93. doi:10.1515/jas-2015-0025

Zaobidna, E. A., Żółtowska, K., & Łopieńska-Biernat, E. (2017). Expression and Activity of Lysozyme in *Apis mellifera* Carnica Brood Infested with *Varroa destructor*. *Journal of Apicultural Science*, *61*(2), 253–256. doi:10.1515/jas-2017-0014

Zhang, Y., Liu, X. J., Zhang, W. Q., & Han, R. C. (2010). Differential gene expression of the honey bees *Apis mellifera* and *A. cerana* induced by *Varroa destructor* infection. *Journal of Insect Physiology*, *56*(9), 1207–1218. doi:10.1016/j.jinsphys.2010.03.019