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# Project Report No.573

# Insights into the defence of honeybees, Apis mellifera L., against insecticides

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# 1. Abstract

There are some contradictory theories on how tolerant honeybees are of pesticides. Since the honeybee genome has been published (Honey bee Genome Sequencing Consortium, 2006), more is known about their metabolic systems, especially the detoxification pathways for potential xenobiotics. Bioassay and biochemical data from various studies have shown that both phase 1 detoxification enzymes (P450s and carboxylesterases) are responsible for pesticide metabolism in honeybees. Here, those metabolic enzymes that confer primary defence to different classes of insecticides (mainly neonicotinoid, thiacloprid) in honeybee were validated. Metabolic enzymes were characterised regarding their ability to interact with the insecticide. No binding was found between honeybee esterases and tested insecticides (tau-fluvalinate, α-cypermethrin, imidacloprid and thiacloprid). Metabolism of tau-fluvalinate and thiacloprid in honeybees is reportedly due to P450 activity, but this metabolism may not be the only reason for the relatively benign action of this insecticide on bees. Honeybees are less sensitive to neonicotinoids containing a cyanoimino pharmacophore than to those with a nitroimino group, however the specific enzymes involved in detoxification remain to be characterised. In this work, pre-treatment of honeybees with a sub-lethal dose of thiacloprid induced protection to the same compound immediately following thiacloprid feeding. Transcriptome profiling, using microarrays, identified a number of genes encoding detoxification enzymes that were overexpressed significantly in insecticide-treated bees compared to untreated controls. These included four candidate P450s, CYP6BE1, CYP305D1, CYP6AS5, CYP315A1 and an esterase CCE8. The four P450s and cytochrome b5 were functionally expressed in *Eschericia coli* and their ability to metabolise thiacloprid examined by LC-MS analysis. There was no obvious metabolism of thiacloprid, thus their role in the metabolism and disposition of thiacloprid is still unclear. CCE8 expression was not achieved using the *E. coli* expression system.

**The main findings from this research were published in Insect Molecular Biology, Volume 25, Issue 2, April 2016 (pages 171-180)**

## 2. Introduction

Honey bees are commercially important beneficial organisms worldwide. Their pollination service has been estimated to be worth £120-200 million and their honey production contributes £10-30 million annually to the UK economy (Defra, 2008). Their contribution to human diet and human health are also very important.

Food demand increases in parallel with rising global population. In this case pollinators, especially honey bees, have an economically and agriculturally important role in crop production as well as a positive effect on biological diversity (Herrera and Pellmyr, 2002). For example, today one third of global food crops are pollinator-dependent, essentially honey bee pollination service-dependent (e.g. berries, nuts, plums, cherries, kiwi, melon) (Klein *et al*., 2007; Aizen *et al*., 2008).

However, a variety of factors represent significant threats to apiculture including disease, parasites and unintended insecticide exposure (Southwick and Southwick, 1992). Chemical control is currently an indispensable input for global agriculture but pesticides are suspected by many to be involved in the disappearance of honey bees since the first report of colony collapse disorder in 2006 (Le Conte *et al*., 2010).

Multiple routes of pesticide exposure are therefore possible for honey bees including both contact (visiting the plants during foraging activity) and oral routes (consuming the infected food source) (Krupke *et al*., 2012). After penetration, an insecticide may be metabolised by detoxification enzymes into a non-toxic molecule or a form that can be eliminated rapidly from the insect body (Fukuto, 1990). As for other insects, honey bees have several superfamilies of enzymes that have the capacity to detoxify xenobiotics including pesticides. These include esterases, P450s and glutathione S-transferases.

The honey bee genome contains a smaller number of genes encoding detoxification enzymes than the published genomes of *Drosophila melanogaster* and *Anopheles gambiae* with only 46 P450 genes, 24 carboxylesterases and 10 glutathione S-transferases present in the honey bee, compared to 85 P450 genes, 35 carboxylesterases and 38 glutathione S-transferases for *D. melanogaster* and 106 P450 genes, 51 carboxylesterases and 31 glutathione S-transferases for *A. gambiae*. It has been suggested that this reduction in detoxification diversity may make honey bees more susceptible to xenobiotics than other insects (Claudianos *et al*., 2006). However, a lower number of detoxification genes does not necessarily correspond to lower detoxification activity (Hardstone and Scott, 2010). Bioassay and biochemical data from various studies have shown that both P450s and carboxylesterases are responsible for pesticide metabolism in honey bees. Esterases and P450s as phase 1 metabolic enzymes (i.e. act directly upon the intact insecticide) have been reported to metabolise a variety of insecticides such as organophosphates,

carbamates, pyrethroids and neonicotinoids (Devonshire *et al*., 1998; Stock *et al*., 2004; Feyereisen, 2005).

The synthetic pyrethroid, tau-fluvalinate, is used widely as an acaricide treatment against bee parasite *Varroa destructor* in apiculture. It is partly less toxic to bees compared to other pyrethroids due to rapid metabolism by P450s (Johnson *et al*., 2006).

The current concern regarding the adverse effects of neonicotinoids on honey bee health is resulting in many studies that investigate the effects of this class of compounds on economically important pollinators. Due to their positive impacts in crop protection including low toxicity to mammals, birds and fish, systemic control of plant-sucking insect pests (aphids, whiteflies, thrips, some coleopteran and lepidopteran) and selective toxicity, the neonicotinoids have rapidly become the preferred new generation of synthetic insecticides (Tomizawa and Casida, 2003). It is therefore vital to understand the effects of neonicotinoids on honey bees to gain advantages from these innovative chemicals with regards to controlling pest species safely and effectively (Atkins, 1992). Cyano-substituted neonicotinoids (e.g thiacloprid) are reportedly less toxic to bees because of P450 metabolism to less toxic metabolites (Iwasa *et al*., 2004). However, the identification of specific enzymes contributing to thiacloprid metabolism is unknown.

The overall aim of this project is to determine metabolic enzymes conferring the primary defence to selected 'less toxic' insecticides, with a focus on esterases and P450s and characterise the molecular and biochemical mechanisms of defence against pesticides in the honeybee:

1) Esterases – use the 'interference assay' (Khot *et al*., 2008) to determine the interactions between esterases and insecticides. Do honey bee esterases have the ability to sequester tested insecticides?
2) P450s – use an induction strategy by sub-lethal concentrations of insecticides, in combination with a range of biological, biochemical and genomic approaches to determine:

    a) Do honey bees have the ability to mount a molecular defence (via gene induction) to a neonicotinoid (thiacloprid) after initial exposure to a sub lethal dose that results in a measurable alteration in phenotype to subsequent exposure?

    b) What are the specific detoxification genes, particularly members of the P450 superfamily, induced by exposure to a neonicotinoid (thiacloprid)?

    c) Do specific candidate detoxification genes have the capacity to metabolise these compounds and explain the differential toxicity of different members of the neonicotinoid class?

# 3. Materials and methods

## 3.1. Honey bee material

*Apis mellifera carnica* was provided by Rothamsted Research. Adult worker bees were collected from the entrance of the hive using a rechargeable vacuum collector and kept in a bee housing cage (Bioquip, California). They were then distributed to plastic containers for assays following anaesthetic ($CO_2$) for no more than 2 minutes. For toxicity and induction experiments, newly emerged worker bees of *A. mellifera carnica* were used which were collected as sealed brood.

## 3.2. Esterase inhibition (Esterase interference assay)

To examine the interactions of purified honey bee esterase with insecticides, an esterase 'interference assay' was carried out as described by Khot *et al*. (2008). Briefly, stock solutions of insecticide (1% in acetone) were pre-incubated with purified esterase for 1 hour at 4°C. Aliquots (15 µL) of esterase and esterase+insecticides were incubated for 1 hour with 60 µL of serial dilutions of azamethiphos in 0.02 M phosphate buffer, pH 7.0, in separate wells of a NUNC microplate. Aliquots (25 µL) of housefly head homogenate (source of AChE) were added to each well and incubated for 15 minutes at room temperature. Esterase in acetone and buffer only served as the positive and negative controls respectively. AChE activity was measured at 405 nm using a Tmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). Readings were taken automatically for 10 minutes at 10 seconds intervals. The rate (mOD $min^{-1}$) was calculated by the integrated software Softmax Pro 5.2. Concentrations to inhibit 50% of the enzyme activity ($IC_{50}$) were calculated using Grafit 3.0 (Leatherbarrow, Erithacus Software). All treatments were performed in triplicate.

## 3.3. Toxicity bioassays

### 3.3.1. Oral toxicity tests

Technical grade thiacloprid was dissolved in acetone and then added to sucrose syrup (50%) in water. Newly emerged worker bees (~10) were transferred to plastic cages after anesthetising with $CO_2$ where they were treated with a range of thiacloprid concentrations for 24 h through oral feeding; the amount of treated diet consumed by each cage was measured by the difference in weight of sucrose syrup before and after the experiment. All treated bees were maintained at 25°C in the dark. All bioassays were scored after 24 h and bees not walking or flying were counted as dead. The data were analysed using a 4 parameter non-linear fit (Grafit 3.0, Leatherbarrow).

### 3.3.2. Contact toxicity tests

Samples were also subjected to a range of thiacloprid concentrations by topical application: newly emerged worker bees were transferred to plastic cages after anesthetising with $CO_2$ (ten bees per

cage, at least three replicates per insecticide dosage). For each cage a reservoir of 50% sucrose in water was available for *ad lib* feeding. Prior to treatment bees were anesthetised by low exposure to $CO_2$. Each bee was topically dosed (1µL) with either thiacloprid in acetone or acetone alone applied to the dorsal thorax using a Burkard microapplicator (Burkard, Rickmansworth, UK). All treated bees were maintained at 25°C in the dark and bioassays were scored after 24 h, bees not walking or flying were counted as dead. The data were analysed using a 4 parameter non-linear fit (Grafit 3.0, Leatherbarrow).

### 3.4. Induction experiment

Thiacloprid toxicity was assayed *in vivo* after exposure to a sub-lethal concentration of thiacloprid, to check for measurable alteration in phenotype. A factorial set of 16 treatments was tested in two repeat experiments. Bees (9–15 per cage = one replicate, 72 cages in total) were fed either a sub-lethal dose ($LD_5$ 0.055 mg/ml) of thiacloprid (dissolved in acetone and then sugar solution) or acetone in sugar solution (controls) for 24 h. At each of 0 h, 48 h, 96 h and 144 h a diagnostic dose of thiacloprid (62.5 µg equating to $LD_{50}$) in acetone was then topically applied to a subset of both the treated and control bees (two cages each in experiment 1, three in experiment 2); the remaining cages (two per feed treatment per experiment) received a topical application of acetone alone (Figure 1). Before and after topical application test/control bees were snap frozen in liquid nitrogen and stored at −80°C for subsequent molecular analyses.

Data from the induction bioassays were analysed in GenStat (14th edition, VSN International) using logistic regression (i.e. a generalized linear model with binomial error and logit link), allowing for differences between experiments before testing treatment effects and with adjustment for over-dispersion.

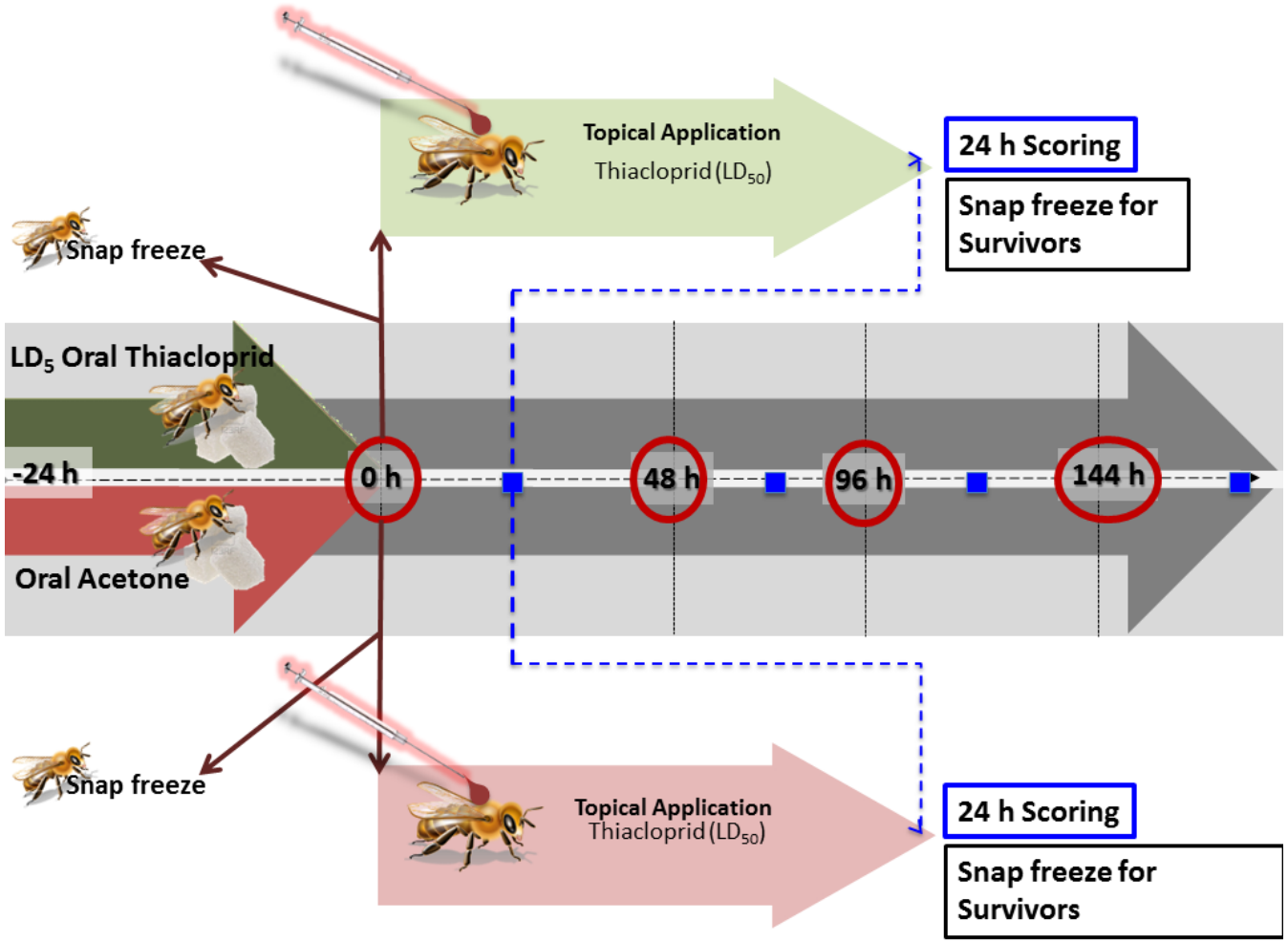


**Figure 1 General scheme for induction experiment.**

### 3.5. Microarray analysis

A custom microarray designed using the Agilent eArray platform (Agilent Technologies) contained 60bp oligonucleotide probes for each of the honey bee consensus gene set (~10,000 genes) derived from the annotated honey bee genome. A SurePrint HD (8×15k) expression array was designed using the Agilent eArray platform. The base composition and the best probe methodologies were selected to design sense orientation 60-mer probes with a 3′ bias. For each contig encoding a detoxification enzyme (P450s, GSTs and CEs) three probes were designed. Additional probe groups for 15 control genes were included.

Groups of four bees per replicate were ground to a fine powder in liquid nitrogen using a pestle and mortar. RNA was extracted from the pooled homogenates using the Bioline Isolate RNA Mini Kit according to the product manual. The quantity of RNA was checked using a nanodrop spectrophotometer and by running an aliquot on a 1.5% agarose gel. For the latter, RNA was mixed with 1x loading buffer (95% formamide; 0.025% xylene cyanol; 0.025% bromophenol blue; 18 mM EDTA; 0.025% SDS), heated for 5 minutes at 65°C and briefly chilled on ice prior to loading. Two micrograms of each RNA was used to generate labelled cRNA, which was hybridised to the arrays, which were then washed and scanned as described in the Agilent Quick Amp Labeling Protocol (Version 5.7). The experiments consisted of four/five biological replicates and for each of these, hybridisations were done in which the Cy3 and Cy5 labels were swapped between samples.

Microarrays were scanned with an Agilent G2565CA scanner and fluorescence intensities of individual spots were obtained using the Agilent Feature Extraction software with default Agilent parameters. Data normalization, filtering, dye flipping and statistical analysis were performed using the GeneSpring GX suite. For statistical analysis, a t-test with null hypothesis of no difference between treatments was used to detect differentially expressed genes. Genes were considered differentially expressed if they had a $p$ value of $<0.05$ and a fold change (up or down) greater than 1.5.

### 3.6. Quantitative PCR

Primers were designed to amplify a fragment ~100 bp using the Primer3 program (Table 1). 4 µg of RNA was used for reverse transcription using Superscript II Reverse Transcriptase and random hexamers (Invitrogen). Each PCR reaction consisted of 4 µl of cDNA (10 ng), 5 µl of SensiMix SYBR Kit (Bioline) and 0.5 µl of each forward and reverse primer (0.25 mM). PCRs were run on a Rotor-Gene 6000 (Corbett Research) with cycling conditions: 10 minutes at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 20 s. A final melt-curve step was included post-PCR (ramping from 72°C to 95°C by 1°C every 5 s) to confirm the absence of any non-specific amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution from

100 ng to 0.01 ng of cDNA. Each qRT-PCR experiment consisted of at least three independent biological replicates with two technical replicates. Data were analysed according to the ΔΔCT method (Pfaffl 2001), using the geometric mean of two selected housekeeping genes (elongation factor and actin) for normalisation according to the strategy described previously (Vandesompele *et al*., 2002).

**Table 1 Oligonucleotide primer sequences used in qPCR**

| Gene | Primer | Sequence (5' - 3') | Product size (bp) |
|---|---|---|---|
| **CYP305D1** | Forward | GGACGTCCTTGGAACGAAT | 108 |
| | Reverse | TCGCATCATCCAATTTCGTA | |
| **CYP315A1** | Forward | CTGGGTCCCGTTTACAAAGA | 101 |
| | Reverse | GGTGTTGACCCTTCAAGTCG | |
| **CYP6AS5** | Forward | CAGGCTCTCCCCAATATTCA | 120 |
| | Reverse | TCGATGGGCTCATTTTTCTC | |
| **CYP6BE1** | Forward | CGAAAGGAACTTGCATAGCC | 120 |
| | Reverse | TCTTCGGAAAATCGTTCTGG | |
| **Cytb5** | Forward | CAGCGGAAGAAGTAGCGAAA | 101 |
| | Reverse | GCCTGGATGTTCGCTTAGAA | |
| **GSTD1** | Forward | AAAAATGCTTGTTATTTTCTGTCTGA | 110 |
| | Reverse | TCAAACGCGTCTTCGAGTATC | |
| **CCE8** | Forward | TCTGCTTGCGCATTCTATTG | 106 |
| | Reverse | CTTTACGCGCTTCTTTGTCC | |

## 3.7. Heterologous expression of candidate genes

### 3.7.1. Cloning CYPs

The candidate honey bee P450s (CYP305D1, CYP315A1, CYP6AS5, CYP6BE1) were amplified from cDNA using KAPA high-fidelity DNA Polymerase (Kapa Biosystems) following the product manual. As a proofreading DNA polymerase was used for amplification, which leaves blunt ended DNA, A-tailing reactions were carried out prior to cloning. After the product was cleaned, it was ligated into the pSC-A-amp/kan cloning vector. For functional P450 expression in *E. coli* the N-terminal coding region of each P450 cDNA was modified: the ompA leader sequence (21 amino acid residues) and two linker amino acid residues (alanine-proline) were added to the 5' end of P450s (ompA+2 strategy) (Pritchard *et al.*, 1997; 2006, McLaughlin *et al.*, 2008). This was achieved by two fusion PCR reactions, carried out using high-fidelity DNA polymerase according to the manufacturer's instructions. In the first PCR, genomic DNA of the *E. coli* JM109 cell line was used as template to amplify a ~100 bp nucleotide fragment (containing the ompA+2 sequence and the first 21 bases of the target P450 gene) preceded by a *NdeI* restriction site using the primers described in Table 2. This intermediate PCR product was purified and then fused to the P450 plasmid template in a second PCR reaction using the same forward and CYP specific reverse primers (Table 2) to generate the full-length ompA-AP-CYP coding sequence flanked by *NdeI* and

*XbaI* restriction sites. The final product was digested and ligated into modified pCW-ori+ vector via *XbaI* and *NdeI* restriction sites and the final sequence were confirmed by sequencing prior to expression.

**Table 2 Primers used for fusion PCRs. All primers are listed in the 5' to 3' direction**

| CYPs[a] | Reverse Primer[b] | CYP Specific Reverse Primer[c] |
|---|---|---|
| ompA-AP-CYP305D1 | ACTATTAACATTATAACAAACAT X | GAATTCTCTAGATTATCGTTTTTCAACTAATACA |
| ompA-AP-CYP315A1 | AATATTTTGCGCAAGATTCAT X | GAATTCTCTAGACTAATTTCTCTCCATCAATTT |
| ompA-AP-CYP6AS5 | AATTTCGAAACTGCTCGCCAT X | GAATTCTCTAGATCATATTTTTGTTATTTTCAAATA |
| ompA-AP-CYP6BE1 | TAACCACGTAGTTAAAAACAT X | GAATTCTCTAGATTATATTGGCTCAATATTTAGA |

[a]The forward primer used for ompA+2 fusion PCR strategy was always 5'-GGAATTCCATATGAAAAAGACAGCTATCGCG -3' with *the Nde*I restriction site underlined.

[b]Reverse complement of the start of CYPs and X represents 5'-CGGAGCGGCCTGCGCTACGGTAGCGAA-3' which corresponds to the reverse complement of proline and alanine codons and the ompa segment sequence.

[c] The region corresponding to the reverse complement of the end of CYPs with *Xba*I restriction site (underlined).

### 3.7.2. Preparation of membranes

Competent *E. coli* JM109 cells were co-transformed with pCWOri+-CYPs and pACYC-AgCPR to enable co-expression of each CYP with the *Anopheles gambiae* CPR following the methods described by Stevenson *et al*. (2012). Plasmids were transformed into JM109 cells and overnight cultures in 200 mL terrific broth incubated at 30°C with shaking. When the cultures reached early log phase growth expression was induced by adding 1 mM IPTG. δ-aminolevulinic acid hydrochloride was added to a final concentration of 0.5 mM at the same time to compensate the low levels of endogenous heme in the bacterial cells. Further incubation was at 24°C for 23 hours before the cells were harvested by centrifugation and membranes prepared as described by Pritchard *et al*. 1998. Membranes were resuspended in ice-cold 1X TSE buffer in a Dounce tissue homogeniser and aliquots stored at -80$^{o}$C. P450 content (Omura and Sato, 1964), total protein concentration (Bradford, 1976) and CPR content (Strobel and Dignam, 1978) were analysed.

### 3.7.3. Cloning cytochrome b5

To simplify the purification of the expressed b5 protein the N-terminal coding region was modified by the addition of six histidine residues (6H) to the 5' end (Holmans *et al*., 1994; Stevenson *et al*., 2011). This was achieved using high-fidelity DNA polymerase and the forward primer 5'-GGAATTCCATATG**CACCATCACCATCACCAC**ATGTCGAAAATTTTTACAGCGGA-3' (*NdeI* restriction site underlined and six histidine codons in bold before start codon) and reverse primer 5'-GAATTCTCTAGATTATGAATACCAAAAATAGTAAAAT-3' (XbaI restriction site underlined). The final product was digested and ligated into the modified pCW-ori+ vector via the *XbaI* and *NdeI* restriction sites with the final sequences confirmed by sequencing prior to expression.

### 3.7.4. Expression and purification of cytochrome b5

The 6H-b5 construct was transformed into JM109 cells and after overnight culture was transferred to 500 mL terrific broth (TB) media containing 50 µg/mL of ampicillin in a 1L flask and shaken at 37°C at 200 rpm and expression induced as described above. Cells were harvested as for the P450s. Pelleted cells were resuspended and treated by the Holmans *et al*. (1994) method with Stevenson *et al*. (2011) modifications. Expression of b5 was checked and quantified by spectrophotometry (Omura and Sato, 1964).

## 3.8. Insecticide metabolism

Insecticide (thiacloprid and imidacloprid) metabolism assays of recombinant bee P450s/CPR/b5 standard reactions were carried out using three replicates for each P450 in the presence or absence of NADPH. A 10 mM stock solution of thiacloprid and imidacloprid were prepared in DMSO and diluted to 100 µM in phosphate buffer (0.1 M, pH 7.6) before adding the reaction to avoid the precipitation of insecticide. Standard reactions consisted of final concentrations of 10 µM insecticide, 100 µl NADPH regeneration system (Promega) (or buffer alone in the case of minus NADPH controls), 0.0117 µM cytochrome b5 and 0.1- 0.4 µM P450 membrane. Reactions (200 µl total) were incubated at $30^0$C, shaking at 1200 rpm for 2 h and then stopped by adding 300 µl of acetonitrile. Samples were then spun at 2000 g for 5 min and 250 µl of supernatant was transferred to HPLC vials and stored at $-20^0$C for LC-MS/MS analysis.

## 3.9. LC-MS analysis

Aliquots of each sample were diluted 50:50 in acetonitrile prior to LC-MS/MS analysis. Separation was achieved using Ultra Performance LC® (ACQUITY UPLC-System; Waters, UK) using an ACQUITY UPLC column (HSS T3, 1.8 µm, 100 x 2.1 mm), with a mobile phase consisting of water (+0.2% formic acid), with a flow rate of 0.6 mL/min. The gradient elution conditions of acetonitrile:water were: 0 min 0:100, 0.5 min 0:100, 3.5 min 95:5, 4.5 min 95:5, 4.6 min 0:100, 5 min 0:100. The mass spectrometer was a Finnigan TSQ Quantum Discovery (Thermo Scientific, UK)

equipped with an Ion Max source operating in positive ion mode. Analytes were detected using selected-reaction-monitoring (SRM), transitions as outlined in Table 3. Quantification was achieved using standard calibration curves constructed in 50:50 acetonitrile:water.

**Table 3 SRM transitions and collision energies**

| Analyte | Molecular Weight (Da) | SRM Transition Parent *m\z* > Product *m\z* | Retention Time (min) |
|---|---|---|---|
| Thiacloprid | 254 | 253 > 126<br>253 > 186 | 4.83 |
| Imidacloprid | 257 | 256 > 175<br>256 > 209 | 4.47 |

# 4. Results

## 4.1. Esterase interferance assay

Esterase involvement in sequestering insecticides can be measured using the 'interference assay' which detects the AChE activity remaining following esterase blockade on azamethiphos. If tested insecticide binds to the esterase, the esterase cannot bind to azamethiphos and as a result AChE activity is present (Khot *et al*., 2008).

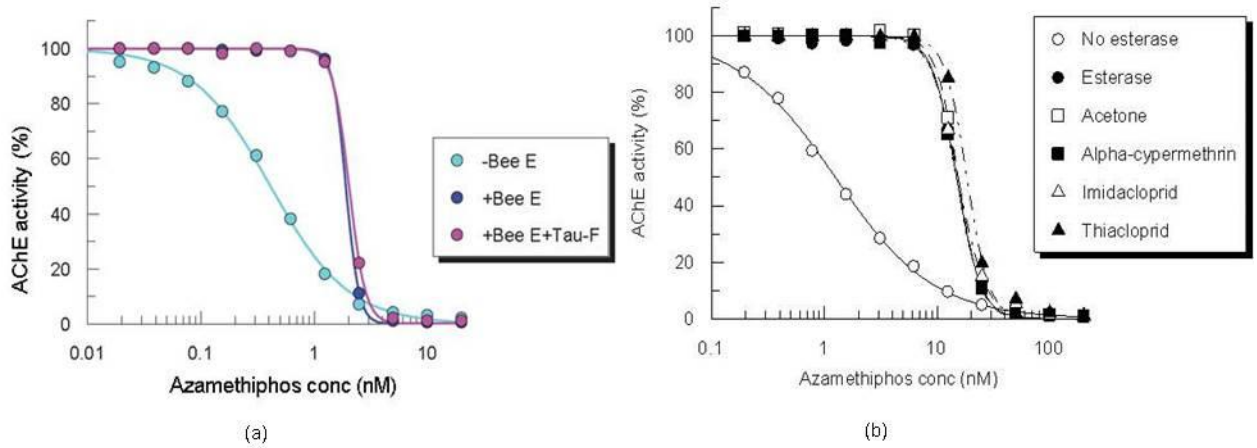


**Figure 2 a) Binding of honey bee esterases with tau-fluvalinate, b) Binding of honey bee esterases to α-cypermethrin, imidacloprid and thiacloprid**

The reduced protection of AChE towards azamethiphos provided by the purified honey bee esterase following pre-incubation with tau-fluvalinate, α-cypermethrin, imidacloprid and thiacloprid is shown in Figure 2. After 1 hour incubation of bee esterase with each neonicotinoid and pyrethroid, no sequestration was observed. Purified honey bee esterase failed to bind to tau-fluvalinate, α-cypermethrin, imidacloprid and thiacloprid (Figure 2). Thus, bee esterase does not provide any protection against tested pyrethroids and neonicotinoids. This is in contrast to herbivorous insects that have been assayed using the same interference assay. The resistance-associated-esterase, E4 from *Myzus persicae* has been found to interact with tau-fluvalinate (Alptekin *et al*., 2015). It is probable this is a result of the relatively toxic secondary chemistry found in plant tissue, from which the insect needs to protect itself. Since the honey bee feeds primarily on nectar, it does not need to protect itself from such an assortment of noxious chemicals.

## 4.2. Toxicity bioassays

Toxicity bioassays were carried out to determine the optimum dose for induction experiment. Full-dose mortality response curves for oral and contact toxicity bioassays with thiacloprid resulted in doses for induction (oral $LD_5$) being calculated as 10 µg/bee and contact toxicity ($LD_{50}$) 62 +/-12 µg/bee. For the oral toxicity assays the $LD_5$ was calculated by assuming average consumption (0.055 mg/ml active) (Figure 3).

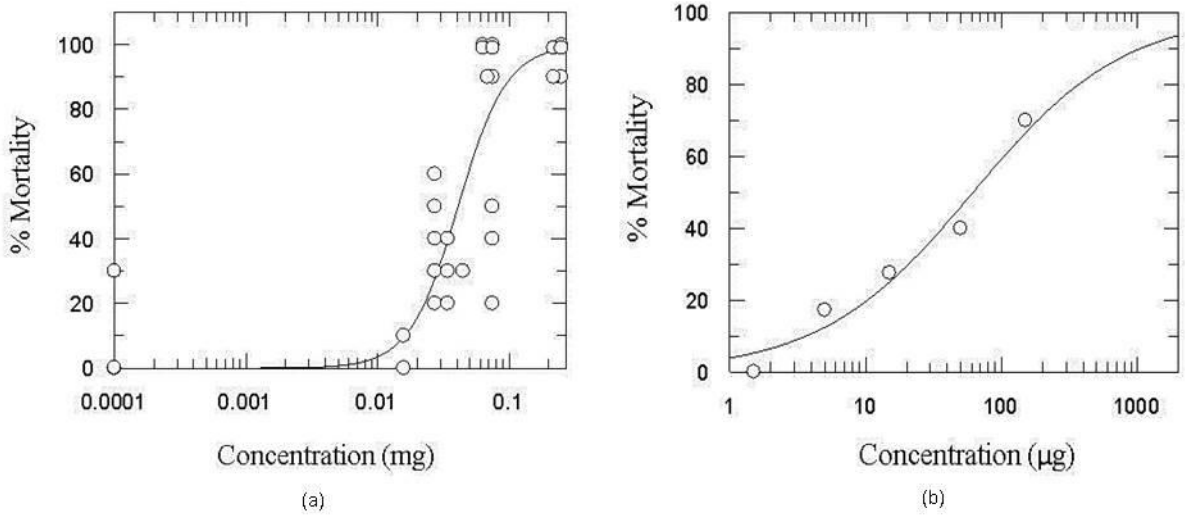


**Figure 3 Results of analysis on oral toxicity (thiacloprid) data (a) and contact toxicity (thiacloprid) data (b) for *A. mellifera carnica*.**

## 4.3. Induction experiment

Induction experiment was used to investigate if honey bees show a measurable alteration in phenotype to the neonicotinoid thiacloprid after initial exposure to a sub-lethal dose of the same compound. Significant differences were identified in the proportion of mortality resulting from topical application (i.e. whether bees were topically applied acetone or thiacloprid); time (i.e. time after pre-treatment that topical application was made); and with both feed and time parameters combined (i.e. within the group topically applied with thiacloprid, time after pre-treatment). Immediately following a pre-treatment time of 24 h, topical application resulted in a significantly increased tolerance to thiacloprid compared to the controls (t-test, $p = 0.006$). 48 h and 144 h following pre-treatment there were no significant differences between a thiacloprid or acetone pre-treatment (t-test, $P > 0.05$). At 96 h there was a significantly increased sensitivity in the bees pre-treated with thiacloprid (t-test, $P = 0.043$) (Table 4, Table 5 and Figure 4).

**Table 4 Comparison of mean mortalities (logit scale) for bees fed either thiacloprid or acetone and subsequently topically applied with acetone at various times post-feeding**

| TIME | 0 h | 48 h | 96 h | 144 h |
|---|---|---|---|---|
| **Difference** | 0.966 | 0.471 | -0.419 | 0.766 |
| **t-statistic** | 0.628 | 0.384 | -0.403 | 0.470 |
| ***P* value** | 0.5327 | 0.7024 | 0.6885 | 0.6403 |

**Table 5 Comparison of mean mortalities (logit scale) for bees fed either thiacloprid or acetone and subsequently topically applied with thiacloprid at various times post-feeding**

| TIME | 0 h | 48 h | 96 h | 144 h |
|---|---|---|---|---|
| **Difference** | -1.351 | -0.628 | 0.978 | 0.789 |
| **t-statistic** | -2.884 | -1.199 | 2.075 | 1.547 |
| ***P* value** | 0.0057 | 0.2360 | 0.0430 | 0.1279 |

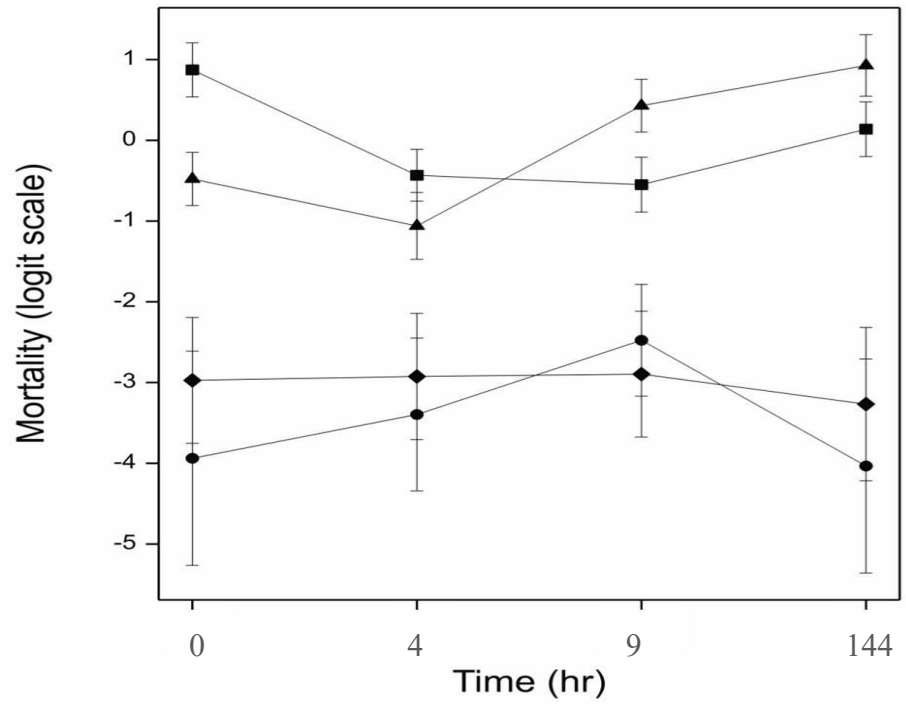


**Figure 4 Predicted treatment mean mortalities (logit scale; n = 1–3 cages of 9–15 bees) from logistic regression (± SE). Fed acetone, topical acetone (circles); fed thiacloprid, topical**

**acetone (diamonds); fed acetone, topical thiacloprid (squares); fed thiacloprid, topical thiacloprid (triangles). Time = delay following 24 h oral pre-treatment.**

## 4.4. Transcriptome profiling

Transcriptome profiling using microarrays was used to compare gene expression in bees fed sucrose-insecticide (treated) and those fed sucrose syrup (control) at each time point. An additional array comparison was conducted comprising bees fed sucrose-insecticide that subsequently survived the 0 h topical bioassay versus the non-treated control from the same time point ('survivor' experiment). In the time course experiment 21 probes (11 up-regulated and 10 down-regulated), 42 probes (21 up-regulated and 21 down-regulated), 27 probes (20 up-regulated and 7 down-regulated) and 13 probes (7 up-regulated and 6 down-regulated) were identified as significantly differentially expressed between control and treated bees at the 0 h, 48 h, 96 h and 144 h time points respectively. In the 'survivor' experiment 96 probes were identified as differentially expressed (57 probes were up-regulated and 39 down-regulated) between treated bees surviving the topical bioassay at 0 h and non-treated controls from the same time point.

Among the differentially expressed probes were several that correspond to genes with putative roles in insecticide metabolism that are potential candidates to explain the alterations seen in phenotype in treated bees compared to controls. In the 0 h comparison two probes representing the P450 gene CYP315A1 were overexpressed (~1.5-fold) and in the 48 h comparison a single probe representing the gene cytochrome b5 was overexpressed 4.5-fold. At the 96 h time point three probes corresponding to the P450 gene CYP9Q1 and a single probe representing the carboxylesterase gene CCE11 were differentially expressed, however in all cases they were down-regulated (-1.5 to -1.7). At the 144 h time point no probes encoding detoxification enzymes were differentially expressed. The 'survivor comparison' displayed the greatest number of differentially expressed probes encoding detoxification genes with in all cases probes up-regulated. In the case of P450s this included four probes encoding CYP6BE1 (1.9-2.2-fold), four probes encoding CYP305D1 (1.8-1.9-fold) and four probes encoding CYP6AS5 (1.6-1.7-fold). For esterases five probes encoding CCE8 were up-regulated 2.1-2.2-fold. In the case of glutathione S-transferases a single probe encoding GSTD1 was over expressed 1.9-fold. Finally a single probe representing the gene cytochrome b5 was overexpressed (1.5-fold).

The expression levels of seven of the detoxification candidate genes from the microarray experiment were validated by qPCR with excellent concordance seen between fold-changes calculated using the qPCR and array approaches (Table 6).

**Table 6 Fold change in expression of candidate genes in treated groups compared to control groups.**

| Gene | Fold change (qPCR) | 95% confidence limits | Fold change (microarray) |
|---|---|---|---|
| **CYP305D1** | 2.4 | 0.5 | 1.8-1.9 |
| **CYP315A1** | 1.8 | 0.6 | 1.5-1.6 |
| **CYP6AS5** | 1.4 | 0.1 | 1.6-1.7 |
| **CYP6BE1** | 1.7 | 0.4 | 1.9-2.2 |
| **Cyt *b5*** | 2 | 0.2 | 1.5 |
| **GSTD1** | 1.1 | 0.3 | 1.9 |
| **CCE8** | 3.3 | 0.4 | 2.1-2.2 |

The qPCR experiments provided confirmation that six of the seven candidate genes were significantly upregulated in treated bees compared to controls with the exception of GSTD1 which was eliminated as a potential candidate as it showed no significant increase in expression in treated bees.

## 4.5. Heterologous expression of candidate genes

Several resistance detoxification genes were shown to be overexpressed or associated with metabolism by synergism studies, however, overexpression does not always mean that these genes are functionally responsible in detoxifying insecticides. Thus, clear identification of such metabolism needs further investigation to prove metabolism.

This study addresses the following question; do specific candidate detoxification genes which are induced upon exposure to a neonicotinoid (thiacloprid) (see section 4.4) have the capacity to metabolise these compounds and explain the differential toxicity of different members of the neonicotinoid class?

In order to determine if CYP305D1, CYP315A1, CYP6AS5, CYP6BE1, cytochrome b5 and CCE8 were involved in neonicotinoid metabolism they were functionally expressed in *E. coli* for further metabolism study.

Variation was observed in the yield of recombinant protein produced for each P450 (Table 7), however, each P450 reduced CO-difference spectra suggesting correctly folded and active enzyme as indicated by significant peaks at 450 with secondary smaller 420 peaks observed (Figure 5). Honey bee cytochrome b5 was also successfully expressed and purified from *E. coli* membranes (0.83 g cytochrome b5/L). It generated a characteristic b5 spectra (Guzov *et al*., 1996), with a peak at 420-430 nm observed when reduced with sodium hydrosulphite (Figure 6). CCE8 expression was not achieved using the *E. coli* system.

**Table 7 P450 concentration and CPR activity in expressed CYPs.**

| CYPs | P450 concentration (nmol P450/mg protein) | CPR activity (nmoles cyt c/min/mg protein) |
|---|---|---|
| CYP305D1 | 0.17 | 8.8 |
| CYP315A1 | 0.116 | 6.9 |
| CYP6AS5 | 0.045 | 4 |
| CYP6BE1 | 0.0518 | 7 |

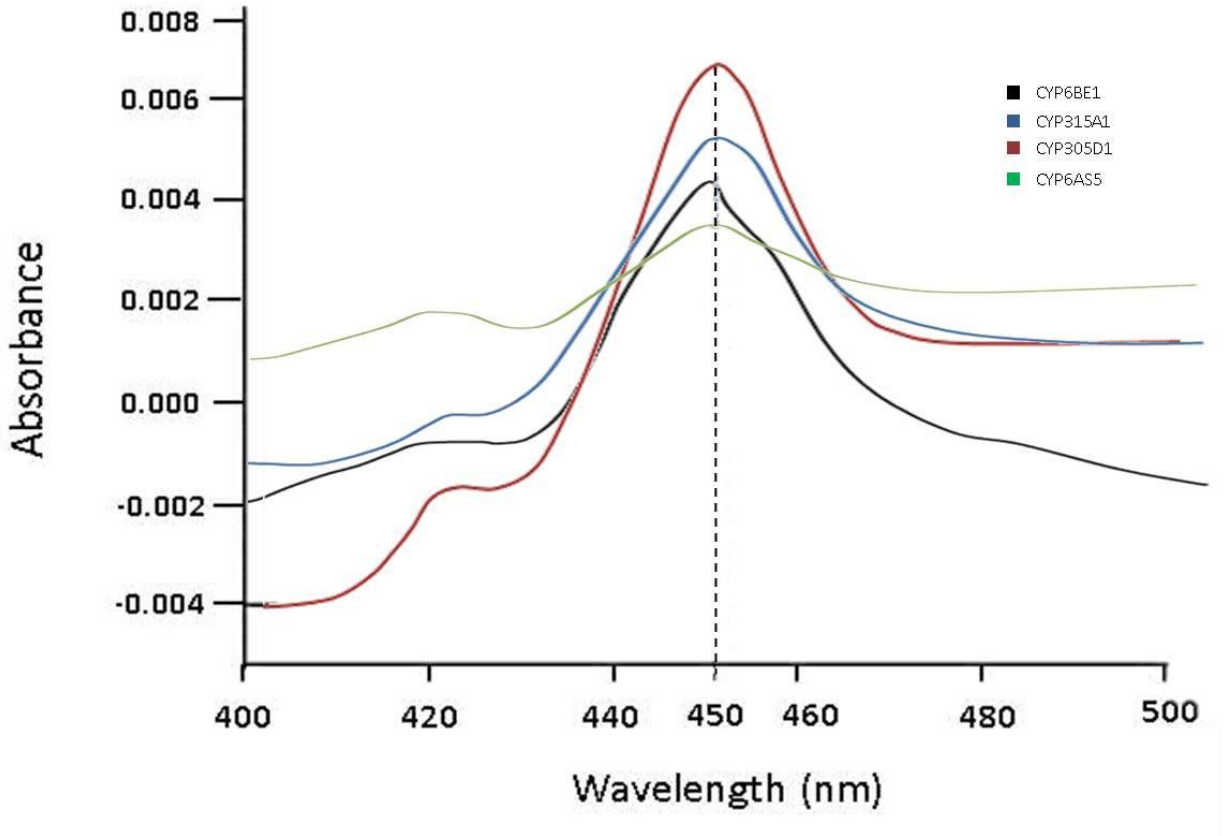


**Figure 5 CO - difference spectra. Four P450s show an absorbance peak at 450 nm indicating.**

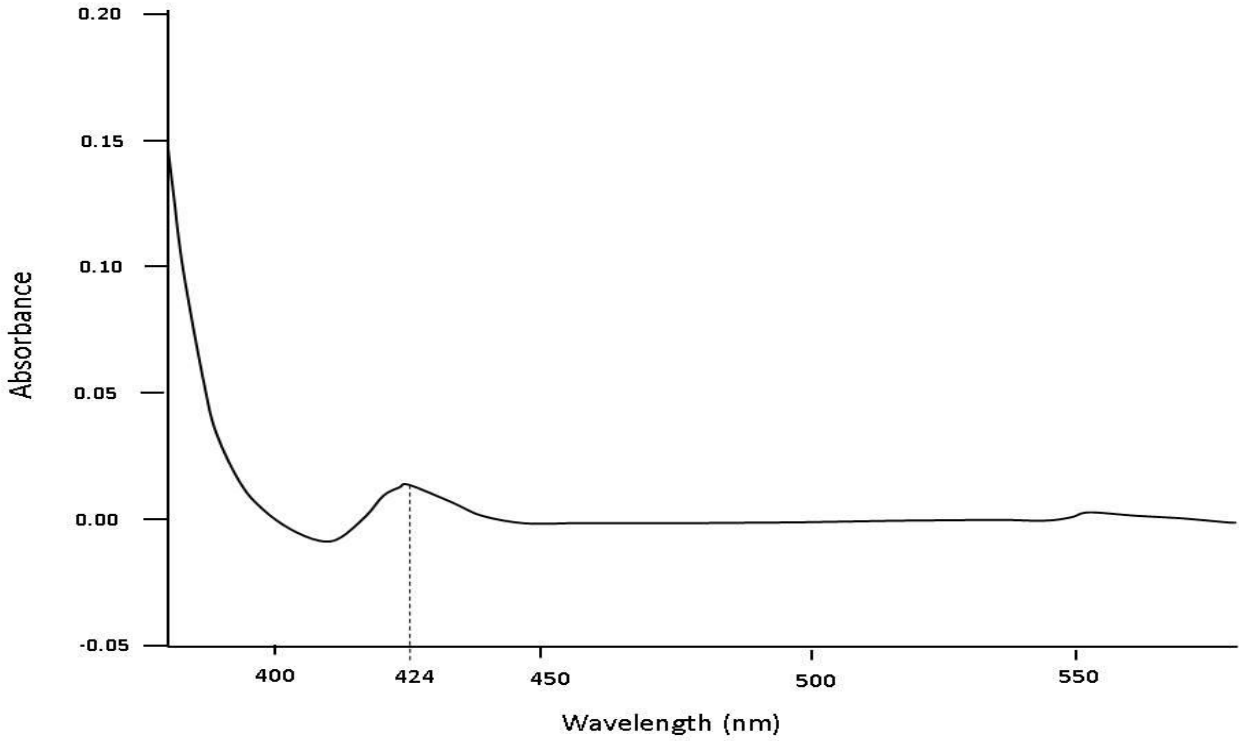


**Figure 6 Absorbance of reduced CO - difference P450 spectrum. Cyt b5 expression is detected at an absorbance of about 424 nm.**

## 4.6. Metabolism assay

The ability of CYP305D1, CYP315A1, CYP6AS5 and CYP6BE1 (in combination with *A. gambiae* CPR and honey bee cytochrome b5) to detoxify thiacloprid and imidacloprid in the presence and absence of NADPH was examined in insecticide metabolism assays. Figure 7 outlines the results of monitoring thiacloprid recovery in the samples using selected-reaction-monitoring (SRM) methods, with quantification against standard calibration curves. NADPH is an obligate cofactor for P450 metabolism as it is required by CPR to deliver electrons to P450 for catalytic activity. Therefore, incubation of substrate was carried out in the presence and absence of NADPH to monitor the NADPH dependant depletion of substrate indicative of P450 metabolism. No significant differences were observed in the thiacloprid recoveries between the +/- NADPH samples for any of the four P450s. Figure 8 outlines the results of monitoring imidacloprid recovery in the samples using SRM methods, with quantification against standard calibration curves. Again no significant differences were observed in the IMI recoveries between the +/- NADPH samples.

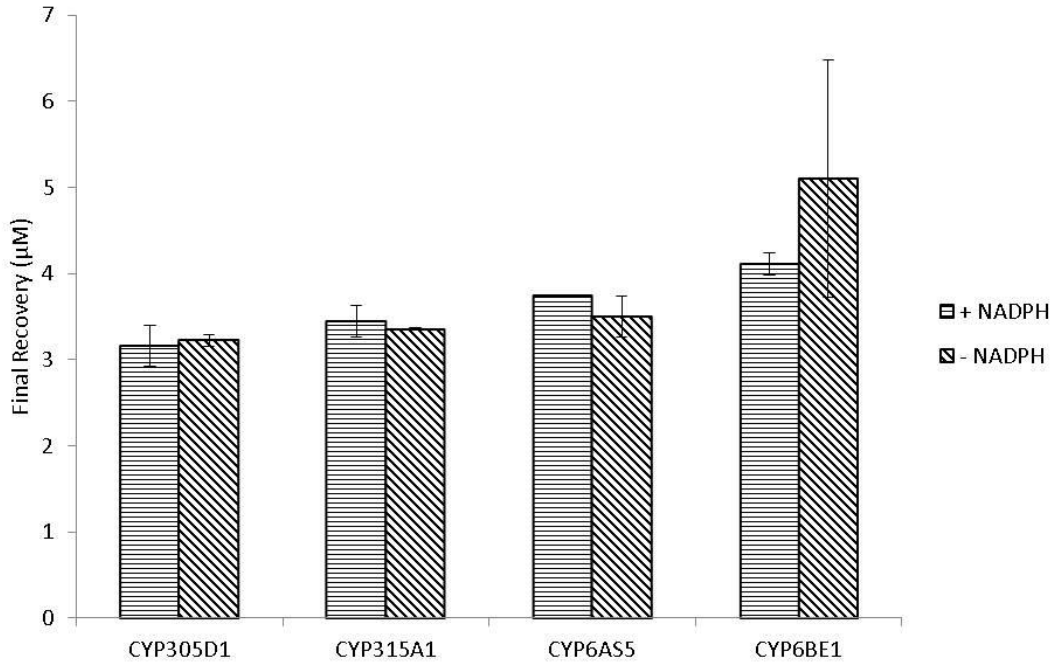


**Figure 7 Recovery of thiacloprid after a 2 h incubation with the four honeybee P450s. Graph represents mean final recovery (µM) ± SE (n = 3).**

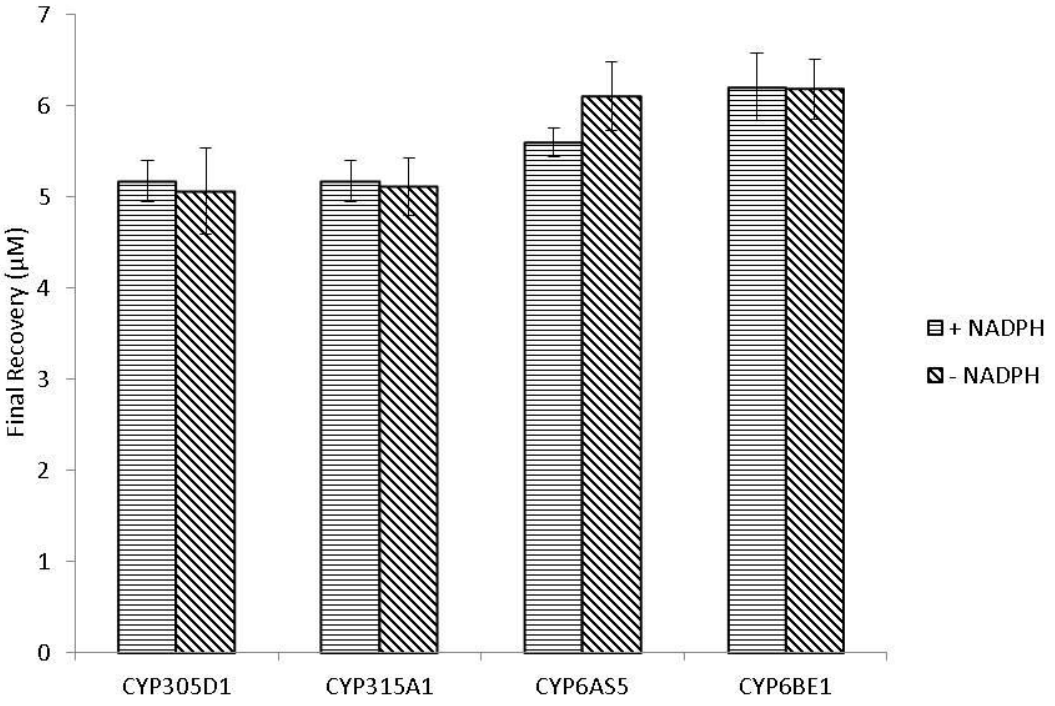


**Figure 8 Recoveries of imidacloprid after a 2 h incubation with the four honeybee P450 expression systems. Graph represents mean final recovery (µM) ± SE (n = 3).**

# 5. Discussion

This project has provided additional evidence for the involvement of phase 1 detoxification enzymes in honey bee 'defence' against the insecticides tau-fluvalinate and thiacloprid. Following the latest incidents of honey bee losses, the role of insecticides has been widely vocalised. However, although it was reported that their metabolic capacity may be limited, an extensive number of studies found honey bees to be no more sensitive to numerous insecticides than other insect species (Hardstone and Scott, 2010). Since the honey bee genome has been published, it is known that detoxification genes are relatively lower in number than other insects but other studies have shown that certain genes play an important role in defence mechanisms of the honey bees against specific insecticides (in-hive). As this project demonstrates, honey bees may have intrinsic mechanisms that provide protection against certain insecticide classes.

Although identification of the specific genes of honey bees that regulate the metabolism of detoxification enzymes is a popular research topic, only a very limited number of studies related to detoxification activity induction have been published. However, several studies, including this report, show that detoxification capacity can be induced by using specific treatments to stimulate the activity. Yu *et al*. (1984) exposed honey bees by ingestion to the sub-lethal doses of 5 insecticides (permethrin, metoxychlor, carbaryl, malathion, diflubenzuron) to assess the effects on the detoxification enzymes. This resulted in failure as dietary exposure did not show any alteration that effected detoxification enzyme activity except with permethrin, which was found to significantly induce GST activity. Kezic *et al.* (1992) investigated P450 induction in bees after the 9$^{th}$ day of dietary exposure to benzo-(α)-pyrene (as itself or through tau-fluvinate or cimiazole); benzo-(α)-pyrene monooxidase activity was increased 5-25 fold in honey bees. In addition, Mao *et al*. (2011) found that CYP9Q3 and CYP9Q2 were induced after exposure to tau-fluvalinate and bifenthrin respectively. Johnson *et al*., (2012) found that when honey bees were exposed to honey extracts, it resulted in the up regulation of CYP6AS sub family.

The present study is the first demonstration with honey bees of a sub-lethal dose of an insecticide providing a protective effect to subsequent exposure of the same insecticide. In a similarly structured study by Johnson *et al*. (2012), no effect on the honey bee toxicity was detected for tau-fluvalinate (pyrethroid) in individuals fed with phenobarbital, xanthotoxin, salicylic acid and indole-3-carbinol; in contrast, quercetin fed individuals showed a reduced sensitivity. However, this study did not include the effect of a tau-fluvalinate feed on subsequent toxicity assays for same chemical compound.

The induction bioassay was planned to monitor the phenotypic effects on honeybees exposed by feeding to sub-lethal doses for 24 hours. This experimental design and the results obtained revealed that 24 hours of exposure reduced bee sensitivity to an immediate subsequent exposure

to a much higher dose of thiacloprid, however this was not a permanent phenomenon and by the 48 hours' time point bees fed thiacloprid were no more tolerant than controls. It would be interesting to explore, in future, if longer/continual initial exposure times resulted in a longer 'protective effect'. This type of longer, low-level exposure more closely resembles the type of exposure scenario bees might encounter in the field, and it would be interesting to carry out similar experiments at the field scale. The findings of such a study could theoretically also inform a new approach in apiculture of designing complementary-diets where honey bees can be fed with sub-lethal doses of certain compounds to establish colony defence to that compound. However, such a strategy would require careful examination of possible sub-lethal effects on bees from the low level exposure doses used and any carry through into bee honey. Although theoretically possible in reality such an approach is unlikely to garner widespread support. Interestingly a protective effect from other stress factors such as diseases, parasites or involvement of other pesticides or phytochemicals could not be demonstrated in the laboratory, so replicating similar studies in field conditions are necessary.

Mao *et al*. (2011) described the importance of such findings for selectively induced P450 genes by a specific induction agent which would help to develop monitoring strategies for honey bee exposure to specific pesticides while foraging and to differentiate between in-hive exposure to non-target acaricides applied to control *Varroa* and to exposure to non-target pesticides used on agricultural crops.

In addition, identification of the specific detoxification genes that metabolise insecticides could be used to design new less toxic pesticides/synergists to minimise severe damage to non-target organisms. These new strategies would aim to design chemicals which have the ability to inhibit pest detoxification activities without damaging the honey bee defence mechanism or possibly even stimulate its detoxification capacity.

In humans a great deal of research focuses on the metabolism of xenobiotics, especially therapeutic drugs, by P450s. As an example human CYP3A4 is the most studied drug-metabolising P450 and has a broad substrate detoxification capacity. Similarly, finding generalist bee detoxification enzymes and identifying a specific model substrate can provide an opportunity to develop high-throughput *in vitro* screening tools which detect insecticides that are substrates/inhibitors and therefore may be metabolised. Furthermore identification of P450s involved in insecticide metabolism would allow the P450/insecticide interaction to be studied using homology modelling. Such studies may guide the synthesis of the new chemical via rational design and allow critical catalytic sites in P450s unique for pests and honey bees to be identified.

In summary identification of the specific honey bee genes that regulate the metabolism of the detoxification enzymes or the alternative target-sites will enable agrochemical R&D to develop safer and target-specific products.

Bee pollinators carry out a vital ecosystem service and their wellbeing is essential to ensure future food security. Because they are insects, bees may be as sensitive to insecticides as the target-pests. However, there are a number of examples where this is not the case, and indeed certain members of the pyrethroid (tau-fluvalinate) and organophosphate (coumaphos) class of insecticides show low toxicity to bees and are used as in-hive treatments to control *Varroa destructor,* a parasitic mite of honey bees. Another example is cyano-substituted neonicotinoids (thiacloprid and acetamiprid) that have been shown to be orders of magnitude less acutely toxic to honey bees than nitro-substituted compounds (imidacloprid, clothianidin, thiamethoxam, dinotefuran, nitenpyram) (Iwasa *et al*., 2004).

Differences between the sub-classes of the neonicotinoids could direct the future focus on insecticide development. Since a nitro group is known to be more toxic to honey bees, by focussing on the development of safer alternatives, it may be possible to create a more suitable environment for the registration of neonicotinoids regarding honey bee health.

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