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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	