Acute Thiamethoxam exposure in *Apis mellifera* : Absence of both stress-induced changes in mRNA splicing and synergistic effects of common fungicide and herbicide

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Abstract

Securing food supply for a growing population is one of the current major challenges and heavily relies on the use of agrochemicals to maximize crop yield. Neonicotinoids are globally one of the most widely used insecticides. It is increasingly recognized, that neonicotinoids have a negative impact on non-target organisms, including important pollinators such as the European honey bee *Apis mellifera*. Toxicity of neonicotinoids may be enhanced through simultaneous exposure with additional pesticides, which could help explain, in part, the global decline of honey bee colonies. Here we examined whether exposure effects of the neonicotinoid Thiamethoxam are enhanced by the commonly used fungicide Carbendazim and the herbicide Glyphosate. For the first time, we also analysed alternative splicing changes upon pesticide exposure in the honey bee. 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, both important genes for neuronal function. Our results indicate that neonicotinoid toxicity applied at sub-lethal doses is not enhanced by Carbendazim nor Glyphosate. Likewise, toxicity of these compounds did not impact on the complex process of spliceosomal-directed joining of exons and non-spliceosomal intron excision in the analysed mRNAs.

INTRODUCTION

Worldwide honeybees and other insects encounter new man-made compounds at potentially harmful concentrations in agricultural landscapes. The combinatorial use of many herbicides, fungicides and pesticides is increasingly recognized for having a negative impact on many pollinators including the honeybee *Apis mellifera* (Johnson et al., 2010; Woodcock et al., 2017). Forager bees may be exposed to chemicals applied to crops during their foraging for nectar, pollen and water (Desneux et al., 2007; Thompson, 2003). Through the contaminated food harvested by bees and brought into the hive, the entire colony can be exposed to complex cocktails of xenobiotics (Devillers et al., 2002). Such exposure to sub-lethal mixtures of pesticides may cause a reduction in vigour and productivity of the hive (Blacquière et al., 2012; Devillers et al., 2002). Indeed, honey bee colonies are in decline in many parts of the world and numerous interacting factors are thought to drive the rates of loss, including pathogens, poor nutrition, environmental stress and crop protection chemicals [reviewed in (Steinhauer et al., 2018)].

One class of insecticides used globally are the neonicotinoids. These nicotine-like neurotoxic insecticides have been linked to declining bee health (Tsvetkov et al., 2017). 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 system (Goulson, 2013; Matsuda et al., 2001). Thiamethoxam is one of the neonicotinoid compounds known to affect honey bees (Friol et al., 2017; Iwasa et al., 2004; Tavares et al., 2015, 2019).

Glyphosate is the most widely applied herbicide worldwide (Benbrook, 2016) and often detected in honey, wax, pollen, and nectar (Boily et al., 2013; Calatayud-Vernich et al., 2018; Mullin et al., 2010). Generally considered harmless to pollinating

insects, glyphosate has been reported to affect larval development and feeding behaviour of honeybees (Liao et al., 2017; Vázquez et al., 2018). Likewise, the fungicide carbendazim can persist in the environment due to its hydrolytically stable properties (Hernandez et al., 1996; Veneziano et al., 2004) and is a frequent contaminant of bee hives (Lambert et al., 2013).

The honey bee genome encodes a comparatively small repertoire of xenobiotic detoxifying enzymes (Claudianos et al., 2006). Consequently, honey bees 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) (Soller, 2006). Alternative splicing in particular enables cells of an organism to alter and expand availability of different transcripts and their encoding protein-isoforms in response to environmental perturbations (Nilsen and Graveley, 2010; Pai and Luca, 2019). Sub-lethal exposure of xenobiotics can, indeed, alter gene expression and induce modulation of splicing reactions (Sumanasekera et al., 2008; Zaharieva et al., 2012). Investigation of potential splicing effects mediated through the action of pesticides may help to clarify how toxic agents interfere with honey bee metabolism.

The *X box binding protein-1* (*Xbp1*) mediates the unfolded protein response (UPR) as a reaction to cellular stress through a splicing mechanism (Calfon et al., 2002; Mitra and Ryoo, 2019; Shen et al., 2001; Yoshida et al., 2001). 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) (Fu and Ares, 2014; Soller, 2006; Wang and Burge, 2008). The most extraordinary example of an alternatively spliced gene is *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 (Hemani and Soller, 2012; Neves et al., 2004; Schmucker et al., 2000; Sun et al., 2013; Watson et al., 2005). *Dscam* alternative splicing has been studied in exon-clusters 4, 6 and 9, which harbour an array of mutually exclusive variable exons and exon selection can be mediated by the splicing regulator Srrm234 in *Drosophila* (Graveley, 2005; Haussmann et al., 2018; Olson et al., 2007; Torres-Méndez et al., 2019; Ustaoglu et al., 2019; Yang et al., 2011).

ELAV (Embryonic Lethal Abnormal Visual System)/HU proteins are important neuronal RNA binding proteins, highly conserved and extensively used as neuronal markers (Hinman and Lou, 2008; Pascale et al., 2008; Soller and White, 2004). ELAV regulates alternative splicing by binding to AU-rich motifs, which are abundant in introns and untranslated regions (Soller and White, 2005). The Drosophila genome has three members of the ELAV family of proteins, while the honey bee genome encodes only one ELAV protein (Haussmann et al., 2011; Zaharieva et al., 2012, 2015). ELAV proteins have prominent roles in regulating synaptic plasticity (Soller et al., 2008, 2010; Haussmann et al., 2008; Soller and Haussmann, 2010; Zaharieva et al., 2015).

Here, we analysed the combined effects of Thiamethoxam, Carbendazim and Glyphosate on worker bee viability. Further, we determined expression and alternative splicing of *Xbp1, Dscam* and *elav* genes in bees and investigated

alternative splicing upon exposure of these commonly used agrochemicals. These experiments could then 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.

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 before dissection. Forager bees for toxicity assays were collected from colonies of the Winterbourne Garden of the University of Birmingham (UK), kept in small cages and fed with water and sucrose (1:1). 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 ten 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. Compounds were diluted in water at their maximal soluble concentration and then the minimum lethal dose was determined by injection. Injections were done with a 10 µl Hamilton syringe and each cold-anaesthetised bee was injected with 2 µl into the abdomen. After injections, bees were kept in an 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 (Koushika et al., 1999) using primer AM Dscam 13R2 (GCCGAGAGTCCTGCGCCGATTCCATTCACAG, 1 pmol/ 20 µl reaction) in combination with an oligo dT primer.). *Xbp1* was amplified with primers Xbp1 AM F2 (CGAATTACCAAAGTCTGTGCCGTTAAG) and Xbp1 AM R2 (GTTCGATATAATCATCTCCTTGGAG) and PCR products were analysed on ethidium bromide stained 3% agarose gels. To amplify the *Dscam* exon 4 cluster, PCR was performed using primers AM Dscam 3F1 (AGTTCACAGCCGAGATGTTAGCGTGAGAGC) and AM Dscam 5R1 (GGAAGGCAGTACCAAGTATTTTC) 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 (GTCGCGGATACTTTGCGACAACATCAC) and elav AM R2 (CCCGGGTAGCATCGAGTTTGCCAATAGATC). For the analysis of *Dscam* and *elav* alternative splicing primers were labeled with ^{32}P gamma-ATP (6000 Ci/ mmol, 25 mM, Perkin Elmer) with PNK to saturation and diluted as appropriate (Iglesias-Gato et al., 2011). From a standard PCR reaction with a ^{32}P labelled forward primer, 10–20% were sequentially digested with a mix of restriction enzymes according to the manufacturer's instructions (NEB) (Iglesias-Gato et al., 2011). PCR reaction and restriction digests were phenol/$CHCl_3$ 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 (Haussmann et al., 2018).

RESULTS

Thiamethoxam toxicity in bees is not enhanced by Carbendazim and Glyphosate

The toxicity of the neonicotinoid was determined by injecting 2 µl of Thiamethoxam solutions into worker bees. The injection volume was adjusted based on our experience from injections in *Drosophila* where an estimated 1/10 of the hemolymph volume is well tolerated (Soller et al., 1997). LD50 for Thiamethoxam was between 1 and 10 µM, and 100 µM resulted in complete lethality (Fig 1). Intriguingly, the commonly used fungicide Carbendazim and herbicide Glyphosate at highest water soluble concentrations of 2 mM and 47 mM were not lethal (Fig 1). Furthermore, combining all of these two compounds with a sub-lethal dose of Thiamethoxam did not enhance its toxicity (Fig 1).

Apis mellifera alternative splicing of the stress sensor *Xbp1* does not change upon exposure to xenobiotics

Non-spliceosomal alternative splicing of the *Xbp1* gene has been associated with cellular stress (Fig 2A and B), but exposure to sub-lethal doses of Thiamethoxam, or the combination of Thiamethoxam with Carbendazim and Glyphosate did not result in apparent changes in the *Xbp1* alternative splicing (Fig 2C).

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 arthopods, the *Dscam* gene (Schmucker et al., 2000). *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 (Lee et al., 2010). 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 and B) (Haussmann et al., 2018). 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, CTGTTTAGAA…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 and 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 were detected (Fig 4).

Apis mellifera elav **alternative splicing does not change upon exposure to xenobiotics**

Next, we examined alternative splicing of *elav* in honey bee workers upon exposure to xenobiotics. To determine alternative splice forms, 5' and 3' ^{32}P labelled PCR products covering the variable region were digested with *Kpn*I and *Fok*I restriction enzymes, respectively, and resolved by denaturing polyacrylamide gels (Fig 5A-C). After exposure 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 and 5C).

DISCUSSION

The low number of detoxifying enzyme genes leaves honey bees and other insects ill-equipped to counter new combinations of agrochemicals present at potentially harmful concentrations in agricultural landscapes. Risks are even higher if they get exposed to combinations of various chemicals as frequently encountered in crops, since it has been shown that certain mixtures enhance the toxicity of xenobiotics in honey bees (Berenbaum and Johnson, 2015; Claudianos et al., 2006).

Here we investigated for the first time whether alternative splicing is a process activated in the nervous system of the honey bee following acute exposure to

xenobiotics. Injection into the hemolymph of forager bees ensured accurate and identical application of the tested pesticides. This is a first 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-experiments (Henry et al., 2015).

Within a 24-hour window of acute exposure we did not observe any synergistic or additive effects on survival rates when sublethal 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.

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 cellular mechanism in response to environmental perturbation (Pai and Luca, 2019). 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 (Sumanasekera et al., 2008; Zaharieva et al., 2012). Since neonicotinoids show neurotoxic features in bees, we reasoned that alternative 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 (Haussmann et al., 2018). 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 (Schmucker et al., 2000).

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 (Iwakoshi et al., 2003). Johnston and colleagues had previously reported a robust UPR activation in the honey bee in response to multiple known stressors, including tunicamycin, a protein glycosylation inhibitor and DTT (Johnston et al., 2016). 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 honey bees. However, we did not detect any changes in the characteristic tRNA-type cytoplasmic splicing event that processes *Xbp1* transcripts in response to cellular accumulation of unfolded proteins.

Overall, our results indicate that acute pesticide exposure in foraging honey bees does not trigger evolutionarily conserved stress-related alternative splicing processes. The increased regulatory plasticity of RNA processing mechanisms available to eukaryotic cells remains unutilized as an adaptive response to these anthropogenic compounds. In combination with a limited detoxification system, this leaves the western honeybee vulnerable to man-made environmental stresses.

Authors' contribution

P.D., 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.

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Figure legends

Figure 1. Viability of *Apis mellifera* exposed to xenobiotics. Means with standard error from three experiments are represented. The percent viability of bees after 24 h is plotted against the concentration of xenobiotics. Bees were injected with 2 µl of water (Control), Carbendazim (C, 2 mM), Glyphosate (G, 47 mM) and/or Thiamethoxam (T) at indicated concentrations.

Figure 2: *Apis mellifera Xbp1* non-spliceosomal intron splicing in worker bees is unaffected by thiamethoxan, carbendazim and glyphosate.

(A) Gene structure of *Apis mellifera Xbp1* depicting the tRNA-type spliced intron and primers used to analyse its splicing (top). (B) To resolve similar sized spliced and unspliced isoforms, the RT-PCR product was digested with *Pst*I, which only cuts the unspliced RT-PCR product. (C) Agarose gel showing the alternative splicing pattern of *Xbp1* by digestion of the RT-PCR product with *Pst*I (P) compared to undigested input (I) 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 (47 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.

Figure 3: *Apis mellifera Dscam* exon 4 alternative splicing during bee development and between castes. (A) Schematic depiction of *Apis mellifera Dscam* exon 4 variable cluster with primers indicated below orange markes corresponding to constant exons 3 and 5. Variable exons 4 are marked in light blue. Newly discovered exon 4.0 is spliced to exon 4.6 (dark blue). An alternative 5' splice site discovered in exon 4.5 is indicated as a line. (B) Denaturing polyacrylamide gels showing the splicing pattern of *Dscam* exon 4 isoform variables on top by digestion of a ^{32}P labeled RT-PCR product with a combination of *Hae*III, *Msp*I, and *Sau3*AI restriction enzymes in embryos (line1), larval brains (line 2), worker brains (line 3), drone brains (line 4) and queen brains (line 5). Exon 4.0, that is close to exon 4.6 in length and

exon 4.0+4.6 are shown from an undigested control (bottom). (C) Table showing the length of variable exons and their length after restriction digest with indicated restriction enzymes. Exon 4.0 is close to exon 4.6 in length and is shown from na undigested control gel in B. (D) Quantification of inclusion levels of individual exons are shown as means with standard error from three experiments for embryos, larval brains, worker brains, drone brains and queen brains.

Figure 4: *Apis mellifera Dscam exon 4* alternative splicing in brains of worker bees is unaffected by thiamethoxan, carbendazim and glyphosate.

Denaturing polyacrylamide gels showing the splicing pattern of *Dscam* exon 4 isoform variables on top by digestion of a ^{32}P labeled RT-PCR product with a combination of *Hae*III, *Msp*I, and *Sau3*AI 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 (47 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.

Figure 5: *Apis mellifera elav* alternative splicing in brains of worker bees is unaffected by thiamethoxan, carbendazim and glyphosate.

(A) Gene structure of *Apis mellifera elav* depicting color-coded functional protein domains with constant exons (1-5, bottom, solid lines) and alternative splicing exons (3a and 4a-d, top, dashed lines). RNA Recognition Motiv 1 (RRM1): light blue, RRM2: dark blue, RRM3: purple, hinge region: red and alternatively spliced parts in red. *Kpn*I and *Fok*I restriction sites used to separate isoforms are indicated below the

gene model. An asterisk indicates isoforms that encode truncated proteins by introducing a frameshift. (B, C) Denaturing polyacrylamide gels showing the alternative splicing pattern of *elav* by digestion of a 5' (B) or 3' (C) ^{32}P labeled RT-PCR product with *Kpn*I (B) and *Fok*I (C) 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 (47 mM, G) 24 h prior dissection. Samples were run on 6% polyacrylamide gel. Ma: DNA marker. The undigested PCR product is shown at the bottom.

Figure 1

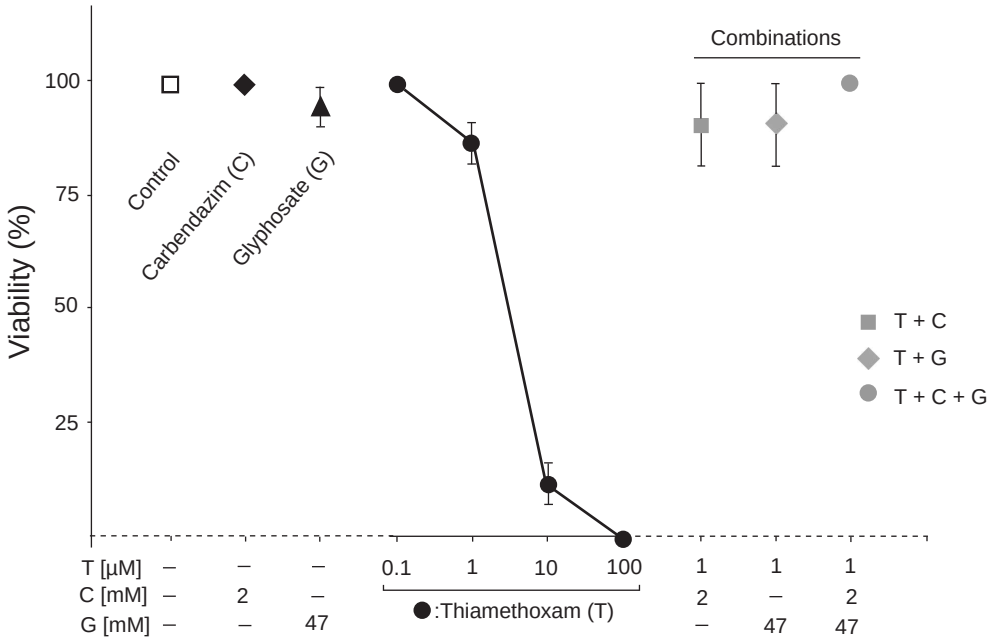


Figure 2

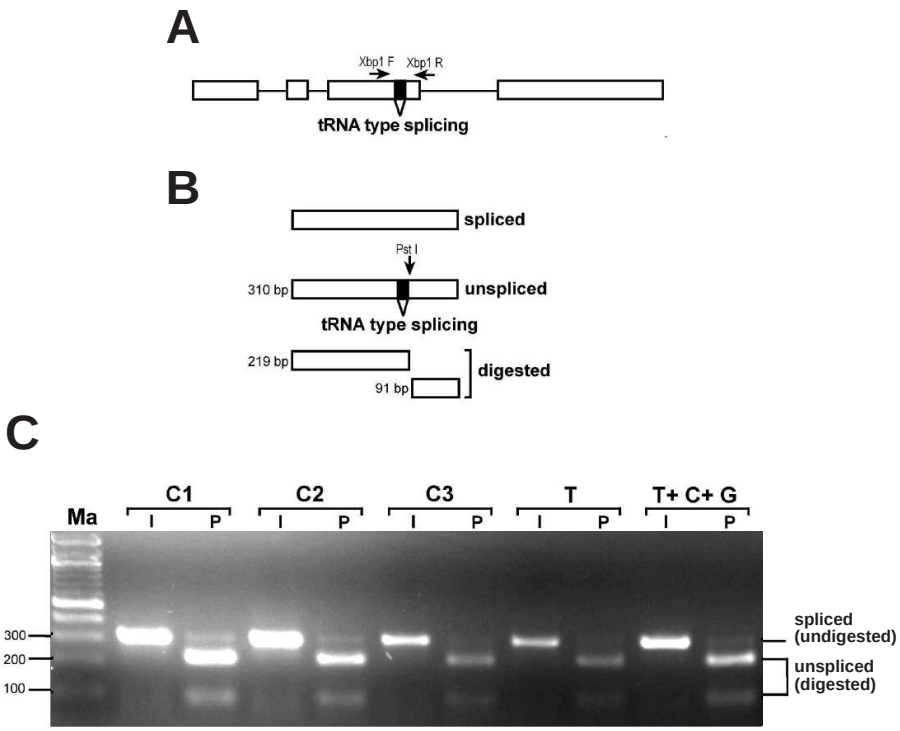
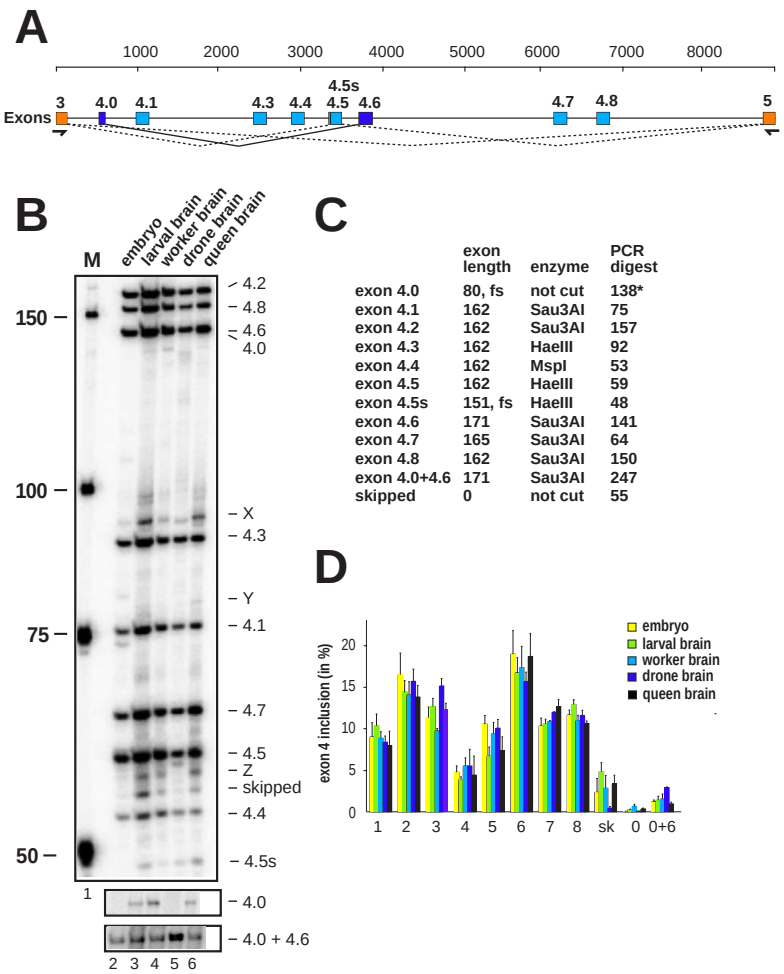


Figure 3



	exon length	enzyme	PCR digest
exon 4.0	80, fs	not cut	138*
exon 4.1	162	Sau3AI	75
exon 4.2	162	Sau3AI	157
exon 4.3	162	HaeIII	92
exon 4.4	162	MspI	53
exon 4.5	162	HaeIII	59
exon 4.5s	151, fs	HaeIII	48
exon 4.6	171	Sau3AI	141
exon 4.7	165	Sau3AI	64
exon 4.8	162	Sau3AI	150
exon 4.0+4.6	171	Sau3AI	247
skipped	0	not cut	55

Figure 4

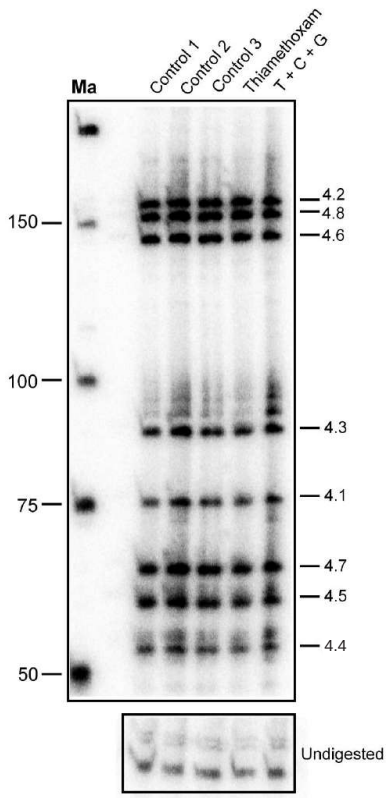


Figure 5