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Molecular effects of neonicotinoids in honey bees (*Apis mellifera*)

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Abstract

Neonicotinoids are implicated in the decline of honey bee populations. As agonists of the nicotinic acetylcholine receptors, they disturb acetylcholine receptor signalling leading to neurotoxicity. Several behavioural studies showed the link between neonicotinoid exposure and adverse effects on foraging activity and reproduction. However, molecular effects underlying these effects are poorly known. 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 neonicotinoids acetamiprid, clothianidin, imidacloporid, 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.* V*itellogenin* 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] pesticides[8] 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 favour 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 honeybees 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, 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] Furthermore, 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 LD_{50} values in the range of a few ng/bee. The toxicity of cyano-substituted neonicotinoids, which include acetamiprid and thiacloprid, is lower with LD_{50} values in the range of μg/bee.[15, 16] There exists a considerable variability in inter-individual sensitivity and between colonies.[17]

Sublethal concentrations of neonicotinoids negatively affected locomotion, behaviour, learning and memory of bees. Imidacloprid, thiamethoxam and clothianidin induced flight muscle paralysis,[18] negatively affected learning,[19] olfactory performance, and foraging behaviour [20]. Similarly, thiamethoxam [21, 22] and acetamiprid [22] 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 semi-field 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 bumblebee 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 nicotinoid-containing nectar (sucrose solution containing imidacloprid or thiamethoxam) and that they cannot identify nicotinoids for avoidance.[28] Exposure to imidacloprid, clothianidin and fipronil led to reductions in the proportion of active bees in the hive, and initiated behaviours 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 behaviour 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 signalling (*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

126

127 **Chemicals**

128 Nicotine (> 99% purity), acetamiprid, clothianidin, imidacloprid and thiametoxam (purities of all
129 > 99%) were purchased from Sigma–Aldrich (Buchs, Switzerland). Stock solutions for each
130 compound were prepared in DMSO and diluted into sucrose-solution (0.1% DMSO).

131

132 **Laboratory exposure experiments**

133 Foraging adult worker honeybees (*Apis mellifera carnica*) of mixed age were collected from
134 frames from an outdoor colony located in a rural site with no agricultural activity and pesticide
135 use in the Black Forest (Germany, GPS: N 47.7667, E 7.8333) from May to July 2014 and
136 2015. All of the bees used in the experiment were from the same colony. The colony had evi-
137 dence o*f Varroa destructor* infestation and was treated with formic acid (August 2014) and
138 oxalic acid (December 2014). Honeybees were collected in small cylindrical plastic containers,
139 cold anaesthetised at 4°C for 60 minutes, and transferred into 16.5 × 11 × 6.5 cm^3 PET bottles
140 with small holes in the lid for gas exchange. A larger hole of 2 mm served for holding an Ep-
141 pendorf microcentrifuge tube filled with 2 mL of a 20% sucrose solution containing nicotine and
142 individual neonicotinoids (concentrations Table S1) or 0.1 % DMSO as solvent control.

143 In a pilot study we investigated possible side effects of DMSO before we started the
144 real experiments. Two exposure experiments were conducted, the first one from April through
145 July 2014, the second one from April through July 2015. Each exposure experiment consisted
146 of three PET bottles with 10 bees per concentration and time point. Two of these three bottles
147 were used to isolate RNA and one of these three bottles was keep frozen as back-up. Each
148 exposure experiment was done twice. As three bees were pooled to obtain one RNA samples,
149 three technical replicates were obtained from one bottle. As RNA was isolated from two bot-
150 tles, there were two biological replicates consisting of 6 technical replicates per experiment. As
151 each exposure experiment was done twice, there were four biological replicates in total con-
152 sisting of 12 technical replicates.

153 Concentrations of neonicotinoids were selected on the basis of environmental realistic
154 levels in nectar, and higher doses were below LD_{50} values. A summary of used concentrations
155 expressed as ng/bee and as ng/ml syrup is shown in Table 1. No compound related mortality
156 occurred during exposure. Ten randomly chosen adult worker bees were placed into each
157 bottle and bottles placed in an incubator (28°C, 60% humidity). Bees were fed ad libitum with
158 sucrose solutions containing the pesticides for 24, 48 and 72 h. Every 24 h, the 2 ml sucrose
159 solution was removed, the amount of sucrose solution taken up by the bees assessed, and
160 replaced by a new solution. The average amount of sucrose solution was 100 μL per bee
161 throughout all exposure experiments. Solvent control and exposed bees were removed at dif-
162 ferent exposure times (24, 48 and 72 h) and stored at -20°C until further analysis. In a 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 one day before spraying neonicotinoid insecticide acetamiprid (brand name Mospilan®, water soluble granulate with 200 g acetamiprid/kg with any adjuvants; 25 g Mospilan were dissolved in 100 L water to get a 0.025 % solution) in the orchard, as well as one, three and seven 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, six biological 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 normalisation 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 analysed 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 ± 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 analysed 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 (Fig. S2). We found a significant increase of *AChRα1*, *AChRα2*, *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 three 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 *nAChRα1* transcript after 48 h and after 72 h, acetamiprid, clothianidin and imidacloprid after 72 h and thiamethoxam after 48 h and 72 h (Fig 1A). Significant induction of *nAChRα2* was detected after exposure to imidacloprid and thiamethoxam after 48 h, but not after nicotine, acetamiprid and clothianidin exposure (Fig. 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 *vitelloginin* transcript after exposure to thiamethoxam in all concentrations after 48 h, and in the two highest concentrations after 72 h exposure (Fig. 2). While nicotine did not induce this transcript, significant increases occurred for acetamiprid and imidacloprid after 24 h and after 48 h (Fig. 2). Clothianidin induced the *vitellogenin* transcript after 24 h (Fig. 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 (Fig. 3). Clothianidin led to decrease of the *pka* transcript at all time points, with strongest effect after 48 h (Fig. 3). Whereas acetamiprid and thiamethoxam did not change 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 (Fig. 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 (Fig. 5, Fig. 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 (Fig. 5). The *defensin-1* transcript was increased at different exposure times upon acetamiprid, clothianidin, imidacloprid and thiamethoxam exposure (Fig. 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 (Fig. S4). Changes for these three transcripts showed only weak significance (Table S2). Thus, the most prominent transcriptional alterations were found for *AChRα1* and *2*, *creb*, *pka* and *vitellogenin.* In particular the up-regulation of *vitellogenin* is specific for neonicotinoids, but not for nicotine.

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 (Fig. 6A). The two low concentrations of thiamethoxam and clothianidin showed very similar expression profiles. The same holds true for the two high thiamethoxam concentrations (Fig. S5). The low imidacloprid concentration showed a similar expression pattern as thiamethoxam and the middle and high concentrations induced a similar pattern as acetamiprid (Fig. S5). Acetamiprid, imidacloprid and thiamethoxam build one group with similar transcriptional alterations, which was distinct from that of clothianidin and of nicotine (Fig S5). Expression profiles of different transcripts show that *nAChRα1* (24 h), *nAChRα2* (24 h), *nAChRα1* (72 h), *nAChRα2* (72 h), *defensin-1* (72 h) and *pka* (72 h) built one group. A second group consists of *nAChRα1* (48 h), *nAChRα2* (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) 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) (Fig. S5).

This heat map analysis indicates that the transcription profiles of nicotine and neonicotinoids induced different expression patterns, although binding to the same receptor. The lowest effect concentrations for clothianidin, imidacloprid and thiamethoxam in our study were below reported residue concentrations in pollen and nectar (Fig. 6B).[27, 28, 29] This indicates that

our observed transcriptional effects occur at environmentally realistic concentrations in case of clothianidin, imidacloprid and thiamethoxam.

Effects of acetamiprid in the field study

Our field study, where acetamiprid (Mospilan®) was applied, allowed a direct comparison to the experimental laboratory exposures. A strong increase in transcript abundance occurred for *nAChRα1* and *nAChRα2*, *vitellogenin* and *catalase* one day after application of the pesticide in bees, while *Apidaecin*, *creb*, *defensin- 1* and *pka* showed no significant changes (Fig. 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 acetamiprid concentrations. The heat map shown in Fig. 7B suggests a similar, but not identical pattern. The transcripts of *nAChRα1*, *nAChRα2* 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 (Fig. 7B). In higher acetamiprid concentrations the transcripts pattern showed larger deviations to the field exposure. The correlation between transcript levels in the low and high acetamiprid concentrations and the field-study on day one after pesticide application was not significant (Fig. 7C). Thus, the field exposure showed much stronger transcriptional changes than laboratory exposures, but some similarities in expression patterns to the experimental acetamiprid 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 *nAChRα1* and *nAChRα2* 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 defence. Effects were stronger for the three more toxic neonicotinoids, clothianidin, imidacloprid and thiamethoxam than for the less toxic acetamiprid. 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 acetamiprid 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*, down-

311 regulation of *apidaecin* and *pka.* We hypothesize that these molecular effects may represent a
312 molecular basis for the previously reported physiological and behavioural effects.
313 The effect concentrations found in our study are much lower than reported LD_{50} values
314 of clothianidin, imidaclorpid and thiamethoxam although different LD_{50} values can be found for
315 these three neonicotinoids in the literature. Laurino et al. determined LD_{50} values of 4.48
316 ng/bee at 24 h, 4.32 ng/bee at 48 h, and 4.21 ng/bee at 72 h f for clothianidin; 183.78 ng/bee
317 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
318 24 h, 3.35 ng/bee at 48 h and 2.88 ng/bee at 72 for thiamethoxam.[33] 2013, different LD_{50} val-
319 ues were determined by the same group for clothianidin, imidaclorid and thiamethoxam.[34] The
320 LD_{50} value of acetamiprid was 14.5 μg/bee.[15] In our study, we did not observe acute toxicity at
321 any of the tested concentrations, so our observed effects seem not off-target effects. The ob-
322 served effects concentrations are also much lower than known IC_{50} values of insect nAChR
323 that are in the mg/mL range.[36, 37] This indicates that the observed effects at low concentrations
324 are due to specific interactions of neonicotinoids with nAChRs. Nevertheless, at higher con-
325 centrations the observed effects could also be due to activation of muscarinic AChR (second-
326 ary effect). To distinguish contributions between nAChR and muscarinic AchR activation, ex-
327 periments with nAChR antagonists would be needed. However, based on the similarity of ef-
328 fects at low and high neonicotinoid concentrations, contributions of muscarinic AChR seem to
329 be of minor importance.
330 We found that nicotine and neonicotinoids led to up-regulation of *nAChR* transcripts in
331 experimental exposures and in our field study in foraging honey bees. Our data confirm those
332 found for nicotine.[40] Nicotine also affected short-term memory.[41]Thus, neonicotinoids have the
333 same effects as nicotine on the expression of *nAChRs*. Upregulation of *nAChRs* in response
334 to neonicotinoids can be regarded as a compensation reaction to the functional loss of these
335 receptors, thus suggesting auto-regulation. Chronic administration of nicotine triggered the up-
336 regulation of *nAChRs* in rats.[42] Alterations in neuronal signalling can have pronounced effects,
337 as indicated by exposure of honey bees to 3.8 ng/bee thiamethoxam, which induced locomotor
338 deficits.[43] Exposure of honey bees to 0.34 ng/bee clothianidin or 0.40 ng/bee imidacloprid or
339 0.48 ng/bee thiamethoxam induced adverse effects on basic motor function.[44]
340 cAMP-dependent kinase (PKA) regulates multiple cellular processes, including the
341 formation of long-term memory in honey bees.[45] Once activated, PKA induces the phosphory-
342 lation of cAMP response element binding protein (Creb). Phosphorylated Creb acts as an ac-
343 tive transcription factor and induces the expression of Creb target genes that are thought to
344 contribute to the formation of long-term synaptic plasticity which is important for long-term
345 memory consolidation.[46] In addition, Creb is known to play a central role in the formation and
346 consolidation of memory.[47, 48, 49] Creb is also involved in the visual and olfactory learning in
347 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 pg/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 µg/µl 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 hormone [55] 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 titre regulates the behavioural shift to foraging, and increases the juvenile hormone level. Juvenile hormone titres 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 non-foraging 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 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. 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 *vitel-*

logenin 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 *apidaecin* and *defensin-1* transcripts.[64] This shows the important role of both proteins to fight against pathogens. Exposure to field realistic concentration of honey bees to 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*, *defensin-1* was upregulated upon neonicotinoid exposure (Fig. 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, 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 sub-chronic toxicity of neonicotinoids.[69, 70] These non-linear patterns could be due to the complex interaction between nAChR binding of neonicotinoids and gene expression regulation that can generate non-classical 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 μg/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 analyse 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 compare it to those of nicotine. Most prominent transcriptional alterations occurred for *AChRα1* and *2*, *creb*, *pka* and *vitellogenin,* which seems selective for the neonicotinoids (but not for nicotine). Clothianidin and thiamethoxam showed dose-response effects for certain transcripts (clothianidin: *AChRα2*, *creb* and *vitellogenin*; thiamethoxam: *AChRα1*, *AChRα2* 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 adverse effects of neonicotinoids at environmental concentrations and help to understand the potential contribution of neonicotinoids to the decline of bee populations.

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Associated content:

Supporting information

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).

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760 Table 1: Concentration of nicotine and the four neonicotinoids used in the present study.
761

Compound	Concentration (ng/bee)	Concentration (ng/ml sugar syrup)
Nicotine	486, 4860 and 48600	4860, 48600 and 486000
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

762

Figure legends

Figure 1

Abundance of transcripts of *nAChRα1* (A) and *nAChRα2* (B) in the brain of honeybees 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.

Figure 2

Abundance of transcripts of *vitellogenin* in the brain of honeybees 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.

Figure 3

Abundance of transcripts of *pka* in the brain of honeybees 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.

Figure 4

Abundance of transcript of *creb* in the brain of honeybees 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 timepoint. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

Figure 5

Abundance of transcripts of *apidaecin* in the brain of honeybees 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.

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 an F-statistic 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.

Figure 7

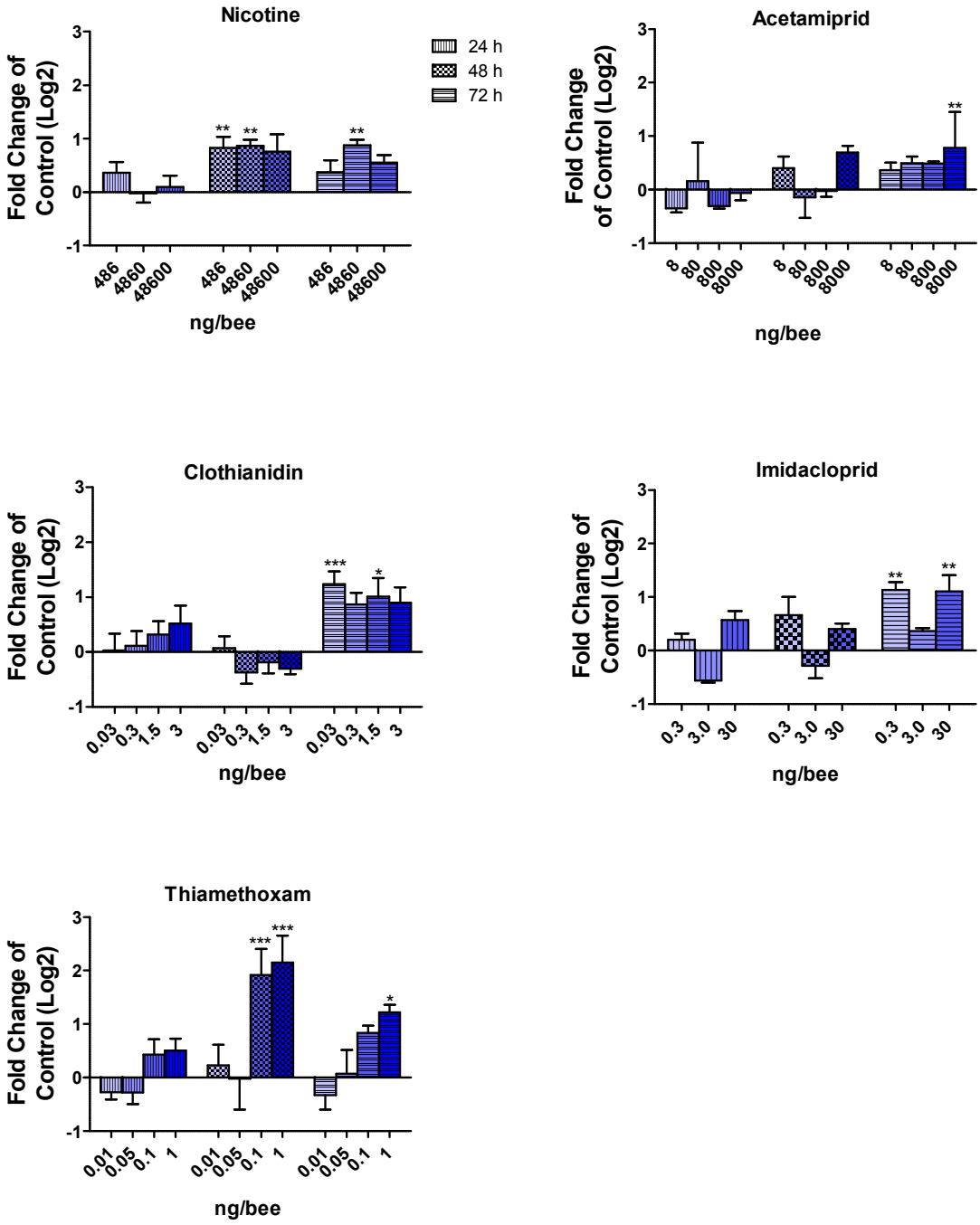
(A) Abundance of transcripts of eight selected genes in the brain of foraging honey bees one day (vertical strips), three days (squares) and seven days (horizontal strips) after exposure to acetamiprid (Mospilon®). (B) Comparison of overall alterations in gene expression after experimental exposure to 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 1 and the lowest (left, r^2: 0.32) and the highest (right, r^2: 0.25) acetamiprid concentration after experimental 24 h exposure.

Figure 8

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

842 Fig. 1:

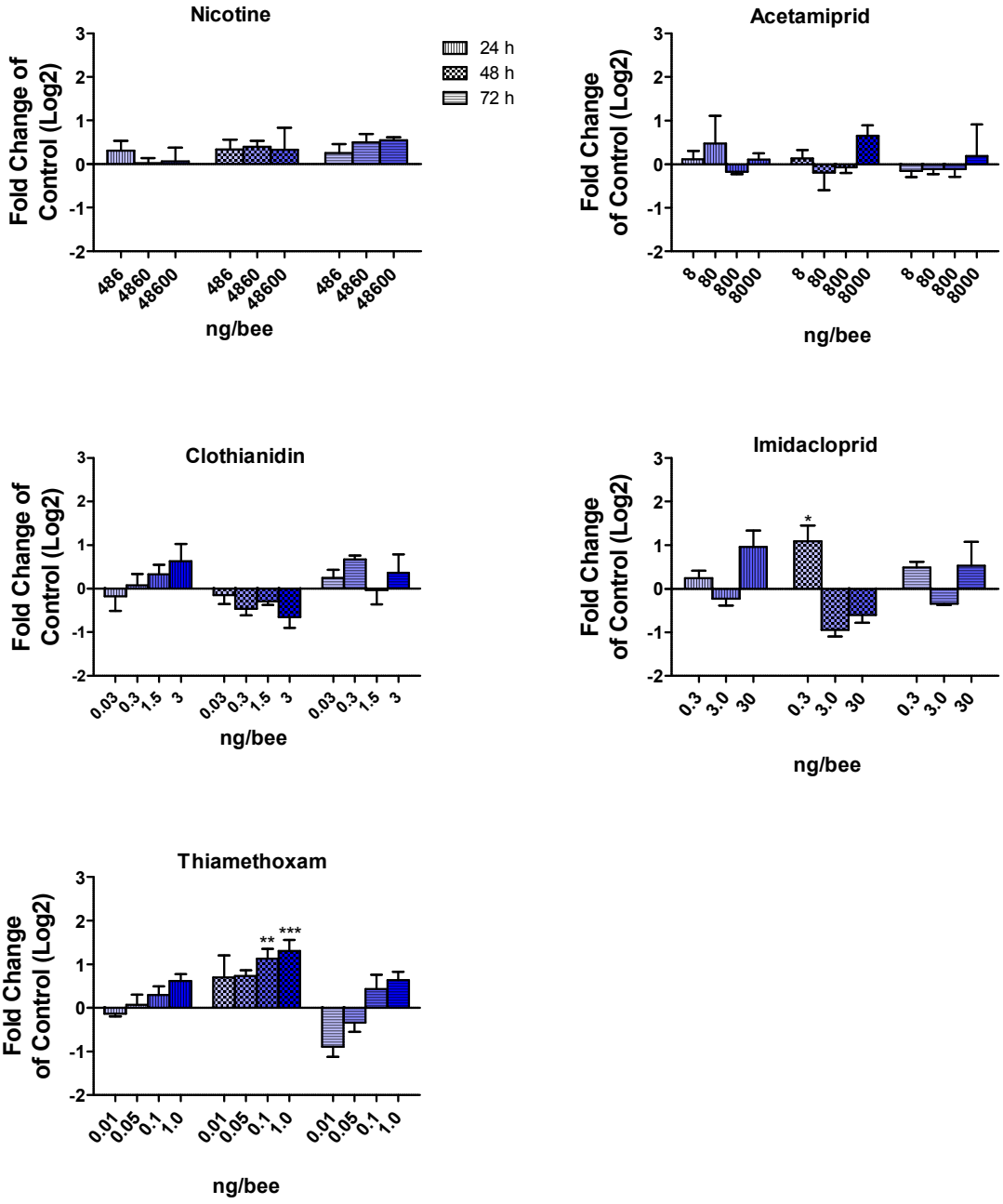
843 **A**



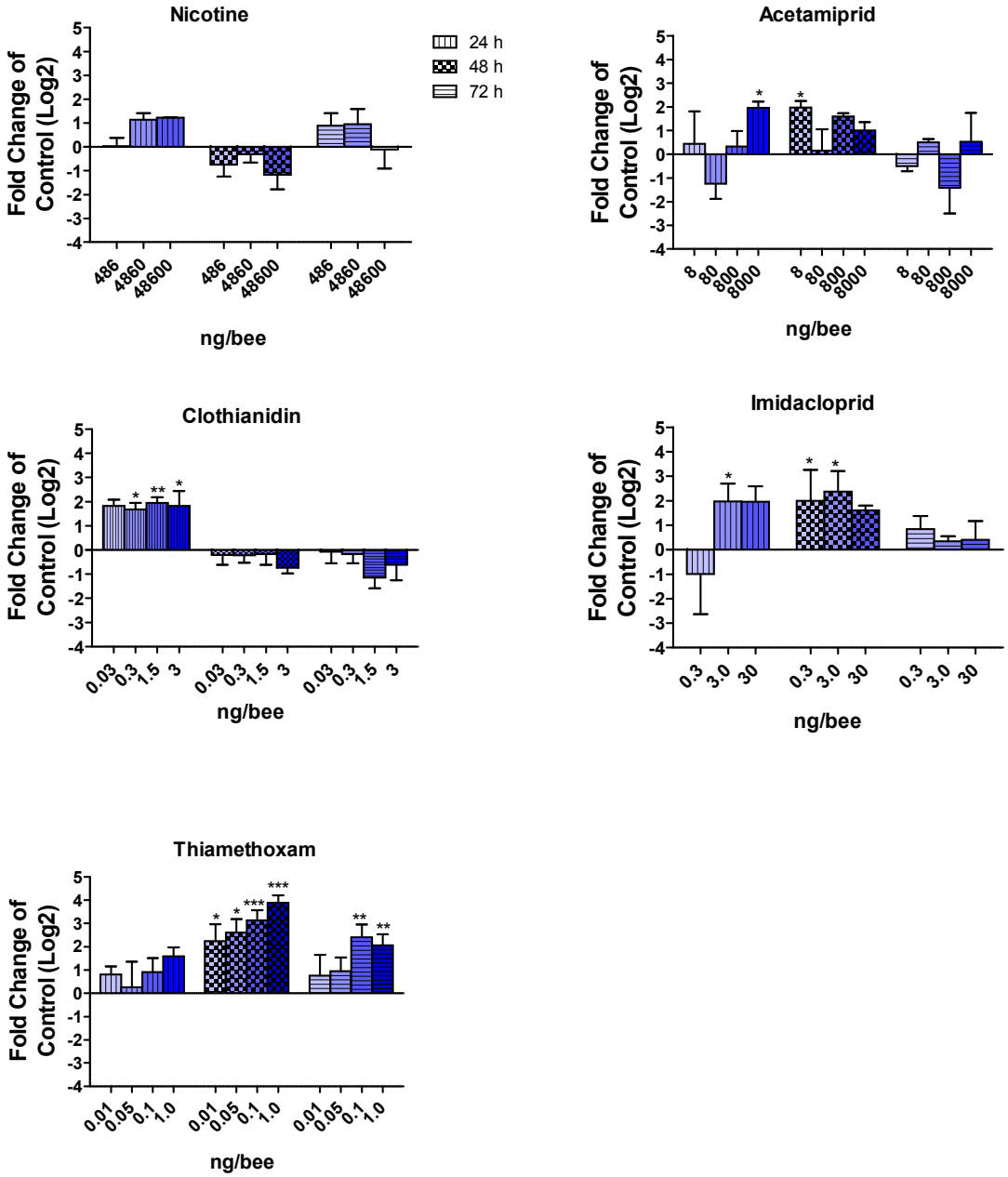
844

845
846
847
848
849
850

B

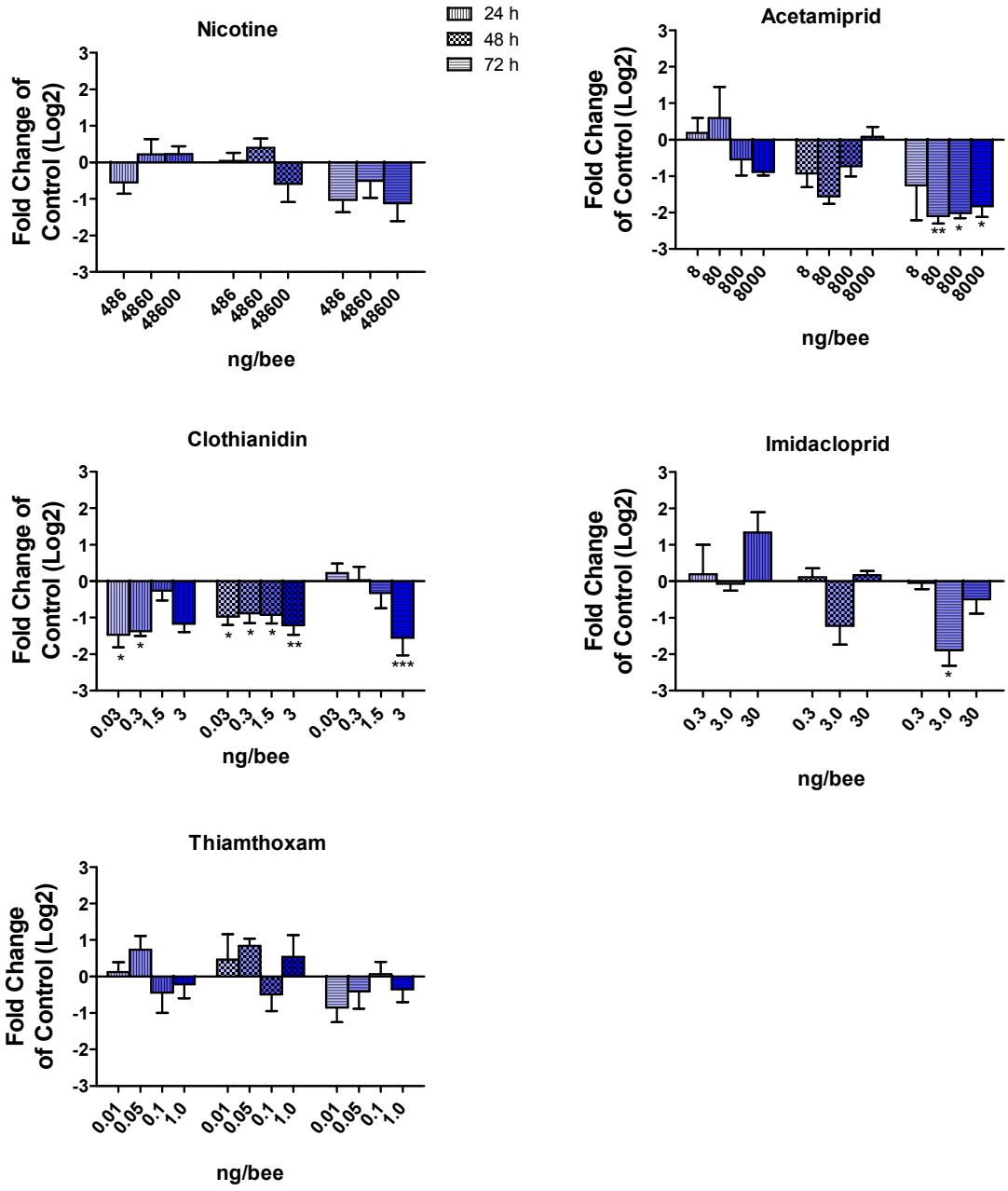

Nicotine
Fold Change of Control (Log2)
24 h
48 h
72 h
486
4860
48600
ng/bee
Acetamiprid
Fold Change of Control (Log2)
8
80
800
8000
ng/bee
Clothianidin
Fold Change of Control (Log2)
0.03
0.3
1.5
3
ng/bee
Imidacloprid
Fold Change of Control (Log2)
*
0.3
3.0
30
ng/bee
Thiamethoxam
Fold Change of Control (Log2)
**
***
0.01
0.05
0.1
1.0
ng/bee

857 Fig. 2:
858



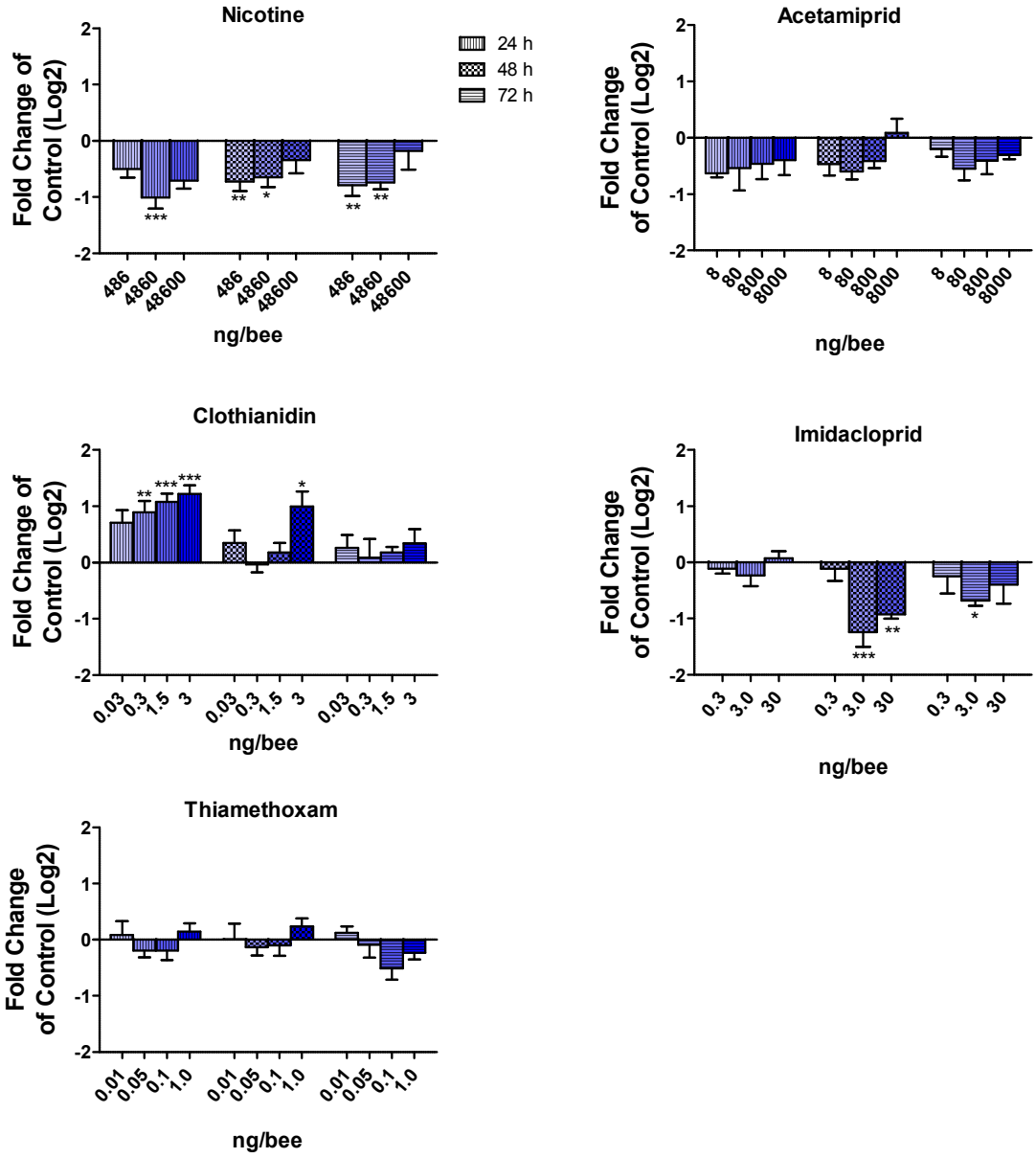
859
860
861

862 Fig. 3:



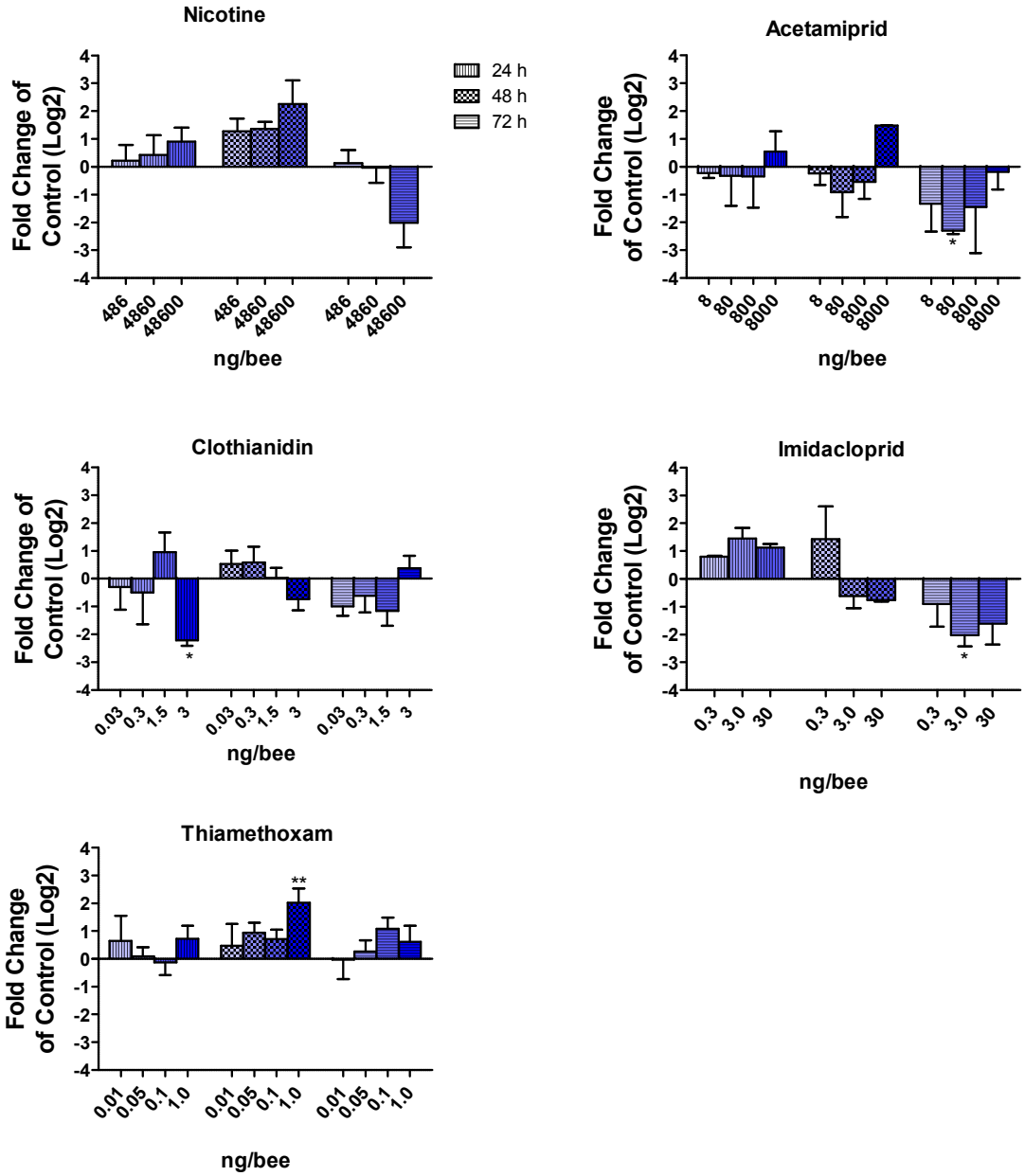
863
864
865

866 Fig. 4:
867



868
869

870 Fig. 5:

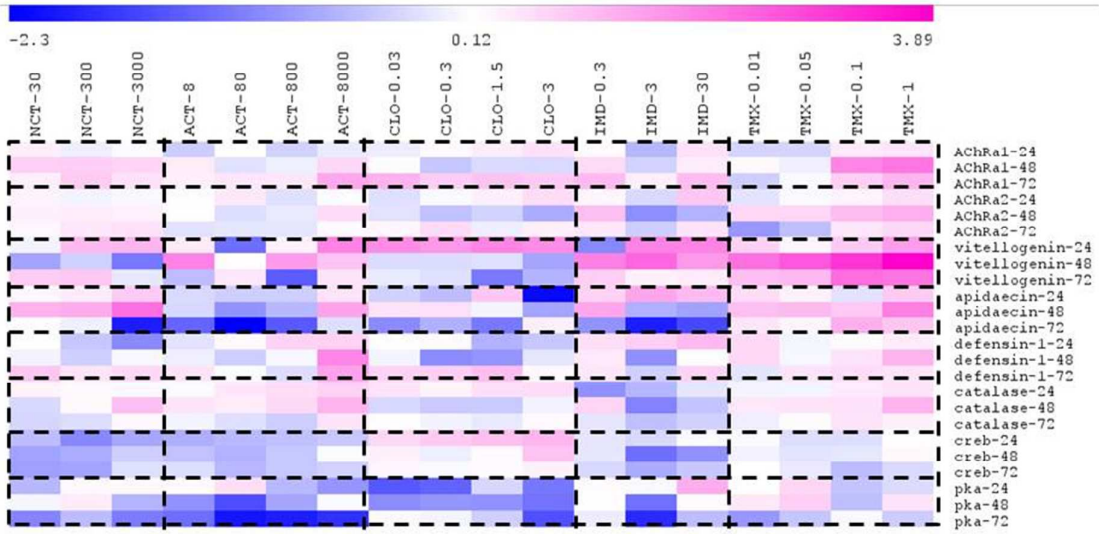


871
872
873

874

Fig. 6:

A:


-2.3
0.12
3.89
NCT-30
NCT-300
NCT-3000
ACT-8
ACT-80
ACT-800
ACT-8000
CLO-0.03
CLO-0.3
CLO-1.5
CLO-3
IMD-0.3
IMD-3
IMD-30
TMX-0.01
TMX-0.05
TMX-0.1
TMX-1
AChRa1-24
AChRa1-48
AChRa1-72
AChRa2-24
AChRa2-48
AChRa2-72
vitellogenin-24
vitellogenin-48
vitellogenin-72
apidaecin-24
apidaecin-48
apidaecin-72
defensin-1-24
defensin-1-48
defensin-1-72
catalase-24
catalase-48
catalase-72
creb-24
creb-48
creb-72
pka-24
pka-48
pka-72

B:

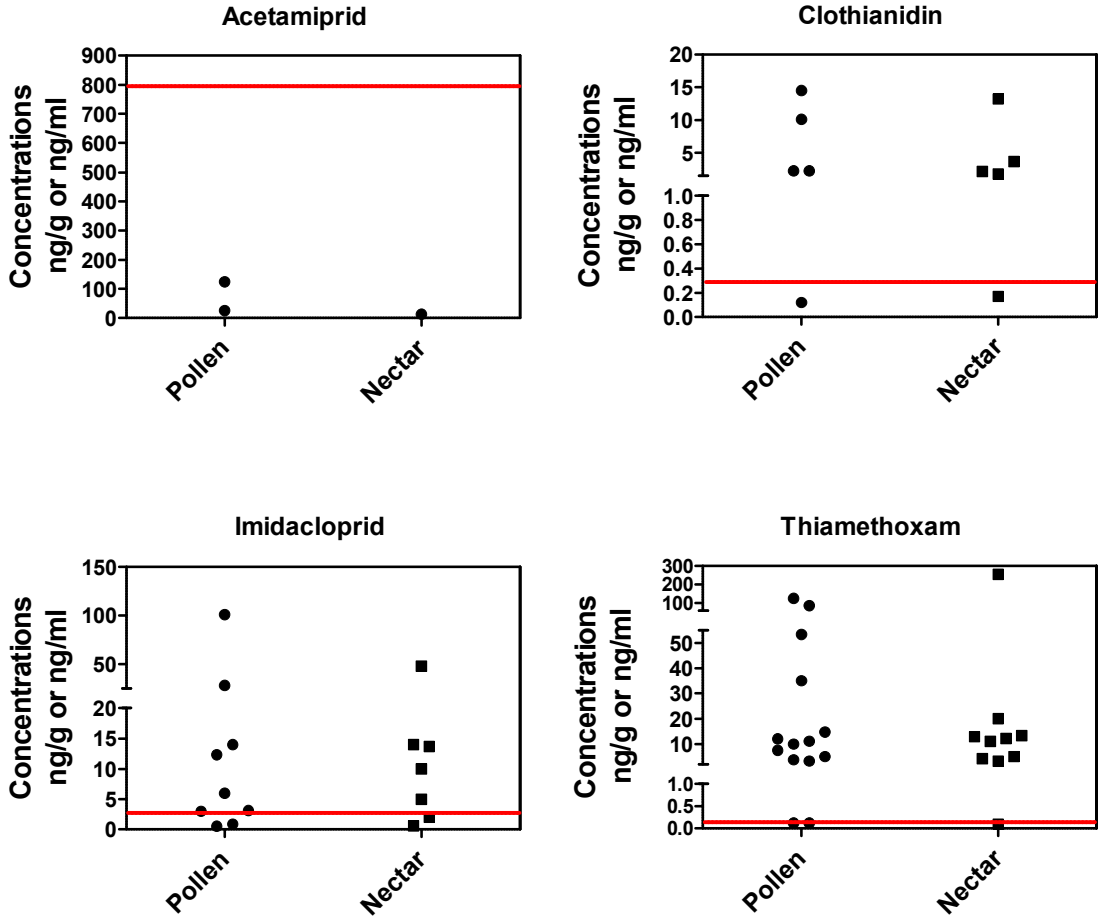
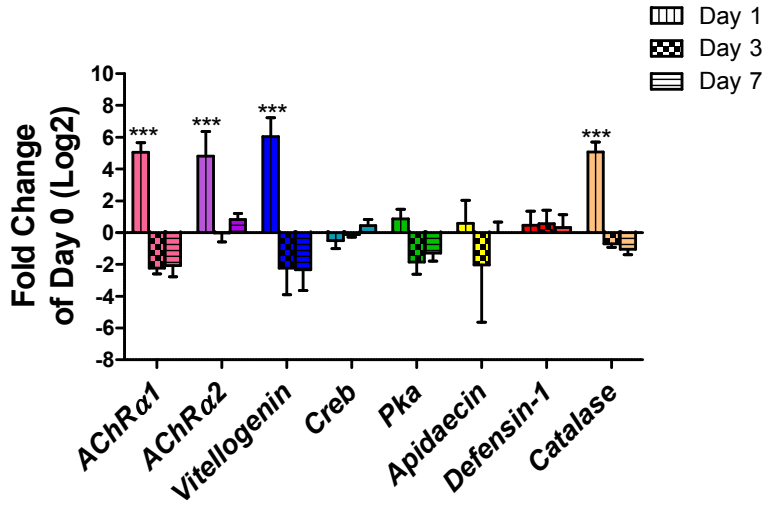
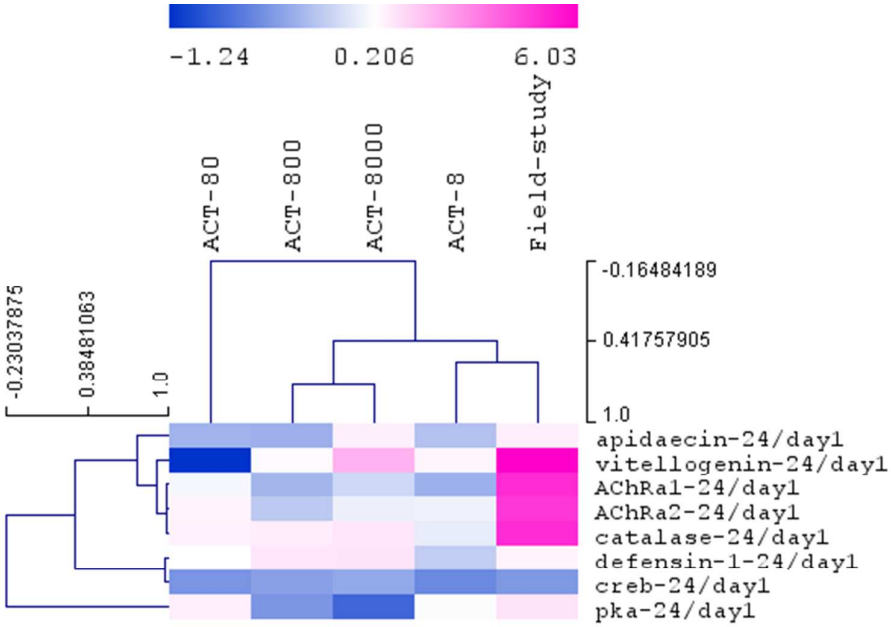

Acetamiprid
Clothianidin
Imidacloprid
Thiamethoxam
Concentrations ng/g or ng/ml
Pollen
Nectar

Fig. 7:

A


Day 1
Day 3
Day 7
Fold Change of Day 0 (Log2)
10
8
6
4
2
0
-2
-4
-6
-8
***
***
***
***
AChRα1
AChRα2
Vitellogenin
Creb
Pka
Apidaecin
Defensin-1
Catalase

B


-1.24
0.206
6.03
ACT-80
ACT-800
ACT-8000
ACT-8
Field-study
-0.23037875
0.38481063
1.0
-0.16484189
0.41757905
1.0
apidaecin-24/day1
vitellogenin-24/day1
AChRa1-24/day1
AChRa2-24/day1
catalase-24/day1
defensin-1-24/day1
creb-24/day1
pka-24/day1

C

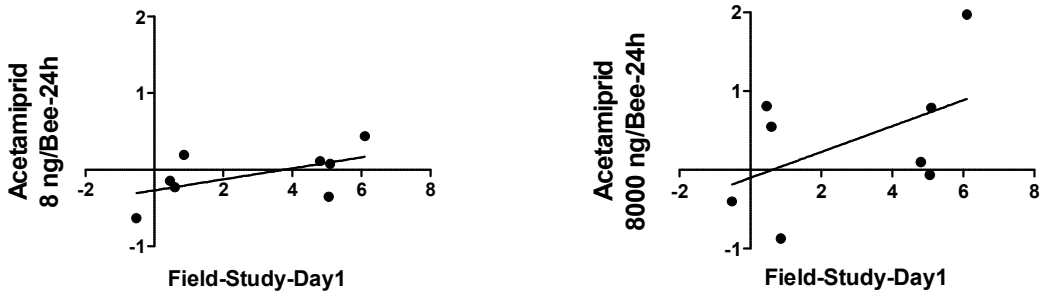
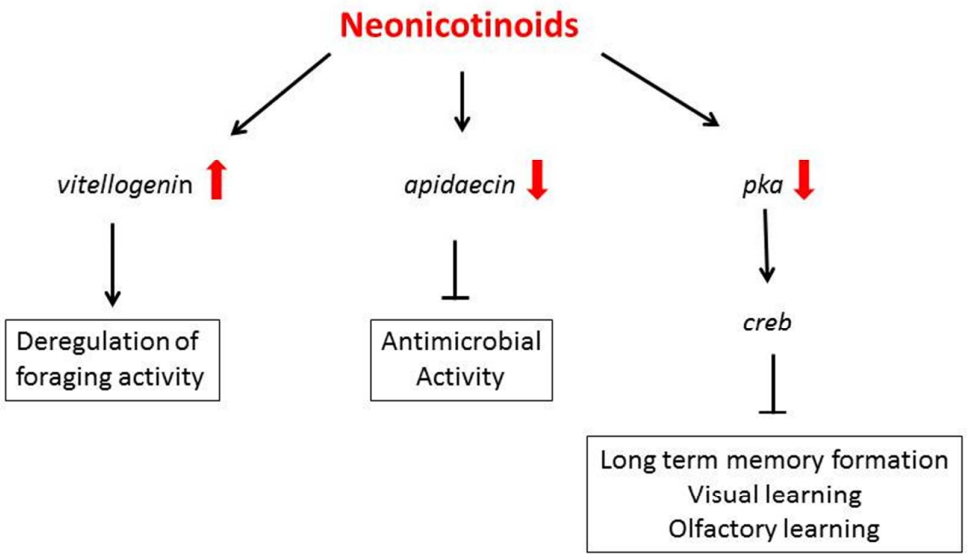

Acetamiprid
8 ng/Bee-24h
Field-Study-Day1
Acetamiprid
8000 ng/Bee-24h
Field-Study-Day1

Fig. 8:


Neonicotinoids
vitellogenin
apidaecin
pka
creb
Deregulation of foraging activity
Antimicrobial Activity
Long term memory formation
Visual learning
Olfactory learning