Insect Molecular Biology

Insect Molecular Biology (2016) **25**(2), 171–180 doi: 10.1111/imb.12211

Induced thiacloprid insensitivity in honeybees (*Apis mellifera* L.) is associated with up-regulation of detoxification genes

S. Alptekin*, C. Bass*, C. Nicholls†, M. J. I. Paine‡, S. J. Clark*, L. Field* and G. D. Moores§

**Department of Biological Chemistry and Crop Protection, Rothamsted Research, Harpenden, UK;* †*AHDB, Stoneleigh Park, Kenilworth, Warwickshire, UK;* ‡*Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK; and* §*ApresLabs Ltd, Research and Innovation Campus, Rothamsted, Harpenden, UK*

Abstract

Honey bees, *Apis mellifera*, are markedly less sensitive to neonicotinoid insecticides containing a cyanoimino pharmacophore than to those with a nitroimino group. Although previous work has suggested that this results from enhanced metabolism of the former by detoxification enzymes, the specific enzyme(s) involved remain to be characterized. In this work, a pretreatment of honey bees with a sublethal dose of thiacloprid resulted in induced insensitivity to the same compound immediately following thiacloprid feeding. A longer pretreatment time resulted in no, or increased, sensitivity. Transcriptome profiling, using microarrays, identified a number of genes encoding detoxification enzymes that were over-expressed significantly in insecticide-treated bees compared with untreated controls. These included five P450s, *CYP6BE1*, *CYP305D1*, *CYP6AS5*, *CYP315A1*, *CYP301A1*, and a carboxyl/cholinesterase (CCE) *CCE8*. Four of these P450s were functionally expressed in *Escherichia coli* and their ability to metabolize thiacloprid examined by liquid chromatography-mass spectrometry (LC-MS) analysis.

Keywords: *Apis mellifera*, induction, P450, esterase, thiacloprid, metabolism.

First published online 20 January 2016.

Correspondence: Graham D. Moores, ApresLabs Ltd, Research and Innovation Campus, Rothamsted, Harpenden, AL5 2JQ, UK. e-mail: graham.moores@apreslabs.co.uk

Introduction

Neonicotinoid insecticides are selective agonists of the invertebrate nicotinic acetylcholine receptor (nAChR), resulting in persistent excitation and ultimately death of the exposed pest insect (Jeschke & Nauen, 2008). Owing to their excellent efficacy in crop protection, high specificity for insects and versatility in application methods they have become the market-leading class of synthetic insecticides (Jeschke *et al*., 2011). There have been concerns, however, over the possible effects of neonicotinoids on nontarget organisms, especially insect pollinators such as honey bees (Goulson, 2013). Seed treatment and soil applications have tended to be the preferred application route for neonicotinoids as they protect young plants via a long-lasting systemic effect, negating the need for foliar spray (Elbert *et al*., 2008). Nontarget arthropods such as bees may be exposed to sublethal doses of these insecticides through both contact and oral routes when they visit plants during foraging activity and consume the affected food source (nectar and pollen; Blacquière *et al*., 2012; Krupke *et al*., 2012). The question of whether the sublethal doses received by pollinators in the field lead to significant impairment in individual and colony performance is a topic of active research and considerable controversy (for a review see Godfray *et al*., 2014).

Cyano-substituted neonicotinoids (thiacloprid and acetamiprid) 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) and bioassays using inhibitors of detoxification enzymes have provided a strong indication that the differential toxicity observed between the two groups of neonicotinoids is a result of increased metabolism of cyano-substituted compounds, rather than intrinsic differences in their affinity for the nAChR (Iwasa *et al*., 2004). The use of synergists has not, however, provided unequivocal evidence as to the primary enzyme system

involved in the enhanced metabolism. It is perhaps more likely to be mediated by cytochrome P450s as pretreatment of honey bees with piperonyl butoxide (inhibitor of P450s and esterases) and other chemically distinct P450 inhibitors was shown to dramatically increase the toxicity of thiacloprid and acetamiprid, whereas no significant differences were observed between bioassays with imidacloprid alone and those pretreated with these inhibitors (Iwasa *et al*., 2004).

It is well known that neonicotinoids can be metabolized by insecticide-resistant insect pests as a result of enhanced expression of specific cytochrome P450s, for example, CYP6CY3 in *Myzus persicae* and CYP6CM1 in *Bemisia tabaci* (Karunker *et al*., 2008; Bass *et al*., 2013). Although honey bees have a relatively low number of CYP genes (46) encoding P450s compared with other insect species (Claudianos *et al*., 2006), Hardstone & Scott (2010) concluded that '(honey bees)...are not a highly sensitive species to insecticides overall, or even to specific classes of insecticides'. The precise P450s involved in the metabolism of cyano-substituted neonicotinoids in honey bees and whether their expression is constitutive or induced on exposure to neonicotinoids are unknown. However, P450 induction by xenobiotics including non-neonicotinoid insecticides has been studied previously. Phenobarbital, a well-known general P450 inducer chemical, failed to induce the expression of any CYP genes in a microarray analysis of honey bees (Johnson *et al*., 2012). By contrast, Kezic̀ *et al*. (1992) reported that benzo(a)pyrene monooxidase activity was induced after exposure of honey bees to benzo-(a)-pyrene (an inducer of human P450 CYP1A1), the pyrethroid insecticide tau-fluvalinate and the miticide cymiazole. More recently, *in vitro* characterization of eight honey bee P450s of the CYP3 clan revealed that three members of the CYP9Q family have the capacity to metabolize the insecticides tau-fluvalinate and coumaphos. Furthermore, the expression of the P450 CYP9Q3 was induced approximately 1.5-fold by tau-fluvalinate and CYP9Q2 by >1.5-fold by bifenthrin (Mao *et al*., 2011). These findings demonstrate that using xenobiotics, particularly insecticides, as inducing factors might increase metabolic activity and allow identification of specific metabolic enzymes from honey bee that are involved in chemical defence.

The aims of this study were to use an induction strategy, in combination with a range of biological, biochemical and genomic approaches, to determine (1) do honey bees have the ability to mount a molecular defence (via gene induction) to a neonicotinoid (thiacloprid) after initial exposure to a sublethal dose that results in a measurable alteration in phenotype to subsequent exposure?; (2) what are the specific detoxification genes, particularly members of the P450 superfamily, induced by exposure to a neonicotinoid (thiacloprid); and (3) do the enzymes induced have the capacity to metabolize the neonicotinoids and explain the differential toxicity of different members of the class?

Table 1. Full dose response bioassays of honey bee against thiacloprid (μg/bee)

Bioassay	LD_5	LD_{50}	CL 95%	Slope ± SE	df	χ^2	No.
Oral	10.1		4.45–16.2	2.29 ± 0.33	13	12.3	150
Contact		61.3	41.0–85.2	2.05 ± 0.32	13	17.3	150

LD_5, lethal dose of thiacloprid to kill 5% of the population; LD_{50}, lethal dose of thiacloprid to kill 50% of the population; CL, confidence limits; df, degrees of freedom; χ^2, chi-square; No., number of bees.

Results

Toxicity bioassays

Full-dose mortality response curves for the oral and contact toxicity bioassays with thiacloprid (Table 1) gave an estimated dose for induction [oral lethal dose of thiacloprid required to kill 5% of the population (LD_5)] of 10 μg/bee and a dose for contact toxicity (LD_{50}) of 61 μg/bee. For the oral toxicity assays the LD_5 was estimated by assuming average consumption (0.055 mg/ml active).

Induction experiment

Bee mortality differed between treatments applied topically (acetone or thiacloprid; $F_{1,52} = 157.73$, $P < 0.001$) and in the timing of topical application following oral pretreatment ($F_{3,52} = 3.45$, $P = 0.023$). There was also an interaction between oral treatment (thiacloprid or acetone) and time ($F_{3,52} = 4.35$, $P = 0.008$). Immediately following a pretreatment time of 24 h, topical application of thiacloprid resulted in increased tolerance compared with the controls (*t*-test, $P = 0.006$). At 48 and 144 h there were no differences between thiacloprid and acetone pretreatment (*t*-test, $P > 0.05$). At 96 h there was a significantly increased sensitivity in the bees pretreated with thiacloprid (*t*-test, $P = 0.043$). There was no significant difference in mortality of bees topically applied with acetone, regardless of whether the oral pretreatment was thiacloprid or acetone (see Table 2, Fig. 1).

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 vs. the nontreated control from the same time point ('survivor' experiment). In the time course experiment 21

Table 2. Comparison of mean mortalities (logit scale) for bees fed either thiacloprid or acetone and subsequently topically applied with either acetone (Ace) or thiacloprid (Thia) at various times postfeeding

	0 h		48 h		96 h		144 h	
Time Topical	Ace	Thia	Ace	Thia	Ace	Thia	Ace	Thia
Difference	0.966	−1.351	0.471	−0.628	−0.419	0.978	0.766	0.789
t-statistic	0.628	−2.884	0.384	−1.199	−0.403	2.075	0.470	1.547
P-value	0.533	−0.006	0.702	0.236	0.688	0.043	0.640	0.128

probes (11 up-regulated and 10 down-regulated), 39 probes (21 up-regulated and 18 down-regulated), 25 probes (18 up-regulated and seven down-regulated) and 13 probes (seven up-regulated and six down-regulated) were identified as encoding sequences significantly differentially expressed between control and treated bees at the 0, 48, 96 and 144 h time points, respectively. In the 'survivor' experiment 95 probes were identified as differentially expressed (57 probes were up-regulated and 38 down-regulated) between treated bees surviving the topical bioassay at 0 h and nontreated controls from the same time point. The full lists of these probes, the genes to which they correspond and the calculated fold-changes are in Supporting Information Table S1. Gene enrichment analysis based on Gene Ontology (GO) revealed the enrichment of a number of GO-terms in the differentially expressed gene sets of each time point (see Figs S1–S5) with terms related to stress response ('innate immune response', 'defense response to bacterium', 'response to oxidative stress', 'antioxidant activity') a common theme observed amongst the time points. A greater number of GO-terms were enriched in the 'survivor' experiment (Fig. S5), with several terms suggestive of enhanced oxidative/P450 activity including 'oxidoreductase activity', 'oxidation-reduction process', 'heme binding' and 'monooxygenase activity'.

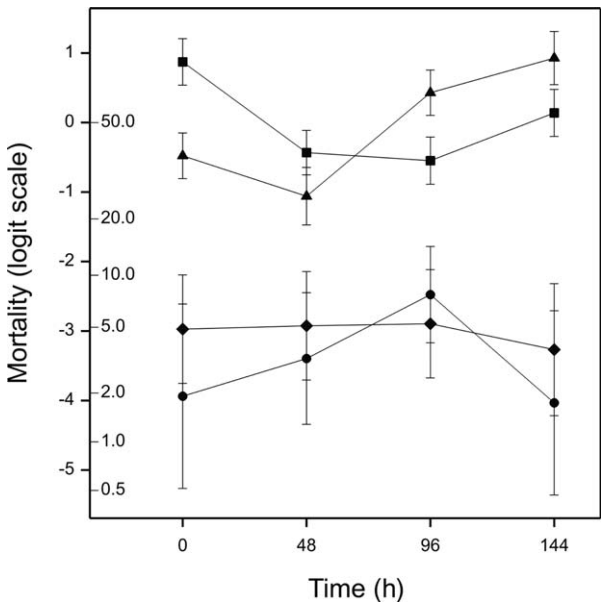

Figure 1. Treatment mean mortalities (logit scale; $n = 1$–3 cages of 9–15 bees) from logistic regression (± SE). Fed acetone, topical acetone (circles); fed thiacloprid, topical acetone (diamonds); fed acetone, topical thiacloprid (squares); fed thiacloprid, topical thiacloprid (triangles). Time = delay following 24 h oral pretreatment. Inner *y*-axis scale represents mortality (%). Mortality was assessed 24 h after topical application.

Amongst the differentially expressed probes were several corresponding to genes with putative roles in insecticide metabolism that are potential candidates to explain the alterations seen in phenotype in the treated bees compared with controls. Thus, in the 0 h comparison two probes representing the P450 gene *CYP315A1* were over-expressed (∼1.5-fold) and in the 48 h comparison a single probe for the gene *cytochrome b5* was over-expressed 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 carboxyl/cholinesterase (*CCE*)*11* were differentially expressed; however, in all cases these were down-regulated (−1.5 to −1.7-fold). At the 144 h time point no probes encoding detoxification enzymes were differentially expressed. The 'survivor comparison' displayed the greatest number of up-regulated probes encoding detoxification genes. This included four P450 genes, four probes for *CYP6BE1* (1.9–2.2-fold), four probes for *CYP305D1* (1.8–1.9-fold), four probes for *CYP6AS5* (1.6–1.7-fold) and a single probe encoding *CYP301A1* (1.6-fold). For esterases five probes encoding *CCE8* were up-regulated 2.1–2.2-fold and for glutathione-s-transferases a single probe encoding the glutathione-S transferase gene (*GSTD1*) was over-expressed 1.9-fold. Finally a single probe representing the gene *cytochrome b5* was over-expressed (1.5-fold).

A number of probes encoding genes associated with the regulation of transcription/signal transduction, which might be involved in the observed induction, were differentially expressed in multiple array comparisons. This included three G-protein-coupled receptor genes (GPCRs) in the survivor comparison (*GB18244-RA*, *GB18304-RA* and *GB17560-RA*), one GPCR related-gene (*GB15369-RA*) at the 0 h time point and one GPCR at the 144 h time point (*GB18786-RA*). Two genes encoding transcription factors (*GB15791-RA* and *GB10501-RA*) were identified as differentially expressed at the 0 h time point, one at the 48 h time point (*GB14951-RA*) and two at the 96 h time point (*GB18833-RA*, *GB12301-RA*).

The expression levels of seven of the detoxification candidate genes from the microarray experiment were validated by qPCR with excellent concordance between fold-changes calculated using the qPCR and array data (see Fig. 2). The qPCR experiments provided

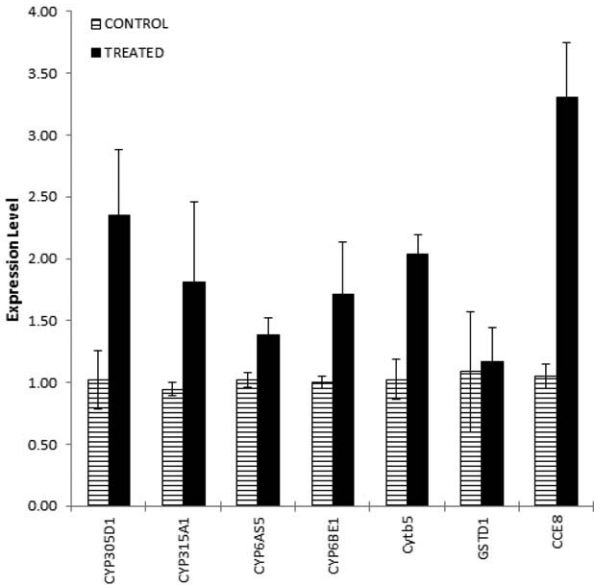

Figure 2. Expression levels determined with qPCR for selected genes using the geometric mean of selected housekeeping genes (elongation factor-1 (*ef1*) and *actin*) (error bars show 95% confidence limits).

confirmation that six of the seven candidate genes were significantly up-regulated in treated bees compared with controls with the exception being the GSTD1, which was eliminated as a potential candidate.

Heterologous expression of candidate genes

For the genes (*CYP305D1*, *CYP315A1*, *CYP6AS5*, *CYP6BE1* and *cytochrome b5*) confirmed as being up-regulated by qPCR, heterologous expression focused on exploring the functional role of the P450s in insecticide detoxification. In order to produce catalytically active P450s, the candidate genes were co-expressed with an *Anopheles gambiae* Cytochrome P450 reductase (CPR) in *Escherichia coli* as previously described (McLaughlin *et al.*, 2008). Variation was observed in the yield of recombinant P450 (Table 3); however, reduced CO-difference spectra suggested correctly folded and active enzyme as indicated by significant peaks at 450 nm and lesser 420 nm peaks (Fig. S6). The cytochrome b5 was also successfully expressed and purified from *E. coli* membranes.

Table 3. 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

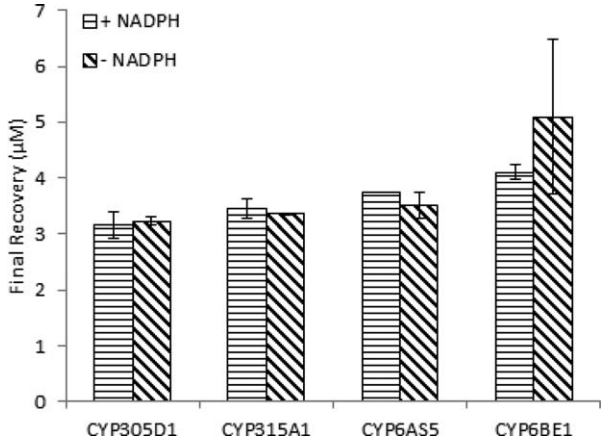

Figure 3. Recovery of thiacloprid after a 2 h incubation with the four honeybee P450s. Graph represents mean final recovery (μM) ± SE ($n = 3$).

Metabolism assays

The ability of CYP305D1, CYP315A1, CYP6AS5 and CYP6BE1 proteins (in combination with the *An. gambiae* CPR and bee cytochrome b5) to detoxify thiacloprid and imidacloprid in the presence and absence of NADPH was examined in insecticide metabolism assays. Figs 3 and 4 outline the results of monitoring thiacloprid and imidacloprid recovery in the samples using selected-reaction-monitoring (SRM) methods, with quantification against standard calibration curves. No significant differences were observed in thiacloprid or imidacloprid recoveries between the +/– NADPH samples for any of the four P450s.

Discussion

Honey bees display profound differences in their susceptibility to different neonicotinoid insecticides, being

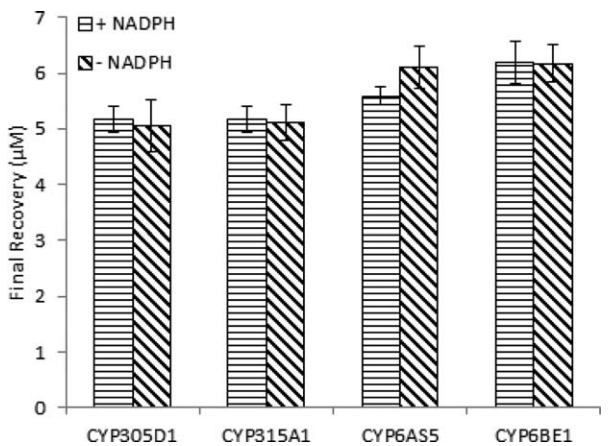

Figure 4. Recovery of imidacloprid after a 2 h incubation with the four honeybee P450 expression systems. Graph represents mean final recovery (μM) ± SE ($n = 3$).

considerably less sensitive to cyano-substituted neonicotinoids such as thiacloprid. Research to date has provided strong indications that this is because of an innate ability of the bees to metabolize neonicotinoids containing a cyano pharmacophore; however, the precise metabolic enzymes involved and whether their expression is constitutive or induced upon exposure to neonicotinoids was unknown. The main aim of the present study was to address these two questions by feeding bees a sublethal dose of thiacloprid and assessing (1) changes in thiacloprid sensitivity in bioassays and (2) changes in gene expression in whole transcriptome microarrays.

The bioassay time course experiment showed that a measurable reduction in thiacloprid sensitivity could be induced in honey bees after exposure to a sublethal dose of this neonicotinoid for 24 h. This effect was time dependent, with increased tolerance only observed immediately following the 24 h of thiacloprid feeding, and treated bees becoming more susceptible than controls at the later time point (96 h). To our knowledge this is the first report with honey bees of a sublethal dose of an insecticide providing a protective effect to subsequent exposure of the same insecticide. Indeed, in a related study on honey bees, no effect on the toxicity of the pyrethroid insecticide tau-fluvalinate was observed in bees fed phenobarbital, xanthotoxin, salicylic acid or indole-3-carbinol compared with controls fed sucrose, although the effect of tau-fluvalinate feeding on subsequent tau-fluvalinate toxicity was not examined (Johnson *et al*., 2012).

One possible explanation for our finding of an induced decrease in sensitivity to thiacloprid in bees is that the sublethal exposure activates the transcription of one or more genes encoding detoxification/defence proteins over the 0–48 h time points and that these subsequently return to constitutive levels or lower than constitutive levels at the later time points. To explore this we carried out a series of microarray comparisons of global gene expression levels in treated vs. control bees over the time series ('time course experiment') and a second experiment in which treated bees surviving the topical bioassay at 0 h were compared with nontreated controls from the same time point ('survivor' experiment). Across all comparisons the number of genes differentially expressed (13–95 probes representing 0.08–0.6% of the 15 737 probes on the array) and the fold changes observed (<8-fold) between treated and control bees were low. Nevertheless, the observed changes were subsequently confirmed by qPCR, with a number of candidate genes being validated as moderately, but significantly, over-expressed in treated bees. GO-term analysis of these differentially expressed genes revealed enriched ontology terms associated with a general stress response and also terms relating to P450-mediated detoxification, the latter resulting from the enhanced expression of a number of probes encoding several honey bee P450s/cytochrome b5. P450 genes were only identified in the earlier time points (0 h, 0 h survivors, 48 h) where altered thiacloprid toxicity was observed in bioassays and the only gene related to detoxification observed in two separate array experiments was cytochrome b5, which can act as an electron donor to P450s. Amongst the CYP genes *CYP315A1* was the only P450 identified as over-expressed in the time course experiment (at the 0 h time point) and this is the orthologue of the *Drosophila melanogaster sad* gene encoding the steroid 2-hydroxylase (Claudianos *et al*., 2006). However, in the 'survivor' experiment in which 'treated bees' were fed thiacloprid for 24 h and then survived a subsequent topical application of thiacloprid (LD_{50}), a number of P450s were identified as being over-expressed. This included two members of the CYP3 clade, *CYP6BE1* and *CYP6AS5*, whose members have been most commonly involved in detoxification of xenobiotics including pesticides in other insects (Li *et al*., 2007). Two further P450s, *CYP305D1* and *CYP301A1*, the latter of which was only represented by a single probe, which belong to the CYP2 and mitochondrial clades, respectively, were also over-expressed. The role of *CYP305D1* is yet to be determined but *CYP301A1* is thought to be involved in ecdysone regulation during adult cuticle formation (Sztal *et al*., 2012). Beyond detoxification genes, several genes involved in the regulation of transcription/signal transduction were also identified as differentially expressed in multiple array comparisons, including both transcription factors and a number of GPCRs. It is possible that these genes may play a role in triggering/regulating the enhanced transcription of the CYP/detox genes. In the case of GPCRs recent work has suggested they may be involved in regulating over-expressed P450s observed in resistant mosquitoes, *Culex quinquefasciatus*, and housefly, *Musca domestica* (Li *et al*., 2013, 2014). For *C. quinquefasciatus* knockdown of four GPCR genes by RNA interference both decreased resistance to permethrin and repressed the expression of four insecticide-resistance related P450 genes (Li *et al*., 2014). It would be interesting to examine the role of these receptors in honey bee gene expression responses to xenobiotics in more detail using a similar approach.

The four P450s, *CYP6BE1, CYP6AS5, CYP315A1* and *CYP305D1*, all of which were represented by multiple over-expressed probes in array comparisons and validated by qPCR, were functionally expressed in *E. coli* in combination with the *An. gambiae* P450 reductase to examine their potential to metabolize thiacloprid. Honey bee cytochrome b5 was also expressed and included in metabolism assays as this enzyme has been

show to modify the catalytic activity of P450s in other insect systems. Although functional P450 proteins were obtained for all four CYP genes, no metabolism of thiacloprid (as assessed by parent compound depletion after incubation of thiacloprid with recombinant P450 in the presence of NADPH) was observed for any of the four P450s. These findings suggest that the P450s induced in our experiments do not have the ability to detoxify thiacloprid and if innate bee tolerance to this compound is indeed mediated by P450s, their expression may be constitutive and hence would not have been detected in our experiments. Alternatively a different enzyme system, such as esterases, may be responsible for thiacloprid metabolism/sequestration. Indeed, the toxicity of the nitro cyano-substituted neonicotinoid acetamiprid was synergized (synergism ratio of 2.96) by the inhibitor S,S,S,-tributyl phosphorotrithioate, suggesting that esterases may play a contributory role in detoxification (Iwasa *et al.*, 2004). In our 'survivor' array comparison five probes representing the esterase CCE8 were overexpressed and this was confirmed by qPCR. This CCE falls into clade A, classified as intracellular enzymes with dietary/detoxification functions (Claudianos *et al.*, 2006). Attempts to functionally express this esterase resulted in nonfunctional enzyme (data not shown) so we were unable to confirm any role of this enzyme in thiacloprid metabolism.

In summary, a number of genes, including several P450s, were induced in honey bees exposed to a sublethal dose of thiacloprid in the present study and this was associated with a measurable, temporary, reduction in toxicity on subsequent thiacloprid exposure. However, a causative role for these P450s in thiacloprid tolerance could not be demonstrated and the specific enzymes involved in the thiacloprid insensitivity remain to be determined.

Experimental procedures

Insect material

Apis mellifera carnica was provided by the AgroEcology Department, Rothamsted Research. All Rothamsted Research hives were treated with Fumidil B (Ceva Animal Health, Amersham, UK) (an antibiotic to treat Nosema disease) in September, and treated with Apiguard (Vita Europe Ltd, Basingstoke, UK) (to reduce *Varroa* mite) every August and September by the beekeeper. The colonies were checked weekly between April and September, given more boxes/space as required, honey taken off for extraction during the summer, and fed sugar syrup in the autumn to maintain the hive through winter. Frames of sealed brood were collected and incubated at 34°C overnight. Emerged worker bees were used for the bioassays.

Reagents

Restriction enzymes were supplied by Promega (Southampton, UK); oligonucleotides, technical insecticides (PESTANAL) and other analytical grade reagents were obtained from Sigma-Aldrich (Dorset, UK).

Toxicity bioassays

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 anaesthetizing 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 the 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.

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 anaesthetizing with CO_2 (10 bees per cage, at least three replicates per insecticide dosage). For each cage a reservoir of 50% sucrose in water was available for *ad libitum* feeding. Prior to treatment bees were anaesthetized by low exposure to CO_2. Each bee was topically dosed (1 μl) 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 and bees not walking or flying were counted as dead.

Induction experiment

Thiacloprid toxicity was assayed *in vivo* after exposure to a sublethal concentration of thiacloprid to check for measurable alteration in phenotype. A factorial set of 16 treatments was tested in two repeat experiments. Bees (nine to 15 per cage = one replicate, 72 cages in total) were fed either a sublethal 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 time point (0, 48, 96 and 144 h), immediately prior to topical application, two cages of oral fed acetone and two cages of oral fed thiacloprid were snap frozen for microarray analysis as described in the Microarray analysis section (16 cages total for each of experiments 1 and 2). At each of 0, 48, 96 and 144 h a diagnostic dose of thiacloprid (61 μg equating to LD_{50}) in acetone or acetone was then topically applied to at least four cages of oral fed acetone and oral fed thiacloprid to give at least two cages of each possible combination (acetone-acetone, acetone-thiacloprid, thiacloprid-acetone, thiacloprid-thiacloprid) at least 32 cages total for each of experiments 1 and 2 (Fig. 5). Samples of bees surviving the treatment ('survivors') were snap frozen and stored at −80°C for subsequent molecular analyses as described in the Microarray analysis section.

Microarray analysis

A custom microarray designed using the Agilent eArray platform (Agilent Technologies, Palo Alto, CA, USA) contained 60-bp oligonucleotide probes for each of the honey bee consensus gene sets (~10 000 genes) derived from the annotated honey bee

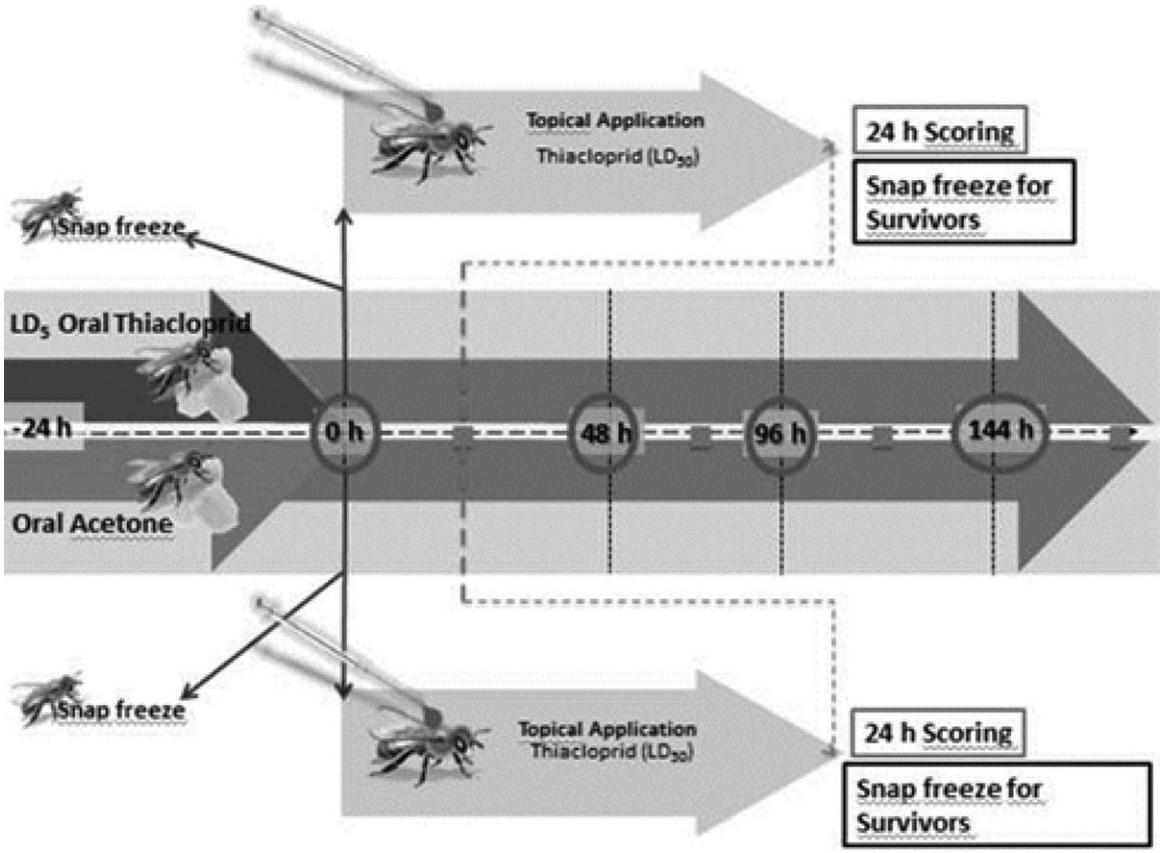

Figure 5. Experimental design. Honey bees were fed either thiacloprid at an estimated lethal dose of thiacloprid required to kill 5% of the population (LD_5) or acetone (time −24 to 0 h). At the times given in the circles (0, 48, 96, 144 h after oral feeding finished) topical application (at estimated LD_{50} dosage or acetone) took place; concurrently, separate cages were taken for microarray analysis. Mortality assessment (scoring) took place 24 h after topical application and survivors were snap frozen.

genome. A SurePrint HD (8 × 15 k) 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. Three probes were designed for each contig encoding a detoxification enzyme (P450s, GSTs and CCEs). 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 an Isolate RNA Mini Kit (Bioline, London, UK) according to the product manual. The quantity of RNA was checked using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and by running an aliquot on a 1.5% agarose gel. For the latter, RNA was mixed with 1× loading buffer (95% formamide; 0.025% xylene cyanol; 0.025% bromophenol blue; 18 mM ethylenediaminetetraacetic acid; 0.025% sodium dodecyl sulphate), heated for 5 min at 65°C and briefly chilled on ice prior to loading. Two micrograms of each RNA was used to generate labelled cRNA, which was hybridized 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, hybridizations were carried out 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 (Agilent Technologies, Cheshire, UK). For statistical analysis, a *t*-test with a 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.

Quantitative PCR

Primers were designed to amplify a fragment ∼100 bp using the Primer3 (Thornton & Basu, 2011) program (Table 4). 4 μg of RNA was used for reverse transcription using Superscript II Reverse Transcriptase and random hexamers (Invitrogen, Carlsbad, CA, USA). Each PCR reaction consisted of 4 μl cDNA (10 ng), 5 μl 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, Cambridge, UK) with the following cycling conditions: 10 min 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 to 95°C by 1°C every 5 s) to confirm the absence of any nonspecific

Table 4. Oligonucleotide primer sequences used in qPCR

Gene	Accession no.	Primer	Sequence (5′–3′)	Product size (bp)
CYP305D1	GB11943	Forward	GGACGTCCTTGGAACGAAT	108
		Reverse	TCGCATCATCCAATTTCGTA	
CYP315A1	GB16447	Forward	CTGGGTCCCGTTTACAAAGA	101
		Reverse	GGTGTTGACCCTTCAAGTCG	
CYP6AS5	GB17434	Forward	CAGGCTCTCCCCAATATTCA	120
		Reverse	TCGATGGGCTCATTTTTCTC	
CYP6BE1	GB14612	Forward	CGAAAGGAACTTGCATAGCC	120
		Reverse	TCTTCGGAAAATCGTTCTGG	
Cytb5	GB12288	Forward	CAGCGGAAGAAGTAGCGAAA	101
		Reverse	GCCTGGATGTTCGCTTAGAA	
GSTD1	GB18045	Forward	AAAAATGCTTGTTATTTTCTGTCTGA	110
		Reverse	TCAAACGCGTCTTCGAGTATC	
CCE8	GB11064	Forward	TCTGCTTGCGCATTCTATTG	106
		Reverse	CTTTACGCGCTTCTTTGTCC	

amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution from 100 to 0.01 ng of cDNA. Each qPCR experiment consisted of at least three independent biological replicates with two technical replicates. Data were analysed according to the delta delta cycle threshold method (Pfaffl, 2001), using the geometric mean of two selected housekeeping genes, elongation factor-1 (*ef1*) and actin, for normalization according to a strategy described previously (Vandesompele *et al.*, 2002).

Heterologous expression of candidate genes

Cloning CYPs

The candidate honey bee P450s (CYP305D1, CYP315A1, CYP6AS5, CYP6BE1) were amplified from cDNA using KAPA high-fidelity DNA Polymerase (Kapa Biosystems, London, UK) 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, AP) were added to the 5′ end of the 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 (Kapa Biosystems, London, UK) 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 *Nde*I restriction site using the primers described in Table 5. 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 5) to generate the full-length ompA-AP-CYP coding sequence flanked by *Nde*I and *Xba*I restriction sites. The final product was digested and ligated into modified pCW-ori+ vector via *Xba*I and *Nde*I restriction sites and the final sequence were confirmed by sequencing prior to expression.

Preparation of membranes

Competent *E. coli* JM109 cells were co-transformed with pCW-ori+CYPs and pACYC-AgCPR to enable co-expression of each CYP with the *An. gambiae* CPR following the methods described by Stevenson *et al.* (2011). Plasmids were transformed into JM109 cells and overnight cultures in 200 ml terrific broth (TB) incubated at 30°C with shaking. When the cultures reached early log phase growth, expression was induced by adding 1 mM IPTG (Isopropyl beta-D-1-thiogalactopyranoside). 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 h before the cells were harvested by centrifugation and membranes prepared as described by

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

CYPs*	Reverse primer†	CYP-specific reverse primer‡
ompA-AP-CYP305D1	ACTATTAACATTATAACAAACAT X	GAATTCTCTAGATTATCGTTTTTCAACTAATACA
ompA-AP-CYP315A1	AATATTTTGCGCAAGATTCAT X	GAATTCTCTAGACTAATTTCTCTCCATCAATTT
ompA-AP-CYP6AS5	AATTTCGAAACTGCTCGCCAT X	GAATTCTCTAGATCATATTTTTGTTATTTTCAAATA
ompA-AP-CYP6BE1	TAACCACGTAGTTAAAAACAT X	GAATTCTCTAGATTATATTGGCTCAATATTTAGA

*The forward primer used for the ompA+2 fusion PCR strategy was always 5′-GGAATTCCATATGAAAAAGACAGCTATCGCG-3′ with the *Nde*I restriction site (underlined).

†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.

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

Pritchard *et al*. (1998). Membranes were resuspended in ice-cold 1× TSE (50 mM Tris-acetate, pH 7.6, 250 mM sucrose, 0.25 mM EDTA) buffer in a Dounce tissue homogenizer and aliquots stored at −80°C. P450 content (Omura & Sato, 1964), total protein concentration (Bradford, 1976) and CPR content (Strobel & Dignam, 1978) were analysed.

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**ATGTCGAAAAT TTTTACAGCGGA-3′ (*Nde*I restriction site underlined and six histidine codons in bold before start codon) and reverse primer 5′-GAATTCTCTAGATTATGAATACCAAAAATAGTAAA AT-3′ (*Xba*I restriction site underlined). The final product was digested and ligated into the modified pCW-ori+ vector via the *Xba*I and *Nde*I restriction sites with the final sequences confirmed by sequencing prior to expression.

Expression and purification of cytochrome b5

The 6H-b5 construct was transformed into JM109 cells and after overnight culture was transferred to 500 ml TB media containing 50 μg/ml ampicillin in a 1 l 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 Holmans *et al*.'s (1994) method with Stevenson *et al*.'s (2011) modifications. Expression of b5 was checked and quantified by spectrophotometry (Omura & Sato, 1964).

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. 10 mM stock solutions of thiacloprid and of imidacloprid were prepared in dimethyl sulphoxide 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°C, shaken at 1200 rpm for 2 h and then stopped by adding 300 μl acetonitrile. Samples were then spun at 2000 *g* for 5 min and 250 μl supernatant was transferred to HPLC vials and stored at −20°C for LC-MS/MS analysis.

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, Elstree, UK) using an ACQUITY UPLC column (HSS T3, 1.8 μm, 100 × 2.1 mm), with a mobile phase consisting of water (+0.2% formic acid) and 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.

Table 6. Selected-reaction-monitoring (SRM) transitions and collision energies

Analyte	Molecular weight (Da)	SRM transition Parent *m**z* > product *m**z*	Retention time (min)
Thiacloprid	254	253 > 126 253 > 186	4.83
Imidacloprid	257	256 > 175 256 > 209	4.47

The mass spectrometer was a Finnigan TSQ Quantum Discovery (Thermo Scientific, Loughborough, UK) equipped with an Ion Max source operating in positive ion mode. Analytes were detected using SRM, with transitions as outlined in Table 6. Quantification was achieved using standard calibration curves constructed in 50:50 acetonitrile : water.

Statistical analysis

Table 1 shows the results of probit analysis of the data from full dose oral and contact bioassays using the statistical program PC Polo Plus (LeOra Software, Berkeley, CA, USA). The LD_5 (oral) and LD_{50} (contact) concentrations were estimated after correcting for control mortality (Abbott 1925; Finney 1971). Data from the induction bioassays were analysed in GenStat (14th edition, VSN International, Hemel Hempstead, UK) using logistic regression (ie a generalized linear model with binomial error and logit link), allowing for differences between experiments before testing treatment effects and with adjustment for overdispersion.

Acknowledgements

This project was partially funded by AHDB Cereals and Oilseeds, a division of the Agriculture and Horticulture Development Board, UK. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the UK.

References

Abbott, W.S. (1925) A method for computing the effectiveness of an insecticide. *J Econ Entomol* **18**: 265–267.

Bass, C., Zimmer, C.T., Riveron, J.M., Wilding, C.S., Wondji, C.S., Kaussmann, M. *et al*. (2013) Gene amplification and microsatellite polymorphism underlie a recent insect host shift. *Proc Natl Acad Sci USA* **110**: 19460–19465.

Blacquière, T., Smagghe, G., Van Gestel, C.A.M. and Mommaerts, V. (2012) Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21**: 973–992.

Bradford, M.M. (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R. *et al*. (2006) A deficit of detoxification enzymes: pesticides sensitivity and environmental response in the honeybee. *Insect Mol Biol* **15**: 615–636.

Elbert, A., Haas, M., Springer, B., Thielert, W. and Nauen, R. (2008) Applied aspects of neonicotinoid uses in crop protection. *Pest Manag Sci* **64**: 1099–1105.

Finney, D.J. (1971) *Probit Analysis*. Cambridge University Press, London.

Godfray, H.C., Blacquière, T., Field, L.M., Hails, R.S., Petrokofsky, G., Potts, S.G. *et al*. (2014) A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc Biol Sci* **281**: 20140558.

Goulson, D. (2013) An overview of the environmental risks posed by neonicotinoid insecticides. *J Appl Ecol* **50**: 977–987.

Hardstone, M.C. and Scott, J.G. (2010) Is *Apis mellifera* more sensitive to insecticides than other insects? *Pest Manag Sci* **66**: 1171–1180.

Holmans, P.L., Shet, M.S., Martin-Wixtrom, C.A., Fisher, C.W. and Estabrook, R.W. (1994) The high-level expression in *Escherichia coli* of the membrane-bound form of human and rat cytochrome b5 and studies on their mechanism of function. *Arch Biochem Biophys* **312**: 554–565.

Iwasa, T., Motoyama, N., Ambrose, J.T. and Roe, R.M. (2004) Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot* **23**: 371–378.

Jeschke, P. and Nauen, R. (2008) Neonicotinoids - from zero to hero in insecticide chemistry. *Pest Manag Sci* **64**: 1084–1098.

Jeschke, P., Nauen, R., Schindler, M. and Elbert, A. (2011) Overview of the status and global strategy for neonicotinoids. *Agric Food Chem* **59**: 2897–2908.

Johnson, R.M., Mao, W., Pollock, H.S., Niu, G., Schuler, M.A. and Berenbaum, M.R. (2012) Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. *PLoS ONE* **7**: e31051 doi:10.1371/journal.pone.0031051.

Karunker, I., Benting, J., Lueke, B., Ponge, T., Nauen, R., Roditakis, E. *et al*. (2008) Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Insect Biochem Mol Biol* **38**: 634–644.

Kezić, N., Lucić, D. and Sulimanović, D. (1992) Induction of mixed function oxidase activity in honey bee as a bioassay for detection of environmental xenobiotics. *Apidologie* **23**: 217–223.

Krupke, C.H., Hunt, G.J., Eitzer, B.D., Andino, G. and Given, K. (2012) Multiple routes of pesticide exposure for honey bees living near agricultural fields. *PLoS ONE* **7**: e29268. doi: 10.1371/journal.pone.0029268.

Li, M., Reid, W.R., Zhang, L., Scott, J.G., Gao, X., Kristensen, M. *et al*. (2013) A whole transcriptomal linkage analysis of gene co-regulation in insecticide resistant house flies, *Musca domestica*. *BMC Genomics* **14**: 803.

Li, T., Liu, L., Zhang, L. and Liu, N. (2014) Role of G-protein-coupled receptor-related genes in insecticide resistance of the mosquito, *Culex quinquefasciatus*. *Sci Rep* **4**: 6474. doi: 10.1038/srep06474.

Li, X., Schuler, M.A. and Berenbaum, M.R. (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52**: 231–253.

Mao, W., Schuler, M.A. and Berenbaum, M.R. (2011) CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). *Proc Natl Acad Sci USA* **108**: 12657–12662.

Mclaughlin, L.A., Niazi, U., Bibby, J., David, J.-P., Vontas, J., Hemingway, J. *et al*. (2008) Characterization of inhibitors and substrates of *Anopheles gambiae* CYP6Z2. *Insect Mol Biol* **17**: 125–135.

Omura, T. and Sato, S.R. (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: E45.

Pritchard, M., Ossetian, R., Li, D.N., Henderson, C.J., Burchell, B., Wolf, C.R. *et al*. (1997) A general strategy for the expression of recombinant human cytochrome P450s in *Escherichia coli* using bacterial signal peptides: expression of CYP3A4, CYP2A6, and CYP2E1. *Arch Biochem Biophys* **345**: 342–354.

Pritchard, M.P., Glancey, M.J., Blake, J.A., Gilham, D.E., Burchell, B., Wolf, C.R. *et al*. (1998) Functional co-expression of CYP2D6 and human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Pharmacogenetics* **8**: 33–42.

Pritchard, M.P., McLaughlin, L. and Friedberg, T. (2006) Establishment of functional human cytochrome P450 monooxygenase systems in *Escherichia coli*. *Methods Mol Biol* **320**: 19–29.

Stevenson, B.J., Bibby, J., Pignatelli, P., Muangnoicharoen, S., O'Neill, P.M., Lian, L.Y. *et al*. (2011) Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. *Insect Biochem Mol Biol* **41**: 492–502.

Strobel, H.W. and Dignam, J.D. (1978) Purification and properties of NADPH-cytochrome P-450 reductase. *Methods Enzymol* **52**: 89–96.

Sztal, T., Chung, H., Berger, S., Currie, P.D., Batterham, P. and Daborn, P.J. (2012) A cytochrome P450 conserved in insects is involved in cuticle formation. *PLoS ONE* **7**: e36544.

Thornton, B. and Basu, C. (2011) Real time PCR (qPCR) primer design using free online software. *Biochem Mol Biol Educ* **39**, 145–154.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Roy, N.V., De Paepe, A. *et al*. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**: research0034.1–0034–research0011.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figures S1–S5. Enriched Gene Ontology (GO) terms in genes differentially expressed in each microarray comparison.

Figure S6. Reduced CO-difference spectra of recombinant *CYP6BE1*, *CYP6AS5*, *CYP315A1* and *CYP305D1*.

Table S1. Lists of differentially expressed probes in all microarray experiments. The genes to which they correspond, *P*-values and the calculated fold-changes are detailed.