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Binary mixtures of neonicotinoids show different transcriptional changes than single neonicotinoids in honeybees (*Apis mellifera*)☆

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ABSTRACT

Among the many factors responsible for the decline of bee populations are plant protection products such as neonicotinoids. In general, bees are exposed to not only one but mixtures of such chemicals. At environmental realistic concentrations neonicotinoids may display negative effects on the immune system, foraging activity, learning and memory formation of bees. Neonicotinoids induce alterations of gene transcripts such as *nicotinic acetylcholine receptor (nAChR)* subunits, *vitellogenin*, genes of the immune system and genes linked to memory formation. While previous studies focused on individual compounds, the effect of neonicotinoid mixtures in bees is poorly known. Here we investigated the effects of neonicotinoids acetamiprid, clothianidin, imidacloprid and thiamethoxam as single compounds, and binary mixtures thereof in honeybees. We determined transcriptional changes of *nAChR* subunits and *vitellogenin* in the brain of experimentally exposed honeybees after exposure up to 72 h. Exposure concentrations were selected on the basis of lowest effect concentrations of the single compounds. Transcriptional induction of *nAChRs* and *vitellogenin* was strongest for thiamethoxam, and weakest for acetamiprid. To a large extent, binary mixtures did not show additive transcriptional inductions but they were less than additive. Our data suggest that the joint transcriptional activity of neonicotinoids cannot be explained by concentration addition. The *in vivo* effects are not only governed by agonistic interaction with nAChRs alone, but are more complex as a result of interactions with other pathways as well. Further studies are needed to investigate the physiological joint effects of mixtures of neonicotinoids and other plant protection products on bees to better understand their joint effects.

1. Introduction

The decline of pollinating insects during the last years is of concern (Potts et al., 2010; Cameron et al., 2011). Honeybees are one of the most important pollinators (Klein et al., 2007), but they suffer from decreases worldwide (Goulson et al., 2015) with negative effects for pollination of many domestic crops (Aizen et al., 2009). The reasons for this decline are not completely understood but are likely caused by multiple factors like pathogens, pesticides and the decrease in wild flowers (Martin et al., 2010; Van der Sluijs et al., 2013). Numerous pesticides have been detected in honey, nectar, pollen and wax (Mullin et al., 2010; Long and Krupke, 2015), and hence, may also contribute to the decline of bee populations.

Systemic pesticides, in particular neonicotinoids, are a preferred class of plant protection products (PPPs) applied in developed countries, often replacing carbamates, pyrethroids and organophosphates, which are still heavily used. Neonicotinoids are neurotoxins targeting the central nervous system by binding to nicotinic acetylcholine receptors leading to overstimulation and paralysis. They are mostly used as seed-coatings to avoid contact with non-target insects (Matsuda et al., 2001). Besides, nectar and pollen are also important sources of neonicotinoids for bees (Van der Sluijs et al., 2013).

Nitro-substituted neonicotinoids including clothianidin (which is also a metabolite of thiamethoxam), imidacloprid and thiamethoxam show high acute toxicity with LD_{50} values in the ng/bee

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Table 1
Concentrations of neonicotinoids used in the present study for single exposures and in binary mixtures.

Compound	Concentration (ng/bee)	Concentration (ng/ml sugar syrup)
Acetamiprid	8	80
Clothianidin	0.3	3
Imidacloprid	0.3	3
Thiamethoxam	0.1	1

range (Nauen et al., 2003). The cyano-substituted neonicotinoids, including acetamiprid, are less toxic with LD_{50} values in the range of μg/bee (Iwasa et al., 2004; Decourtye and Devillers, 2010). At sublethal concentrations, neonicotinoids negatively affect locomotion, behaviour, learning, orientation and memory of bees (Guez et al., 2001; Decourtye et al., 2003; El Hassani et al., 2008; Aliouane et al., 2009). In addition, neonicotinoids negatively influence the foraging activity of worker bees (Bortolotti et al., 2003; Yang et al., 2008). Honeybees are also attracted from nectar containing neonicotinoids (Kessler et al., 2015).

Molecular effects triggered by neonicotinoids are poorly known. Recently, we showed alterations in gene expression, including nicotinic acetylcholine receptors (nAChR), *vitellogenin*, immune system genes, and genes involved in memory formation in the brain of honeybees after oral exposure to environmental realistic concentrations. Strong effects were induced by clothianidin, imidacloprid and thiamethoxam, but acetamiprid had lower effects (Christen et al., 2016). Generally, bees are exposed to different pesticides via pollen and nectar at the same time (Long and Krupke, 2015). Binary mixtures of acetamiprid and thiamethoxam showed additive mortality in silkworms (Yu et al., 2016), but mixtures of imidacloprid and thiacloprid did not in case of *Chironimus riparius* larvae (Kunce et al., 2015). However, the joint activity of neonicotinoid mixtures is unknown in bees, in particular on molecular and physiological levels.

In the present study, we evaluated the molecular effects of binary mixtures of neonicotinoids on two target genes affected by neonicotinoids (Christen et al., 2016), the *nAChRs* and *vitellogenin*, the latter having multiple important functions such as regulation of life span and foraging activity. Neonicotinoids interact agonistically with nAChRs. In theory, the mixture activity can be described by the concentration addition (CA) model due to the identical mode of action of these compounds (agonistic interaction with these receptors). We demonstrated the applicability of the CA model for many different compounds having similar modes of action *in vitro* (Christen et al., 2012, 2014). However, we also showed that the *in vivo* activity could deviate from additivity, due to the complex *in vivo* interactions, and additional biological pathways affected (Rossier et al., 2016). The aim of our present work was to test the hypothesis that binary mixtures show additive interactions on the transcriptional expression of these target genes in bees. As bees may be exposed not only to one but mixtures of plant protection products, we also aimed to get getter insights into the mixture activity of pesticides.

2. Material and methods

2.1. Chemicals

Acetamiprid, clothianidin, imidacloprid and thiametoxam (purities of all > 99%) were purchased from Sigma–Aldrich (Buchs, Switzerland). Stock solutions for each compound were prepared in DMSO and diluted into 20% sucrose-solution to a final concentration of 0.1% DMSO.

2.2. Experimental design of laboratory exposures

Generally, the dose response curves of single compounds serve as a basis for the mixture design and mixture analysis of joint activities according to the concept of concentration addition (CA). First, we assessed transcriptional changes of single compounds to confirm our previous data (Christen et al., 2016), where we showed that transcriptional effects of neonicotinoids cannot generally be described by monotonic dose response curves. Furthermore, activities of binary mixtures are often determined at concentrations of individual compounds that show equal activity (equal effect study design). However, in our present study the definition of equi-effective concentrations was not feasible due to the lack of the dose-response curves for many transcripts (Christen et al., 2016). Therefore, our design for the mixture experiments was based on the lowest effect concentration (LOEC) of each single neonicotinoid for significant alterations in gene expression. Thus, the compounds were mixed at their LOECs for transcriptional changes. This design seems justified for our analysis, as three of the four tested neonicotinoids, clothianidin, imidacloprid and thiamethoxam, showed a rather similar (but not identical) potency in their transcriptional activities, and thus, compounds of almost similar activity were mixed. In contrast, the activity of acetamiprid was lower, and here, the equi-effective mixture design did not apply.

Adult forager honeybees (*Apis mellifera carnica*) of mixed age were obtained from frames from an outdoor colony placed at a location with no agricultural activity and pesticide use in the Black

Table 2
Primer sequences used for quantitative qPCR analysis.

Primer name	Sequence 5′ > 3′	Accession number	Source
ribosomal protein L32 forward	CGTCATATGTTGCCAACTGGT	NM_001011587 XM_016914656	Becker et al., 2016
ribosomal protein L32 reverse	TTGAGCACGTTCAACAATGG	NM_001011587 XM_016914656	
nAhR alpha 1 subunit forward	GAAATACGTGGCGATGGTGC	NM_001098220 XM_001121970	Christen et al., 2016
nAhR alpha 1 subunit reverse	GTGGTATCGTACGGCTCGG	NM_001098220 XM_001121970	
nAhR alpha 2 subunit forward	CCGAACTCTACGTACCGAGC	NM_001011625 XM_392547	Christen et al., 2016
nAhR alpha 2 subunit reverse	TCGAACGTCTATCTCGCACG	NM_001011625 XM_392547	
vitellogenin forward	GCAGAATACATGGACGGTGT	NM_001011578 XM_392349	Pankiw and Page, 2000
vitellogenin reverse	GAACAGTCTTCGGAAGCTTG	NM_001011578 XM_392349	

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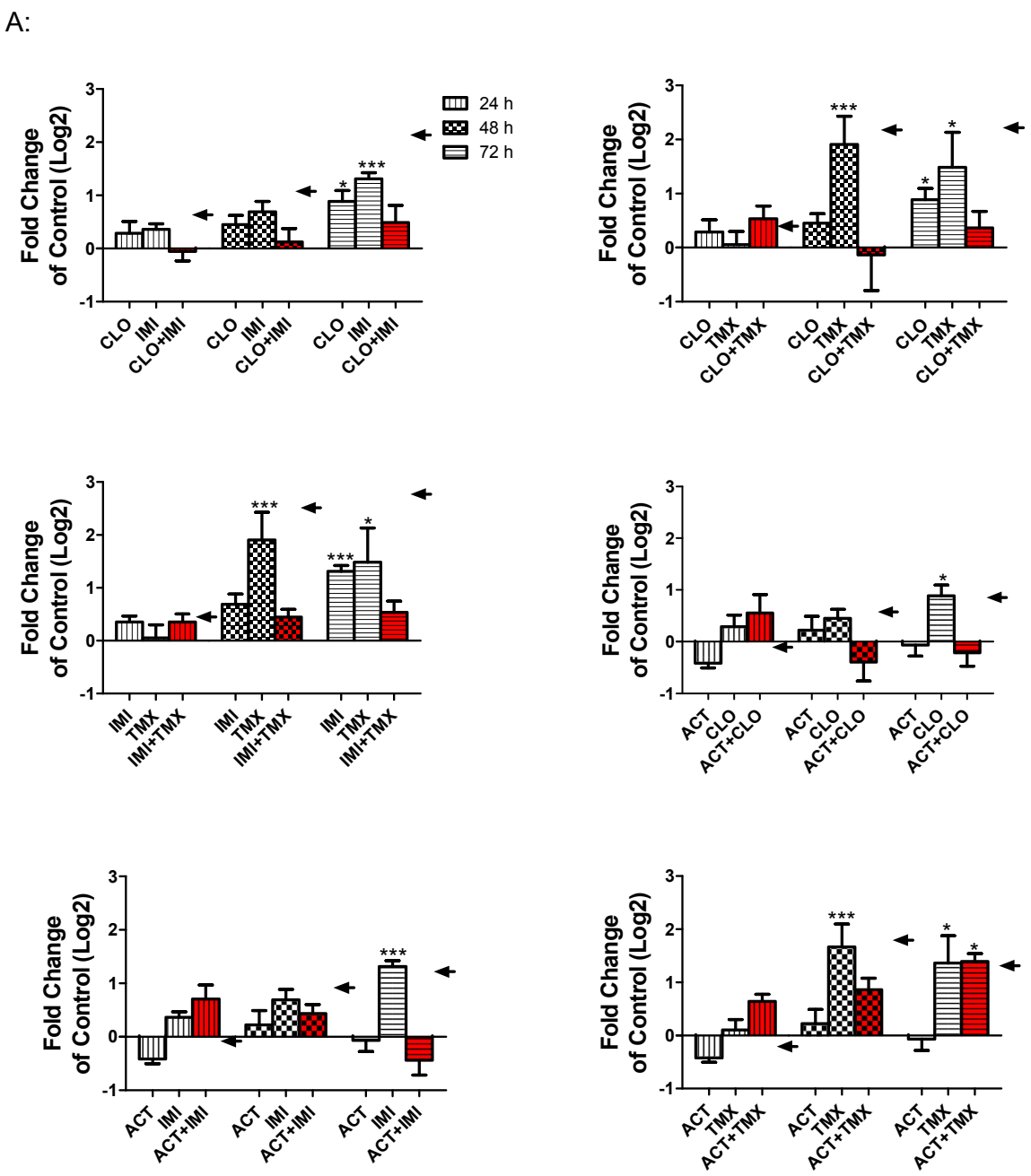

Fig. 1. Abundance of transcripts of *nAChRα1* (A) and *nAChRα2* (B) in the brain of honeybees following exposure to 8 ng/bee acetamiprid (ACT), 0.3 ng/bee clothianidin (CLO), 0.3 ng/bee imidacloprid (IMI), 0.1 ng/bee thiamethoxam (TMX), 8 ng ACT + 0.3 ng/bee CLO, 8 ng/bee ACT + 0.3 ng/bee IMI, 8 ng/bee ACT + 0.1 ng/bee TMX, 0.3 ng/bee CLO + 0.3 ng/bee IMI, 0.3 ng/bee CLO + 0.1 ng/bee TMX and 0.3 ng/bee IMI + 0.1 ng/bee TMX for 24 h (vertical strips), 48 h (squares) and 72 h (horizontal strips). Bars represent experimental transcriptional level of *nAChRα1* (A) and *nAChRα2* (B), and arrows depict estimated additive transcriptional changes assuming equal activity of the single compounds according to concentration addition. Shown are the mean results including standard error of means of four biological replicates per concentration and time-point for the mixtures and two biological replicates (with 6 technical replicates) per concentration and time-point for the single substances. Significant differences to controls with p-value of $\leq$0.05 are marked with asterisks.

Forest (Germany, GPS: N 47.7667, E 7.8333) from May to August 2016. All used bees were from the same hive. The colony had signs of *Varroa destructor* affection and was handled with formic acid (August 2015) and oxalic acid (December 2015). Honeybee collection, transportation to the laboratory, distribution into plastic bottles and exposure to sugar solution with or without test substance were done according to Christen et al. (2016).

Each exposure experiment for individual neonicotinoids and their mixtures consisted of three PET bottles with 10 bees per concentration and time point. Two of these three bottles were used to isolate RNA and one bottle was kept frozen as back-up. Each exposure experiment was done twice. Three bees were pooled to obtain one RNA samples, three technical replicates were obtained from one bottle. Two bottles were analysed yielding two biological replicates out of 6 technical replicates per experiment. As each exposure experiment was done twice, there were four biological replicates in total consisting of 12 technical replicates. For the single substances, one bottle per experiment with 10 bees was exposed to single neonicotinoids resulting in two biological replicates consisting of 6 technical replicates.

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B:

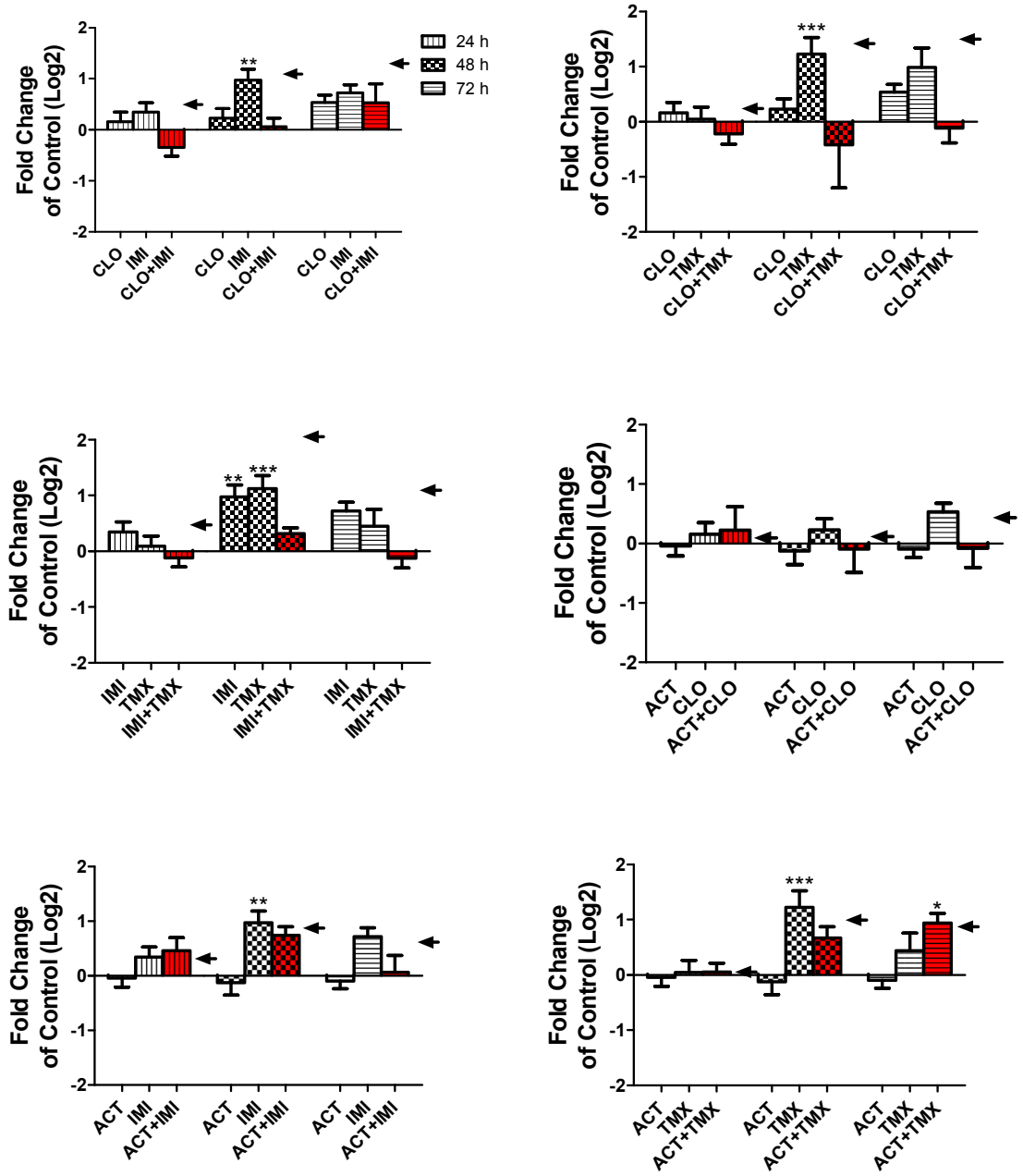

Fig. 1. (*continued*).

The concentrations of neonicotinoids used for single exposures and mixture experiments were selected on the basis of our previous study (Christen et al., 2016). The LOEC of each neonicotinoid eliciting a significant transcriptional effect was chosen to perform mixture experiments. A summary of used concentrations expressed as ng/bee and as ng/ml sugar syrup is shown in Table 1. No compound related mortality occurred during exposure. The exposure was performed similarly as described before (Christen et al., 2016).

2.3. RNA isolation, reverse transcription, and quantitative (q)PCR

RNA isolation, cDNA synthesis and quantitative PCR were performed according to Christen et al. (2016). The sequences of used qPCR primers are shown in Table 2.

2.4. 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 $0.001 > p > 0.0001$.

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3. Results and discussion

First, honeybees were exposed to individual neonicotinoids, which resulted in similar transcriptional alterations as previously, thus confirming our previous data (Christen et al., 2016). Thiamethoxam showed the strongest induction for all analysed transcripts followed by imidacloprid. Clothianidin showed lower changes and acetamiprid affected expression of *vitellogenin* only (Figs. 1A and B, 2 and 3). The strongest alterations were detected for *vitellogenin*, thus strengthening our hypothesis that induction of this transcript may be used as a biomarker for neonicotinoid exposure. The cyano-substituted neonicotinoid acetamiprid exhibited the weakest effects. Overall, transcriptional alterations induced by clothianidin, imidacloprid and thiamthoxam were in a similar range. The present data confirm the transcriptional alterations previously described (Christen et al., 2016). Furthermore, alterations occurred at environmentally relevant concentrations except for acetamiprid, where effect concentrations were above concentrations found in nectar,

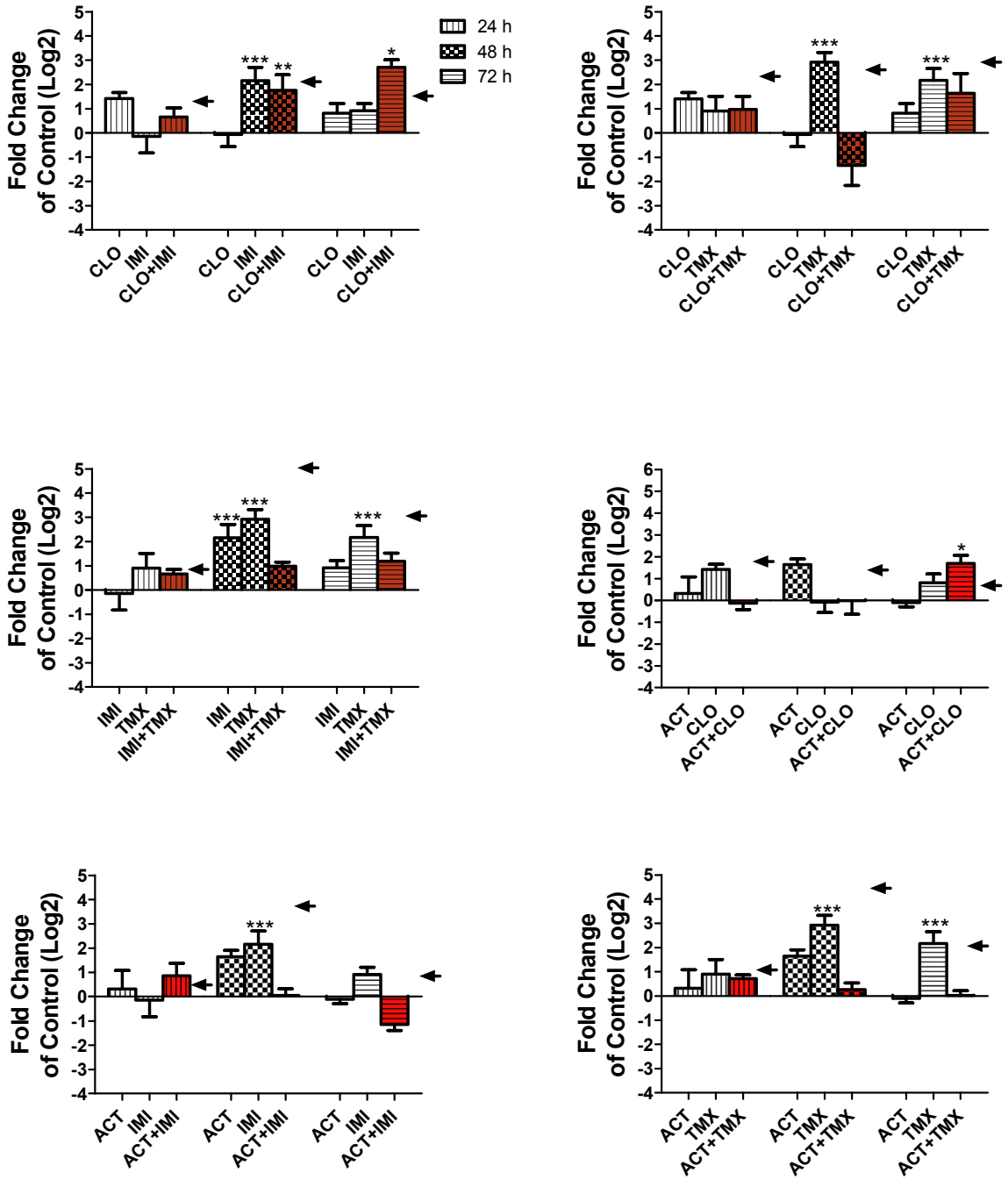

Fig. 2. Abundance of transcripts of *vitellogenin* in the brain of honeybees following exposure to 8 ng/bee ACT, 0.3 ng/bee CLO, 0.3 ng/bee IMI, 0.1 ng/bee TMX, 8 ng ACT +0.3 ng/bee CLO, 8 ng/bee ACT +0.3 ng/bee IMI, 8 ng/bee ACT +0.1 ng/bee TMX, 0.3 ng/bee CLO +0.3 ng/bee IMI, 0.3 ng/bee CLO +0.1 ng/bee TMX and 0.3 ng/bee IMI +0.1 ng/bee TMX for 24 h (vertical strips), 48 h (squares) and 72 h (horizontal strips). Bars represent experimental transcriptional level of *vitellogenin*, and arrows depict estimated additive transcriptional changes assuming equal activity of the single compounds according to concentration addition. Shown are the mean results including standard error of means of four biological replicates per concentration and time-point for the mixtures and two biological replicates (with 6 technical replicates) per concentration and time-point for the single substances. Significant differences to controls with p-value of ≤0.05 are marked with asterisks.

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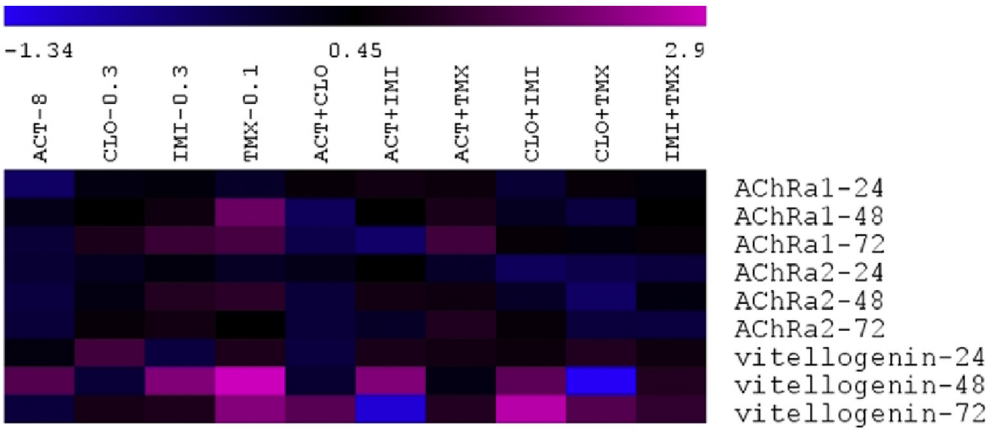

Fig. 3. Heat map of all obtained transcriptional alterations of *nAChRa1*, *nAChRα2* and *vitellogenin* for single compounds and binary mixtures shown for different exposure times of 24, 48 and 72 h. Key: acetamiprid at 8 ng/bee (ACT-8), clothianidin at 0.3 ng/bee (CLO-0.3), imidacloprid at 0.3 ng/bee (IMI-0.3), thiamethoxam at 0.1 ng/bee (TMX-0.1), acetamiprid at 8 ng/bee + clothianidin at 0.3 ng/bee (ACT + CLO), acetamiprid at 8 ng/bee + imidacloprid at 0.3 ng/bee (ACT + IMI), acetamiprid at 8 ng/bee + thiamethoxam at 0.1 ng/bee (ACT + TMX), clothianidin at 0.3 ng/bee + imidacloprid at 0.3 ng/bee (CLO + IMI), clothianidin at 0.3 ng/bee + thiamethoxam at 0.1 ng/bee (CLO + TMX), and imidacloprid at 0.3 ng/bee + thiamethoxam at 0.1 ng/bee (IMI + TMX).

pollen or in bees (Christen et al., 2016).

Neonicotinoids differ in their binding affinity to nAChRs with thiacloprid showing higher affinity than imidacloprid (Selvam et al., 2015). A study with membrane preparation from the pea aphid *Acythosiphon pisum* revealed that there exist α-bungarotoxin (nicotin antagonist) binding sites and high and low affinity binding sites for imidacloprid. Thiamethoxam showed a higher affinity to the α-bungarotoxin binding site than imidacloprid, while clothianidin showed the same affinity to both binding sites (Taillebois et al., 2014).

For the mixtures, we hypothesized that the individual compounds act jointly according to the CA model showing an additive behaviour. However, due to regulation of gene transcription by diverse and interacting processes, deviation from additivity can occur. Figs. 1 and 2 show that transcriptional alterations in the six analysed mixtures were generally different than those of the individual neonicotinoids alone. For the *nAChRα1* transcript, an additive transcriptional response was only detected for the mixture of acetamiprid and thiamethoxam at 72 h, and clothianidin and thiamthoxam at 24 h (Fig. 1A). All other mixtures showed lower transcriptional alterations than the single compound (Fig. 1A). An additive activity was also shown for the mixtures of acetamiprid and imidacloprid at 48 h, and of acetamiprid and thiamethoxam at 48 h for the expression of *nAChRα2*. All other mixtures showed lower than additive expressional changes for this transcript (Fig. 1B).

For the transcriptional induction of *vitellogenin*, most mixtures exhibited different induction than the single compounds (Fig. 2), except for mixtures of clothianidin and imidacloprid, and acetamiprid and clothianidin that showed additivity at 48 h, and stronger than additive induction at 72 h. The heat map in Fig. 3 depicts the overall changes for all transcripts. The *vitellogenin* transcript showed the strongest alterations, and transcriptional changes vary during exposure times. For individual neonicotinoids, thiamethoxam showed the strongest inductions for all transcripts. The clothianidin and imidacloprid mixture showed the strongest induction of *vitellogenin*. Mixtures of both acetamiprid and clothianidin with a second neonicotinoid showed different transcription patterns, which was dependent on the second neonicotinoid.

To our knowledge, this is the first demonstration of mixture activity of neonicotinoids in honeybees. Previous studies analysed the mortality of binary mixtures of neonicotinoids with other pesticides in honeybees and in other organisms. Honeybees exposed to binary mixtures of neonicotinoids and fungicides suggested that the fungicides increased the acute toxicity of some but not all mixtures (Iwasa et al., 2004). Similar to our present study in honeybees, marine molluscs exposed to imidacloprid and thiacloprid, either alone or in combination, showed different transcriptional changes when tested individually than as mixture. It was suggested that the mode of action on the molecular level may be distinct and that the mixtures behave according to the independent action model (Dondero et al., 2010).

The unexpected different activity of binary mixtures compared to single compounds in our present study could be explained in several ways. The observed lack of dose-effect relationships of the single neonicotinoids is reproducible as we found the same reactions previously (Christen et al., 2016). The transcriptional changes behaved as rather all or nothing reactions than showing monotonic dose response relationships. Only clothianidin and thiamethoxam exhibited several dose-dependent alterations (Christen et al., 2016). These findings are in line with previous data on sub-lethal toxicity of neonicotinoids (Pankiw and Page, 2000; Schneider et al., 2012). The non-linear effects could be due to the complex interactions between the binding of neonicotinoids on the nAChRs and regulation of gene expression that can cause non-classical dose relationships (Charpentier et al., 2014). Due to the lack of dose related effects of single substances, it was not possible to predict mixture activities following the classical CA model (Christen et al., 2012). However, our approach to estimate the additive reaction based on the response of single compounds at each exposure time point, seems justified for assessing, whether or not, the reaction in binary mixtures is additive.

Overall, our data do not support our working hypothesis as binary mixtures mostly did not show additive effects as hypothesized. Reasons for the lack of additive interaction are as follows. First, neonicotinoids with not only similar, but also different potencies were tested, as for example thiamethoxam, showing high, and acetamiprid lower activity. Second, neonicotinoids have different affinities to the nAChRs; thiacloprid has a higher binding affinity than imidacloprid (Selvam et al., 2015). Third, complex signalling pathways and additional regulatory mechanisms such as receptor cross talk, adaptive responses and feedback loops can be expected in transcriptional responses. Thus, additive reactions, as for instance on the nAChR alone, cannot necessarily be expected.

Different activity of compounds in mixtures acting by the same mode of action was previously shown for other compounds. In a

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receptor based study using yeast cells transfected with the human progesterone receptor binary mixtures of different progestins showed different activity than the single substances (Rossier et al., 2016). Another receptor based study with MDA-kb2 cells transfected with the human androgen receptor investigating the anti-androgenic activity of phthalates showed different activity of some binary mixtures at low concentrations (Christen et al., 2012). All these studies indicate that even if the compounds in a mixture have the same mode of action, their joint activity cannot be assumed as being always additive. Thus, experimental evidence should be obtained for mixture activities rather than model estimates alone.

4. Conclusions

In the present study we confirmed previous data on transcriptional alteration induced by neonicotinoids in the brain of honeybees. We demonstrated that induction of the multifunctional protein vitellogenin is an important effect of neonicotinoids, and thus may function as a biomarker for exposure for this class of pesticides. Our data suggest that transcriptional changes occur at environmentally relevant concentrations of neonicotinoids. Furthermore, our analysis on the joint action of binary mixtures suggests that they do not simply act additively. Despite their identical mode of action, they showed lower activity than expected according to concentration addition.

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