A mechanism-based approach unveils metabolic routes potentially mediating chlorantraniliprole synergism in honey bees, *Apis mellifera* L., by azole fungicides

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**Abstract**

BACKGROUND: Almond production in California is an intensively managed agroecosystem dependent on managed pollination by honey bees, *Apis mellifera* L. A recent laboratory study reported synergism in honey bees between chlorantraniliprole, a common diamide insecticide used in almond orchards, and the fungicide propiconazole. Indeed, there is an emerging body of evidence that honey bee cytochrome P450 monooxygenases of the CYP9Q subfamily are involved in the detoxification of insecticides across a diverse range of chemical classes. The objective of the present study was to unveil the molecular background of the described synergism and to explore the potential role of CYP9Q enzymes in diamide detoxification.

RESULTS: Our study confirmed the previously reported synergistic potential of propiconazole on chlorantraniliprole in acute contact toxicity bioassays, whereas no synergism was observed for flubendiamide. Fluorescence-based biochemical assays revealed an interaction of chlorantraniliprole, but not flubendiamide, with functionally expressed CYP9Q2 and CYP9Q3. These findings were validated by an increased chlorantraniliprole tolerance of transgenic *Drosophila* lines expressing CYP9Q2/3, and an analytically confirmed oxidative metabolism of chlorantraniliprole by recombinantly expressed enzymes. Furthermore, we showed that several triazole fungicides used in almond orchards, including propiconazole, were strong nanomolar inhibitors of functionally expressed honey bee CYP9Q2 and CYP9Q3, whereas other fungicides such as iprodione and cyprodinil did not inhibit these enzymes.

CONCLUSION: Honey bee CYP9Q enzymes are involved in chlorantraniliprole metabolism and inhibited by triazole fungicides possibly leading to synergism in acute contact toxicity bioassays. Our mechanistic approach has the potential to inform tier I honey bee pesticide risk assessment.

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**Keywords:** honey bee; mixture toxicity; chlorantraniliprole; flubendiamide; fungicides; cytochrome P450

1 INTRODUCTION

Tree nut production in California’s Central Valley is among the world’s leading nut production areas with an estimated production area of 1 600 000 acres for almonds alone. 1 Among many insect pest species in almonds, the navel orangeworm *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), and especially the peach twig borer *Anarsia lineatella* (Lepidoptera: Gelechiidae) are considered important primary pests. 2 Larvae are damaging the developing nut directly and adults may cause fungal infestation followed by aflatoxin production during oviposition, making insecticidal sprays during bloom an essential element of integrated pest management (IPM) practices. 2 This poses a challenge as almonds are also heavily dependent on pollination services provided by more than a million honey bee colonies moved to California from all over the United States during almond bloom every year. 3 Therefore, only insecticides with a low-risk profile for bees are registered for use during almond bloom.

Two of those low-risk insecticides are the diamide insecticides flubendiamide (FLB, registration withdrawn in U.S. in 2016 due to concerns regarding aquatic invertebrates) and chlorantraniliprole (CPR) which are considered moderately to practically non-toxic to honey bees based on Tier I acute contact and oral toxicity studies. 4,5 Diamide insecticides can
be divided into two structurally different subtypes: phthalic (e.g. flubendiamide) and antranilic (e.g. chlorantraniliprole)\(^{26,27}\) diamides, however, both are selective ryanodine receptor (RyR) activators. RyRs are large homotetrameric calcium-release channels located in endo- and sarcoplasmic reticulum. By triggering calcium release from internal stores, calcium homeostasis is disrupted leading to specific symptoms such as feeding cessation, paralysis, and muscle contraction, eventually leading to death. While mammals encode three different RyR isoforms expressed in different tissues, insects possess only one RyR gene.\(^8\) Recently, the structural basis for ryanodine receptor modulation by chlorantraniliprole has been elucidated using the RyR1 isoform purified from rabbit skeletal muscle tissue.\(^9\) As previously implied by studies linking amino acid substitutions to diamide resistance in insect pest species,\(^{10-14}\) functional studies\(^{15,16}\) and computational modeling approaches,\(^{17,18}\) the binding site was found to be located in the transmembrane region with a shared binding pocket for CPR and FLB,\(^9\) albeit species specific differences have been reported.\(^{19,20}\) From several amino acid residues in the diamide binding pocket, I4790 and G4946 (numbering based on *Plutella xylostella* RyR) are of particular interest. Whereas G4946 seems highly conserved across insect taxa, I4790 is order-specific in insects with a methionine demonstrated to be present in insects other than Lepidoptera. I4790M substitutions in resistant strains of lepidopteran pest species provide circumstantial evidence for the reduced binding affinity associated with methionine at this position.\(^{10,12,14,21,22}\) Functional studies\(^{23,24}\) confirmed that a methionine at this position moderately reduced binding affinity/toxicity of CPR and FLB contributing to the high specificity of diamide insecticides against lepidopteran pests.\(^{25,26}\) In the western honey bee, *Apis mellifera* L., radio-ligand binding studies revealed a higher selectivity for FLB over antranilic diamides,\(^{20}\) proposing that lower target site affinity contributes to the low bee toxicity of FLB, whereas the mechanisms driving CPR selectivity remained elusive. However, Wade *et al.*\(^{27}\) recently reported synergism between CPR and the fungicide propiconazole, a known inhibitor of honey bee cytochrome P450s involved in insecticide detoxification,\(^{28,29}\) thus raising the question on the importance of oxidative detoxification for the pharmacokinetic behavior of diamides in honey bees, and possible issues of bee safety. Especially since P450 inhibition and its toxicokinetic implications is the most prominent mechanism of synergistic mixture toxicity reported in bees.\(^{30}\)

Here, we investigated whether honey bee P450 genes such as CYP9Q2 and CYP9Q3, which have been previously shown to be rather promiscuous in their capacity to detoxify different insecticidal chemotypes,\(^{31-33}\) are also involved in the detoxification of diamide insecticides. Furthermore, we mechanistically assessed the risk for synergism of commonly applied fungicides in California almond orchards in combination with CPR using a recently described molecular risk assessment approach\(^{29}\) providing an example for its utility in an intensively managed agricultural cropping system. The results provide insights into the molecular aspects of diamide toxicology and pharmacology while strengthening the evidence for the importance of cytochrome P450-mediated insecticide detoxification in honey bees.

## 2 MATERIALS & METHODS

### 2.1 Chemicals

Chlorantraniliprole (98% purity, CAS 500008-45-7) and flubendiamide (98.1%, CAS 272451-65-7) were obtained in-house at Bayer AG. Cyprodinil (99%, CAS 121552-61-2), iprodione (99.6%, CAS 1215631-57-4), boscalid (99.5%, CAS 188425-85-6), pyraclostrobin (99.9%, CAS 175013-18-0), chlorothalonil (99%, CAS 1897-45-6), difenoconazole (99.5%, CAS 119446-68-3), fenbuconazole (99%, CAS 114369-43-6), metconazole (98.9%, CAS 125116-23-6) and propiconazole (99%, CAS 60207-90-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescent probe 7-benzyloxyxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC; CAS 277309-33-8) was synthesized by Enamine Ltd. (Riga, Latvia) with a purity of 95%. HPLC gradient grade acetonitrile was purchased from Merck (Darmstadt, Germany). Unless otherwise mentioned all other reagents were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2 Drosophila bioassay

Transgenic Drosophila bioassays were conducted as recently described.\(^{33}\) Briefly, UAS-strains carrying either the *A. mellifera* gene CYP9Q2 or CYP9Q3 and a control strain (same genetic background but lacking the transgene) were crossed with a GAL4-Hsp70 strain. After incubating the F1 flies thrice for 30 min at 37 °C the day before, flies were once again incubated at 37°C immediately before starting the bioassay. Bioassays were conducted in 12-well plates (Greiner Bio-One, Kremnäüster, Austria) with 2 mL artificial diet per well (Jazz-Mix™ Drosophila Food, Thermo Fisher Scientific, Waltham, MA, USA). Chlorantraniliprole and flubendiamide were dissolved and diluted in acetone and each dilution was further diluted 1:2 in ddH₂O containing 0.1% (w/v) Triton X-100 before dispersing the insecticide over the diet surface. Mortality was scored after 48 h and LC₅₀ values calculated by probit analysis using PoloPlus 2.0 (LeOra Software, Petaluma, CA, USA).

### 2.3 Bee acute contact toxicity and synergism study

*A. mellifera* worker bees of mixed age were randomly collected from the honey super of three queen-right colonies located in Monheim am Rhein, Germany. The colonies had not received chemical treatment for at least 6 months and their health-status was weekly checked by visual inspection. Acute contact insecticide toxicity assays on honey bees were performed following the OECD guideline no. 214,\(^{34}\) with some modifications to adjust the application of the potential synergist propiconazole. Synergist studies with propiconazole were performed exactly as previously described using application rates of 10 μg/bee.\(^{29}\) Mortality was assessed after 48 h. Control bees treated with acetone or propiconazole only remained unaffected for the test period. Statistical analysis was performed using GraphPad Prism v8.3 (GraphPad Software Inc., San Diego, CA, USA).

### 2.4 Fluorescent substrate assays

Honey bee cytochrome P450 proteins CYP9Q2 (Accession No.: XP_392000.1), CYP9Q3 (Accession No.: XP_006562363.1) and CYP6AQ1 (Accession No.: NP_001191991.1) were obtained by functional expression in High Five insect cells co-infected with *A. mellifera* NADPH-dependent cytochrome P450 reductase (Accession No.: XP_006569769.1) as previously described.\(^{31,35}\) Protein concentration was determined using Bradford reagent.

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\(\text{Accession No.: XP_006569769.1}\) as previously described.\(^{31,35}\)
(Bio-Rad, CA, USA) and bovine serum albumin (BSA) as a reference.

The inhibitory potential (IC50 values) of diamide insecticides and fungicides on recombinantly expressed honey bee P450s was tested as previously described using BOMFC as the probe substrate at a fixed concentration around the apparent Ki value (CYP9Q3: 6.5 μM, CYP9Q2 15 μM, CYP6AQ1: 20 μM). Four μg of microsomal protein was used per reaction. Fluorescence was measured using a microplate reader (Tecan Spark, Tecan Group Ltd., Männedorf, Switzerland) at an emission wavelength of λem 510 nm (20 nm band width) while excited at λex 405 nm (20 nm band width). Fungicides and insecticides were dissolved in dimethyl sulfoxide (DMSO) and tested using a 5-fold dilution series starting from 100 μM to 0.032 μM. To ensure solubility, fungicides and flubendiamide were tested with a final DMSO concentration of 2% (chlorantraniliprole and azole fungicides: 1%). Appropriate DMSO controls were included in each measurement.

Kinetic inhibition studies were performed according to Haas & Nauen29 with BOMFC concentrations ranging from 200 to 0.2 μM and increasing concentrations of chlorantraniliprole. Measurements were analyzed for the respective inhibition type using non-linear regression assuming Michaelis–Menten kinetics and reversible inhibition according to Fowler & Zhang.36 Data were analyzed and IC50-values calculated using a four-parameter non-linear regression fitting routine in GraphPad prism v8.3 (GraphPad Software Inc., San Diego, CA, USA).

2.5 Metabolite analysis

For CPR metabolite identification microsomal High-5 cell preparations of recombinantly expressed CYP9Q2 and CYP9Q3 (80 μg protein)28 were incubated with 50 μM chlorantraniliprole in 100 μL reactions at 30°C for 2 h (0.1 M potassium phosphate buffer, pH 7.6, NADPH regenerating system (Promega, WI, USA), 1% DMSO, 0.05% BSA). Microsomes incubated without NADPH regenerating system and cells infected with an empty plasmid virus served as controls. Reactions were stopped by adding 400 μL ice-cold acetonitrile. Sample were stored overnight at 4°C for protein precipitation and afterwards centrifuged at 4°C and 3200 g for 30 min. The supernatant was transferred to a 96 well collection plate (1 mL, Waters Corporation, MA, USA) and subsequently analyzed via UPLC-TOF-MS employing an Acquity UPLC I-Class system coupled to a cyclic IMS mass spectrophotometer (Waters Corporation, MA, USA). A Zorbax Eclipse Plus C18 (1.8 μm, 100 × 2.1 mm) (Agilent Technologies, CA, USA) column was used with a column oven temperature of 60°C. The mobile phase consisted of acetonitrile/0.25% formic acid (eluient A) and water/0.25% formic acid (eluient B) in gradient mode and a flow rate of 0.6 mL min−1 with eluent B starting at 90% for 4.5 min, decreasing to 5% for 2.5 min and increasing to 90% again for 1 min. The mass spectrometer operated in positive ion mode with a full scan resolution of 60 000 fwhm (full width at half maximum). Measurements and metabolite search were conducted with MassLynx and Molblynx software (Waters Corporation, MA, USA).

2.6 CYP9Q3 homology model and docking

A CYP9Q3 homology model was created using the Maestro suite software (Schrodinger Inc., NY, USA). The native sequence was queried by a simple BLAST against the homologue database and human CYP3A4 co-crystallized with imidazole (PDB-ID: 4D6Z) was chosen as a template followed by structure-based sequence realignment. The resulting raw model was energy-minimized by 2500 steps of a conjugate gradient procedure to remove local disorder within the chain. Subsequent docking was performed using AutoDock Vina37 embedded in UCSF Chimera software (v1.14, UCSF, CA, USA). Input comprised the created CYP9Q3 homology model as receptor, CPR and FLB as ligands and a docking box surrounding the heme iron center of the enzyme. The obtained output comprised a list of the top five binding poses ranked by ΔG, the predicted binding energy in kcal mol−1 (score = ΔG). The exhaustiveness of search parameter was set at 8. CPR isosurface plots of the Fukui function for an attack by electrophile were calculated according to Parr & Yang.38

3 RESULTS

3.1 Synergist bioassays

Pretreatment of honey bees with propiconazole in laboratory bioassays 1 h prior to insecticide application significantly increased the acute contact toxicity of CPR but not FLB (Fig. 1), thus confirming a previously reported synergism between CPR and propiconazole.27 While topical application of 2 μg/bees and 0.2 μg/bees CPR alone resulted in mortality of <15% (in accordance with the reported LD50 of >4 μg/bees), honey bee pretreatment with 10 μg/bees propiconazole significantly increased the mortality of 2 μg CPR and 0.2 μg CPR (applied per bee) from 12% ± 7.6% to 100% ± 0% and 3.6% ± 2.3% to 69% ± 13% (unpaired t-test, P < 0.01), respectively. The observed synergism mediated by propiconazole proposed a role for P450s in CPR metabolism in vivo. FLB acute contact toxicity, however, was not influenced by propiconazole pretreatment, and honey bee mortality was less than 5% even at FLB doses of 100 μg/bees.
are also involved in diamide metabolism, we tested the recombinantly expressed honey bee CYP9Q2 and CYP9Q3 was CYP9Q3-mediated BOMFC metabolism using increasing concentrations of CPR. Data are mean values ± SD (n = 4).

Table 1. Inhibitory potential (IC50-values) of the diamide insecticides chlorantraniliprole and flubendiamide against the honey bee P450 enzymes CYP9Q2, CYP9Q3 and CYP6AQ1 using a fluorescence-based assay with 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) as a probe

| Diamide          | CYP9Q3 IC50 [μM] | 95% CI | CYP9Q2 IC50 [μM] | 95% CI | CYP6AQ1 IC50 [μM] |
|------------------|-----------------|--------|-----------------|--------|-------------------|
| Chlorantraniliprole | 17.4           | 15.3–19.8       | 93.4           | 79.4–114             | >100       |
| Flubendiamide    | >100            | —              | >100            | —                  | >100       |

† 95% Confidence Interval.

3.2 Fluorescent probe assays with recombinantly expressed honey bee P450s

To test whether specific honey bee P450s previously associated with insecticide detoxification are also involved in diamide metabolism we used a recently published fluorescent probe (BOMFC) assay with three functionally expressed honey bee P450s: CYP9Q2, CYP9Q3 and CYP6AQ1. While CYP6AQ1-mediated BOMFC metabolism was not inhibited by CPR at a concentration of 100 μM, BOMFC metabolism by CYP9Q2 was only weakly inhibited by CPR (Fig. S1, Table 1). In contrast, transgenic flies expressing CYP9Q3 were only 1.2-fold less tolerant to CPR than control flies with the same genetic background, but not expressing these P450s. Full dose–response biosays revealed that fly lines expressing CYP9Q2 or CYP9Q3 are significantly more tolerant to CPR than a control strain with the same genetic background, but not expressing these P450s (Table 2).

In line with the in-vitro fluorescent probe assay results presented above, transgenic flies expressing CYP9Q3 are significantly less sensitive to CPR (LC50 value 155 ppm (CI95: 126–191)) compared to control flies (LC50 of 22.2 ppm (CI95: 19.7–24.9)) (Fig. 3(A)). In contrast, transgenic flies expressing CYP9Q2 were only 1.2-fold less tolerant to FLB compared to control flies, confirming a minor (if any) role of CYP9Q3 in FLB metabolism (Fig. 3(B)). The calculated LC50 value of FLB against control flies was 147 ppm (CI95: 136–156), and the obtained LC50 value for flies expressing CYP9Q3 was similar, i.e. 179 ppm (CI95: 166–193) (Table 2). Transgenic flies expressing CYP9Q2 under the control of the Hsp70 promoter followed the same trend with a marked decrease in sensitivity against CPR, but not FLB (Table 2).

3.3 Diamide sensitivity of transgenic Drosophila lines expressing CYP9Q2 or CYP9Q3

To determine whether the observed CPR interaction in-vitro with recombinantly expressed honey bee CYP9Q2 and CYP9Q3 was indeed indicative for oxidative CPR metabolism, we tested the effect of CPR (and FLB) on transgenic Drosophila lines ectopically expressing either CYP9Q2 or CYP9Q3 in comparison with flies not expressing these honey bee P450s. Full dose–response biosays revealed that fly lines expressing CYP9Q2 or CYP9Q3 are significantly more tolerant to CPR than a control strain with the same genetic background, but not expressing these P450s (Table 2).

Figure 2. Effect of diamides on coumarin fluorescent probe metabolism by CYP9Q3. (A) Inhibitory potential of the diamide insecticides flubendiamide (FLB) and chlorantraniliprole (CPR) against functionally expressed honey bee CYP9Q3 using a fluorescence-based assay with 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) as a probe at a fixed concentration (6.5 μM) around the apparent Km value. (B) Michaelis–Menten kinetics of CYP9Q3-mediated BOMFC metabolism using increasing concentrations of CPR. Data are mean values ± SD (n = 4).
Table 2. Chlorantraniliprole and flubendiamide acute toxicity against adults of transgenic *Drosophila melanogaster* strains expressing honey bee CYP9Q2 and CYP9Q3, respectively, in comparison to a reference control strain with the same genetic background (Empty)

| Insecticide + crossing | LD$_{50}$ 48 h [ppm] | 95% CI | Slope | RR$^1$ | 95% CI$^2$ | Chi² (df) |
|------------------------|-----------------------|--------|--------|--------|------------|----------|
| Chlorantraniliprole     |                       |        |        |        |            |          |
| Empty × Hsp70           | 22.2                  | 19.7–24.9 | 3.09   | —      | —          | 1.55 (4) |
| CYP9Q2 × Hsp70          | 96.2                  | 71.9–127 | 1.74   | 4.34   | 3.6–5.3    | 9.22 (5) |
| CYP9Q3 × Hsp70          | 155                   | 126–191 | 2.86   | 6.99   | 5.9–8.3    | 8.93 (5) |
| Flubendiamide           |                       |        |        |        |            |          |
| Empty × Hsp70           | 147                   | 136–156 | 8.78   | —      | —          | 0.77 (7) |
| CYP9Q2 × Hsp70          | 253                   | 216–292 | 4.62   | 1.73   | 1.54–1.94  | 12.59 (7) |
| CYP9Q3 × Hsp70          | 179                   | 166–193 | 6.97   | 1.23   | 1.11–1.36  | 2.90 (7) |

Expression was driven by the GAL4/UAS system using the GAL4-Hsp70 driver line.$^{44}$

$^1$ Resistance ratio: LD$_{50}$ of transgenic strain divided by the LD$_{50}$ of reference strain (Empty × Hsp70).

$^2$ Confidence Interval 95%.

Figure 3. Diamide toxicity against Drosophila lines in diet overlay bioassays. Toxicity of (A) chlorantraniliprole and (B) flubendiamide against transgenic *Drosophila* adults ectopically expressing honey bee CYP9Q3 and a control strain (Empty) with the same genetic background. Data are mean values ± SEM (n = 3).

Figure 4. Computational modelling and chlorantraniliprole (CPR) metabolism by CYP9Q3. (A) Most favorable docking poses of CPR in the catalytic pocket of a CYP9Q3 homology model based on human CYP3A4 with methylphenyl carbon and (B) the N-methyl carbon of the anthraniloyl moiety oriented towards the heme iron center. (C) Isosurface of the Fukui function highlighting potential sites for oxidative attack (in green) suggesting methylphenyl hydroxylation as well as N-demethylation as most probable oxidative sites of attack. (D) UPLC-TOF/MS analysis confirming the formation of a hydroxylated M + 16 metabolite of CPR after the incubation with functionally expressed CYP9Q3 in vitro.
and the chlorine of the anthraniloyl moiety, which is oriented towards V371, S310 and I491 of CYP9Q3. Both, the methylphenyl and the N-methyl carbon are putative sites for oxidative attack leading to methylphenyl-hydroxylation or N-demethylation, respectively (Fig. 4(C)). In this context, for CPR, the isosurface plots of the Fukui function for the attack of an electrophile at the respective positions supported the docking results (Fig. 4(C)). FLB docking into the catalytic pocket of CYP9Q3 did not result in energetically favorable poses (Fig. S2), supporting the observed lack of interaction with CYP9Q3 in different bioassays conducted in this study.

3.5 CPR metabolism by recombinantly expressed CYP9Q enzymes

To confirm the generation of hydroxy- or N-desmethyl CPR metabolites we incubated recombinantly expressed CYP9Q3 (and CYP9Q2) with 50 μM of CPR and subsequently searched for metabolites using UPLC-TOF-MS. Indeed, we were able to identify a M + 16 metabolite as the main metabolite after CPR incubation with CYP9Q3 corresponding to a hydroxylation event (Fig. 2(E)). Due to missing reference substances, we could not determine whether the hydroxylation occurred at the methylphenyl or rather at the N-methyl carbon. Interestingly, the main metabolite identified after CYP9Q2 incubation with CPR was a M-31 metabolite (Fig. S3), which might correspond to a cyclization reaction with loss of water after hydroxylation at the N-methyl carbon as previously reported in lactating goats.39

3.6 Inhibition of honey bee CYP9Q enzymes by fungicides registered in Californian almonds

Our results suggest that CPR pharmacokinetics in honey bees depends to some extent on oxidative degradation mediated by CYP9Q2 and CYP9Q3. Therefore, we tested on these P450s the inhibitory effect of nine different fungicides (incl. propiconazole) registered for use in Californian almond orchards as reported by Wade et al.,27 and according to the Californian Pesticide Information Portal (https://calipip.cdpr.ca.gov/main.cfm). All azole fungicides tested were strong inhibitors of CYP9Q2 and CYP9Q3, respectively, with IC50 values in the nanomolar range, thus suggesting potential to synergize insecticides detoxified by these enzymes (Table 3). The lowest IC50 value of approximately 30 nM was obtained for difenoconazole against both CYP9Q2 and CYP9Q3. All other fungicides – of different chemical classes – were not inhibitory at concentrations up to 10 μM, except the carbamoyl boscalid (IC50: 4.13 μM) and the strobilurin pyraclostrobin (IC50: 7.84 μM) which showed weak inhibitory effects towards CYP9Q3, but not CYP9Q2, however micromolar concentrations are very unlikely to be relevant in vivo.29

4 DISCUSSION

California almond is among those crops heavily reliant on pollination by honey bees, but also on treatments with plant protection products to particularly combat insect pests and diseases. Therefore, registered insecticides for use during almond bloom must have a favorable bee safety profile such as the diamide CPR, which has a broad spectrum of insecticidal efficacy including lepidopteran, coleopteran, dipteran, and isopteran pests.40,41 Whereas CPR is registered and frequently used in almonds, the registration of FLB, the second diamide insecticide included in this study, has been discontinued in 2016. It has recently been suggested that the honey bee risk of CPR may need to be managed when combined with the azole fungicide propiconazole due to synergistic effects resulting in increased acute toxicity to honey bees.27 Similar propiconazole-mediated synergistic effects have been described earlier for other insecticides such as the N-cyanoamidine neonicotinoids thiacloprid and acetamiprid and linked to the inhibition of honey bee P450s,28 particularly CYP9Q2 and CYP9Q3.29 These P450s, known to rapidly degrade N-cyanoamidine neonicotinoids and butenolides by oxidative attack in vitro and in vivo31,33 were demonstrated to be molecular determinants of insecticide selectivity and highly sensitive to azole-mediated inhibition.29,33 Here we provided several lines of evidence that also explain the recently described synergistic effect between propiconazole and CPR is most likely conferred by the inhibition of CYP9Q2 and CYP9Q3, which both contribute to the oxidative degradation of CPR.

First, our finding that the anthranilic diamide insecticide CPR was metabolized by recombinantly expressed CYP9Q2/3 isoforms is of particular interest, as these honey bee P450s were already shown to have the catalytic capacity to metabolize compounds from four other chemical classes of insecticides: neonicotinoids,31 pyrethroids,32 organophosphates,32 and butenolides.32 Another P450, CYP6A9Q1, recently shown to hydroxylate

| Table 3. Inhibitory potential (IC50-values) of commonly applied fungicides (e.g. in Californian almond orchards27) against honey bee P450 enzymes CYP9Q2 and CYP9Q3 using a fluorescence-based assay with 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) as a probe |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Fungicide**   | **Class**       | **CYP9Q3**      | **CYP9Q2**      |
|                 | **IC50 [μM]**   | **95% CI**      | **Hill slope**  | **Adj. R²**     | **IC50 [μM]**   | **95% CI**      | **Hill slope**  | **Adj. R²**     |
| Propiconazole*  | Triazole        | 0.072           | 0.065–0.081     | −1.10           | 0.99            | 0.151           | 0.136–0.169     | −0.88           | 0.99            |
| Difenconazole   | Triazole        | 0.032           | 0.028–0.037     | −1.02           | 0.97            | 0.033           | 0.028–0.039     | −0.81           | 0.98            |
| Fenbuconazole   | Triazole        | 0.058           | 0.043–0.079     | −0.86           | 0.93            | 0.041           | 0.033–0.058     | −0.67           | 0.95            |
| Metconazole     | Triazole        | 0.057           | 0.049–0.066     | −1.03           | 0.97            | 0.033           | 0.027–0.039     | −0.6            | 0.98            |
| Cypriodin       | Anilino-Pyrimidine | >10               | —               | —               | —               | >10               | —               | —               |
| Iprodione       | Dicarboximide   | >10               | —               | —               | —               | >10               | —               | —               |
| Boscalid        | Carboxamide     | 4.13             | 3–5.76         | −1.05           | 0.90            | >10               | —               | —               |
| Pyraclostrobin  | Strobilurin     | 7.84             | 6.04–10.20     | −0.56           | 0.93            | >10               | —               | —               |
| Chlorothalonil  | Chloronitrile   | >10               | —               | —               | —               | >10               | —               | —               |

The values for propiconazole (marked with *) were taken from Haas et al. (2021)33

1 95% Confidence Interval.
The butenolide flupyradifurone,33 did not interact with CPR in fluorescence probe assays; so, it was excluded from additional experiments. In contrast, CYP9Q3 – and to a lesser extent CYP9Q2 – showed a clear interaction with CPR in a fluorescent probe assay recently introduced for mechanistic risk assessment purposes at the molecular level.29 This finding suggests a certain level of promiscuity of the CYP9Q subfamily, particularly CYP9Q3, and fuels previous claims about their general involvement in xenobiotic defense in honey bees,40 i.e. accepting a rather diverse range of substrates.29,33 The structural basis for the observed ligand promiscuity in CYP9Q3 remains elusive due to the lack of crystal structures in complex with a chemically diverse range of ligands. However, the non-typical kinetic data obtained for some insecticide ligands, suggests that the binding cavity of CYP9Q3 might undergo conformational changes upon binding of these ligands.29 Such conformational changes in structure have been demonstrated in human CYP3A4 and are considered the major driver of its ligand promiscuity, and explaining its important role in the detoxification of the majority of drugs in humans.43

Next, we assessed the efficacy of CPR and FLB against transgenic *Drosophila* lines expressing either CYP9Q2 or CYP9Q3.31 These fly lines were recently developed and used in predictive screens for the assessment of insecticide selectivity and pesticide–pesticide interactions.31,13,44 Transgenic flies expressing CYP9Q2 and CYP9Q3 were significantly less susceptible to CPR when compared to control flies, suggesting a pivotal role for these P450s in CPR toxicokinetics. A significantly reduced susceptibility in CYP9Q2/3 expressing flies was also reported for other insecticides shown to be readily metabolized by recombinantly expressed honey bee CYP9Q enzymes, e.g. thiacloprid and flupyradifurone.31,33 These transgenic CYP9Q fly lines remain almost completely susceptible to FLB, supporting our biochemical data obtained in fluorescence probe assays, suggesting no detoxification capacity of CYP9Q on FLB. Indeed, control (wildtype) *Drosophila* were significantly less sensitive to FLB than CPR, a fact recently linked to selectivity issues on the RyR receptor level in dipteran species.23 The difference in sensitivity between FLB and CPR was partially explained by the presence of a methionine residue, M4790 (diamondback moth RyR numbering; isoleucine in lepidopteran species) located in the RyR transmembrane helix S2 and supposed to be involved in diadime binding, which differs slightly between benzenediacarbamimide-type diamides such as FLB and anthracilic diamides like CPR.17,45 This view was partially confirmed by a study introducing the isoleucine residue into the *Drosophila* RyR via CRISPR/Cas9 genome editing leading to a >10-fold increase in FLB susceptibility compared to CPR33 and stronger resistance towards FLB when the I4790M mutation was introduced in a susceptible *P. xylostella* strain.44 Honey bee RyR were shown to be much less sensitive to FLB than CPR,46 indicating a much weaker binding of FLB which contributes to its classification as practically non-toxic to honey bees in acute toxicity bioassays. Sublethal effects of CPR on honey bees were linked to internal calcium store releases indicating RyR activation.46,47 However, these effects, as well as honey bee symptoms of poisoning after CPR exposure were described to be transient,3 suggesting that pharmacokinetics plays a major role in CPR clearance from its sites of action, an assumption supported by data obtained in this study.

In *silico* docking and mass spectral data from samples analyzed after the incubation of CPR with recombinantly expressed honey bee P450s indicated that a primary site of attack for oxidative CPR metabolism by CYP9Q3 and CYP9Q2 is the anthraniloyl moiety, particularly the methylphenyl or N-methyl carbon, respectively. Our hypothesis regarding CPR metabolite formation by methylphenyl-hydroxylation or N-demethylation is supported by an earlier study in lactating goats.39 The metabolic fate of CPR in lactating goats is dominated by oxidative metabolites formed after N-demethylation, methylphenyl hydroxylation, and further oxidation to the carboxylic acid, whereas various cyclic metabolites resulted from the loss of water from the N-hydroxymethyl group. Information about the metabolic fate of CPR in insects is elusive, and only a few studies identified possible routes of metabolically mediated oxidative resistance to CPR in pest insects.38-50 The present study is, to the best of our knowledge, the first which provided functional evidence for CPR metabolite formation by an insect P450.

Finally, it is evident from the data presented here that CYP9Q2 and CYP9Q3 play an important role in CPR metabolism and detoxification, thus explaining to some extent the honey bee friendly profile of CPR. Fungicides are known as potential insecticide synergists in honey bees based on their potential to inhibit P450s for a long time.51,52 The strength of synergism is correlated with the importance of inhibited P450 isoforms for the detoxification of the applied insecticide, as recently confirmed for the chemical class of neonicotinoids. *N*-cyanoamidine-substituted chemotypes were shown to be much more affected by azole-mediated synergism than *N*-nitro substituted neonicotinoids,28,29 because the latter chemotype is hardly metabolized by honey bee CYP9Q3.30 A number of fungicide-insecticide combinations have been shown to be synergistic, not just in laboratory bioassays,35 but also under field conditions,53 rendering mixture toxicity a topic of regulatory concern.30,54 The strength of the synergistic potential of propiconazole in combination with CPR in a laboratory worst-case scenario on both honey bee larvae and adults suggests that mixture toxicity under applied conditions cannot be excluded and possibly warrants scrutiny.27 Our molecular study unveiled two known honey bee P450 isoforms possibly driving the observed synergism, thus allowing the use of a recently described molecular risk assessment approach.29,30 This mechanistic approach allowed us to rapidly assess the inhibitory potential of commonly applied fungicides in almond orchards, and thus identifying those which pose no risk to CYP9Q2 and CYP9Q3 which were shown to be involved in the oxidative degradation of CPR. Not surprisingly, all azole fungicides tested here are nanomolar inhibitors of CYP9Q2/3, thus deserving further investigation regarding their synergistic potential in combination with CPR. Interestingly, it was recently shown that not all azole fungicides share the same high inhibitory potential towards honey bee P450, for example prothioconazole.29,32 Tested fungicides of chemical classes other than azoles did not show any inhibition towards CYP9Q2/3, suggesting that they are not interfering with P450-mediated CPR metabolism, which is supported by the lack of synergism between CPR and several fungicides tested.27

In conclusion, our case study provides a practical example of the utility of an *in-vitro* screening approach for mechanistic risk assessment to rapidly screen mixture partners regarding their potential risk for increased mixture toxicity. Thus, allowing the identification of candidates which could be tested in higher tier studies to characterize potential synergistic interaction at the organism level under field conditions. We think that our approach followed here is an example of how the mechanistic understanding of pesticide pharmacology in honey bees together with molecular medicine approaches can help to complement existing risk assessment measures and thus improving bee safety.55
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CONFLICT OF INTEREST
JG, UK and RN are employed by Bayer AG, a manufacturer of pesticides.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION
Supporting information may be found in the online version of this article.

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