

Molecular Effects of Neonicotinoids in Honey Bees (*Apis mellifera*)

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S Supporting Information

ABSTRACT: Neonicotinoids are implicated in the decline of bee populations. As agonists of nicotinic acetylcholine receptors, they disturb acetylcholine receptor signaling leading to neurotoxicity. Several behavioral studies showed the link between neonicotinoid exposure and adverse effects on foraging activity and reproduction. However, molecular effects underlying these effects are poorly understood. Here we elucidated molecular effects at environmental realistic levels of three neonicotinoids and nicotine, and compared laboratory studies to field exposures with acetamiprid. We assessed transcriptional alterations of eight selected genes in caged honey bees exposed to different concentrations of the neonicotinoids acetamiprid, clothianidin, imidacloprid, and thiamethoxam, as well as nicotine. We determined transcripts of several targets, including *nicotinic acetylcholine receptor α 1* and *α 2 subunit*, the multifunctional gene *vitellogenin*, immune system genes *apidaecin* and *defensin-1*, stress-related gene *catalase* and two genes linked to memory formation, *pka* and *creb*. *Vitellogenin* showed a strong increase upon neonicotinoid exposures in the laboratory and field, while *creb* and *pka* transcripts were down-regulated. The induction of *vitellogenin* suggests adverse effects on foraging activity, whereas *creb* and *pka* down-regulation may be implicated in decreased long-term memory formation. Transcriptional alterations occurred at environmental concentrations and provide an explanation for the molecular basis of observed adverse effects of neonicotinoids to bees.



■ INTRODUCTION

Recent reports on global pollinator declines^{1,2} are alarming, especially with respect to the increasing demands for pollination services.³ Honey bees are the most economically valuable pollinators.⁴ However, the number of managed honey bees decreased by one-fourth in Europe between 1985 and 2005,⁵ and by more than one-half in North America between 1947 and 2005.⁵ This decline represents a major challenge for beekeepers and scientists. The reasons are poorly understood. Several studies highlighted the impact of pathogens,⁷ pesticides⁸ and also the lack of wild flowers. Declines similarly occur for wild bees and bumblebees. Multiple chemical residues have been detected inside honey bee hives.⁹ However, no individual factor such as lack of flowers, pesticides or pathogens seems to act as principal driver of colony collapse disorder or other honey bee losses. Thus, the global decline of bee populations can be considered as a multifactorial phenomenon driven by a combination of parasites, pesticides and shortage of (wild) flowers.¹⁰ The decline of bee populations has significant negative implications for plant pollination, including many domesticated crops, and therefore, limitation of crop yields.¹⁰

Pesticides are currently a debated cause of bee declines. Over the years, the classes of pesticide used in agriculture and their application methods have shifted substantially. Carbamates, pyrethroids and organochlorides have been less used for the

favor of new classes of systemic insecticides, in particular neonicotinoids. Three neonicotinoids, thiamethoxam, imidacloprid, and clothianidine, and two organophosphates (phosmet and chlorpyrifos), are thought to pose the highest risk to honey bees on a global scale.¹¹

Neonicotinoids are commonly applied as seed coatings to limit contact with insects and prevent losses. They are neurotoxins that target the central nervous system. Mainly acting as specific agonists by binding to acetylcholine receptors (AChR), neonicotinoids are highly effective in disrupting central nervous system function by overstimulation and paralysis, and are thus efficiently used for controlling insect pests.¹² In addition to very high toxicity, systemic compounds like neonicotinoids can be particularly problematic for pollinating insects through exposure to residues in nectar and pollen of treated crops.⁸ Recent studies have demonstrated that the hive products of honey bee colonies located in agricultural environments across Europe and North America are contaminated by various pesticides, including neonicotinoids.¹³

Received: February 8, 2016

Revised: March 16, 2016

Accepted: March 18, 2016

Published: March 18, 2016

Furthermore, some neonicotinoids are highly persistent in soil and soil water.¹³

Neonicotinoids show high acute toxicity to honey bees. Particularly the nitro-substituted compounds, clothianidin, imidacloprid and thiamethoxam (metabolized to clothianidin in plants and insects),¹⁴ show very high toxicity with LD₅₀ values in the range of a few ng/bee. The toxicity of cyano-substituted neonicotinoids, which include acetamiprid and thiacloprid, is lower with LD₅₀ values in the range of µg/bee.^{15,16} There exists a considerable variability in interindividual sensitivity and between colonies.¹⁷

Sublethal concentrations of neonicotinoids negatively affected locomotion, behavior, learning and memory of bees. Imidacloprid, thiamethoxam and clothianidin induced flight muscle paralysis,¹⁸ and negatively affected learning,¹⁹ olfactory performance, and foraging behavior.²⁰ Similarly, thiamethoxam^{21,22} and acetamiprid²² decreased memory and olfactory learning capacity. Importantly, neonicotinoids negatively affected orientation and foraging of worker bees.^{15,23,24} These effects were also observed in semifield conditions. Adverse effects of neonicotinoids on wild bees were shown under field conditions. Rape seed coated with clothianidin reduced wild bee density, solitary bee nesting, and bumble bee colony growth and reproduction.²⁵ Field realistic doses of imidacloprid alters foraging activity and decreases avoidance of predators of honey bees.²⁶ Bumble bee colonies exposed to clothianidin-treated weedy turf showed delayed weight gain and produced no new queens.²⁷ Recently, bees were shown to become attracted to neonicotinoid-containing nectar (sucrose solution containing imidacloprid or thiamethoxam), and that they cannot identify neonicotinoids for avoidance.²⁸ Exposure to imidacloprid, clothianidin, and fipronil led to reductions in the proportion of active bees in the hive, and initiated behaviors that reduced the efficiency of foraging flights. Also queens of honey bees are negatively affected by neonicotinoids. Experimental exposure reduced their reproductive anatomy and physiology.²⁹ Thus, it seems likely that bees living in farmland and exposed to neonicotinoids suffer from sublethal effects and reduced survival.

Thus, far, studies on adverse effects of neonicotinoids focused on orientation, and foraging behavior and memory formation, as well as on population relevant traits, including colony size and survival.^{29,30} Surprisingly, molecular effects of neonicotinoids were very little regarded (if at all), and thus poorly understood. Exposure to clothianidin led to a nAChR-dependent rapid mitochondrial depolarization in cultured neurons.³¹ Therefore, the aim of our study was to assess molecular effects of four neonicotinoids to better understand the molecular basis of their toxic actions in honey bees. We compared transcriptional effects on selected target genes of nicotine with those of nitro- (clothianidin, imidacloprid, and thiamethoxam) and cyano-substituted (acetamiprid) neonicotinoids to identify common and compound-specific adverse effect pathways. To this end, we focused on a series of different toxicologically relevant pathways, including neuronal signaling (*nicotinic acetylcholine receptor alpha 1 and 2 subunits*, *nAChRα1*, and *nAChRα2*), long-term memory formation (*creb* and *pka*), life-span (*vitellogenin*), immune system response (*apidaecin* and *defensin-1*), and stress response (*catalase*). In addition to experimental laboratory exposures, we assessed effects of acetamiprid in the field. Our study revealed for the first time important molecular effects at environmentally relevant concentrations that may explain physiological adverse effects

on the bees, and contribute to the understanding of the decline of bee populations.

MATERIALS AND METHODS

Chemicals. Nicotine (>99% purity), acetamiprid, clothianidin, imidacloprid, and thiamethoxam (purities of all >99%) were purchased from Sigma–Aldrich (Buchs, Switzerland). Stock solutions for each compound were prepared in DMSO and diluted into sucrose-solution (0.1% DMSO).

Laboratory Exposure Experiments. Foraging adult worker honey bees (*Apis mellifera carnica*) of mixed age were collected from frames from an outdoor colony located in a rural site with no agricultural activity and pesticide use in the Black Forest (Germany, GPS: N 47.7667, E 7.8333) from May to July 2014 and 2015. All of the bees used in the experiment were from the same colony. The colony had evidence of *Varroa destructor* infestation and was treated with formic acid (August 2014) and oxalic acid (December 2014). Honey bees were collected in small cylindrical plastic containers, cold anaesthetised at 4 °C for 60 min, and transferred into 16.5 × 11 × 6.5 cm³ PET bottles with small holes in the lid for gas exchange. A larger hole of 2 mm served for holding an Eppendorf microcentrifuge tube filled with 2 mL of a 20% sucrose solution containing nicotine and individual neonicotinoids (concentrations Table 1) or 0.1% DMSO as solvent control.

Table 1. Concentration of Nicotine and the Four Neonicotinoids Used in the Present Study

compound	concentration (ng/bee)	concentration (ng/mL sucrose solution)
nicotine	486, 4860, and 48 600	4860, 48 600, and 486 000
acetamiprid	8, 80, 800, and 8000	80, 800, 8000, and 80000
clothianidin	0.03, 0.3, 1.5, and 3	0.3; 3; 15, and 30
imidacloprid	0.3, 3, and 30	3, 30, and 300
thiamethoxam	0.01, 0.05, 0.1, and 1	0.1, 0.5, 1, and 10

In a pilot study, we investigated potential side effects of DMSO before we started the experiments. Two exposure experiments were conducted, the first one from April through July 2014, the second one from April through July 2015. Each exposure experiment consisted of three PET bottles with 10 bees per concentration and time point. Two of these three bottles were used to isolate RNA from bees, and one of these three bottles was kept frozen as back-up. Each exposure experiment was performed twice. As three bees were pooled to obtain one RNA sample, three technical replicates were obtained from one bottle. As RNA was isolated from two bottles, there were two biological replicates consisting of six technical replicates per experiment. As each exposure experiment was done twice, there were four biological replicates in total consisting of 12 technical replicates.

Concentrations of neonicotinoids were selected on the basis of environmental realistic levels in nectar, and higher doses were below LD₅₀ values. A summary of used concentrations expressed as ng/bee and as ng/mL sucrose solution is shown in Table 1. No compound related mortality occurred during exposure. Ten randomly chosen adult worker bees were placed into each bottle and bottles placed in an incubator (28 °C, 60% humidity). Bees were fed ad libitum with sucrose solutions containing the pesticides for 24, 48, and 72 h. Every 24 h, the 2 mL sucrose solution was removed, the amount of sucrose

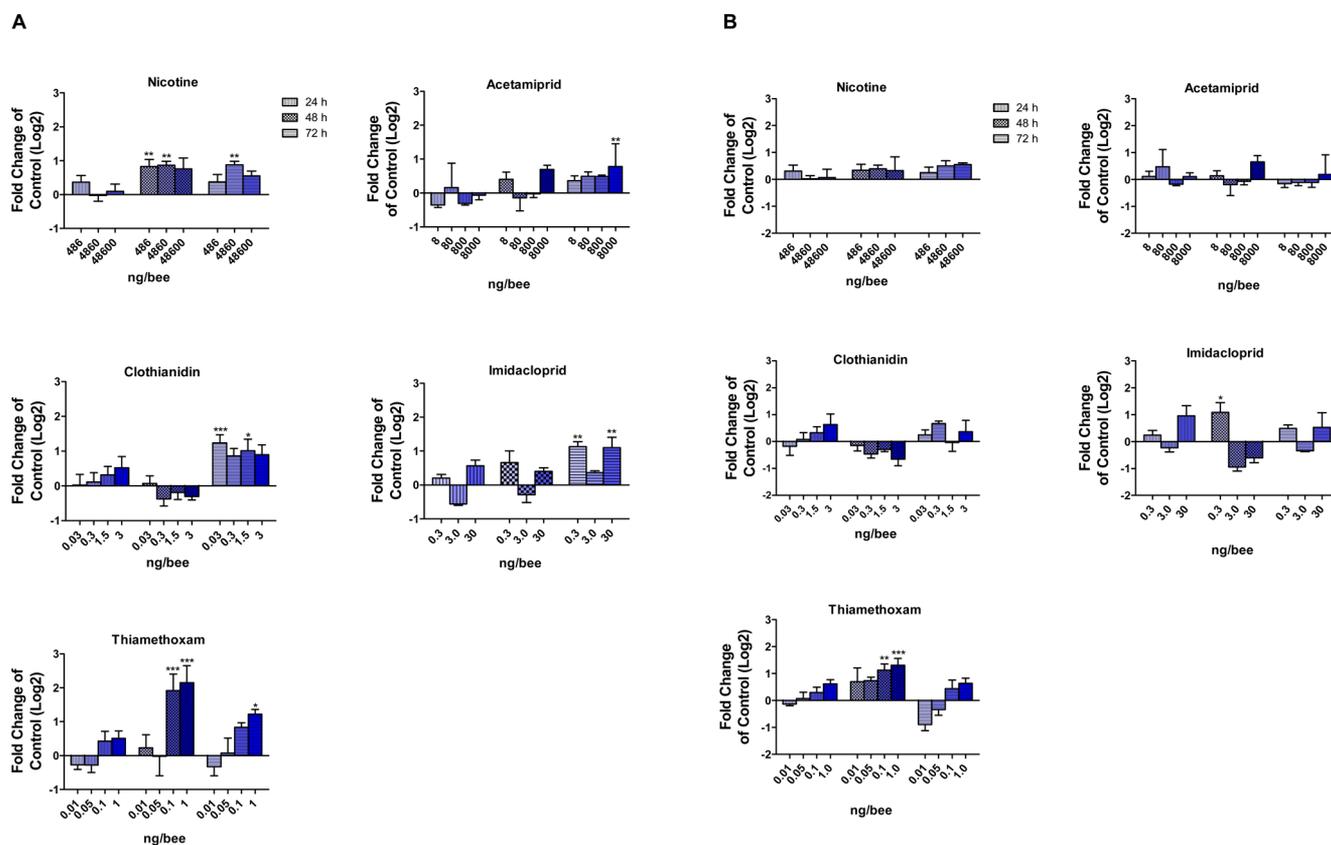


Figure 1. Abundance of transcripts of *nAChRα1* (A) and *nAChRα2* (B) in the brain of honey bees following exposure to different concentrations of nicotine, acetamiprid, clothianidin, imidacloprid, and thiamethoxam for 24 h (vertical strips), 48 h (squares) and 72 h (horizontal strips). Shown are the results of four biological replicates per concentration and time-point. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

solution taken up by the bees assessed, and replaced by a new solution. The average amount of sucrose solution was 100 μ L per bee throughout all exposure experiments. Solvent control bees and exposed bees were removed at different exposure times (24, 48, and 72 h) and stored at -20 °C until further analysis. In the pilot study, we found no effects of the solvent control (DMSO) on expressional changes in the honey bees.

Field Study. From May to July 2014, one bee hive (colony size around 50 000 bees) was placed into an orchard, where plums were grown, near to the Swiss border in the Rhine valley (Blansingen, GPS: N 47.689882, E 7.538758). Approximately 50–70 foraging bees were collected 1 day before spraying neonicotinoid insecticide acetamiprid (brand name Mospilan, water-soluble granulate with 200 g acetamiprid/kg without any adjuvants; 25 g Mospilan were dissolved in 100 L water to obtain a 0.025% solution) in the orchard, as well as 1, 3, and 7 days after spraying. Pesticide application occurred on 16th of July 2014, employing a concentration of 0.125 kg/ha. Collected bees were transported to the laboratory and immediately frozen at -20 °C until RNA isolation.

RNA Isolation, Reverse Transcription, And Quantitative (q)PCR. The brain of frozen bees was removed in total by opening the cranium using a scalpel and forceps. The thorax of frozen bees was removed by opening the chitin layer of the thorax using a scalpel, and removing the whole tissue in the thorax with forceps. The thorax was only removed from bees of the study conducted 2014. Total RNA of three pooled bee brains or thorax samples, respectively, was isolated using Trizol reagent according to the manufacturer's instructions. Per each condition, four biological replicates, consisting of 12 technical

replicates, were isolated. 1000 ng RNA was reverse transcribed as described before.³² qPCR based on SYBR green fluorescence (SYBR green PCR master mix; Roche) was performed as previously.³² Primer sequences were taken from literature or newly designed using the NCBI primer BLAST tool, and sequences of used primers are given in Table S1. For all performed analysis *ribosomal protein L32* (*rpl32*) was used as house-keeping gene for normalization due to constant expression in bees, similar as in other systems.³² All samples showed constant *rpl32* expression (Figure S1). Alterations of mRNA abundance in neonicotinoid exposed samples were always compared against the solvent (DMSO) control samples to determine the effect of pesticides.

Data Processing and Statistical Analysis. Heat maps of expressional changes were designed by importing analyzed qPCR data into MEV 4.9 (Multi Experiment Viewer) software. Differences between treatments were assessed by one way ANOVA followed by a Bonferroni's multiple comparison test to compare treatment means with respective controls. Results are given as means \pm standard error of means. Differences were considered statistically significant with one asterisk at $0.05 > p > 0.01$, two asterisks at $0.01 > p > 0.001$, and three asterisks at $0.001 > p > 0.0001$. All statistical data of the one way ANOVA are provided in Table S2. Correlation between field data and laboratory data were analyzed by linear regression.

RESULTS

First, we evaluated whether transcriptional changes induced by thiamethoxam were similar in brain and thorax of honey bees. To this end transcript of six genes were compared in bees

exposed to 0.1, 1, 2.5, and 5 ng/bee thiamethoxam for 24, 48, and 72 h (Figure S2). We found a significant increase of *AChR α 1*, *AChR α 2*, *vitellogenin* and *catalase* transcripts in both tissues. *Apidaecin* was significantly up-regulated in the brain only. As both tissues showed almost similar transcriptional alterations, subsequent analyses were focused solely on the brain as target organ of neonicotinoids, although extrapolation from one tissue to another is difficult.

We then compared effects of nicotine to those of the four neonicotinoids, acetamiprid, clothianidin, imidacloprid, and thiamethoxam, focusing on different toxicological pathways in the brain. Some of the effects of nicotine and all four neonicotinoids are shared, while some compound-specific patterns occurred in some of the target genes.

Transcriptional Changes on Target Genes and Pathways. We assessed the expression of eight selected target genes in the brain of honey bee workers after exposure for 24, 48, and 72 h to nicotine and the four neonicotinoids. Nicotine led to induction of the *nAChR α 1* transcript after 48 h and after 72 h, acetamiprid, clothianidin and imidacloprid after 72 h, and thiamethoxam after 48 and 72 h (Figure 1A). Significant induction of *nAChR α 2* was detected after exposure to imidacloprid and thiamethoxam after 48 h, but not after nicotine, acetamiprid and clothianidin exposure (Figure 1B). The strongest significance for both transcripts was found for thiamethoxam with $F(14, 165) = 6.9$ and 5.327 (Table S2).

We observed strongest and dose-related increases of the *vitellogenin* transcript after exposure to thiamethoxam in all concentrations after 48 h, and in the two highest concentrations after 72 h exposure (Figure 2). While nicotine did not induce this transcript, significant increases occurred for acetamiprid and imidacloprid after 24 h and after 48 h (Figure 2). Clothianidin induced the *vitellogenin* transcript after 24 h (Figure 2). Thiamethoxam showed the strongest significance with $F(14, 165) = 6.745$ (Table S2).

Nicotine and thiamethoxam did not alter the amount of the *pka* transcript, while acetamiprid and imidacloprid lowered its amount after 72 h (Figure 3). Clothianidin led to decrease of the *pka* transcript at all time points, with strongest effect after 48 h (Figure 3). Whereas acetamiprid and thiamethoxam did not change the abundance of the *creb* transcript, imidacloprid led to a decrease after 48 and 72 h, and nicotine led to a decrease after all time points. In contrast, clothianidin led to an increase after 24 h (Figure 4). Clothianidin showed for both transcripts the strongest significant changes with $F(14, 165) = 5.261$ for *pka* and 5.906 for *creb* (Table S2).

Nicotine had no effect on the amount of *apidaecin* and *defensin-1* transcripts (Figure 5, Figure S3). Acetamiprid and imidacloprid lowered the amount of *apidaecin* transcript after 72 h, clothianidin after 24 h, while thiamethoxam led to an increase after 48 h (Figure 5). The *defensin-1* transcript was increased at different exposure times upon acetamiprid, clothianidin, imidacloprid and thiamethoxam exposure (Figure S3). In case of the *catalase* transcript, acetamiprid and thiamethoxam led to an increase after 48 h, and imidacloprid led to concentration-dependent alterations, whereas nicotine and clothianidin had no effect on the expression of this transcript (Figure S4). Changes for these three transcripts showed only weak significance (Table S2). Thus, the most prominent transcriptional alterations were found for *AChR α 1* and 2, *creb*, *pka*, and *vitellogenin*. In particular the up-regulation of *vitellogenin* is specific for neonicotinoids, but not for nicotine.

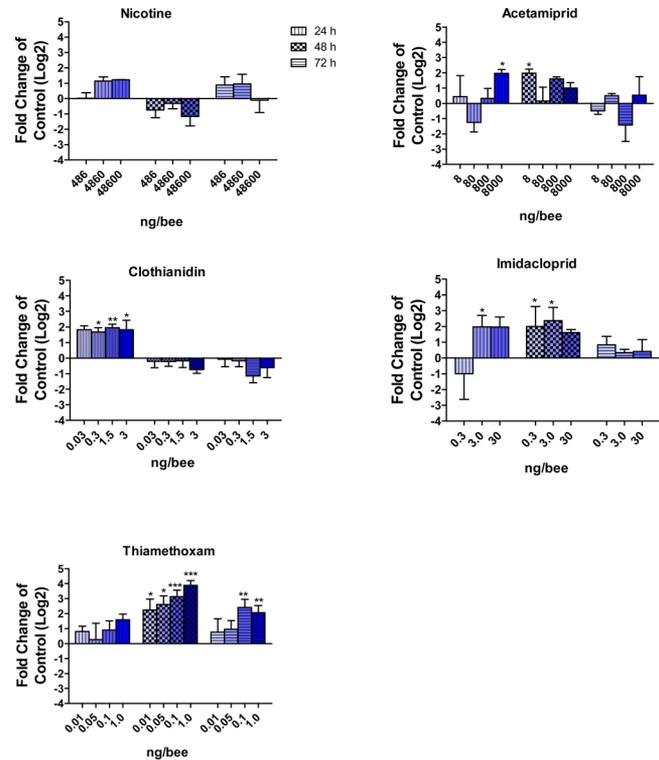


Figure 2. Abundance of transcripts of *vitellogenin* in the brain of honey bees following exposure to different concentrations of nicotine, acetamiprid, clothianidin, imidacloprid, and thiamethoxam for 24 h (vertical strips), 48 h (squares) and 72 h (horizontal strips). Shown are the results of four biological replicates per concentration and time-point. Significant differences with p -value of ≤ 0.05 are marked with asterisks.

Overall Change and Transcripts Profiles of Nicotine and Neonicotinoids. To compare and summarize overall changes in gene expression, a heat map analysis was performed. Thereby, changes during different exposure times are visible for each transcript. The strongest transcriptional down-regulations were found for acetamiprid and clothianidin, and the strongest transcriptional induction was found for thiamethoxam (Figure 6A). The two low concentrations of thiamethoxam and clothianidin showed very similar expression profiles. The same holds true for the two high thiamethoxam concentrations (Figure S5). The low imidacloprid concentration showed a similar expression pattern as thiamethoxam, and the middle and high concentrations induced a similar pattern as acetamiprid (Figure S5). Acetamiprid, imidacloprid and thiamethoxam build one group with similar transcriptional alterations, which was distinct from that of clothianidin and of nicotine (Figure S5). Expression profiles of different transcripts show that *nAChR α 1* (24 h), *nAChR α 2* (24 h), *nAChR α 1* (72 h), *nAChR α 2* (72 h), *defensin-1* (72 h), and *pka* (72 h) built one group. A second group consists of *nAChR α 1* (48 h), *nAChR α 2* (48 h), *apidaecin* (72 h), *catalase* (48 and 72 h), *defensin-1* (48 h), *pka* (48 h), and *vitellogenin* (72), and the transcripts of *catalase* (24 h), *creb* (24, 48, and 72 h) and *vitellogenin* (24 h) built a third group. These three groups built again one group with similar expression pattern. Very different expression profiles were found for *apidaecin* (24 h), *defensin-1* (24 h), *pka* (24 h) and *vitellogenin* (48 h) (Figure S5).

This heat map analysis indicates that the transcription profiles of nicotine and neonicotinoids are different, although

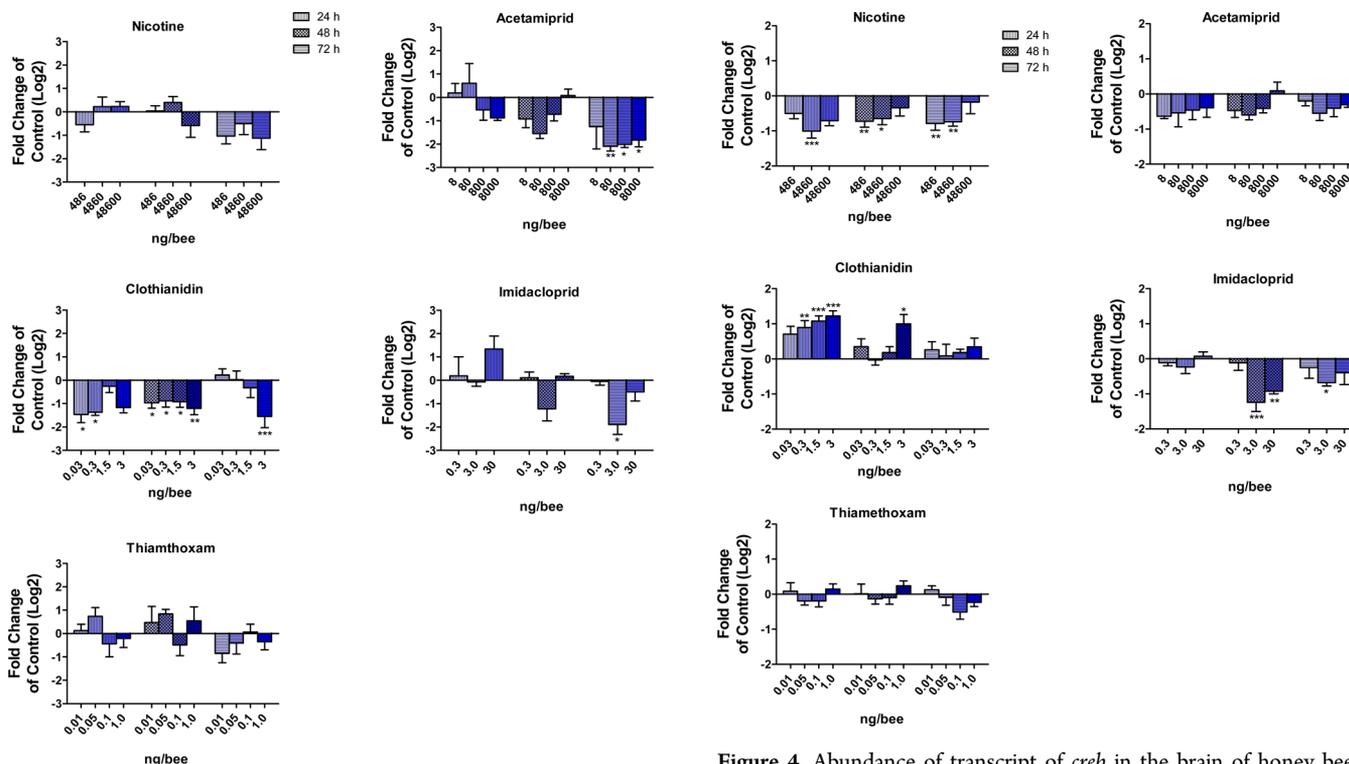


Figure 3. Abundance of transcripts of *pka* in the brain of honey bees following exposure to different concentrations of nicotine, acetamidrid, clothianidin, imidacloprid and thiamethoxam for 24 h (vertical strips), 48 h (squares) and 72 h (horizontal strips). Shown are the results of four biological replicates per concentration and time-point. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

they bind to the same receptors. The lowest effect concentrations for clothianidin, imidacloprid and thiamethoxam in our study were below reported residue concentrations in pollen and nectar (Figure 6B).^{27–29} This indicates that our observed transcriptional effects occur at environmentally realistic concentrations in case of clothianidin, imidacloprid and thiamethoxam.

Effects of Acetamidrid in the Field Study. Our field study, where acetamidrid (Mospilan) was applied, allowed a direct comparison to the experimental laboratory exposures. A strong increase in transcript abundance occurred for *nAChR α 1* and *nAChR α 2*, *vitellogenin*, and *catalase* one day after application of the pesticide in bees, while *apidaecin*, *creb*, *defensin-1*, and *pka* showed no significant changes (Figure 7A). The detected alterations in gene expression were highly significant with $F(31, 160) = 16.29$ (Table S2). We compared transcriptional changes in bees in the field study after one day to those found in bees after 24 h of laboratory exposures to low acetamidrid concentrations. The heat map shown in Figure 7B suggests a similar, but not identical pattern. The transcripts of *nAChR α 1*, *nAChR α 2*, and *catalase* reacted very similarly. Along with *vitellogenin* and *apidaecin*, they built one cluster. *Creb* and *defensin-1* also showed similar expression profiles, while *pka* was different (Figure 7B). In higher acetamidrid concentrations, the transcripts pattern showed larger deviations to the field exposure. The correlation between transcript levels in the low and high acetamidrid concentrations, and the field-study on day one after pesticide application was not significant (Figure 7C). Thus, the field exposure showed much stronger transcriptional changes than laboratory exposures, but some similarities in

Figure 4. Abundance of transcript of *creb* in the brain of honey bees following exposure to different concentrations of nicotine, acetamidrid, clothianidin, imidacloprid and thiamethoxam for 24 h (vertical strips), 48 h (squares) and 72 h (horizontal strips). Shown are the results of four biological replicates per concentration and time point. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

expression patterns to the experimental acetamidrid exposures occurred.

DISCUSSION

Here we showed for the first time significant molecular effects of nicotine and neonicotinoids in the brain of honey bee workers in experimental laboratory and field exposures. Transcripts of *nAChR α 1* and *nAChR α 2* were induced by nicotine and neonicotinoids, while *vitellogenin* was induced by neonicotinoids only. In addition to affecting the neuronal system, neonicotinoids led to expressional changes of immune system related genes, suggesting adverse effects on brain function and immune system defense. Effects were stronger for the three more toxic neonicotinoids, clothianidin, imidacloprid and thiamethoxam, than for the less toxic acetamidrid. Our data also indicate that clothianidin as reactive metabolite of thiamethoxam resulted in similar but faster and more significant transcriptional alterations; they occurred at a shorter exposure time than those of thiamethoxam. We also showed that similar molecular effects of acetamidrid occurred in field exposures as in the laboratory. Effects observed in the field exposure were stronger but transient. A schematic overview of the effects of neonicotinoids is given in Figure 8. They are characterized by induction of *vitellogenin*, and down-regulation of *apidaecin* and *pka*. We hypothesize that these molecular effects may represent a molecular basis for the previously reported physiological and behavioral effects.

The effect concentrations found in our study are much lower than reported LD₅₀ values of clothianidin, imidacloprid, and thiamethoxam, although different LD₅₀ values can be found for these three neonicotinoids in the literature. Laurino et al.

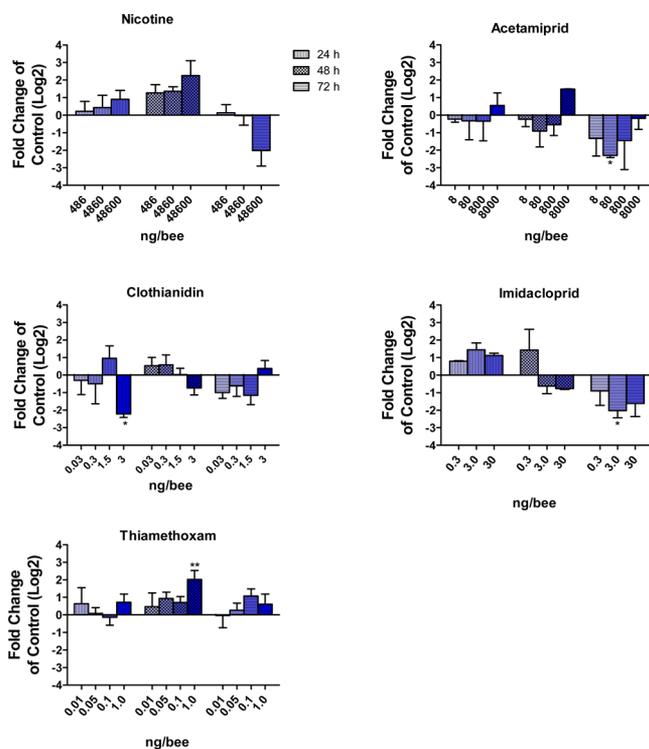


Figure 5. Abundance of transcripts of *apidaecin* in the brain of honey bees following exposure to different concentrations of nicotine, acetamiprid, clothianidin, imidacloprid and thiamethoxam for 24 h (vertical strips), 48 h (squares) and 72 h (horizontal strips). Shown are the results of four biological replicates per concentration and time-point. Significant differences with p -value of ≤ 0.05 are marked with asterisks.

(2010) determined LD_{50} values of 4.48 ng/bee at 24 h, 4.32 ng/bee at 48 h, and 4.21 ng/bee at 72 h for clothianidin; 183.78 ng/bee at 24 h, 104.12 ng/bee at 48 h and 72.94 ng/bee at 72 h for imidacloprid, and 3.55 ng/bee at 24 h, 3.35 ng/bee at 48 h and 2.88 ng/bee at 72 h for thiamethoxam.³³ In 2013, different LD_{50} values were determined by the same group for clothianidin, imidacloprid and thiamethoxam.³⁴ The LD_{50} value of acetamiprid was 14.5 $\mu\text{g}/\text{bee}$.¹⁵ In our study, we did not observe acute toxicity at any of the tested concentrations, so our observed effects seem not off-target effects. The observed effects concentrations were also much lower than known IC_{50} values of insect nAChR that are in the mg/mL range.^{36,37} This indicates that the observed effects at low concentrations are due to specific interactions of neonicotinoids with nAChRs. Nevertheless, at higher concentrations the observed effects could also be due to activation of muscarinic AChR (as a secondary effect). To distinguish contributions between nAChR and muscarinic AChR activation, experiments with nAChR antagonists would be needed. However, based on the similarity of effects at low and high neonicotinoid concentrations, contributions of muscarinic AChR seem to be of minor importance.

We found that nicotine and neonicotinoids led to up-regulation of nAChR transcripts in experimental exposures and in our field study in foraging honey bees. Our data confirm those found for nicotine.⁴⁰ Nicotine also affected short-term memory.⁴¹ Thus, neonicotinoids have the same effects as nicotine on the expression of nAChRs. Upregulation of nAChRs in response to neonicotinoids can be regarded as a

compensation reaction to the functional loss of these receptors, thus suggesting autoregulation. Chronic administration of nicotine triggered the up-regulation of nAChRs in rats.⁴² Alterations in neuronal signaling can have pronounced effects, as indicated by exposure of honey bees to 3.8 ng/bee thiamethoxam, which induced locomotor deficits.⁴³ Exposure of honey bees to 0.34 ng/bee clothianidin, 0.40 ng/bee imidacloprid or 0.48 ng/bee thiamethoxam induced adverse effects on basic motor function.⁴⁴

cAMP-dependent kinase (PKA) regulates multiple cellular processes, including the formation of long-term memory in honey bees.⁴⁵ Once activated, PKA induces the phosphorylation of cAMP response element binding protein (Creb). Phosphorylated Creb acts as an active transcription factor and induces the expression of Creb target genes that are thought to contribute to the formation of long-term synaptic plasticity which is important for long-term memory consolidation.⁴⁶ In addition, Creb is known to play a central role in the formation and consolidation of memory.^{47–49} Creb is also involved in the visual and olfactory learning in bees.⁵⁰ The observed down-regulation of *creb* upon neonicotinoid exposure in our experiments could be involved in the adverse effects on memory formation. Inhibition of Creb in rats was responsible for the nicotine-induced impairment of hippocampal plasticity.⁴⁸ Likewise, down-regulation of *creb* in honey bees could lead to reduction of plasticity of the neuronal system. We found a down-regulation of *creb* by nicotine and imidacloprid, and a down-regulation of *pka* by acetamiprid, clothianidin, and imidacloprid. This could partly explain the negative effects of neonicotinoids on long-term memory formation and learning. It is known that 6.12 ng/bee imidacloprid and 0.69 $\mu\text{g}/\text{bee}$ thiamethoxam impaired short-term olfactory memory in foraging honey bees.⁵¹

Vitellogenin is a female-specific glucolipoprotein produced under hormonal control, and produced by the reproductive queen, but also by worker bees, particularly hive bees. When hive bees develop into foragers, vitellogenin production is ceased. Vitellogenin is predominantly found in the hemolymph of queens, hive bees, and winter bees. The levels are highest in the longest-lived winter bee workers (up to 60–90 $\mu\text{g}/\mu\text{L}$ hemolymph), and lowest in short-lived foragers.⁵² In addition to reproductive function (queen) vitellogenin can be characterized as a protein being used for different metabolic purposes, and it also shields cells from oxidative damage and protects against oxidative stress.⁵² The protectant role and its increase with aging, led to the hypothesis that vitellogenin serves as a regulator of honey bees lifespan.^{52,53} Furthermore, vitellogenin can suppress juvenile hormone in worker bees,⁵⁴ which acts as pro-aging hormone⁵⁵ and promotes foraging. Down-regulation of vitellogenin by RNA interference led to increased levels of juvenile hormone and accelerated transition to the short-lived forager stage.⁵⁵ A drop in vitellogenin titer regulates the behavioral shift to foraging, and increases the juvenile hormone level. Juvenile hormone titers of hive bees, which are not foraging, were 10–100 ng/mL, and of foragers, 200–600 ng/mL.^{56,57} RNAi-mediated silencing of vitellogenin gene function turns honey bee workers into extremely precocious foragers. This suggests that vitellogenin is a primary switch signal.⁵⁸ An increased expression of vitellogenin was observed in nonforaging worker bees in queen-less colonies.⁵⁹

In addition, vitellogenin regulates foraging performance in worker bees. With high levels, workers search more for pollen, and with low levels, for nectar.⁶⁰ Exposure of honey bees to

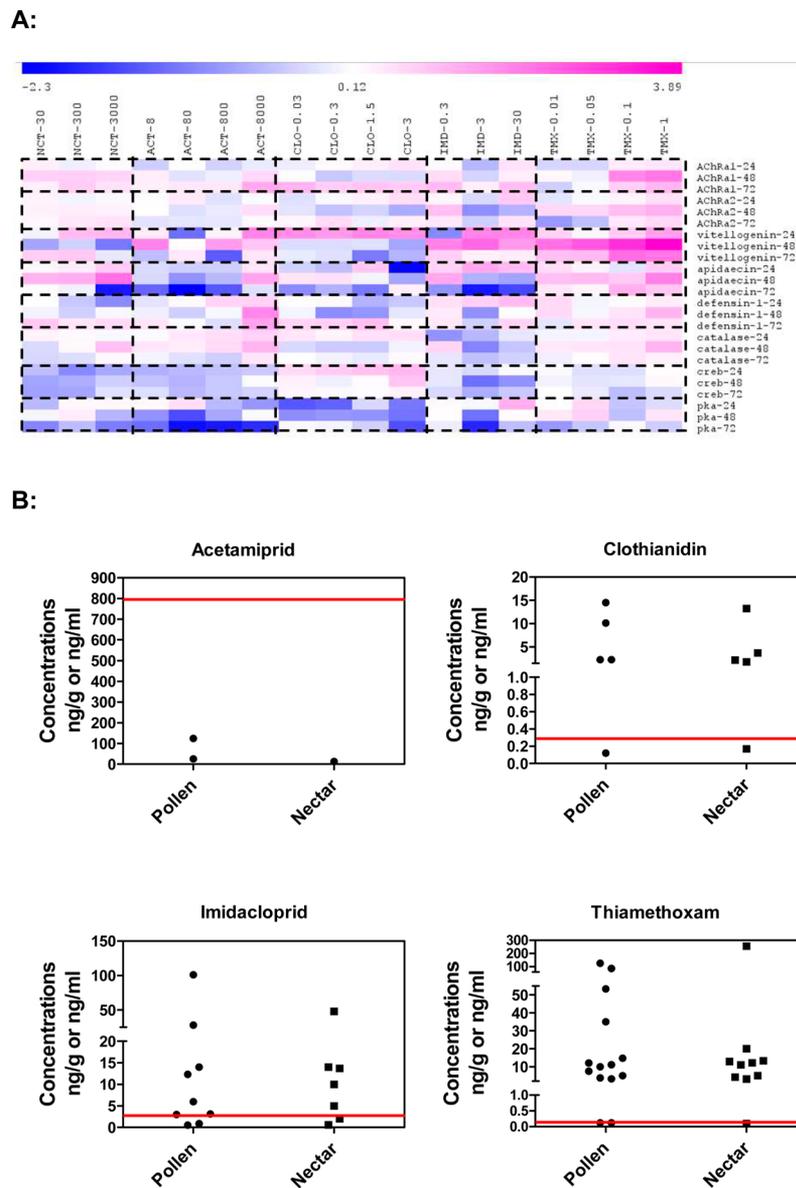


Figure 6. Expression profiles in the brain of honey bees following exposure to nicotine, acetamiprid, imidacloprid and thiamethoxam for different times and concentrations. (A) Heat map of all obtained transcriptional alterations for each compound and concentration shown for different exposure times of 24, 48, and 72 h. (B) Comparison of effect levels found in the present study (only significant effects with F-statistics above 3) with reported pollen and nectar concentrations of acetamiprid, clothianidin, imidacloprid and thiamethoxam according to^{35,38,39} Red line shows the lowest observed effect concentrations (LOEL) observed in the present study.

clothianidin and imidacloprid in the low ng/bee range reduced foraging activity.⁶¹ So far, the decrease in foraging activity upon neonicotinoid exposure was linked to disturbed memory formation. The neonicotinoids clothianidin, imidacloprid and thiamethoxam were thought to either block the retrieval of exploratory navigation memory, or altered this form of navigation memory.⁶² The up-regulation of vitellogenin after neonicotinoid exposure observed in our study could be another explanation for the previously shown reduced foraging activity of honey bee workers after neonicotinoid exposure.⁶¹ Most notably, the strong induction of vitellogenin detected in bees exposed to acetamiprid in our field study may be an explanation for transient negative effects of neonicotinoids on foraging activity of honey bees. The importance of *vitellogenin* as a biomarker for neonicotinoid exposure is supported by comparison with nicotine; *vitellogenin* was the only gene

studied that was strongly up-regulated upon experimental neonicotinoid exposure. Strong increase was also found in our field study. Therefore, the expression of *vitellogenin* may serve as a good biomarker candidate for the exposure of honey bees to neonicotinoids. Further analysis addressing this in more depth, as well as studies on the vitellogenin protein levels would help to understand this important effect.

Insect immunity shows many parallels to the innate immune response of vertebrates, involving a diverse set of actions including the secretion of antimicrobial peptides, phagocytosis, melanisation, and the enzymatic degradation of pathogens.⁶³ Apidaecin and defensin-1 are members of the Toll like receptor pathway and function as antimicrobial effectors.⁶¹ Injection of *E. coli* or the bee parasite *Paenibacillus larvae* into adult worker bees lead to the strong induction of the *apidaecin* and *defensin-1* transcripts.⁶⁴ This shows the important role of both proteins to

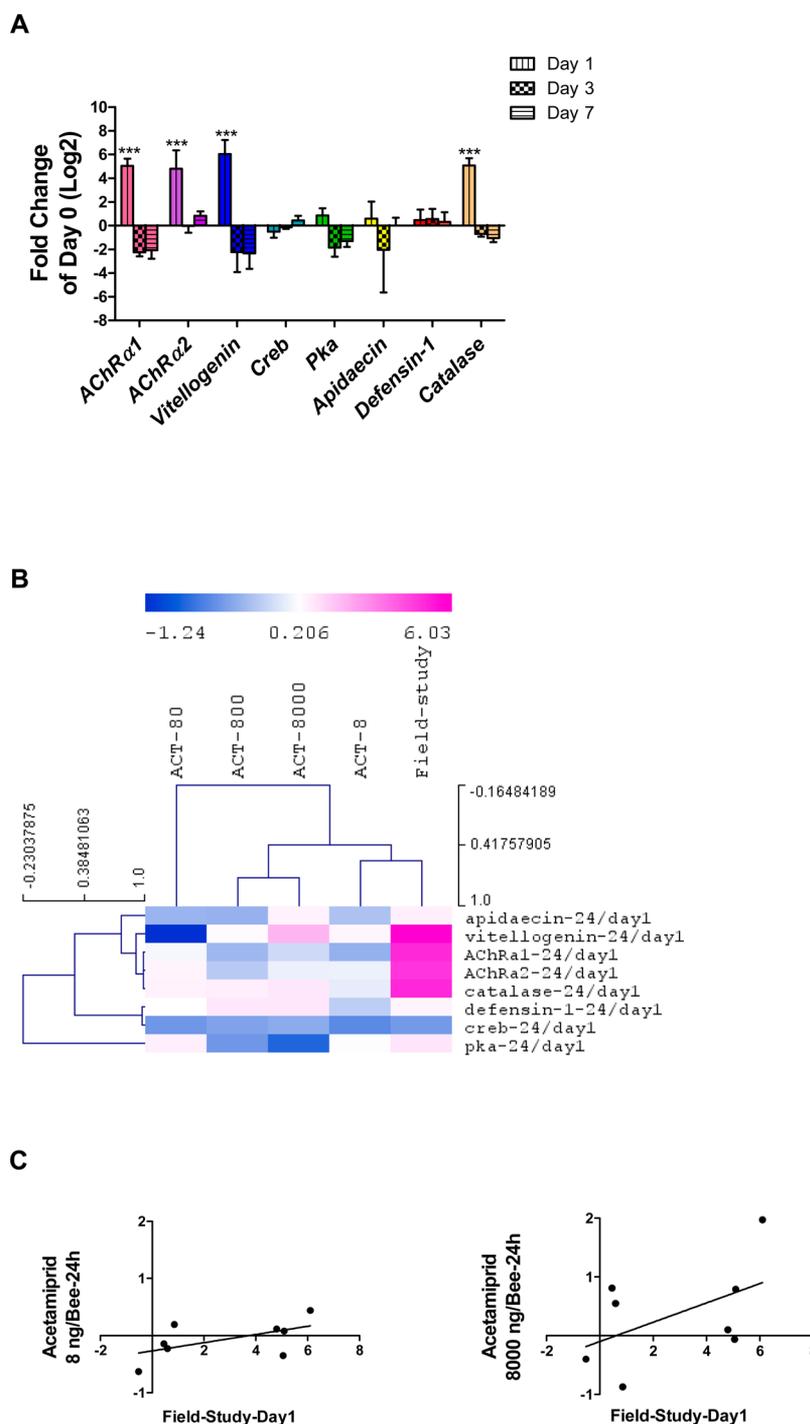


Figure 7. (A) Abundance of transcripts of eight selected genes in the brain of foraging honey bees one day (vertical strips), 3 days (squares) and 7 days (horizontal strips) after exposure to acetamiprid (Mospilon). (B) Comparison of overall alterations in gene expression after experimental exposure to acetamiprid for 24 h and field exposure to acetamiprid (Mospilon) for one day in the field study by heat map analysis. Shown are the results of six pooled samples per concentration and time-point. Significant differences with p -value of ≤ 0.05 are marked by asterisks. (C) Correlation between alteration in gene expression (fold-changes) for determined transcripts between field-study-day one and the lowest (left, r^2 : 0.32), and the highest (right, r^2 : 0.25) acetamiprid concentration after experimental 24 h exposure.

fight against pathogens. Exposure of honey bees to field realistic concentrations of imidacloprid decreased hemocyte density, encapsulation response, and antimicrobial activity.⁶⁵ Adverse effects of nicotine on the immune system were previously reported.⁶⁶ Decreased transcripts of *apidaecin* found in our experiments upon exposure to acetamiprid, clothianidin and imidacloprid may be implicated in negatively effects on the

immune system. In contrast to *apidaecin*, the *defensin-1* transcript was upregulated upon neonicotinoid exposure (Figure S3). Defensin is produced as antimicrobial peptide upon infection with pathogens.⁶⁷ There is a negative correlation between the expression of defensin-1 and the expression of storage proteins like apoLp-III,⁶⁸ thus neonicotinoid-related up-regulation of defensin-1 may affect this process. Interestingly,

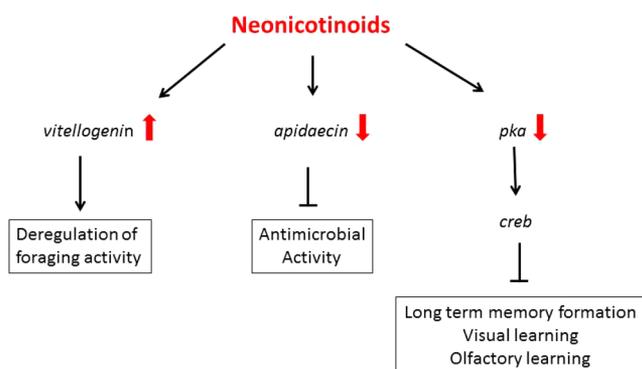


Figure 8. Schematic overview of neonicotinoid-induced molecular effects in honey bees on the transcriptional level, and associated physiological and behavioral consequences. Red arrows point out up- and down-regulation of the investigated transcripts.

nicotine and neonicotinoid-induced transcriptional changes did not show clear dose–effect relationships, but can rather be regarded as an all or nothing reaction. Only clothianidin and thiamethoxam showed some dose-dependent effects. Moreover, sometimes strongest reaction occurred in the lowest dose. This is in line with previous data on subchronic toxicity of neonicotinoids.^{69,70} These nonlinear patterns could be due to the complex interaction between nAChR binding of neonicotinoids and gene expression regulation that can generate nonclassical dose response relationships.⁷¹

Especially for the three highly toxic neonicotinoids, clothianidin, imidacloprid, and thiamethoxam, effects occurred at very low ng/bee (or low ng/mL) levels. These concentrations are environmentally relevant. Reported maximal concentrations of imidacloprid and thiamethoxam in wild flowers collected adjacent to recently planted crop fields were 48 ng/g and 256 ng/g.⁷² In pollen of squash, imidacloprid and thiamethoxam concentrations were up to 28 ppb (28 ng/mg) and 35 ppb (35 ng/mg), respectively. The maximal found imidacloprid and thiamethoxam concentrations in nectar of squash were 14 ppb (14 ng/mL) and 20 ppb (20 ng/mL), respectively.³⁵ In pollen of rape flowers, mean clothianidin and thiamethoxam concentrations were 2.27 ng/g and 3.26 ng/g, respectively. In pollen of wild flowers grown at the margins of oilseed rape fields, mean thiamethoxam concentrations were 14.81 ng/g.⁷³ In addition to nectar and pollen, puddle water is a source for neonicotinoid uptake; thiamethoxam concentrations of up to 63.4 $\mu\text{g/L}$ (63.4 ng/mL) were found.⁷⁴ Thus, environmental concentrations of clothianidin, imidacloprid, and thiamethoxam are in the same range of the here reported concentrations that induced alterations in gene expression. Clothianidin and imidacloprid induced the expression of *vitellogenin* at 3 ng/mL, and thiamethoxam at 0.1 ng/mL. Therefore, the observed effects in our study occurred at environmental realistic concentrations. The fact that our field study revealed strong transcriptional changes that were similar to the laboratory study with acetamiprid, namely upregulation of *AChRs*, *vitellogenin*, and *catalase*, strengthens the implication that our experimental laboratory data can be extrapolated to the field. However, our study focused on the transcriptional level, and it is currently not clear, how the transcriptional alterations translate to the protein level and to physiological effects. This limitation should be addressed in forthcoming studies by analyses on the protein level and by linking molecular with physiological effects.

In conclusion, here we report for the first time molecular effects on key neuronal processes in the brain of honey bees exposed to four neonicotinoids, and we compare them to those of nicotine. Most prominent transcriptional alterations occurred for *AChR α 1* and 2, *creb*, *pka* and *vitellogenin*, the latter of which seems selective for the neonicotinoids (but not for nicotine). Clothianidin and thiamethoxam showed dose–response effects for certain transcripts (clothianidin: *AChR α 2*, *creb*, and *vitellogenin*; thiamethoxam: *AChR α 1*, *AChR α 2*, and *vitellogenin*), confirming that they are compound-related. The observed transcriptional effects may represent a basis for previously reported adverse effects of neonicotinoids on the immune system, foraging behavior and memory in bees, and provide preliminary insights into the molecular basis of such physiological effects. Particularly the induction of *vitellogenin* may have physiological implications and can serve as potential biomarker for neonicotinoid exposure. Bees exposed to neonicotinoids showed reduced foraging activity and high *vitellogenin* levels (normally found in winter bees). Therefore, the observed overexpression of the *vitellogenin* transcript could be one of the molecular bases for the alteration in foraging activity upon neonicotinoid exposure. The alterations in *creb* and *pka* expression may represent a molecular basis for the observed negative effects of neonicotinoids on honey bee memory formation. Therefore, our work may provide some first hints on the molecular basis of the observed physiological changes of honey bees upon neonicotinoid exposure. How these transcriptional alterations will translate to adverse effects in bees living in neonicotinoid contaminated environments should be addressed in forthcoming studies. Our data clearly show transcriptional effects of neonicotinoids at environmental concentrations and help to understand the potential contribution of neonicotinoids to the decline of bee populations.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b00678.

Information about primer sequences used for quantitative real-time PCR analysis and statistical values of the ANOVA analysis (Tables S1 and S2). Expression of reference gene *rpl32* (Figure S1), results of the laboratory study performed in 2014 (Figure S2), abundance of transcript *defensin-1* after exposure to nicotine and neonicotinoids (Figure S3), abundance of transcript *catalase* after exposure to nicotine and neonicotinoids (Figure S4), and overall gene expression analysis of the laboratory study (Figure S5) (PDF)

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Notes

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■ ACKNOWLEDGMENTS

We thank Linda Haltinger for technical assistance and Eva Reinhard, Swiss Federal Office for Agriculture (BLW), Bern, for support and for reading the manuscript. The study was funded by the BLW and internal funding by the University of Applied Sciences and Arts Northwestern Switzerland.

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