Acute thiamethoxam toxicity in honeybees is not enhanced by common fungicide and herbicide and lacks stress-induced changes in mRNA splicing

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Securing food supply for a growing population is a major challenge and heavily relies on the use of agrochemicals to maximize crop yield. It is increasingly recognized, that some neonicotinoid insecticides have a negative impact on non-target organisms, including important pollinators such as the European honeybee Apis mellifera. Toxicity of neonicotinoids may be enhanced through simultaneous exposure with additional pesticides, which could help explain, in part, the global decline of honeybee colonies. Here we examined whether exposure effects of the neonicotinoid thiamethoxam on bee viability are enhanced by the commonly used fungicide carbendazim and the herbicide glyphosate. We also analysed alternative splicing changes upon pesticide exposure in the honeybee. In particular, we examined transcripts of three genes: (i) the stress sensor gene X box binding protein-1 (Xbp1), (ii) the Down Syndrome Cell Adhesion Molecule (Dscam) gene and iii) the embryonic lethal/abnormal visual system (elav) gene, which are important for neuronal function. Our results showed that acute thiamethoxam exposure is not enhanced by carbendazim, nor glyphosate. Toxicity of the compounds did not trigger stress-induced, alternative splicing in the analysed mRNAs, thereby leaving dormant a cellular response pathway to these man-made environmental perturbations.

Worldwide honeybees and other insects encounter new man-made compounds at potentially harmful concentrations in agricultural landscapes. The combinational use of many herbicides, fungicides and pesticides is increasingly recognized for having a negative impact on non-target organisms including the honeybee Apis mellifera1,2. During their foraging for nectar, pollen and water, forager bees can be exposed to agrochemicals which have been applied to crops3,4. Through the contaminated food harvested by bees and brought into the hive, the entire colony can be exposed to complex cocktails of xenobiotics5. Such exposure to sub-lethal mixtures of pesticides may cause a reduction in vigour and productivity of the hive6,7. Indeed, honeybee colonies are in decline in many parts of the world and numerous interacting factors drive the rates of loss, including pathogens, poor nutrition, environmental stress and crop protection chemicals [reviewed in7].

One class of insecticides used globally are the neonicotinoids. These nicotine-like neurotoxic insecticides have been linked to declining bee health8. At high levels, neonicotinoids lead to paralysis and death of target and non-target insects by binding to nicotinic acetylcholine receptors (nAChRs) which are expressed in the insect nervous system9,10. Thiamethoxam is one of the neonicotinoid compounds known to affect honeybees11–14.

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Glyphosate is the most widely applied herbicide worldwide and often detected in honey, wax, pollen, and nectar. Generally considered harmless to pollinating insects, glyphosate has been reported to affect larval development and feeding behaviour of honeybees. Likewise, the fungicide carbendazim can persist in the environment due to its hydrolytically stable properties and is a frequent contaminant of bee hives.

The honeybee genome encodes a comparatively small repertoire of xenobiotic detoxifying enzymes. Consequently, honeybees may have only limited physiological and cellular response options when confronted with different mixtures of agrochemicals. A potential cellular strategy to rapidly respond to such environmental stressors would be differential expression and processing of messenger RNAs (mRNAs). Alternative splicing in particular enables cells of an organism to alter and expand availability of different transcripts and their encoding protein-isomers in response to environmental perturbations. Sub-lethal exposure of xenobiotics can, indeed, alter gene expression and induce modulation of splicing reactions. Investigation of potential splicing effects mediated through the action of pesticides may help to clarify how toxic agents interfere with honeybee metabolism.

The X box binding protein-1 (Xbp1) mediates the unfolded protein response (UPR) as a reaction to cellular stress through a splicing mechanism. The Xbp1 mRNA contains a retained intron that prevents expression of functional Xbp1 protein. This intron is spliced through a mechanism normally operative in tRNA genes leading to expression of the full length Xbp1 transcription factor, which then triggers the UPR.

Alternative mRNA splicing is particularly abundant in the brain and most elaborate in ion channels and cell adhesion molecule genes. The most extraordinary example of an alternatively spliced gene is the Drosophila melanogaster gene Down syndrome cell adhesion molecule (Dscam) gene in the fruitfly Drosophila melanogaster. Dscam can encode 38,016 alternatively spliced mRNAs. Dscam plays important roles in neuronal wiring and axon guidance in the nervous system and in phagocytosis of pathogens in the immune system. Moreover, genetic variants of Dscam have been linked to insecticide resistance in Drosophila. Dscam alternative splicing has been studied in exon-clusters of 4, 6 and 9, which harbour an array of mutually exclusive variable exons and exon selection can be mediated by the splicing regulator Srrm2.

ELAV (Embryonic Lethal Abnormal Visual System)/HU proteins are important neuronal RNA binding proteins, highly conserved and extensively used as neuronal markers. ELAV regulates alternative splicing by binding to AU-rich motifs, which are abundant in introns and untranslated regions. The Drosophila genome has three members of the ELAV family of proteins, while the honeybee genome encodes only one ELAV protein. ELAV proteins have prominent roles in regulating synaptic plasticity.

Here, we analysed the combined effects of thiamethoxam, carbendazim and glyphosate on worker bee viability. Further, we examined the expression and alternative splicing of the genes Xbp1, Dscam and elav and investigated alternative splicing upon exposure of these commonly used agrochemicals. These experiments could reveal possible indicators of the toxicity of these pesticides. The search for biomarkers and information about the effects of pesticides on the neuronal system of bees is of great importance, aiming to contribute to the characterization of exposure to these xenobiotics at the molecular level.

**Results**

**Thiamefoxam toxicity in bees is not enhanced by carbendazim and glyphosate.** To determine the toxicity of thiamethoxam we injected this neonicotinoid into worker bees. Injection is the most accurate method of delivery and allowed us to administer an identical dose to each worker bee (Supplemental Fig. 2). We chose an injection volume of 2 µl based on our previous experience from injections with Drosophila, where an estimated 1/10 of the hemolymph volume is well tolerated. LD50 for thiamethoxam was between 1 and 10 µM. Thorough exposure to xenobiotics at the molecular level.

**Apis mellifera alternative splicing of the stress sensor Xbp1 does not change upon exposure to xenobiotics.** Upon cellular stress, the unfolded protein response (UPR) is triggered by non-spliceosomal tRNA-type alternative splicing of the Xbp1 gene. To confirm that we can induce UPR, we injected Dithiothreitol (DTT) and followed Xbp1 splicing by RT-PCR over 24 hours at selected time-points (Fig. 2A–D). Twenty-four hours after injection of 2 µl of a 20 mM DTT solution, splicing of Xbp1 increased about two-fold (Fig. 2C,D). In contrast, injection of sub-lethal doses of thiamethoxam, or the combination of thiamethoxam and herbicide glyphosate, both at highest water soluble concentrations of 2 mM and 32 mM were not lethal (Fig. 1). Furthermore, combining these two compounds with a sub-lethal dose of thiamethoxam did not enhance its toxicity (Fig. 1). Initially, we assessed viability rates after 24 hours of exposure. We then repeated these injection experiments and determined viability also after 48 hours of exposure to these combinations of agrochemicals and did not observe significant differences between the two time points (Fig. 1).

**Apis mellifera Dscam exon 4 alternative splicing does not change during development, in adults and upon exposure to xenobiotics.** To examine potentially toxic effects of thiamethoxam on alternative splicing regulation in bees, we chose to analyse the splicing pattern in one of the most complex genes in arthropods, the Dscam gene. Dscam in bees has three variable clusters of mutually exclusive exons which are the exon 4 cluster with 8 annotated variables, the exon 6 cluster with 45 variables and the exon 9 cluster with 17 variables. We chose the exon 4 cluster because we could separate all variable exons after digestion based on annotated sequences with a combination of restriction enzymes on denaturing polyacrylamide gels, whereby exons 4.1, 4.2, 4.6, 4.7, 4.8 were resolved by Sau3AI, exons 4.3, 4.5 by HaeIII and exon 4.4 by MspI (Fig. 3A,B). Since the splicing pattern has not been characterized before, we then determined whether all eight annotated exons 4 were present in bees (Fig. 3A).
Indeed, we could detect all annotated eight exons, but in addition, we also detected an additional exon, termed exon 4.0 (80 nts, CTGTTAGAA...TACAGACACG), that is mostly spliced to exon 4.6 and a recessed alternative 5′ splice site in exon 4.5. In addition, a number of bands were evident (X-Z, Fig. 3B), that could not be further identified using separation and excision of bands on agarose gels for sequencing. In contrast to Drosophila, only eight of the 12 exons are present in the bee Apis mellifera (Fig. 3B,C).

Inclusion levels of variable exons were determined for bee embryos, larval brains, and brains from foragers, drones and queens. The inclusion of annotated exon 4 variants (exons 4.1–4.8) revealed no apparent differences in these five developmental stages (Fig. 3D).

After exposure to sub-lethal doses of thiamethoxam, or the combination of thiamethoxam with carbendazim and glyphosate, no apparent changes in the Dscam exon 4 splicing pattern in the brain were detected 5h, 10h or 24h after injection (Fig. 4 and Supplemental Fig. 2).

Apis mellifera elav alternative splicing does not change upon exposure to xenobiotics. Next, we examined alternative splicing of elav in honeybee workers upon exposure to xenobiotics. To determine alternative splice forms, 5′ and 3′32P labelled PCR products covering the variable region were digested with KpnI and FokI restriction enzymes, respectively, and resolved by denaturing polyacrylamide gels (Fig. 5A–C). After exposure for 24 hours to sub-lethal doses of thiamethoxam, or the combination of thiamethoxam with carbendazim and glyphosate, no apparent changes were detected in the elav splicing pattern in the brain (Fig. 5B,C).

Discussion

Like other insects, honeybees are exposed to complex combinations of agrochemicals present at potentially harmful concentrations in agricultural landscapes61. Honeybees display marked variation in sensitivity to different insecticides62,63, which may be influenced by different types of pesticide-pesticide cocktails64,65,66.

Here we investigated for the first time whether alternative splicing of important stress and neuronal genes is activated in the nervous system of the honeybee following acute exposure to xenobiotics. Injection into the hemolymph of forager bees ensured accurate and identical application of the tested pesticides. This is an initial approach to explore molecular effects of compounds on individual forager bees before evaluating exposure outcomes at the colony-level in cost- and labor-intensive field-experiments65.

Within a 48 hour window after acute exposure we did not observe any synergistic or additive effects on survival rates when sub-lethal doses of either glyphosate or carbendazim, or both, were administered along with the neonicotinoid thiamethoxam. Phenotypic outcomes of these particular combinations of insecticide, herbicide and fungicide have not been reported previously. As we only examined the viability of individual forager bees following short-term exposure we cannot rule out other, more subtle phenotypic effects nor predict what the synergistic impacts of chronic exposure to these pesticide combinations would be on individual bees or on a hive level. For example, gene expression alterations have been detected following 48 hour and 72 hour thiamethoxam exposures at concentrations which are similar to the sub-lethal dose tested here, however these changes after such prolonged time are likely indirect66.

Alternative splicing is a mechanism by which the exons are spliced in different ways to generate multiple transcripts from one mRNA precursor. This process contributes to protein diversity by generating different types of proteins and has been recognised as a rapid cellular mechanism in response to environmental perturbation27.
From our experience with *Drosophila*, alternative splicing differences are most pronounced 24 hours after xeno- 
biotic exposure; for this reason we chose this time point to investigate RNA splicing in honeybees. Alternative 
splicing is further thought to provide means for adaption to environmental changes, but given the 
complexity of the splicing process involving hundreds of proteins alternative splicing likely is also susceptible to 
interference by xenobiotics. Since neonicotinoids show neurotoxic features in bees, we reasoned that alter -
native splicing of *elav* might be altered when bees are exposed to thiamethoxam alone, or in combination with 
glyphosate and carbendazim. Therefore, we analyzed the inclusion levels of *elav* variable exons through a novel 
method. Our results revealed minimal changes in *elav* splicing in the presence of the abiotic pesticide stressors 
when compared to the control groups.

Similar to *elav*, the same pesticide dosages and exposure conditions revealed no significant changes in splicing 
patterns of *Dscam* exon 4. This lack of alternative splicing changes was unexpected as an enormous *Dscam* 
diversity is generated by mutually exclusive splicing in the *Drosophila* nervous and immune systems.

The third gene we investigated for alternative splicing was *Xbp1*. *Xbp1* is involved in the unfolded protein 
response (UPR), which is activated during stress conditions. Johnston and colleagues had previously reported a 
robust UPR activation in the honeybee in response to multiple known stressors, including tunicamycin, a protein 
glycosylation inhibitor and DTT. Based on these findings we reasoned that *Xbp1* might serve as a key molecular 
component in mediating individual and combined effects of environmental stressors in honeybees. However, we 
did not detect changes in the characteristic tRNA-type cytoplasmic splicing event that processes *Xbp1* transcripts 
in response to cellular accumulation of unfolded proteins.

Although our study is limited in that we evaluated potential alternative splicing events in transcripts of 
only three genes, namely *Xbp1*, *Dscam* and *elav*, these genes are important representatives of regulators of a 
stress-response, neuronal wiring, the immune response and synaptogenesis important for behavioural perfor -
ance during foraging. In this study the acute exposure of bees to agrochemicals does not trigger the alternative 
splicing of the conserved stress-induced *Xbp1*, *Dscam* and *elav* genes in the brain. It remains to be shown by
large-scale analysis of alternative splicing from whole genome RNA sequencing whether the lack of activation of this RNA processing mechanism is a general feature in honeybees upon acute exposure. However, long-term chronic low dose exposure to Thiamethoxam did not reveal significant changes in alternative splicing at global levels. If alternative splicing remains underused as an adaptive response, this would leave the western honeybee more vulnerable to man-made environmental stresses.

**Materials and Methods**

**Toxicity assays.** For developmental expression studies, bees (Apis mellifera) of different castes and developmental stages were taken from the experimental apiary of the University campus in Toulouse (France), and cold-anesthetized by placing them on ice before dissection. Forager bees for toxicity assays were collected from colonies of the Winterbourne Garden of the University of Birmingham (UK) and kept in small round cages used for food storage (500 ml) with holes for air circulation. Bees were fed daily from a sucrose (1:1) filled 2 ml Eppendorf tube with small holes inserted into the lid of the container through a hole. Water was provided by a paper tissue saturated daily with water (Evian). For each experimental group 30 bees were collected (replicates with ten individuals in each group). To ensure that laboratory conditions were not stressful for bees, three groups of 10–12 bees were used. Bees from control group 1 were dissected immediately after collection, and their brains extracted. Bees from control group 2 were fed and dissected after 24 hours. Bees from control group 3 were injected with water into abdomen. Thiamethoxam (Sigma-Aldrich, #37942, PESTANAL analytical standard), carbendazim (Sigma-Aldrich, #45368, PESTANAL analytical standard) and glyphosate (Round-up, Bayer) were diluted in water at their maximal soluble concentration and then the minimum lethal dose was determined by injection. A 20 mM water-dissolved Dithiothreitol (DTT) solution was prepared and 2 µl used for injections.

For injections, bees were cold-anaesthetised by placing them on ice. Once immobilised, the bees were transferred onto a custom-made metal pad connected to a tube with circulating ice-water, which was driven by an aquarium pump. This set-up kept the animals anaesthetised during injections and allowed them to recover...
afterwards at 35°C. Injections were done with a 10 µl Hamilton syringe and each cold-anaesthetised bee was injected with 2 µl of individual xenobiotics or a mixture thereof with indicated concentrations into the left side of the abdomen between abdominal segment 2 and 3 (Supplemental Fig. 1). After injections, bees were kept in a humidified incubator at 32°C and viability was scored after 24 hours.

RNA extraction, reverse transcription (RT) and polymerase chain reaction (PCR) and analysis of alternative splicing. RNA extraction was done using Tri-reagent (SIGMA) and reverse transcription was done with Superscript II (Invitrogen) as previously described using primer AM Dscam 13R2 (GCCGAGAGTCCTGC GCCGATTCCATTCACAG, 1 pmol/20 µl reaction) in combination with an oligo dT primer. 1–2 µl of the RT-PCR mix was used in 50 µl PCR reaction using Fermentas Taq (Thermo Scientific) according to the manufacturer's instructions. Xbp1 was amplified with primers Xbp1 AM F3 (GAAGAAACTGTTCGAAGGTTAAGGGAAC) and Xbp1 AM R2 (GTTCGATATAATCATCTCCTTGGAG) or Xbp1 AM R1 (TCAAGAGGAAGTAGATGGTCAGAA). For the Pst I digestion of the PCR amplicon, 40 units of the restriction enzyme were added to the PCR amplicon solution, following PCR amplification; the PCR-Pst I mix was digested for 1 hour at 37°C. PCR products were then analysed on ethidium bromide stained 3% agarose gels. To amplify the Dscam exon 4 cluster, PCR was performed using primers AM Dscam 3F1 (AGTTCACAGCCGAGATGTTAGCAGTGAGGC) and AM Dscam 5R1 (GGAAGGCATACCAAGTATTTTC) for 37 cycles with 1 µl of cDNA. New variables of Dscam exon 4 were gel purified and determined by sequencing (exon 4.0 and 4.0 + 4.6) or by the annotated sequence (Exon 4.5 recessed alternative 5’ splice site). Apis elav was amplified with primers elav AM F2

Figure 4. Apis mellifera Dscam exon 4 alternative splicing in brains of worker bees is unaffected by thiamethoxan, carbendazim and glyphosate. Denaturing polyacrylamide gels (6%) showing the splicing pattern of Dscam exon 4 isoform variables on top by digestion of a 32P labeled RT-PCR product with a combination of HaeIII, MspI, and Sau3AI restriction enzymes in control bees dissected immediately after collection (Control 1), control bees fed with water and sucrose for 24 h (Control 2) and control bees injected with water (Control 3) compared to bees injected with thiamethoxam (1 µM) and bees injected with a mixture of thiamethoxam (1 µM, T), carbendazim (2 mM, C) and glyphosate (32 mM, G) 24 h prior dissection. Samples were run on 8% polyacrylamide gel. Ma: DNA marker. The undigested PCR product is shown at the bottom.
(GTCGGTGATTTGCGACAACATCAC) and elav AM R2 (CCCAGGTACGATCGATTTGCCAATAC). For the analysis of Dscam and elav alternative splicing primers were labeled with 32P gamma-ATP (6000 Ci/mm, Perkin Elmer) with PNK to saturation and diluted as appropriate. From a standard PCR reaction with a 32P labeled forward primer, 10–20% were sequentially digested with a mix of restriction enzymes according to the manufacturer’s instructions (NEB). PCR reaction and restriction digests were phenol/CHCl3 extracted, ethanol precipitated in the presence of glycogen (Roche) and analyzed on standard 6% sequencing type denaturing polyacrylamide gels. After exposure to a phosphoimager (BioRad), individual bands were quantified using ImageQuant (BioRad) and inclusion levels for individual variable exons were calculated from the summed up total of all variables. Statistical analysis was done by one-way ANOVA followed by Tukey–Kramer post-hoc analysis using Graphpad prism. Percent inclusion levels were calculated from the total sum of variables as described.

Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
R.S., T.C.R. and M.S. conceptualized the project. P.D. and P.U. performed the experiments, T.C.R., O.M., J.M.D., R.S. and M.S. supervised experiments and analyzed data, R.S. and M.S. wrote the manuscript with help from P.D., P.U. and J.M.D.

Competing interests
The authors declare no competing interests.

Additional information
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