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# Pesticide risk assessment at the molecular level using honey bee cytochrome P450 enzymes: A complementary approach

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ABSTRACT

Honey bee (*Apis mellifera*) first-tier pesticide risk assessment is largely based on standardized laboratory toxicity bioassays after both acute and chronic exposure. Recent research on honey bee cytochrome P450 monooxygenases (P450s) uncovered CYP9Q3 as the molecular determinant mediating neonicotinoid insecticide selectivity and explaining why certain neonicotinoids such as thiacloprid show > 1000-fold lower acute toxicity than others (e.g. imidacloprid). Here this knowledge is leveraged for mechanistic risk assessment at the molecular level using a fluorescence-based high-throughput *in vitro* assay, predicting the interaction of diverse pesticidal chemotypes, including azole fungicides, with recombinantly expressed honey bee CYP9Q enzymes, known to metabolize thiacloprid, acetamiprid and *tau*-fluvalinate. Some azole fungicides were shown to be synergistic in combination with certain insecticides, including neonicotinoids and pyrethroids, whereas others such as prothioconazole were not. We demonstrate that biochemical CYP9Q2/CYP9Q3 inhibition data of azoles revealed a striking correlation with their synergistic potential at the organismal level, and even allow to explain combined toxicity effects observed for tank mixtures under field conditions. Our novel toxicogenomics-based approach is designed to complement existing methods for pesticide risk assessment with unprecedented screening capacity, by utilizing honey bee P450 enzymes known to confer pesticide selectivity, in order to biochemically address issues of ecotoxicological concern.

## 1. Introduction

Pollination of wild and cultivated plants is an indispensable service provided by a diverse range of free-living organisms and commercially managed bee species such as the western honey bee (*Apis mellifera* L.), the most important managed crop pollinator globally (Klein et al., 2007; Potts et al., 2016). While global agriculture is expected to see an increase in pollination-dependent production (Aizen et al., 2008), parts of the world are facing a decrease in bee abundance and diversity (Potts et al., 2010), raising concern about a possible short-coming of pollination services. Potential factors disproportionately contributing to insect pollinator decline include parasites, pathogens, climate change, habitat loss, diseases and pesticides (Goulson et al., 2015; Potts et al., 2016). Particularly risks related to unintended bee pollinator exposure to insecticides, applied by farmers to keep destructive crop pests under economic damage thresholds, is of major concern (Benuszak et al., 2017; Gill et al., 2012; Johnson, 2015). Many insecticides are acutely toxic and have side-effects on honey bees, especially those addressing neuronal target sites such as voltage-gated and ligand-gated ion channels, which are known to be rather conserved among insects.

A strong research focus in terms of bee safety issues is on neonicotinoids (Connolly, 2013; Cressey, 2017; Godfray et al., 2014; Lundin et al., 2015; Stanley et al., 2015), a systemic class of at least seven commercial insecticides globally used to control some of the world's most devastating pests such as virus-transmitting whiteflies and aphids (Jeschke et al., 2011; Jeschke and Nauen, 2008). Neonicotinoid insecticides are agonists selectively targeting insect nicotinic acetylcholine receptors (nAChR) – located in the central nervous system – by reversibly binding to the orthosteric site (Casida, 2018; Jeschke et al., 2013). Insect nAChR across species are conserved with regard to high-affinity neonicotinoid binding (Taillebois et al., 2018), including honey bees (Nauen et al., 2001). However, earlier studies revealed that neonicotinoids show marked differential toxicity to honey bees after acute contact exposure (Iwasa et al., 2004). Some of them such as clothianidin, dinotefuran, imidacloprid and thiamethoxam are highly toxic in acute toxicity tests conducted according to OECD guidelines, a

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regulatory requirement in a tiered risk assessment approach (OECD, 1998a, 1998b; US EPA, 2014). Whereas, cyanoimine neonicotinoids such as thiacloprid and acetamiprid are considered practically non-toxic to honey bees based on standardized acute toxicity tests (Casida, 2018; Iwasa et al., 2004); the classification "practically non-toxic" is based on $LD_{50}$-values ≥ 11 μg/bee according to EPA guidance for assessing pesticide risks to bees (US EPA, 2014). Neurotoxic insecticides including neonicotinoids are also known to be able to induce sublethal effects in bee pollinators with implications for e.g. cognitive functions, olfaction, gustation, orientation and foraging behavior (Belzunces et al., 2012; Blacquière et al., 2012).

An earlier study reviewed the sensitivity of honey bees to 62 insecticides from several mode of action classes, including neonicotinoids, and concluded that honey bees were not more sensitive to insecticides (Hardstone and Scott, 2010) in comparison to other insect species, despite the fact that the xenobiotic detoxification capacity of honey bees rely on a rather limited detoxification gene inventory (Berenbaum and Johnson, 2015; Claudianos et al., 2006; Gong and Diao, 2017). The most important enzyme superfamily involved in oxidative xenobiotic detoxification in insects, including honey bees, is the cytochrome P450 monooxygenases (P450s) (Berenbaum and Johnson, 2015; Dermauw et al., 2020; Gong and Diao, 2017). The honey bee P450s CYP9Q2 and CYP9Q3 were recently identified as the molecular determinants of bee selectivity towards the neonicotinoids thiacloprid and acetamiprid (Manjon et al., 2018). Both P450s are highly expressed in honey bee brain and Malpighian tubules, the insect equivalent of mammalian kidney, and readily detoxify thiacloprid and acetamiprid (but not imidacloprid) by hydroxylation and *N*-demethylation, respectively (Manjon et al., 2018).

This finding opens novel molecular options for, a) mechanistic pesticide risk assessment in honey bees using individual P450s (López-Osorio and Wurm, 2020), particularly investigating the impact of mixture partners such as azole fungicides, known to inhibit P450s (Berenbaum and Johnson, 2015; Egbuta et al., 2014; Gong and Diao, 2017; Iwasa et al., 2004), and b) to biochemically explore (un)known field-relevant synergistic insecticide/fungicide interactions of ecotoxicological concern (Carnesecchi et al., 2019; Johnson et al., 2013; Robinson et al., 2017; Wernecke et al., 2019), similar to drug-drug interaction (DDI) studies conducted in the pharmaceutical industry with human CYP3A4 and other P450s to exclude adverse effects of new chemical entities (Fowler and Zhang, 2008; Kosaka et al., 2017; Wang et al., 2014). In an ecotoxicological context such a molecular toxicogenomic approach to investigate the P450-mediated detoxification of single compounds and adverse pharmacokinetic interactions of mixtures would complement established procedures in bee pollinator pesticide risk assessment.

The objectives of the present study were (1) to develop a simple and rapid fluorescence based screening method for the kinetic analyses of metabolic substrate interaction with recombinantly expressed honey bee CYP9Q2/3, (2) to explore the synergistic potential and inhibitory action of common azole fungicides on cyanoimine neonicotinoid toxicity *in vivo* and CYP9Q2/3 *in vitro*, respectively, and (3) to investigate whether the molecular approach could provide a first line of evidence for potential synergistic toxicity effects described for neonicotinoid / fungicide tank mixtures.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals, technical pesticides and reagents used were of analytical grade and include: prothioconazole (CAS 178928-70-6, ≥99%, Sigma Aldrich PESTANAL® analytical standard), propiconazole (CAS 60207-90-1, ≥99%, Sigma Aldrich PESTANAL® analytical standard), prochloraz (CAS 67747-09-5, ≥98%, Sigma Aldrich PESTANAL® analytical standard), azoxystrobin (CAS 131860-33-8, ≥98%, Sigma Aldrich PESTANAL® analytical standard), epoxiconazole (CAS 133855-98-8, ≥99%, Sigma Aldrich PESTANAL® analytical standard), uniconazole (CAS 83657-22-1, ≥98, Sigma Aldrich PESTANAL® analytical standard), triflumizole (CAS 68694-11-1, ≥99%, Sigma Aldrich PESTANAL® analytical standard), triadimefon (CAS 43121-43-3, ≥99%, Sigma Aldrich PESTANAL® analytical standard), thiacloprid (CAS 111988-49-9, ≥99%, Sigma Aldrich PESTANAL® analytical standard), imidacloprid (CAS 138261-41-3, >98%, Dr. Ehrenstorfer GmbH), acetamiprid (CAS 160430-64-8, Sigma Aldrich PESTANAL® analytical standard), thiamethoxam (CAS 153719-23-4, ≥99%, Sigma Aldrich PESTANAL® analytical standard), 7-benzyloxy-4-trifluoromethylcoumarin (≥99% Sigma Aldrich), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, CAS 6381-92-6, ≥98.5%, Sigma Aldrich), DL-dithiothreitol (DTT, CAS 3483-12-3, ≥99.5%), Zwittergent 3-10 Detergent (CAS 15163-36-7, Sigma Aldrich), L-glutathione oxidized (CAS 27025-41-8, Sigma Aldrich) and β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (CAS 2646-71-1 anhydrous, ≥93%, Sigma Aldrich).

### 2.2. Honey bees and insect cells

Adult worker honey bees (*Apis mellifera* L.) used for toxicity and synergism tests in this study were of mixed age and collected from queen-right colonies, maintained pesticide-free and managed according to standard beekeeping practice. The health status of the colonies was weekly checked by visual inspection. The colonies had not received chemical treatments for at least six months before testing. Worker bees were randomly collected from the honey super of 15 different hives.

Sf9 and High5 insect cell lines were maintained in suspension culture under serum-free conditions at 27 °C, 120 rpm in SF-900 SFM II and Express-Five SFM medium (Thermo Fisher Scientific, MA, USA) containing 10 μg $mL^{-1}$ gentamycin, respectively. Express-Five SFM medium was further supplemented by 18 mM GlutaMAX (Gibco, Thermo Fisher Scientific, MA, USA) and 10 U $mL^{-1}$ heparin

### 2.3. Recombinant expression of honey bee P450s

Functional expression of *A. mellifera* CYP9Q2 (GenBank Accession No.: XP_392000) and CYP9Q3 (Accession No.: XP_006562363) was performed in High5 cells co-infected with *A. mellifera* NADPH-dependent cytochrome P450 reductase (CPR) (Accession No.: XP_006569769.1) using the Bac-to-Bac baculovirus expression system (Thermo Fisher Scientific, MA, USA) as described previously (Manjon et al., 2018). Cells were harvested after 52 h, washed with Dulbecco's phosphate-buffered saline, centrifuged and the cell pellet stored at −80 °C until microsomal membrane preparation according to standard procedures (Janmohamed et al., 2006), with minor changes. Briefly, cell pellets were homogenized for 30 s in ice-cold 0.1 M potassium phosphate buffer, pH 7.6 containing 1 mM EDTA, 1 mM DTT, 200 mM sucrose and one cOmplete™ EDTA-free Protease Inhibitor Cocktail tablet per 50 mL buffer, using a FastPrep-24 5G instrument (MP Biomedicals, Irvine, CA, USA) and centrifuged (10 min, 700*g*, 4 °C). The supernatant is then again centrifuged at 100,000*g* for 1 h at 4 °C and the pellet subsequently resuspended in 0.1 M potassium phosphate buffer (pH 7.6, 1 mM EDTA, 1 mM DTT, 5% glycerol) using a Dounce tissue grinder. Protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as a reference.

### 2.4. Enzyme kinetics and honey bee cytochrome P450 inhibition assays

Michaelis-Menten kinetics for both recombinantly expressed CYP9Q2 and CYP9Q3 were conducted with the recently described probe substrate 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) (Manjon et al., 2018). Assays were performed in flat-back, black 384-well microplates with 50 μL total reaction volume and 4 technical replicates per data point. The chosen assay conditions were optimized for

linearity with time and protein content of 7-hydroxy-4-(trifluoromethyl) coumarin (HC) fluorescent product formation at 20 ± 1 °C. Each reaction consists of 25 µL BFC (final concentration range: 0.2–200 µM) prepared in DMSO and further diluted in 0.1 M potassium phosphate buffer (pH 7.6) plus a competing pesticide at varying concentrations, NADPH (1 mM) and 25 µL enzyme dissolved in 0.1 M potassium phosphate buffer (pH 7.6, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 0.05% (w/v) BSA, 0.01% (w/v) Zwittergent 3–10). Final protein concentration of microsomal preparations was 0.16 mg $mL^{-1}$ corresponding to 4 µg per reaction for CYP9Q3 and 0.32 mg $mL^{-1}$ (8 µg per reaction) for CYP9Q2, as it is less active with the chosen substrate BFC. Controls included reactions without BFC and without NADPH. Reactions were incubated for maximum 60 min (20 min recommended) at 20 ± 1 °C in the dark and stopped by the addition of 50 µL stop solution (45% DMSO, 45% 0.1 M Tris-HCL pH 10, 5 mM glutathione oxidized, 4U $mL^{-1}$ glutathione-reductase from *S. cerevisiae*). P450-mediated product formation (HC) was detected using a microplate reader (Tecan Spark, Tecan Group Ltd., Männedorf, Switzerland, Fig. 1A). The controls lacking NADPH and BFC were subtracted from each data point. A standard curve for the probe substrate BFC was generated using 7-hydroxy-4-(trifluoromethyl)-coumarin (HC) in order to calculate the reaction velocity in pmol HC formed / min × mg protein. The data were analyzed for competitive, non-competitive and mixed-type inhibition by non-linear regression assuming Michaelis-Menten kinetics using GraphPad Prism v8.3 (GraphPad Software Inc., San Diego, CA, USA).

For the determination of $IC_{50}$-values the probe substrate BFC was used at single concentrations around the apparent $K_m$ value, i.e. 10 µM and 40 µM for CYP9Q3 and CYP9Q2, respectively (Figure S1, $K_m$ value = Michaelis-Menten constant, i.e. the substrate concentration at which reaction velocity (V) is half of $V_{max}$; $V_{max}$ = rate of reaction when the enzyme is fully saturated with substrate). Microsomal membrane protein amounts used in the 50 µL reactions were 4 µg and 8 µg for CYP9Q3 and CYP9Q2, respectively. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and a serial dilution ranging from 10 to 0.00064 mM was prepared. Each DMSO dilution was then further diluted in potassium phosphate buffer (pH 7.6) resulting in a final inhibitor concentration range of 50 to 0.0032 µM (in total seven concentrations were tested and replicated four times). The final DMSO concentration was 1% (v/v), except for azoxystrobin and epoxiconazole (2%; to prevent precipitation at the highest testing concentration). Appropriate DMSO controls were included in each measurement. The respective recombinant P450 enzyme was pre-incubated with the inhibitor (20 ± 1 °C) for 10 min

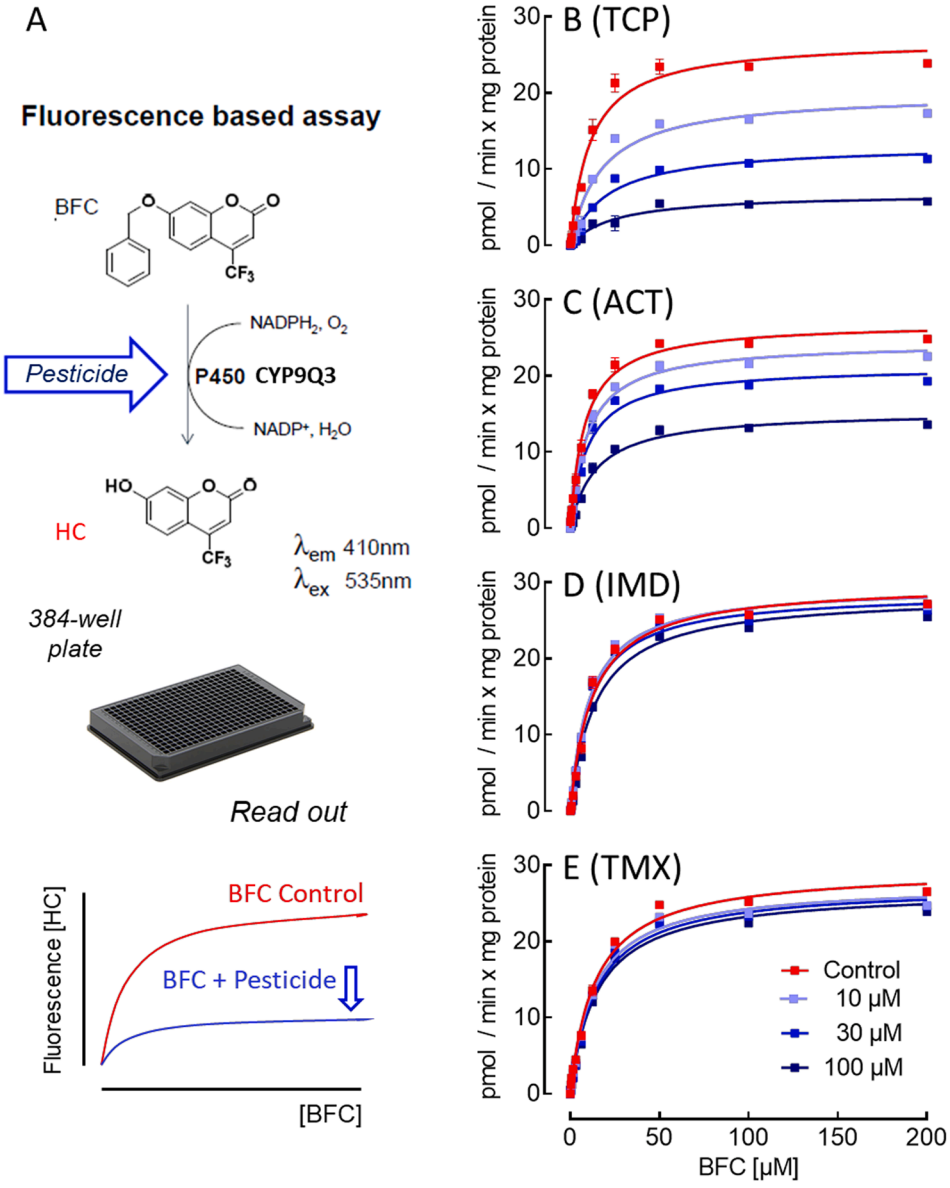


**Fig. 1.** Basic principle (A) of the fluorescence-based screening assay measuring the pesticide mediated inhibition of 7-hydroxy-4-(trifluoromethyl)coumarin (HC) formation by recombinantly expressed honey bee CYP9Q3 incubated with different concentrations of (B) thiacloprid (TCP), (C) acetamiprid (ACT), (D) imidacloprid (IMD) and (E) thiamethoxam (TMX) and the probe substrate 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC). Details on Michaelis-Menten kinetic based data analysis are given in the Supporting Information, Table S1. Data are mean values ± SD (n = 4).

before adding 25 μL of the substrate solution (10 μM BFC (CYP9Q3) and 40 μM BFC (CYP9Q2), respectively, in potassium phosphate buffer, pH 7.6, 0.5 mM NADPH). The reaction was stopped after maximum 60 min incubation (usually 20 min work well) and the formation of 7-hydroxy-4-(trifluoromethyl)-coumarin was measured and calculated as described above. Remaining P450 activity (% of control) was plotted against inhibitor concentration and analyzed using a four-parameter logistic nonlinear fitting routine using GraphPad Prism software v8.3 (GraphPad Software Inc., San Diego, CA, USA).

### 2.5. Honey bee acute contact toxicity and synergism studies

Acute contact toxicity assays were performed according to official OECD guidelines (OECD, 1998b) with slight adaptations to enable to test the hypothesis of synergism by fungicides. Briefly, bees were collected in the morning and kept under test conditions (25 °C, 70% RH) until treatment in the afternoon. Sucrose solution (50% w/v) was provided ad libitum. For fungicide/insecticide synergist bioassays worker bees were anaesthetized with $CO_2$ and treated with 1 μL acetone containing 10 μg of the respective fungicide onto the dorsal thorax one hour prior to insecticide application. Afterwards, bees were again anaesthetized, and the insecticide to be synergized was applied in acetone at different concentrations for dose-response analysis (Table S2). Control bees were treated with acetone only. In synergist bioassays an additional control group was treated with the respective fungicide as pre-treatment followed by acetone 1 h later. Control mortality was <10% in all cases and did not differ between 10 μg fungicide or acetone pre-treatment. Mortality was scored after 24 and 48 h. $LD_{50}$-values and 95% confidence intervals (95% CI) were calculated by probit analysis using PoloPlus 2.0 (LeOra Software, Petaluma, CA, USA). Synergistic ratios (SR) were calculated by dividing the $LD_{50}$-value of the insecticide solo treatment by the $LD_{50}$-value of the insecticide/fungicide combination treatment and was also performed by using the PoloPlus 2.0 software. All bioassays were performed at least twice with three replicates (n = 10 bees) per concentration.

### 2.6. Correlation analysis

$LD_{50}$-values and $IC_{50}$ values were log transformed before Pearson correlation analysis (two-tailed, $\alpha = 0.05$). Additionally, a simple linear regression was performed to generate a best-fit line. Analysis was carried out using GraphPad Prism software v8.3 (GraphPad Software Inc., San Diego, CA, USA)

## 3. Results and discussion

### 3.1. Insecticide interaction with CYP9Q3 employing a fluorescence-based microplate assay

It was recently demonstrated that honey bee CYP9Q3 (and CYP9Q2) – as well as closely related orthologs in other managed bee pollinators – provide protection to the commercial *N*-cyanoimine neonicotinoids thiacloprid and acetamiprid (Beadle et al., 2019; Manjon et al., 2018), complementing earlier findings on the involvement of CYP9Q enzymes in the detoxification of the varroacides *tau*-fluvalinate and coumaphos (Mao et al., 2011). Here, we developed a fluorescence-based microplate assay to rapidly screen compounds for interaction with recombinantly expressed CYP9Q3 (Fig. 1A). The O-dearylation of BFC by CYP9Q3 (resulting in the fluorescent product HC) follows Michaels-Menten kinetics and revealed a $K_m$ value of 11.3 μM (CI95% 10.5–12.1), whereas CYP9Q2 did not strictly follow single substrate binding kinetics with BFC (Figure S1). CYP9Q3 co-incubation of BFC with different concentrations of thiacloprid and acetamiprid strongly interferes with HC formation (Fig. 1B and C), resulting in significantly decreased $V_{max}$ values and increased $K_m$ values, indicating a mixed type of competitive/non-competitive inhibition (Table S1).

No inhibition of BFC metabolism was observed with increasing concentrations of imidacloprid and thiamethoxam (Fig. 1D and E, Table S1), thus supporting previous claims that CYP9Q3 lacks the capacity to metabolize these highly bee toxic neonicotinoids (Manjon et al., 2018). Incubation of CYP9Q3 with different concentrations of thiacloprid at a fixed concentration of 10 μM BFC for different time intervals revealed a depletion of thiacloprid, indicated by a significant time-dependent increase in HC formation ($P < 0.0001$; F 24.12 (5, 154)). The calculated $IC_{50}$ values for thiacloprid shifted significantly from 3.9 μM (CI95%: 2.90–5.22) after ten minutes incubation to 14.1 μM (CI95%: 11.9–16.7) when incubated for 60 min – thus suggesting CYP9Q3-mediated thiacloprid metabolism over time (Fig. 2).

The pyrethroid *tau*-fluvalinate is another insecticide previously described to be detoxified by CYP9Q enzymes (Mao et al., 2011), and indeed, the molecular probe assay revealed competitive inhibition of CYP9Q3-mediated BFC metabolism by increasing concentrations of *tau*-fluvalinate, as demonstrated by a significant increase in $K_m$ value with no significant change in $V_{max}$ (Fig. 3A, Table S1). We further analyzed the observed inhibition pattern by linearizing the Michaelis-Menten equation employing a Hanes-Woolf plot, thus confirming competitive inhibition by *tau*-fluvalinate (Fig. 3B). Whereas the Hanes-Woolf conversion of thiacloprid data revealed an allosteric effect and cooperative behavior - indicating heterotropic interaction between BFC and TCP at the active site of CYP9Q3 (Fig. 3C). The observed differences in substrate interaction may be linked to the variable molecular size of the different compounds (Fig. 3D); *tau*-fluvalinate is much larger than thiacloprid and its molecular size may not allow multiple substrate binding, thus showing fully competitive inhibition. Cooperativity is a common phenomenon also reported for several human P450s involved in drug metabolism such as CYP3A4 (Denisov et al., 2009; Shou et al., 1999). Multiple binding sites are thought to be correlated with broad substrate- and regioselectivity, typical for P450s involved in xenobiotic metabolism (Korzekwa et al., 1998), thus supporting the special role of the CYP9Q enzymes in honey bee defense against rather diverse chemical classes of insecticides. The described assay can discriminate between known substrates and non-substrates as well as between different inhibition patterns and is considered a powerful tool to screen a diverse range of compounds and their interaction with these enzymes.

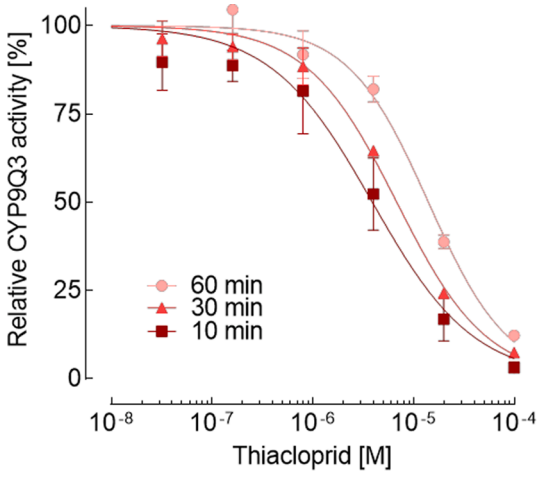


**Fig. 2.** Increase in $IC_{50}$-values for thiacloprid at different elapsed time intervals when incubated with recombinantly expressed honey bee CYP9Q3 suggesting thiacloprid depletion by metabolism. The inhibition of 7-hydroxy-4-(trifluoromethyl)coumarin generation was measured by using the probe substrate BFC at 10 μM. Data are mean values ± SD (n = 4). The calculated $IC_{50}$-values of 3.9 μM (CI95%: 2.90–5.22), 6.82 μM (CI95%: 6.08–7.65) and 14.1 μM (11.9–16.7) after 10, 30 and 60 min are significantly different for each data set ($P < 0.0001$; F 24.12 (5, 154)).

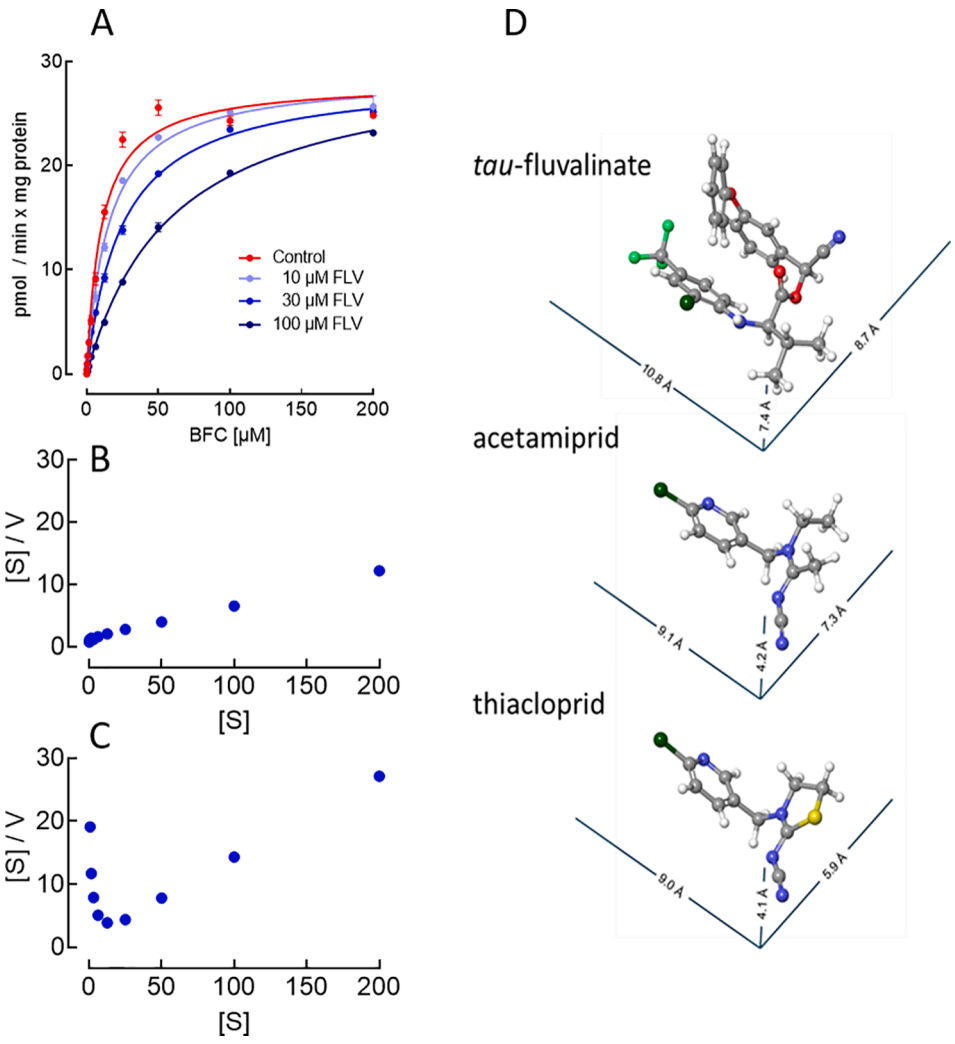


**Fig. 3.** Fluorescence-based inhibition assay measuring the pesticide mediated inhibition of 7-hydroxy-4-(trifluoromethyl)coumarin (HC) formation by recombinantly expressed honey bee CYP9Q3 incubated with different concentrations of (A) *tau*-fluvalinate (FLV). Linearization of the Michaelis-Menten equation by Hanes-Woolf plots showing the competitive inhibition of CYP9Q3 mediated formation of HC by (B) 30 µM *tau*-fluvalinate, and (C) mixed-type inhibition by 30 µM thiacloprid (TCP). [S] = BFC concentration in µM, V = velocity in pmol HC formed / min × mg protein. Details on Michaelis-Menten kinetic data analysis are given in the Supporting Information, Table S1. Data are mean values ± SD (n = 4). (D) Three-dimensional ball-and-stick models of *tau*-fluvalinate, acetamiprid and thiacloprid in standard normalized orientation for the comparison of their molecular size. The molecules were generated using the software package Maestro (Schrödinger Release 2020-1: Maestro, Schrödinger, LLC, New York, NY, 2020).

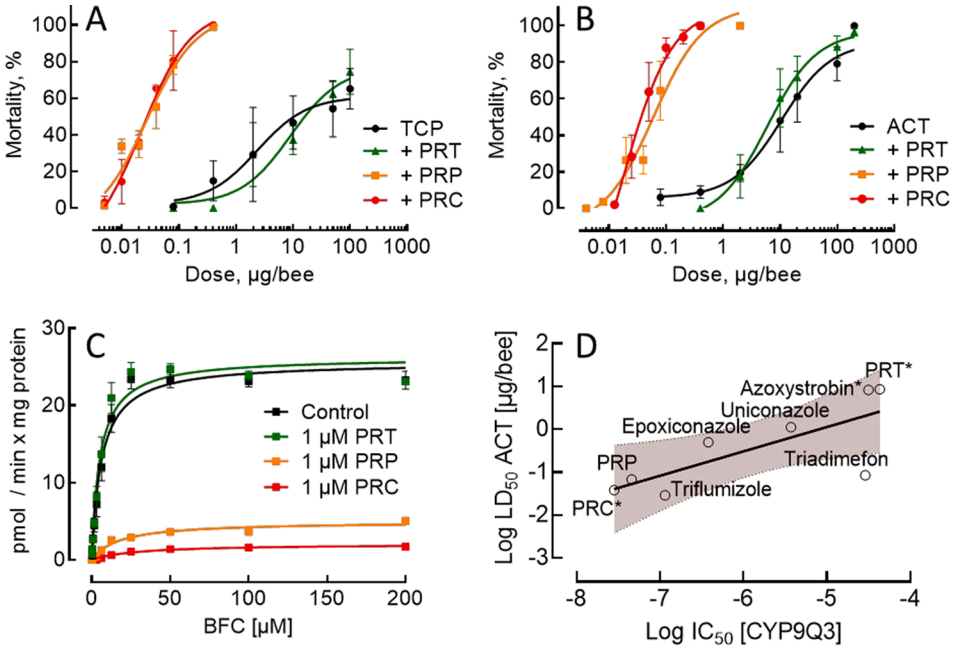


**Fig. 4.** Dose-response relationship and synergism of (A) thiacloprid (TCP) and (B) acetamiprid (ACT) toxicity when topically applied to honey bees either alone or pre-treated with the azole fungicides prothioconazole (PRT), propiconazole (PRP) and prochloraz (PRC). Data are mean values ± SEM (n = 2–4). (C) Fluorescence-based CYP9Q3 inhibition assay with 1 µM of different azole fungicides. An analysis of the kinetic data is given in the Supporting Information, Table S2. (D) Pearson correlation analysis (r = 0.76) between *in vitro* $IC_{50}$ values and *in vivo* $LD_{50}$ values obtained from CYP9Q3 fungicide inhibition assays and honey bee acute contact bioassays with acetamiprid in combination with fungicides, respectively. LD50-values for acetamiprid in combination with fungicides were taken from Iwasa et al. (2004) or generated in this study (those marked with an asterisk). The shaded area displays the 95% confidence limits of the fitted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.2. Neonicotinoid synergism by azole fungicides in vivo is mediated by CYP9Q inhibition

To address another objective of our study we utilized the developed assay to biochemically explore the impact of insecticide / fungicide interactions on honey bees because mixture toxicity is considered an underestimated issue in pollinator risk assessment, and of regulatory concern (Carnesecchi et al., 2019; Johnson et al., 2013; López-Osorio and Wurm, 2020; Robinson et al., 2017). Synergistic effects between insecticides and P450 inhibitors such as azole fungicides or piperonyl butoxide have been described to increase acute honey bee toxicity under laboratory conditions (Han et al., 2019; Iwasa et al., 2004; Johnson et al., 2006), but also under applied conditions, e.g. for thiacloprid/prochloraz mixtures (Wernecke et al., 2019). To explore this in more detail, we first tested the synergistic potential of three common azole fungicides on the acute toxicity of thiacloprid and acetamiprid *in vivo*. The bioassays confirmed strong synergism of *N*-cyanoimine neonicotinoid honey bee toxicity by prochloraz and propiconazole, but not prothioconazole (Fig. 4A and B). Expectedly the acute contact $LD_{50}$ values for thiacloprid and acetamiprid dropped significantly (>100-fold) when combined with either prochloraz or propiconazole (Table S2).

Next, we determined the inhibitory potential of the fungicides at a concentration of 1 μM on recombinantly expressed honey bee CYP9Q3 using the established fluorescence probe assay. Both prochloraz and propiconazole were strong CYP9Q3 inhibitors and exhibited a remarkable depletion of fluorescence (i.e. HC formation), whereas prothioconazole proved to be ineffective at the tested concentration (Fig. 4C, Table S3). Our biochemical results provide a compelling line of evidence that the observed synergism *in vivo* is mediated by the inhibition of CYP9Q3, affecting the pharmacokinetics of otherwise practically non-toxic *N*-cyanoimine neonicotinoids, and leading to a cumulation of insecticidal parent compound with strong toxicodynamic implications, resulting in enhanced acute toxicity.

In order to further test our hypothesis, we biochemically screened five additional fungicides against CYP9Q3, all of them previously described to synergize acetamiprid acute contact toxicity in honey bees at varying levels, except azoxystrobin (Iwasa et al., 2004). Indeed, we found a strong correlation ($r = 0.76$) between the inhibition of CYP9Q3 by fungicides and their previously reported synergistic potential on acetamiprid toxicity *in vivo* (Fig. 4D, Table 1), underpinning the predictive value of the biochemical assay developed and validated in this study. The strongest inhibition of CYP9Q3 was obtained with prochloraz ($IC_{50}$ 29 nM (CI95%: 25–32 nM)), followed by triflumizole ($IC_{50}$ 63 nM (CI95%: 54–74 nM)) and propiconazole ($IC_{50}$ 93 nM (CI95%: 74–110 nM)). In contrast, prothioconazole showed a > 1,000-fold lower inhibition of CYP9Q3 ($IC_{50}$ 43 μM (CI95%: 37–52 μM)), likewise azoxystrobin ($IC_{50}$ 32 μM (CI95%: 24–47 μM)). While the oxidative degradation of thiacloprid almost completely depends on CYP9Q3, it has been recently demonstrated that the metabolism of acetamiprid depends on the *N*-demethylation by CYP9Q3, but particularly CYP9Q2 (Manjon et al., 2018).

Therefore, we additionally tested the inhibition potential of the same fungicides on recombinantly expressed CYP9Q2 and obtained a similar trend and ranking as measured for CYP9Q3 (Table 1). We observed an even stronger correlation ($r = 0.85$) between CYP9Q2 inhibition by fungicides and their synergistic potential *in vivo* (Figure S2), thus confirming the importance of CYP9Q2 in acetamiprid detoxification in honey bees. Prochloraz has long been known to synergize the toxicity of the pyrethroid λ-cyhalothrin in honey bees (Pilling et al., 1995). Later studies confirmed its synergistic potential in combination with *tau*-fluvalinate and coumaphos (Johnson et al., 2013), but also thiacloprid (Wernecke et al., 2019). Here without doubt we provided strong evidence that these observations are most likely linked to the inhibition of CYP9Q3 (and CYP9Q2) and thus resolved a longstanding problem at the molecular level, i.e. to identify the molecular determinant driving the extent of synergism between azole fungicides and a number of different insecticides. The work carried out here can be easily expanded to orthologous P450 enzymes described in other bee species and known to confer tolerance to pesticides, such as CYP9Q6 and CYP9BU1/2 in the buff-tailed bumblebee *Bombus terrestris* and the solitary red mason bee *Osmia bicornis*, respectively (Beadle et al., 2019; Troczka et al., 2019). Thus, contributing to the understanding of effects of simultaneous exposure of bees to pesticide mixtures (David et al., 2016; López-Osorio and Wurm, 2020).

## 4. Conclusion

Our findings for the very first time illustrate a causality between fungicide mediated synergism of thiacloprid and acetamiprid acute contact toxicity *in vivo* and the inhibition of individual, recombinantly expressed honey bee P450s, i.e. CYP9Q3 and CYP9Q2. We conclude from our studies that the developed fluorescence-based screening assay utilizing recombinantly expressed honey bee CYP9Q enzymes provides a powerful tool to complement and support bee pollinator pesticide risk assessment. The possible implementation and utilization of such a novel molecular approach to address issues of regulatory concern remains to be elucidated and depends on the importance and capacity of CYP9Q enzymes to degrade respective insecticidal chemotypes of interest. The approach can be extended to other bee pollinator P450 enzymes of interest such as bumble bee CYP9Q6 (Troczka et al., 2019), but strongly depends on the possibility to functionally express larger amounts of these P450s by appropriate methods (Nauen et al., 2021), and the identification of suitable model substrates allowing the type of screening described. However, we are convinced that applying molecular medicine approaches to evaluate pesticides and their risks posed to bee

**Table 1**
Acute contact toxicity of acetamiprid in combination with different fungicides against honey bees and inhibitory potential of those fungicides against honey bee CYP9Q3 and CYP9Q2, shown to be involved in acetamiprid detoxification (Manjon et al., 2018). The fungicide P450 inhibitory potential ($IC_{50}$-values) was measured in a fluorescence-based assay using BFC as a probe substrate at fixed concentrations of 10 μM and 40 μM for CYP9Q3 and CYP9Q2, respectively. Acute contact toxicity data ($LD_{50}$-values) were taken from Iwasa et al. (2004) or generated in this study (marked with *).

| Insecticide + synergist | $LD_{50}$ 48 h [μg/bee] | 95% CI[a] | CYP9Q3 $IC_{50}$ [μM] | 95% CI | CYP9Q2 $IC_{50}$ [μM] | 95% CI |
|---|---|---|---|---|---|---|
| Acetamiprid | | | | | | |
| Alone* | 8.81 | 4.40–15.2 | – | – | – | – |
| +Triflumizole | 0.029 | 0.008–0.10 | 0.063 | 0.054–0.074 | 0.040 | 0.028–0.056 |
| +Propiconazole | 0.0675 | 0.037–0.08 | 0.093 | 0.074–0.11 | 0.33 | 0.29–0.37 |
| +Triadimefon | 0.0844 | 0.043–0.18 | 27 | 20–38 | 0.95 | 0.74–1.2 |
| +Epoxiconazole | 0.5 | 0.156–1.66 | 4.0 | 2.9–5.4 | 0.087 | 0.063–0.12 |
| +Uniconazole-P | 1.12 | 0.270–4.96 | 3.5 | 3.0–4.1 | 1.2 | 0.91–1.5 |
| +Prothioconazole* | 8.57 | 4.53–14.38 | 43 | 37–52 | 16 | 15–17 |
| +Prochloraz* | 0.038 | 0.03–0.046 | 0.029 | 0.025–0.032 | 0.083 | 0.074–0.093 |
| +Azoxystrobin* | 8.31 | 5.37–11.5 | 32 | 24–47 | 5.4 | 3.8–7.6 |

[a] 95% CI: 95% Confidence Intervals.

pollinator is of benefit in the regulatory landscape as recently suggested (López-Osorio and Wurm, 2020), but we are also aware it wońt completely replace higher tier laboratory and field testing.

## CRediT authorship contribution statement

**Julian Haas:** Methodology, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Ralf Nauen:** Conceptualization, Supervision, Methodology, Data curation, Visualization, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. RN is employed by Bayer AG, a manufacturer of pesticides.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106372.

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