Ecotoxicology and Environmental Safety 217 (2021) 112247

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

A toxicogenomics approach reveals characteristics supporting the honey bee (*Apis mellifera* L.) safety profile of the butenolide insecticide flupyradifurone

Julian Haas [a,b,1], Marion Zaworra [b,1], Johannes Glaubitz [b], Gillian Hertlein [b,2], Maxie Kohler [b], Andreas Lagojda [b], Bettina Lueke [b], Christian Maus [b], Maria-Teresa Almanza [b], T. G. Emyr Davies [c], Chris Bass [d], Ralf Nauen [b,*,3]

[a] *Institute of Crop Science and Resource Conservation, University of Bonn, 53115 Bonn, Germany*
[b] *Bayer AG, Crop Science Division, R&D, D-40789 Monheim, Germany*
[c] *Department of Biointeractions and Crop Protection, Rothamsted Research, Harpenden, United Kingdom*
[d] *College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn, United Kingdom*

ARTICLE INFO

Edited by: Dr. Caterina Faggio

Keywords:
P450
Risk assessment
Pollinators
Detoxification
Synergism
Fungicides

ABSTRACT

Flupyradifurone, a novel butenolide insecticide, selectively targets insect nicotinic acetylcholine receptors (nAChRs), comparable to structurally different insecticidal chemotypes such as neonicotinoids and sulfoximines. However, flupyradifurone was shown in acute toxicity tests to be several orders of magnitude less toxic to western honey bee (*Apis mellifera* L.) than many other insecticides targeting insect nAChRs. The underlying reasons for this difference in toxicity remains unknown and were investigated here. Pharmacokinetic studies after contact application of [^{14}C]flupyradifurone to honey bees revealed slow uptake, with internalized compound degraded into a few metabolites that are all practically non-toxic to honey bees in both oral and contact bioassays. Furthermore, receptor binding studies revealed a lack of high-affinity binding of these metabolites to honey bee nAChRs. Screening of a library of 27 heterologously expressed honey bee cytochrome P450 enzymes (P450s) identified three P450s involved in the detoxification of flupyradifurone: CYP6AQ1, CYP9Q2 and CYP9Q3. Transgenic *Drosophila* lines ectopically expressing CYP9Q2 and CYP9Q3 were significantly less susceptible to flupyradifurone when compared to control flies, confirming the importance of these P450s for flupyradifurone metabolism in honey bees. Biochemical assays using the fluorescent probe substrate 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) indicated a weak, non-competitive inhibition of BOMFC metabolism by flupyradifurone. In contrast, the azole fungicides prochloraz and propiconazole were strong nanomolar inhibitors of these flupyradifurone metabolizing P450s, explaining their highly synergistic effects in combination with flupyradifurone as demonstrated in acute laboratory contact toxicity tests of adult bees. Interestingly, the azole fungicide prothioconazole is only slightly synergistic in combination with flupyradifurone – an observation supported by molecular P450 inhibition assays. Such molecular assays have value in the prediction of potential risks posed to bees by flupyradifurone mixture partners under applied conditions. Quantitative PCR confirmed the expression of the identified P450 genes in all honey bee life-stages, with highest expression levels observed in late larvae and adults, suggesting honey bees have the capacity to metabolize flupyradifurone across all life-stages. These findings provide a biochemical explanation for the low intrinsic toxicity of flupyradifurone to honey bees and offer a new, more holistic approach to support bee pollinator risk assessment by molecular means.

* Correspondence to: Bayer AG, Crop Science Division, R&D, Building 6260, Alfred Nobel Str. 50, D40789 Monheim, Germany.
E-mail address: ralf.nauen@bayer.com (R. Nauen).
[1] Joint first authors.
[2] Present address: idalab GmbH, 10785 Berlin, Germany.
[3] ORCID: 0000-0002-7525-8589.

https://doi.org/10.1016/j.ecoenv.2021.112247
Received 18 January 2021; Received in revised form 1 April 2021; Accepted 8 April 2021
Available online 23 April 2021

1. Introduction

Flupyradifurone, 4-[(2,2-difluoroethyl)amino]-2(5H)-furanone (FPF) - known under the major brand name Sivanto® - is a novel butenolide insecticide developed for foliar, soil and seed treatment applications (Nauen et al., 2015). FPF was commercially introduced to the market in 2014 as an integrated pest management (IPM)-friendly tool (Bordini et al., 2021), and is registered for use in a wide variety of fruit and vegetable crops and defined broad acre crops. It targets some of the world's most destructive sucking pests including aphids, psyllids, scales, leafhoppers, mealy bugs, and is particularly important for the control of whiteflies such as *Bemisia tabaci*, a vector of serious phytopathogenic viruses such as tomato yellow leaf curl virus and cucurbit yellow stunting disorder virus (Castle et al., 2017; Roditakis et al., 2017). FPF acts as a partial agonist of insect nicotinic acetylcholine receptors (nAChR) by reversible binding at the orthosteric site (Casida, 2018; Nauen et al., 2015). The electronegative butenolide pharmacophore of FPF is derived from the natural plant alkaloid stemofoline (Sakata et al., 1978), and possesses a pronounced dipole moment (Fig. S1). This is important as electrostatic interactions are one of the key determinants of selective binding of such ligands to insect over vertebrate nAChRs (Casida and Durkin, 2013; Jeschke et al., 2013; Beck et al., 2015).

Butenolides are sometimes confused with neonicotinoids, a different chemical class of insecticides that also act as selective agonists at the orthosteric site of insect nAChR, but originating from the synthetic nitromethylene heterocycle nithiazine (Jeschke and Nauen, 2008; Soloway et al., 1978). Some well-known insecticides such as imidacloprid, thiamethoxam and clothianidin belong to the chemical class of neonicotinoids and carry an *N*-nitroguanidine pharmacophore (Casida, 2018). They were launched in the early 1990s and have the greatest current market value of any insecticide class followed by the pyrethroids (Sparks et al., 2020). Neonicotinoids are known to be highly effective insecticides particularly when applied as a seed treatment (Jeschke et al., 2011), however, concerns were raised about possible environmental and ecotoxicological risks (Goulson, 2013; Hladik et al., 2018; Pagano et al., 2020; Stara et al., 2020a, 2020b), and their potential role in recent bee pollinator declines (Blacquière et al., 2012; Godfray et al., 2014; Lundin et al., 2015; Potts et al., 2016). In 2013 the European Commission (EC) suspended the use of imidacloprid, thiamethoxam and clothianidin seed treatments in bee-attractive crops such as oilseed rape (EU, 2013), followed by their complete ban for all outdoor uses in 2018, because according to the European Food Safety Authority (EFSA), these neonicotinoids pose a high risk to honey bees. While the *N*-nitroguanidine neonicotinoids are as intrinsically toxic to honey bees as to the insect pests they target (Iwasa et al., 2004), the *N*-cyanoimine neonicotinoid insecticides, such as thiacloprid and acetamiprid, are much less toxic to honey bees (Iwasa et al., 2004), and were exempt from the EU ban in 2018. Based on rather high acute LD_{50}-values (> 11 μg/bee) thiacloprid is classified as 'practically non-toxic' to honey bees (Schmuck, 2001; US EPA, 2014), and a recent assessment identified no critical issues of ecotoxicological concern (EFSA, 2019a). However, this compound was a candidate for substitution and its registration in EU-27 was not renewed in April 2020. Like the *N*-cyanoimine neonicotinoids the butenolide FPF is several 100-fold less toxic to honey bees than imidacloprid, thiamethoxam and clothianidin (Casida, 2018; Nauen et al., 2015), suggesting that modulators of insect nAChRs are not inherently problematic to bees, just because they target a conserved receptor site in insects (Casida, 2018). Indeed, differential bee toxicity has been described for several classes of insecticides, independent of the target-site addressed by these insecticides (Hardstone and Scott, 2010; Reid et al., 2020).

In order to explain the differential toxicity of insecticides addressing the same mode of action to bee pollinators, it is important to identify and understand the mechanisms underpinning selectivity. Honey bees as well as other insects recruit biochemical defense mechanisms based on metabolic enzymes to facilitate the detoxification of xenobiotic compounds such as plant secondary metabolites, and also pesticides (Johnson, 2015). Gene superfamilies expressing detoxification enzymes such as cytochrome P450-monooxygenases (P450s) are key to overcoming or reducing the toxic effects mediated by foreign compounds (Gong and Diao, 2017; Panini et al., 2016; Rane et al., 2019). P450s, by far the most important group of detoxification enzymes, are involved in oxidative Phase I metabolism of a diverse range of xenobiotics, and are known to confer insecticide resistance (Dermauw et al., 2020; Feyereisen, 2011). The detoxification gene inventory of honey bees, including P450s, is reduced compared to other insects (Claudianos et al., 2006), and it has been speculated that this deficit may render them more sensitive to pesticides and lead to synergistic interactions among them (Berenbaum and Johnson, 2015). However, honey bees have been previously shown to exhibit marked tolerance to some pesticides such as *tau*-fluvalinate, a pyrethroid used in apiaries to control *Varroa* mites, an ectoparasite of increasing concern due to its involvement in global honey bee colony losses (Stokstad, 2019). It was found that the degradation of *tau*-fluvalinate in honey bees is mediated by P450 enzymes and in particular the CYP9Q subfamily by forming metabolites susceptible to further cleavage by esterases (Johnson et al., 2006; Mao et al., 2011). Interestingly members of the same P450 subfamily were recently shown to be involved in the differential toxicity of neonicotinoid insecticides against honey bees. Manjon et al. (2018) demonstrated that CYP9Q3 expressed in honey bee brain and Malpighian tubules, the insect equivalent of vertebrate kidneys, rapidly degraded thiacloprid but not imidacloprid. The authors functionally expressed all 27 honey bee P450 enzymes of the CYP 3 clade in insect cells and provided several lines of evidence that CYP9Q3 is the molecular determinant of thiacloprid selectivity in honey bees. CYP9Q3 orthologs rapidly metabolizing thiacloprid were subsequently also identified and characterized in buff-tailed bumble bee, *Bombus terrestris* (Manjon et al., 2018; Troczka et al., 2019) and red mason bee, *Osmia bicornis*, a solitary bee species (Beadle et al., 2019).

Honey bees are insects and adverse intrinsic effects upon contact to insecticides targeting a phylogenetically conserved and sensitive receptor site are therefore not surprising. As previously suggested, this innate risk needs to be mitigated by appropriate measures (Biddinger and Rajotte, 2015; Connolly, 2013; Hladik et al., 2018), helping farmers to protect their crops while avoiding harmful effect on off-target insects. The butenolide FPF was officially approved by the EU in 2015 (EU, 2015), and is considered bee-safe according to standard regulatory pesticide risk assessment (EFSA, 2015). FPF has also been shown to be honey bee-safe under field conditions (EFSA, 2015; Campbell et al., 2016), and an effective alternative solution for several highly destructive sucking pests formerly covered by neonicotinoid registrations (Nauen et al., 2015). FPF has a rather low acute contact toxicity to honey bees of > 100 μg/bee, and was shown to have a much lower impact on honey bee behavior at sublethal and field-relevant rates upon both acute and chronic exposure when compared to other modulators of nAChRs (Bell et al., 2020; Hesselbach et al., 2020; Hesselbach and Scheiner, 2018, 2019; Tong et al., 2019; Wu et al., 2021). However, sublethal effects such as poor coordination and hyperactivity were recently described in honey bees when this compound was tested in combination with the fungicide propiconazole, suggesting that the inhibition of honey bee P450s by azole fungicides has the potential to synergize FPF toxicity (Tosi and Nieh, 2019). Indeed the flupyradifurone (Sivanto®) label in the U.S. already contains language prohibiting mixing of flupyradifurone with azole fungicides during bloom period (https://www.cropscience.bayer.us/products/insecticides/sivanto/labels-msds).

The objective of the present study was to uncover the mechanistic and molecular drivers that contribute to the low toxicity of FPF to honey bees. We used a honey bee toxicogenomics approach to study the pharmacokinetics, toxicodynamics and metabolic fate of FPF, in order to decipher and understand the molecular determinants of FPF selectivity. Our aim was to develop a detailed biochemical and physiological understanding of how FPF interacts with honey bees at the molecular level

and demonstrate the potential of this approach to complement existing methods for bee pollinator pesticide risk assessment.

2. Materials and methods

2.1. Chemicals

[^{3}H]imidacloprid (specific activity 1.406 GBq/μmol), flupyradifurone (FPF) (Fig. S1), [^{14}C]-flupyradifurone (FPF) (label position furanone-4-[^{14}C], specific activity 4.24 MBq/mg), FPF-4-[(2,2-difluoroethyl)amino]-furanone (FPF-AF), FPF-acetic acid (FPF-AA), FPF-difluoroethanamine (FPF-DFEA) and FPF-hydroxy (FPF-OH) were of analytical grade and obtained in-house (Bayer AG, Monheim, Germany). The fluorescent probe 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC; CAS 277309-33-8) was custom synthesized by Enamine Ltd. (Kiev, Ukraine) with a purity of 95%. Purchased technical pesticides 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) and imidacloprid (CAS 138261-41-3, > 98%, Dr. Ehrenstorfer GmbH). HPLC gradient grade acetonitrile was purchased from Merck (Darmstadt, Germany). Unless otherwise mentioned all other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Honey bees

Adult worker honey bees (*Apis mellifera*) used in the toxicity assays, pharmacokinetic and radioligand binding studies were of mixed age and collected from queen-right colonies located in Monheim, Germany (GPS: N 51.0750, E 6.8984), maintained pesticide-free and managed according to standard beekeeping practice. The health status of the colonies was regularly checked by visual inspection. The colonies were not treated for Varroa mite control for at least three months prior to bee collection and testing. Honey bees used for gene expression analysis were collected from three different hives at the same location as mentioned above. Defined development stages were obtained by caging the queen and collecting larvae at 4, 6, 8, and 11 days post oviposition. Pupae were collected 18 days post oviposition. Adult bees were collected from frames containing brood (nurses), frames from the honey super (workers) and in front of the entrance (foragers). The samples were taken at two time points during the summer season and immediately snap frozen in liquid nitrogen and stored at − 80 °C until further use.

2.3. Toxicity bioassays

The acute oral and contact toxicity data of FPF and its respective metabolites against adult worker bees were assessed according to OECD guideline 213 (OECD, 1998a) and 214 (OECD, 1998b), and as recently published by the European Food Safety Authority (EFSA, 2015). We strictly followed the OECD guidelines without modification, *i.e.* all official criteria defined in the guidelines were met (particularly for oral assays), so studies were GLP compliant and meeting the EU regulatory requirements for plant protection product registration. Acute mixture contact toxicity assays were performed according to official OECD guideline 214 (OECD, 1998b) with slight adaptations to enable to test the hypothesis of synergism by fungicides. Briefly, bees were randomly collected from the honey super of different colonies in the morning and kept under test conditions (25 °C, 70% RH, complete darkness, in metal cages (L 8.5 x W 4.5 x H 6.5 cm (Fig. S2)) lined with filter paper) 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 technical fungicide onto the dorsal thorax one hour prior to insecticide application. Afterwards, bees were again anaesthetized and FPF was applied in acetone at different concentrations for dose-response analysis. Control bees were treated with acetone only. In synergist bioassays an additional control group was treated with the respective fungicide as a pre-treatment followed by acetone 1 h later. Applications were performed using a Hamilton syringe (Model 701N, Hamilton Company, Reno, NV, USA) 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 thrice with three replicates (n = 10 bees) per concentration.

2.4. Flupyradifurone pharmacokinetics in vivo and metabolite analysis

The pharmacokinetics and *in vivo* metabolism of FPF in worker bees was tracked by a [^{14}C]-label at the furanone-4 position as recently described (Zaworra et al., 2019). Briefly: worker bees were collected from hives, randomly placed in metal cages in groups of five bees and kept in a darkened laboratory at room temperature for 24 h prior to treatment. Sucrose solution (500 g/L Apipuder (Südzucker, Mannheim, Germany)) was constantly provided *ad libitum* by syringes. After 24 h 900 ng [^{14}C]-FPF (approx. 227,000 dpm) dissolved in 2 μL acetone was applied onto the dorsal thorax of bees anesthetized with CO_2. A group of control bees were treated with solvent only to check for solvent effects. Pharmacokinetic parameters were assessed 0, 2, 4 and 24 h after topical application in the treatment groups consisting of three replicates per time point. The subsequent sample preparation and metabolite analysis using LC-MS/MS was done exactly as described elsewhere (Zaworra et al., 2019). The electro-spray ionization MS spectra (ESI) for the extracted metabolites [^{14}C]FPF, [^{14}C]FPF-OH, [^{14}C]FPF-AF and [^{14}C]FPF-AA (Figs. S3–S6) were obtained with a Q Exactive mass spectrometer (Thermo, San Jose, CA, U.S.A.) 24 h after FPF application.

2.5. Receptor binding studies

Nicotinic acetylcholine receptor (nAChR) binding affinity of FPF and its metabolites was measured by the displacement of [^{3}H]-imidacloprid. Radioligand binding studies were performed using honey bee head membranes prepared from frozen (− 80 °C) honey bee heads following previously published protocols (Manjon et al., 2018). I_{50}-values and corresponding 95% confidence limits (CL 95%) obtained from radioligand displacement data were calculated using a four-parameter logistic non-linear fitting routine using GraphPad Prism software v8.3 (GraphPad Software Inc., San Diego, CA, USA).

2.6. Honey bee cytochrome P450 expression library

Twenty-seven honey bee clade 3 cytochrome P450 proteins (Table S1) used in this study were obtained by functional recombinant expression in High Five insect 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, Waltham, MA, USA) as recently described (Manjon et al., 2018; Nauen et al., 2021). Briefly: 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 5 G instrument (MP Biomedicals, Irvine, CA, USA) and centrifuged (10

min, 700 g, 4 °C). The supernatant was 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.

2.7. Flupyradifurone metabolism by honey bee P450s and UPLC MS/MS analysis

Functionally expressed honey bee P450s in isolated microsomes of High Five cells (2 mg mL^{-1} protein) were incubated with 10 μM FPF for a quantitative parent compound depletion screening. Incubations were carried out in 0.1 M potassium phosphate buffer pH 7.6 containing an NADPH-regenerating system (Promega, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, and 0.4 U/mL glucose-6- phosphate dehydrogenase) for 2 h at 30 °C in triplicate in 100 μL reaction volumes (40 μL microsomes, 10 μL 100 μM FPF and 50 μL buffer including NADPH regeneration system). Microsomes incubated without NADPH and cells infected with a mock virus served as controls. For Michaelis-Menten kinetics with recombinantly expressed CYP6AQ1, CYP9Q2 and CYP9Q3 (for GenBank accession numbers refer to Table S1) FPF was used in concentrations between 100 μM and 0.0317 μM employing the same assay conditions as described above with an incubation time of 1 h. In all cases the reaction was stopped with the addition of 400 μL ice cold acetonitrile. The samples were then stored overnight at 4 °C for protein precipitation. Afterwards they were centrifuged at 3200 g for 30 min at 4 °C and the supernatant subsequently analyzed by UPLC-MS/MS with slight modifications according to a previously published protocol (Manjon et al., 2018). Briefly, for the chromatography on an Agilent 1290 Infinity II, a Waters Acquity HSS T3 column (2.1 × 50 mm, 1.8 mm) with acetonitrile/water/1% formic acid as the eluent in gradient mode was employed. After positive electrospray ionization, ion transitions were recorded on a Sciex API6500 Triple Quad. FPF, FPF-OH, FPF-AF, FPF-AA and FPF-DFEA were measured in positive ion mode (ion transitions: FPF 289 > 126, FPF-OH 305 > 126, FPF-AF 164 > 146, FPF-AA 265 > 126, FPF-DFEA 207 > 126). The peak integrals were calibrated externally against a standard calibration curve. The linear range for the quantification of FPF, FPF-OH, FPF-AF, FPF-AA and FPF-DFEA was 0.1–200 ng/mL, 0.1–200 ng/mL, 2–100 ng/mL, 0.5–200 ng/mL and 0.5–200 ng/mL, respectively. Samples were diluted prior to measurement if needed. Recovery rates of parent compound using microsomal fractions with recombinantly expressed P450s without NADPH were normally close to 100%.

2.8. RNA isolation and RT-qPCR

Total RNA of honey bee larvae was isolated from pools of five individuals. RNA from adults and pupae was isolated from individual animals and pooled afterwards. At least two pools per hive, sampling time and developmental stage were used for gene expression analysis. In total we analyzed more than 96 samples comprised of eight different life stages collected from three different hives at two time points (four-week interval). The snap-frozen samples were ground using a stainless-steel bead with four disruption cycles at 20 Hz for 30 s in a Mixer Mill MM 300 (Retsch GmbH, Haan, Germany). RNA from first instar larvae was isolated using the PicoPure isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA from older larvae was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). An on-column RNase-Free DNase (Qiagen, Hilden, Germany) digest was included in both isolation procedures. Disrupted pupal and adult tissue was lysed using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA), and crude RNA was isolated using phenol-chloroform extraction. The RNA was further purified from the aqueous phase *via* magnetic beads using the Agencourt RNAdvance Tissue Kit (Beckman Coulter, Brea, CA, USA), followed by a DNase I digest (Agilent Technologies, Santa Clara, CA, USA). RNA was quantified by spectrophotometry (NanoQuant Infinite 200, Tecan, Switzerland) and its integrity verified by an automated gel electrophoresis system, according to CM-RNA and CL-RNA methods (QIAxcel RNA QC Kit v2.0, Qiagen, Hilden, Germany). For quantitative PCR (RT-qPCR) iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) was used for cDNA generation with 750 ng RNA used per reverse transcription reaction. Real-time PCR was performed in triplicate using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with 2.5 ng cDNA and 0.25 μM of each primer (Table S2) in a total reaction volume of 10 μL using a CFX384™ Real-Time system (Bio-Rad), and non-template mixtures as negative controls. The PCR program was as follows: 95 °C for 30 s; 95 °C for 15 s; 64 °C for 15 s; 60 °C for 15 s, plate read; steps 2–5 were repeated 30 times followed by a final melt-curve post-PCR (ramping from 65 °C to 95 °C by 0.5 °C every 5 s) to check for non-specific amplification. The amplification efficiency was determined for each primer pair and inter-run controls were included in each run to minimize plate/run specific effects. For normalization two reference genes, *polyubiquitin-A* and *tbp-association factor* were selected. These have been validated in previous studies (Cornman et al., 2013; Lourenço et al., 2008) and showed good stability across life stages and tissues ($M < 1$; $CV < 0.5$). Gene expression analysis was performed using qbase + software version 3.1 (Biogazelle, Zwijnaarde, Belgium) (Hellemans et al., 2007).

2.9. Transgenic Drosophila bioassays and microsomal isolation

Bioassays were conducted with Drosophila lines generated previously (Manjon et al., 2018, Table S3). Flies were reared in standard vials containing artificial diet (Jazz-Mix™ Drosophila Food, Thermo Fisher Scientific, Waltham, MA, USA). UAS-strains carrying the gene CYP9Q2 or CYP9Q3 and a control strain generated with an empty plasmid were crossed with the Hsp70-GAL4 strain. Four to eight-day old female flies of the F1 generation were incubated at 37 °C three times for 30 min with 1-hour intervals the day before starting the bioassay. Just prior to starting the bioassay, the flies were incubated once again.

Bioassays were carried out in 12-well plates (Greiner Bio-One, Kremsmünster, Austria) with 2 mL artificial diet per well. FPF was dissolved and diluted in pure acetone. Each dilution was then further diluted 1:2 in ddH_2O containing 0.1% Triton X-100. 50 μL of each concentration was transferred to a well (3 wells per concentration) and dispersed over the entire surface of the diet. Plates were left to dry completely before starting the bioassay. Ten flies were placed in each well. Full dose-response bioassays were repeated thrice. Mortality was scored after 48 h and 72 h. LD_{50} values were generated by probit analysis using PoloPlus 2.0 (LeOra Software, Petaluma, CA, USA).

For microsomal isolation adult flies were snap frozen after the heat shock procedure was conducted as described above and stored at −80 °C. Approximately 1 g of adult flies were homogenized for 30 s (4 times) in 50 mL ice-cold 0.1 M potassium phosphate buffer (pH 7.6; 1 mM EDTA; 1 mM DTT; 200 mM sucrose; one cOmplete™ EDTA-free Protease Inhibitor Cocktail tablet) using a FastPrep-24 5 G instrument (MP Biomedicals, Santa Ana, CA, USA). The homogenate was filtered through one layer of Miracloth (Merck Millipore, Burlington, MA, USA) and the microsomal fraction was obtained by differential centrifugation (10 min at 3000 g; 15 min at 10,000 g; 60 min at 100,000 g) at 4 °C. The resulting pellet was finally 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. Activity of microsomal fractions was confirmed against a selection of coumarin model substrates and FPF metabolism and quantification of metabolites was performed exactly as described above for recombinant honey bee P450s (Section 2.7).

2.10. Honey bee P450 inhibition kinetics

Michaelis-Menten kinetics for the recombinantly expressed honey bee P450s CYP9Q2, CYP9Q3 and CYP6AQ1 were conducted as previously described (Haas and Nauen, 2021) with slight modifications. Briefly, 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) was used as a suitable fluorescent probe substrate for all three enzymes. The formation of 7-hydroxy-4-(trifluoromethyl)coumarin was linear with time and BOMFC concentration (data not shown). Final concentrations of microsomal preparations were 0.16 mg mL^{-1} for CYP9Q2 and CYP9Q3 and 0.8 mg mL^{-1} for CYP6AQ1. Fluorescence measurements were done using a microplate reader (Tecan Spark, Tecan Group Ltd., Männedorf, Switzerland) at an emission wavelength of λ_{em} 510 nm (20 nm band width) while excited at λ_{ex} 405 nm (20 nm band width). All other parameters were the same as described elsewhere (Haas and Nauen, 2021).

For IC_{50} determinations a single BOMFC concentration close to its K_m-value for the respective P450 enzyme was used, *i.e.* 6.5 μM, 15 μM and 20 μM for CYP9Q3, CYP9Q2 and CYP6AQ1, respectively (Fig. S7). The microsomal protein amount in 50 μL reaction volumes was 4 μg for measurements with CYP9Q2 and CYP9Q3, but 40 μg for CYP6AQ1. Azole fungicides were tested using a 5-fold dilution series ranging from 50 to 0.0032 μM, whereas FPF and its metabolites were tested using a 5-fold dilution series ranging from 100 μM to 0.032 μM. All other parameters were the same as recently described (Haas and Nauen, 2021).

2.11. Statistical analysis

Significant differences ($p < 0.05$) in gene expression between life-stages was analyzed by one-way ANOVA with *post hoc* Tukey's Honest Significant Difference (HSD) test. Further information on statistical data analysis is given in respective figure legends where appropriate. Enzymatic data obtained from substrate and/or inhibitor incubations with recombinantly expressed P450s 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). All other experimental data were analyzed and visualized using GraphPad Prism v8.3 unless otherwise stated.

3. Results

3.1. Pharmacokinetics and metabolic fate of [^{14}C]-FPF following contact exposure

The pharmacokinetic behavior of [^{14}C]-FPF in honey bees at different elapsed time intervals post-exposure was studied upon topical application of 900 ng a.i./bee, *i.e.* a dose 100-times lower than the contact LD_{50}-value of FPF (> 100 μg/bee; Table 1). All bees survived the treatment and showed no symptoms of poisoning or behavioral abnormalities at any assessed time point. FPF penetrated the honey bee cuticle relatively slowly with 86.4 ± 3.75% and 76.2 ± 5.09% of the radiolabel recovered from the external wash 4 h and 24 h after application, respectively (Fig. 1). Twenty-four hours after application 12.4 ± 0.74% of the applied [^{14}C]-FPF equivalents were extracted from bee tissue (internal), and 11.3 ± 4.95% collected as excreta, thus indicating clearance of almost 50% of the radiolabel within 24 h of cuticular uptake (Fig. 1). Qualitative HPLC ESI-MS analysis of honey bee tissue extracts revealed that the parent FPF dominates the recovered compounds, followed by FPF-AF. Other metabolites identified were FPF-AA and FPF-OH, while some of the other smaller peaks could not be clearly identified (Fig. 2).

3.2. Acute honey bee toxicity and nAChR binding of FPF metabolites

The FPF metabolites FPF-OH, FPF-AF and FPF-AA identified *in vivo* were practically non-toxic to worker bees when tested in standard OECD acute contact and oral toxicity bioassays (Table 1). Even at the highest metabolite dose tested no symptoms of poisoning were observed. The resulting LD_{50}-values were > 100 μg/bee and > 81.5 μg/bee after contact and oral application, respectively. Considering the oral route of exposure, all metabolites are practically non-toxic when compared to FPF (LD_{50}-value: 1.2 μg/bee), thus indicating an effective metabolic detoxification of FPF in honey bees. These bioassay findings are

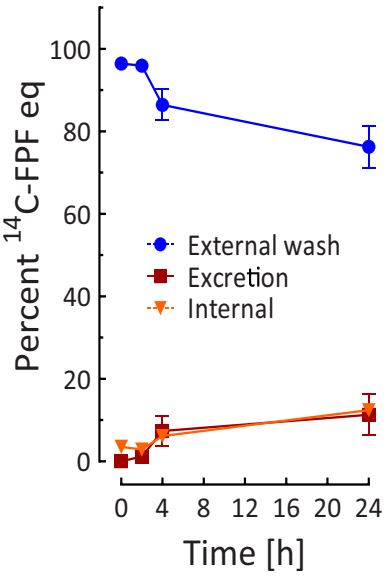

Fig. 1. Uptake and distribution of [^{14}C]-FPF-equivalents expressed as percent recovered radioactivity at different elapsed time intervals after topical application of honey bee adults with [^{14}C]-FPF (900 ng). Data are mean values ± SEM (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Inhibition of [3H]imidacloprid binding (I50) to honey bee head membrane nAChR preparations by the butenolide insecticide flupyradifurone and its metabolites compared with the neonicotinoid imidacloprid and one of its main metabolites, 5-hydroxy-imidacloprid (IMD-OH).

		nAChR binding studies			LD_{50} (μg a.i./bee)	
Chemical class	Compound	I_{50} [nM]	CL 95%	Ratio[a]	Contact	Oral
Butenolide	Flupyradifurone (FPF)	7.6	5.8–9.9	–	> 100	1.2
Neonicotinoid	Imidacloprid (IMD)	1.2	0.93–1.6	–	0.0251	0.0037
Butenolide	FPF-OH	1700	430–6800	224	> 100	> 105
	FPF-amino-furanone (FPF-AF)	> 10,000	–	> 1300	> 100	> 81.5
	FPF-acetic acid (FPF-AA)	> 10,000	–	> 1300	> 100	> 90
	FPF-difluoroethanamine (FPF-DFEA)	> 10,000	–	> 1300	> 100	nd
Neonicotinoid	IMD-OH	24	15–37	20	–	0.159

Acute honey bee toxicity data were taken from Nauen et al. (2001), Nauen et al. (2015) and EFSA (2015), except for FPF-AA and FPF-DFEA (this study).
[a] Binding affinity relative to the parent compound

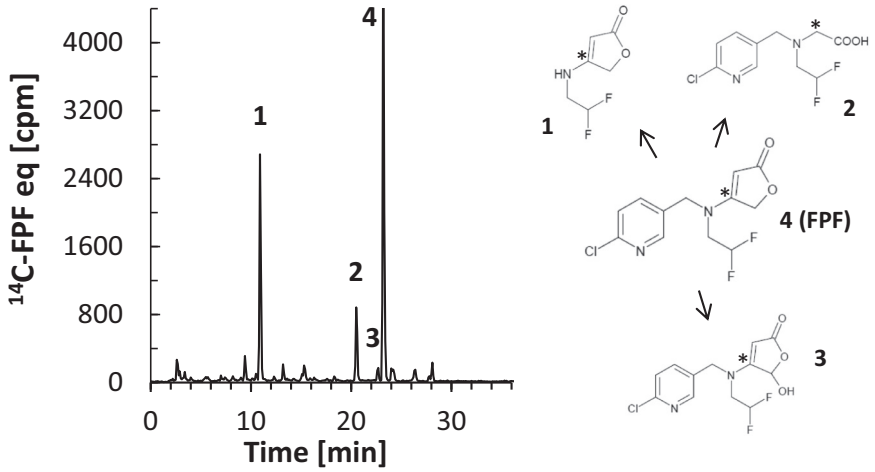

Fig. 2. HPLC radiohistogram of a sample extracted from honey bees topically treated with 900 ng [^{14}C]-FPF 24 h prior to extraction for qualitative metabolite profiling using ESI-MS. Reference standards allowed the identification of three metabolites: (1) FPF-(difluoroethyl)amino-furanone (FPF-AF), (2) FPF-acetic acid (FPF-AA) and (3) FPF-5hydroxy (FPF-OH). The [^{14}C]-label position is indicated by an asterisk.

supported by the lack of competitive high-affinity binding of any of the tested metabolites to [^{3}H]imidacloprid sensitive nAChRs in honey bee head membrane preparations when compared to FPF, which showed nanomolar affinity in radioligand binding studies (Table 1). This is in strong contrast to one of the major metabolites of the neonicotinoid imidacloprid after contact application, IMD-OH (Zaworra et al., 2019), which shows high-affinity binding to honey bee nAChRs in the nanomolar range (I_{50} 24 nM) and an oral LD_{50}-value of 0.159 µg/bee (Table 1). FPF-OH, the hydroxylated butenolide variant, and minor metabolite detected *in vivo* (Fig. 2), binds with much lower affinity (71-fold) to honey bee nAChRs and is practically non-toxic (> 105 µg/bee) in acute oral bioassays. The major FPF metabolite detected in our pharmacokinetic study, FPF-AF, did not bind to nAChRs at concentrations as high as 10,000 nM (Table 1). Finally, our data revealed a significant 6-fold lower binding affinity of FPF to honey bee nAChR preparations than the neonicotinoid insecticide imidacloprid (I_{50}-values of 7.6 and 1.2 nM, respectively; Table 1).

3.3. Cytochrome P450-mediated degradation of FPF

In order to investigate the oxidative metabolic fate of FPF at the molecular level we recombinantly expressed 27 different CYP3 clade honey bee P450 enzymes individually in High Five cells and examined their capacity to metabolize FPF *in vitro*. Many of the honey bee P450s

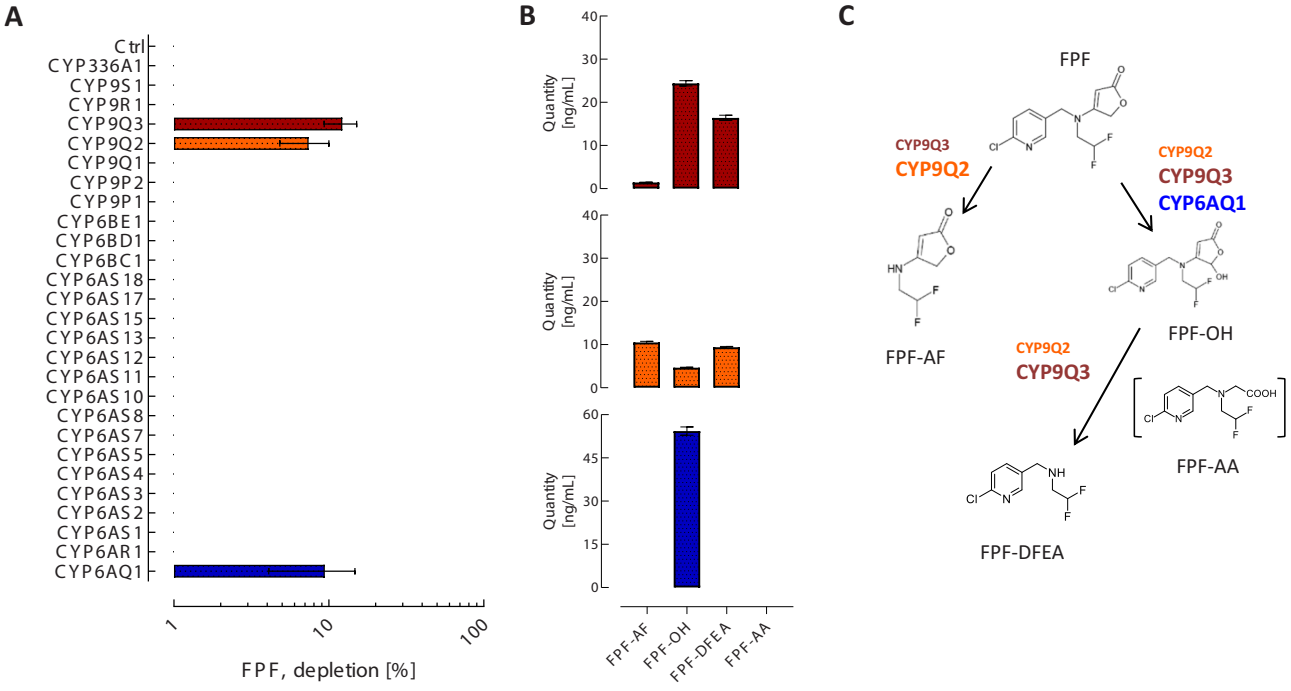

Fig. 3. (A) Flupyradifurone (FPF) depletion by 27 recombinantly expressed cytochrome P450s of the honey bee CYP3 clade measured by UPLC-MS/MS analysis and (B) quantity of metabolites detected after the incubation of FPF with recombinantly expressed honey bee CYP9Q3 (top), CYP9Q2 (center) and CYP6AQ1 (bottom). Data are mean values ± SD (n = 3). FPF-(difluoroethyl)amino-furanone (FPF-AF), FPF-5hydroxy (FPF-OH), FPF-difluoroethanamine (FPF-DFEA) and FPF-acetic acid (FPF-AA). (C) Proposed scheme of oxidative metabolic fate of FPF mediated by honey bee cytochrome P450s. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

tested, and mock cell microsomal preparations, did not metabolize FPF. However, a significant depletion of FPF was observed after incubation of FPF with functionally expressed CYP9Q2, CYP9Q3 and CYP6AQ1 (Fig. 3A), identifying them as strong candidate enzymes driving the oxidative metabolism of FPF *in vivo*. A more detailed quantitative UPLC-MS/MS analysis revealed that the hydroxylation of the furanone moiety - resulting in FPF-OH - is catalyzed by all three P450s at varying levels and follows Michaelis-Menten kinetics, with highest V_{max} values obtained for CYP6AQ1 (Fig. S8). However, the cleavage of the 6-chloro-pyridinylmethylamine bridge forming FPF-AF (and its counterpart 6-chloro-2-picolyl alcohol) is in particular mediated by CYP9Q2 (Fig. 3B). We identified a third metabolite, FPF-DFEA, most likely resulting from the oxidative degradation of the FPF(-OH) furanone moiety catalyzed by CYP9Q2 and CYP9Q3 (Fig. 3B and C). The metabolite FPF-DFEA was not detected during the *in vivo* pharmacokinetic study with radiolabeled FPF, because the applied FPF was labeled at the [furanone-4-^{14}C] position (Fig. 2). We hypothesize that FPF-AA (detected *in vivo*) and its unstable oxidized derivative FPF-AA-2-oxo, respectively, are potential intermediates resulting in FPF-DFEA (Fig. 3C).

3.4. Enzyme kinetics, biochemical characterization and validation of FPF-metabolizing P450s

We identified BOMFC as an appropriate probe substrate to measure the activity of the FPF-metabolizing and recombinantly expressed P450s CYP9Q2, CYP9Q3 and CYP6AQ1. The P450 catalyzed formation of the fluorescent product, 7-hydroxy-4-(trifluoromethyl)coumarin (HC), can be easily followed facilitating high-throughput rapid enzyme kinetic measurements. CYP9Q3-mediated fluorescent product formation is inhibited by increasing concentrations of FPF (Fig. 4A), and Michaelis-Menten kinetics revealed significantly decreased V_{max} values and unchanged K_m values, indicating non-competitive inhibition of HC formation by FPF (Table S4). Similar results were obtained for CYP9Q2 and CYP6AQ1 and are summarized in Table S4. Full dose response analysis revealed a rather weak inhibition of BOMFC metabolism by FPF (Fig. 4B) as well as FPF-OH (Fig. 4C) for all three P450 enzymes, as demonstrated by I_{50}-values of > 10 μM (Table S5). Based on the fluorescence assays with BOMFC we noticed that FPF-OH showed the highest affinity to CYP9Q3 (I_{50} 20.9 μM), followed by CYP9Q2 (I_{50} 69.2) – whereas CYP6AQ1 is hardly affected by FPF-OH ($I_{50} > 300$ μM), which is in contrast to its much more pronounced affinity to FPF (I_{50} 17.0 μM) (Table S5). These enzyme kinetic data strongly support the analytical results on the metabolic detoxification of FPF we obtained for the individual P450s (Fig. 3C). Particularly, the interaction of FPF-OH with the different P450s shown in Fig. 4C correlates with the extent of FPF-DFEA formation by recombinantly expressed CYP9Q3 and CYP9Q2 when directly incubated with FPF-OH (Fig. 4D), thus supporting the proposed oxidative metabolic fate of FPF shown in Fig. 3C. Recombinantly expressed CYP6AQ1 did not form FPF-DFEA, a finding strongly supported by the lack of binding of FPF-OH to CYP6AQ1 (Fig. 4C and Table S5).

In order to provide a further line of evidence underpinning the importance of the identified P450s for the oxidative metabolism of FPF, we employed two lines of transgenic *Drosophila* ectopically expressing the honey bee genes CYP9Q2 and CYP9Q3, respectively. No such transgenic line was available for CYP6AQ1. Transgenic flies expressing CYP9Q2 or CYP9Q3 under the control of a HSP70 promoter were

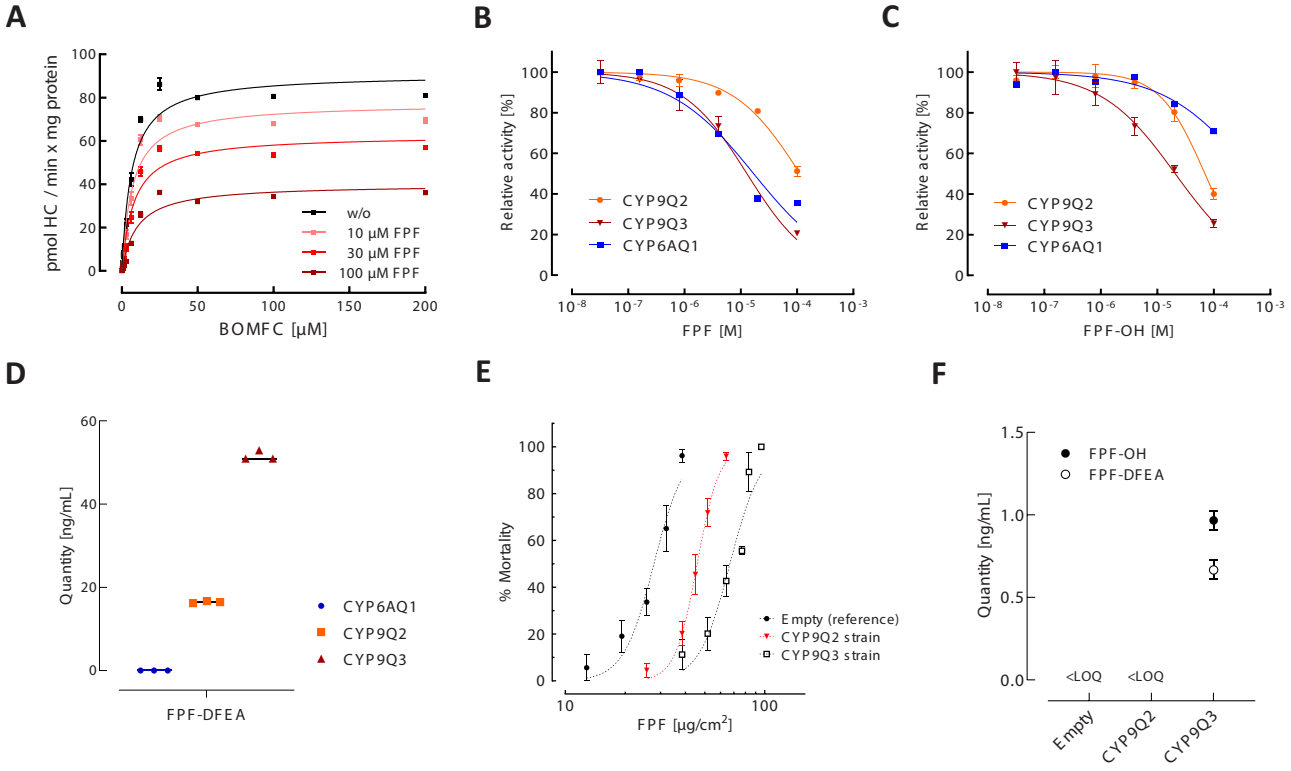

Fig. 4. (A) Steady-state kinetics of 7-hydroxy-4-(trifluoromethyl)coumarin (HC) formation by CYP9Q3 using BOMFC as a substrate in the presence of flupyradifurone (FPF). (B) Effect on P450-mediated BOMFC metabolism by increasing concentrations of FPF and (C) FPF-OH; data are mean values ± SD (n = 4). (D) Formation of FPF-difluoroethanamine (FPF-DFEA) by honey bee P450s incubated with FPF-OH (n = 3). (E) Efficacy of FPF against transgenic *Drosophila* strains ectopically expressing CYP9Q2 and CYP9Q3, respectively, in comparison to a control strain (Empty); data are mean values ± SEM (n = 3). (F) Quantification of FPF-OH and FPF-DFEA after incubation of FPF with microsomal preparations of transgenic flies expressing honey bee CYP9Q2 and CYP9Q3 in comparison to control flies; data are mean values ± SD (n = 3). LOQ = limit of quantitation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

significantly less sensitive to FPF compared to a control strain lacking the transgenes (Fig. 4E). The LD_{50}-value of FPF against flies expressing CYP9Q3 and CYP9Q2 was 65.7 μg/cm^2 (CI95% 55.3–81.6) and 45.3 μg/cm^2 (CI95% 39.8–51.7), respectively, when compared to a control strain (LD_{50} 26.5 μg/cm^2; CI95% 20.1–36.0) (Table S6). This finding demonstrates the potential of CYP9Q2 and CYP9Q3 to confer FPF tolerance *in vivo*. Based on the calculated LD_{50}-values and non-overlapping confidence intervals (95%) CYP9Q3 expressing flies were significantly less sensitive to FPF than CYP9Q2 expressing flies. This is consistent with the findings obtained for the recombinantly expressed enzymes in the biochemical and analytical assays described above. Furthermore, we incubated FPF with microsomal fractions prepared from transgenic *Drosophila* and subsequently analyzed them by UPLC-MS/MS for the presence of FPF metabolites. We detected the formation of FPF-OH and FPF-DFEA in microsomes from flies expressing CYP9Q3, whereas the metabolite levels in microsomal preparations from all other lines were below the limit of quantification (Fig. 4F). The detection of both FPF-OH and FPF-DFEA in microsomal preparations from CYP9Q3 expressing flies is in line with our findings obtained from FPF incubations with recombinantly expressed CYP9Q3 (Fig. 3B).

3.5. P450 gene expression profiling across honey bee life stages

We used RT-qPCR to determine the level of expression of the identified FPF-degrading P450s at eight different time points across honey bee life-stages, covering early to late larval instars, early and late pupal stages after brood cell capping, as well as adults divided into nurse, worker (in hive) and foragers, collected at the hive entrance. The highest expression levels for all three P450 genes was observed in late larvae and adults, suggesting a high potential to detoxify FPF in these life stages. CYP6AQ1 and CYP9Q2 were expressed 1000-fold and 100-fold higher in adults compared to first instar larvae respectively (Fig. 5). Overall, the expression level followed a similar pattern for the three P450 genes implicated in FPF-metabolizing, *i.e.* showing rather high expression levels before brood cell capping, a significant decline during pupation and a marked, highly significant increase after eclosion (Fig. 5). Based on the rather low P450 transcript levels at the early larval stages, *i.e.* four and six days after oviposition, we assume that these stages have the lowest capacity to detoxify FPF. In contrast, adults, that collect and process pollen as food for larval consumption, have the greatest capacity to metabolize FPF based on the high expression levels of CYP6AQ1, CYP9Q2 and CYP9Q3 in this life stage.

3.6. P450 inhibition by azole fungicides and FPF synergism

Having deciphered the honey bee P450s involved in the detoxification of FPF we tested their sensitivity to three common azole fungicides in order to predict their potential to synergize FPF acute honey bee toxicity under laboratory conditions. Such a mechanistic approach at the molecular level can be used to rapidly uncover possible toxicity risks of applying FPF as mixtures with these fungicides. We utilized the fluorescent probe kinetic assay described above and measured the inhibition of P450-mediated BOMFC metabolism by prochloraz, propiconazole and prothioconazole (Fig. 6A). Prochloraz strongly inhibited CYP9Q3, CYP9Q2 and CYP6AQ1 activity exhibiting I_{50}-values of 13 nM, 29 nM and 6.8 nM, respectively (Table S7). Propiconazole also inhibited CYP9Q3 and CYP9Q2 in the nanomolar range exhibiting I_{50}-values of 72 nM and 160 nM, respectively. It was a less effective inhibitor of CYP6AQ1, as demonstrated by an I_{50}-value of 1100 nM. In contrast to prochloraz and propiconazole, prothioconazole was a very weak inhibitor of CYP9Q3 and CYP9Q2, showing I_{50}-values of 39,000 nM and 19,000 nM, respectively. However, it was somewhat more active against CYP6AQ1 (I_{50} 3700 nM), but still significantly less effective when compared to the other azole fungicides (Table S7).

Honey bee contact toxicity bioassays revealed a strong synergism of FPF acute toxicity by propiconazole and prochloraz, but not prothioconazole (Fig. 6B). FPF was synergized by > 243-fold and > 112-fold when pre-treated with prochloraz and propiconazole, respectively (Table S8), whereas, the synergistic ratio observed in combination with prothioconazole was much lower (>1.15). Thus, the capacity of these fungicides to synergize the toxicity of FPF in *in vivo* is entirely consistent with the ability of these compounds to inhibit key FPF-metabolizing P450 enzymes.

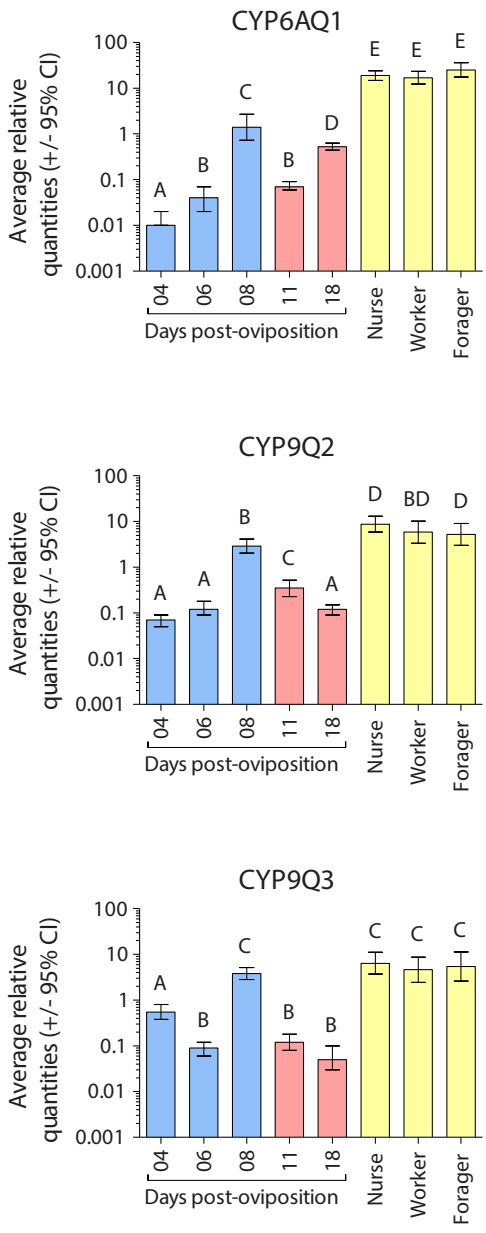

Fig. 5. Relative expression measured by quantitative PCR of honey bee P450 genes involved in the oxidative detoxification of flupyradifurone. Significant differences ($p < 0.05$) in expression between life-stages are denoted by different letters above bars as determined by one-way ANOVA with *post hoc* Tukey HSD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

4. Discussion

FPF belongs to the new class of butenolide insecticides that selectively target insect nAChRs, with a similar mode of action as other commercial competitive modulators acting on nAChRs such as neonicotinoids and sulfoximines (Nauen et al., 2015; Casida, 2018). Our

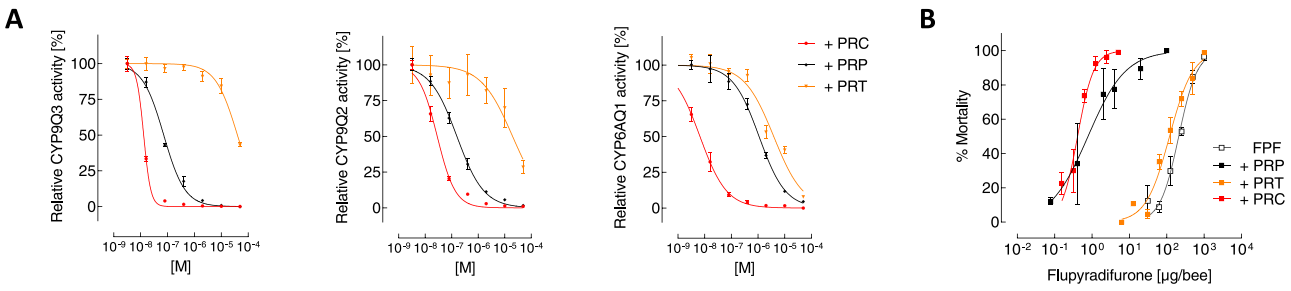

Fig. 6. (A) Inhibition of recombinantly expressed CYP9Q3, CYP9Q2 and CYP6AQ1 by three azole fungicides using BOMFC as a substrate. Data are mean values ± SD (n = 4). (B) Dose-response relationship and synergism of flupyradifurone (FPF) when topically applied to honey bees either alone or pre-treated with the azole fungicides propiconazole (PRP), prothioconazole (PRT) and prochloraz (PRC). Data are mean values ± SEM (n = 3–4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

radioligand binding studies confirm this mode of action, although FPF showed a slightly, but significantly lower binding affinity to honey bee nAChR preparations than the neonicotinoid insecticide imidacloprid. However, this finding is likely explained by the chemical differences between these insecticides, with FPF characterized by its novel butenolide pharmacophore, as previously demonstrated using cheminformatics (Jeschke et al., 2015; Nauen et al., 2015). This is further supported by homology modeling and docking approaches at the level of calculated electron densities, which revealed slight differences between nAChR modulators in their binding topology at the orthosteric site of nAChRs (Beck et al., 2015). However, despite its nanomolar receptor binding affinity, FPF is significantly less acutely toxic to honey bees compared to most neonicotinoid insecticides and sulfoximines (Iwasa et al., 2004; EFSA, 2015; EFSA, 2019b). In order to uncover the underlying physiological and biochemical principles explaining the low acute honey bee toxicity of FPF, we combined pharmacokinetic and toxicogenomic approaches as a complement to standard regulatory bee pollinator pesticide risk assessment (López-Osorio and Wurm, 2020). Indeed, such approaches have already been successfully employed to decipher the molecular determinants of neonicotinoid selectivity in different bee pollinator species (Manjon et al., 2018; Beadle et al., 2019; Zaworra et al., 2019), and to understand the biochemical mechanisms of pesticide synergism in honey bees (Haas and Nauen, 2021).

Our pharmacokinetic studies revealed a slow uptake of topically applied [^{14}C]FPF through the honey bee cuticle over 24 h (23.7%). This is less than that recorded for the neonicotinoids thiacloprid (38%) and imidacloprid (60%) over the same time interval (Zaworra et al., 2019). Additionally, half of the amount of [^{14}C]FPF equivalents taken up by honey bees were excreted within 24 h, suggesting a rapid clearance of parent compound as well as radiolabeled FPF metabolites. Zaworra et al. (2019) showed in an almost identical experimental set-up that [^{14}C] imidacloprid equivalents accumulated in honey bees and were only slowly excreted. However, the authors showed that imidacloprid treated bees exhibited neurotoxic symptoms of poisoning, most likely slowing down functional metabolism and excretion, as reported in earlier studies (Suchail et al., 2004). Based on the pharmacokinetic results obtained here, we suggest that limited penetration and rapid clearance are factors contributing to the classification of FPF as “practically non-toxic” (U.S. EPA, 2014) upon contact application in acute tier I honey bee toxicity assays (LD_{50} >100 µg/bee). It is worth mentioning that cuticle-applied thiacloprid showed a pharmacokinetic behavior comparable to FPF (Zaworra et al., 2019), but its affinity to honey bee nAChRs is 7-fold higher compared to FPF (Manjon et al., 2018). This suggests a pharmacokinetically driven toxicodynamic component is involved in the differential acute toxicity between FPF and neonicotinoid insecticides.

The acute oral toxicity of FPF (LD_{50} 1.2 µg/bee) is > 80-fold higher than its acute contact toxicity, suggesting a quicker absorption and distribution *via* this exposure route. However, in contrast to the neonicotinoid imidacloprid, the oral toxicity of the butenolide FPF is several hundred-fold lower (Table 1). This is not explained by differences in their physicochemical properties or their affinity to honey bee nAChRs. HPLC analysis of homogenized honey bee tissue samples taken from our pharmacokinetic experiment with [^{14}C]FPF suggest that the low acute oral toxicity of FPF is most likely based on its *in vivo* metabolic fate. This results in the generation of practically non-toxic FPF metabolites that lack high affinity nAChR binding properties. We clearly identified three [^{14}C]-labeled metabolites: FPF-AF, FPF-AA and FPF-OH, most likely generated by, (1) cleavage of the 6-chloro-pyridinylmethylamine bridge, (2) oxidative degradation of the butenolide moiety and (3) hydroxylation of the butenolide moiety, respectively. These empirically identified sites of oxidative attack match those computationally predicted by local reactivity descriptors using Fukui functions (Beck, 2005; Fig. S1). FPF-OH formed the smallest fraction of the metabolites identified *in vivo*. Previous pharmacokinetic studies with the neonicotinoids imidacloprid and thiacloprid detected hydroxy-imidacloprid and hydroxy-thiacloprid as major metabolites, respectively (Suchail et al., 2004; Zaworra et al., 2019). Of these hydroxylated imidacloprid has been shown to bind strongly to insect nAChRs and to be highly toxic to honey bees (Table 1), suggesting that its formation as a major metabolite facilitates toxicity (Nauen et al., 2001; Suchail et al., 2001, 2004). In contrast, FPF-OH binds only weakly to honey bee nAChR preparations, and, as demonstrated here, binding is too weak to result in measurable acute oral and contact toxicity. As we used furanone-4-[^{14}C] radiolabeled FPF we failed to detect any expected (major) non-labeled metabolites resulting from its degradation pathway, such as 6-chloropicolyl alcohol and its further oxidized derivative 6-chloronicotinic acid, however, both these metabolites are practically non-toxic to honey bees as they lack the attached butenolide pharmacophore (EFSA, 2015).

The pharmacokinetic data presented here strongly suggested oxidative degradation as the key pathway for the metabolic fate of FPF. Therefore, we employed a recently constructed P450 library of the honey bee CYP 3 clade to identify potential candidate genes mediating FPF metabolism (Manjon et al., 2018). We incubated FPF *in vitro* with 27 different honey bee P450 enzymes recombinantly expressed in insect cells and identified three candidate P450s involved in the depletion of FPF, namely CYP9Q2, CYP9Q3 and CYP6AQ1. Both CYP9Q2 and CYP9Q3 are involved in the formation of FPF-AF (and 6-chloropicolyl alcohol) as well as FPF-OH, whereas CYP6AQ1 selectively hydroxylates FPF. We also demonstrated that CYP9Q2 and CYP9Q3, but not CYP6AQ1, are involved in consecutive oxidative reactions leading to FPF-DFEA, a metabolite lacking the butenolide pharmacophore, which is therefore inactive. This metabolite is possibly formed *via* FPF-AA as an intermediate product, which we have not detected *in vitro* (see Fig. 3). On the other hand, we did not detect FPD-DFEA (but FPF-AA) in our *in vivo* pharmacokinetic experiment, given that we applied [furanone-4-^{14}C]-FPF and not [6-chloro-pyridinylmethyl-^{14}C]-FPF.

CYP9Q2 and CYP9Q3 have been previously described as the molecular determinants of neonicotinoid selectivity in honey bees, *i.e.* explaining the practically non-toxic behavior of *N*-cyanoimine neonicotinoids such as thiacloprid and acetamiprid by mediating

hydroxylation and *N*-demethylation, respectively (Manjon et al., 2018). The same P450 subfamily was already shown to be involved in *tau*-fluvalinate, coumaphos and quercetin metabolism in honey bees (Mao et al., 2011). Furthermore, it was demonstrated that the expression of CYP9Q2 and CYP9Q3 in the honey bee brain is induced upon exposure to insecticidal organophosphates (Christen and Fent, 2017). Taken together, these studies provide a growing body of evidence that P450s of the CYP9Q subfamily play a crucial role in the detoxification of diverse chemical classes of xenobiotics in honey bees, including several insecticides. Indeed, this claim is further supported by studies demonstrating that functionally expressed CYP9Q orthologs from bumblebees (*Bombus terrestris*), *i.e.* CYP9Q4, CYP9Q5 and CYP9Q6 (Manjon et al., 2018; Troczka et al., 2019), and red mason bees (*Osmia bicornis*), *i.e.* CYP9BU1 and CYP9BU2 (Beadle et al., 2019), metabolized *N*-cyanoimine neonicotinoids. It remains to be shown if these orthologs of honey bee CYP9Q genes also mediate FPF metabolism. However, recently published FPF acute contact toxicity LD_{50}-values are comparatively low ($LD_{50} > 11$ μg/bee) against both *B. terrestris* and *O. bicornis,* possibly indicating an evolutionary conserved role for these CYP9Q orthologs in xenobiotic detoxification and thus FPF selectivity across several bee species. This conclusion is further supported by a recent study linking the increased sensitivity of the alfalfa leafcutter bee, *Megachile rotundata,* towards the above-mentioned compounds (including FPF) to the lack of CYP9Q orthologs in its genome (Hayward et al., 2019).

The constitutive expression of honey bee *CYP9Q* genes has been investigated extensively, revealing that it is highest in detoxification-relevant tissues and life stages. For example, CYP9Q transcripts have been found at high levels in the brain and Malpighian tubules (Manjon et al., 2018; Vannette et al., 2015), and shown to be elevated in mandibular and hypopharyngeal glands, as well as in antennae and legs of honey bee foragers when compared to nurse bees (Mao et al., 2015; Vannette et al., 2015). Our study revealed constitutive expression of CYP9Q2, CYP9Q3 and CYP6AQ1 across developmental stages with highest levels in those stages primarily exposed to xenobiotics, *e.g.* adults, but also late larvae. The observed expression profile, particularly in motile stages, likely mirrors a general protective role of these P450s in xenobiotic defense in honey bees. The lower expression in (early) larval stages might suggest that larvae are more susceptible to flupyradifurone than adults, but this is not supported by *in-vivo* data where a comparable toxicity between adults and larvae is observed (EFSA, 2015). However, we still think it is an interesting topic for future research. Little is known about CYP6AQ1 and its role in xenobiotic detoxification, however, a recent study described its transcriptional regulation after exposure to a pyrethroid insecticide (Wieczorek et al., 2020). Interestingly, significant up- and/or down-regulation of P450 gene expression after FPF exposure was not found in honey bee larvae (Kablau et al., 2020) or adults (Wu et al., 2021), suggesting a minor, if any, role in honey bee P450 induction by FPF. However, P450 induction and possible consequences on the detoxification of the respective inducing agent needs to be interpreted with care. For example, none of the P450s induced by honey bee exposure to thiacloprid metabolized thiacloprid when functionally expressed in *E. coli* (Alptekin et al., 2016). This finding demonstrates the importance of functionally validating the detoxification role of genes upregulated upon exposure to chemicals such as insecticides. Recently, a fluorescent probe-based assay of honey bee P450 enzymes has been described that allows the rapid identification of P450-insecticide interactions (Haas and Nauen, 2021). In the present study, steady-state kinetics of 7-hydroxy-4-(trifluoromethyl)coumarin formation by CYP9Q3, CYP9Q2 and CYP6AQ1 using BOMFC as a substrate in the presence of FPF unambiguously confirmed its binding to the catalytic site of these enzymes. Similar assays have been used for many years in the pharmaceutical industry to screen for adverse effects of drugs on human P450 enzymes (Fowler and Zhang, 2008; Kosaka et al., 2017), and it has been recently proposed that similar molecular approaches can complement current bee pollinator pesticide risk assessment (López-Osorio and Wurm, 2020). Here, transgenic *Drosophila* ectopically expressing honey bee CYP9Q2 and CYP9Q3 (McLeman et al., 2020) were employed to provide an additional line of evidence for the importance of these enzymes in FPF selectivity. As shown earlier for thiacloprid (Manjon et al., 2018), these transgenic flies were also significantly more tolerant to FPF than wildtype flies, underpinning the crucial role for CYP9Q2 and CYP9Q3 for the observed FPF tolerance in honey bees. We also confirmed FPF-OH and FPF-DFEA as the main metabolites generated by microsomal preparations from transgenic flies expressing CYP9Q3. Interestingly, no FPF metabolites were detected in microsomal preparations of control flies that lack a transgene, demonstrating that the microsomal P450 gene inventory of *Drosophila*, even if more diverse compared to honey bees (Berenbaum and Johnson, 2015), lacks the capacity to detoxify FPF.

Finally, we were able to show that the identified honey bee P450s mediating FPF metabolism are strongly inhibited by commonly used fungicides such as propiconazole and prochloraz, and to a much lesser extent by prothioconazole. This finding explains at the molecular level the synergistic effects recently described when FPF was co-applied with the azole fungicide propiconazole (Tosi and Nieh, 2019). The synergistic effect demonstrated by the authors is most likely based on the inhibition of the individual P450s identified here that are directly involved in the metabolic fate of FPF. Similar synergistic effects, leading to increased honey bee toxicity, were demonstrated with thiacloprid/prochloraz mixtures (Wernecke et al., 2019). In this case it was shown, using the recently proposed molecular pesticide risk assessment approach, that the observed synergism is driven by the inhibition of CYP9Q3 (Haas and Nauen, 2021). Interestingly, intrinsic synergistic effects of prothioconazole are very weak *in vivo* (Table S8; supported by rather low P450 inhibition *in vitro*) and may not even qualify as synergism according to the definitions that have been used to constitute a synergistic effect (Cedergreen, 2014; Belden and Brain, 2018; Carnesecchi et al., 2019). Use restrictions are in place regarding tank mixtures of flupyradifurone with azole fungicides during bloom and the obtained data demonstrate the importance of pesticide applicators adhering to that guidance to mitigate the risk of synergistic interaction. Our results, however, raise the question if all azole fungicides can be considered equal when it comes to their inhibitory potential towards essential cytochrome P450s.

Synergistic interactions between pesticides in bees have been known for a long time (Pilling and Jepson, 1993; Pilling et al., 1995; Johnson et al., 2006; Iwasa et al., 2004), but are now an issue of growing regulatory concern (Johnson et al., 2013; Robinson et al., 2017; Sgolastra et al., 2017; Carnesecchi et al., 2019). However, as demonstrated here for FPF, if the molecular basis driving synergistic effects for a given insecticide is known, biochemical assays can provide a useful complement to existing risk assessment approaches by allowing a better understanding of the mechanistic basis of potential adverse interactions (López-Osorio and Wurm, 2020; Haas and Nauen, 2021). It is, however, important to acknowledge that higher tier studies under field-applied conditions at realistic exposure scenarios remain important to elucidate the impact of potential harmful interactions identified in the laboratory (Schmuck et al., 2003; Thompson et al., 2014).

In conclusion, our pharmacokinetic and toxicogenomic approach has provided new insights into the molecular mechanisms contributing to the honey bee safety profile of the butenolide insecticide FPF. We propose that the data gathered using such a mechanistic pesticide risk assessment approach has strong potential to significantly complement that generated in whole-organism studies as part of existing regulatory requirements.

CRediT authorship contribution statement

Ralf Nauen: Conceptualization, Supervision, Methodology. **Julian Haas, Gillian Hertlein, Maxie Kohler, Andreas Lagojda, Bettina Lueke, Marion Zaworra**: Methodology, Investigation, Data curation, Visualization. **Julian Haas, Ralf Nauen, Marion Zaworra**: Writing - original draft preparation. **Chris Bass, Emyr Davies**: Resources. All

authors: Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Some of the authors are employed by Bayer AG, a manufacturer of pesticides (a declaration of interest note was also added to the manuscript).

Acknowledgements

The authors are grateful to Mark Miles for critically reading the manuscript. Rothamsted Research receives grant aided support from the Biotechnology and Biological Sciences Research Council, UK.

Declaration of competing interest

RN, MZ, BL, JG, MK, CM, MTA and AL are employed by Bayer, a manufacturer of pesticides.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112247.

References

Alptekin, S., Bass, C., Nicholls, C., Paine, M.J.I., Clark, S.J., Field, L., Moores, G.D., 2016. Induced thiacloprid insensitivity in honeybees (Apis mellifera L.) is associated with up-regulation of detoxification genes. Insect Mol. Biol. 25, 171–180.

Beadle, K., Singh, K.S., Troczka, B.J., Randall, E., Zaworra, M., Zimmer, C.T., Hayward, A., Reid, R., Kor, L., Kohler, M., Buer, B., Nelson, D.R., Williamson, M.S., Davies, T.G.E., Field, L.M., Nauen, R., Bass, C., 2019. Genomic insights into neonicotinoid sensitivity in the solitary bee Osmia bicornis. PLoS Genet. 15, e1007903.

Belden, J.B., Brain, R.A., 2018. Incorporating the joint toxicity of co-applied pesticides into the ecological risk assessment process. Integr. Environ. Assess. Manag. 14, 79–91.

Bell, H.C., Benavides, J.E., Montgomery, C.N., Navratil, J.R.E., Nieh, J.C., 2020. The novel butenolide pesticide flupyradifurone does not alter responsiveness to sucrose at either acute or chronic short-term field-realistic doses in the honey bee, Apis mellifera. Pest Manag. Sci. 76, 111–117.

Berenbaum, M.R., Johnson, R.M., 2015. Xenobiotic detoxification pathways in honey bees. Curr. Opin. Insect Sci. Soc. Insects Vectors Med. Vet. Entomol. 10, 51–58.

Biddinger, D.J., Rajotte, E.G., 2015. Integrated pest and pollinator management—adding a new dimension to an accepted paradigm. Curr. Opin. Insect Sci. Soc. Insects Vectors Med. Vet. Entomol. 10, 204–209.

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

Bordini, I., Ellsworth, P.C., Naranjo, S.E., Fournier, A., 2021. Novel insecticides and generalist predators support conservation biological control in cotton. Biol. Control 154, 104502.

Campbell, J.W., Cabrera, A.R., Stanley-Stahr, C., Ellis, J.D., 2016. An evaluation of the honey bee (hymenoptera: apidae) safety profile of a new systemic insecticide, flupyradifurone, under field conditions in Florida. J. Econ. Entomol. 109, 1967–1972.

Carnesecchi, E., Svendsen, C., Lasagni, S., Grech, A., Quignot, N., Amzal, B., Toma, C., Tosi, S., Rortais, A., Cortinas-Abrahantes, J., Capri, E., Kramer, N., Benfenati, E., Spurgeon, D., Guillot, G., Dorne, J.L.C.M., 2019. Investigating combined toxicity of binary mixtures in bees: Meta-analysis of laboratory tests, modelling, mechanistic basis and implications for risk assessment. Environ. Int. 133, 105256.

Casida, J.E., 2018. Neonicotinoids and other insect nicotinic receptor competitive modulators: progress and prospects. Annu. Rev. Entomol. 63, 125–144.

Casida, J.E., Durkin, K.A., 2013. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. Annu. Rev. Entomol. 58, 99–117.

Castle, S., Palumbo, J., Merten, P., Cowden, C., Prabhaker, N., 2017. Effects of foliar and systemic insecticides on whitefly transmission and incidence of Cucurbit yellow stunting disorder virus. Pest Manag. Sci. 73, 1462–1472.

Cedergreen, N., 2014. Quantifying synergy: a systematic revuew of mixture toxicity studies within environmental toxicology. PLoS ONE 9 (5), e96580.

Christen, V., Fent, K., 2017. Exposure of honey bees (Apis mellifera) to different classes of insecticides exhibit distinct molecular effect patterns at concentrations that mimic environmental contamination. Environ. Pollut. 226, 48–59.

Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 15, 615–636.

Connolly, C.N., 2013. The risk of insecticides to pollinating insects. Commun. Integr. Biol. 6, e25074.

Cornman, R.S., Lopez, D., Evans, J.D., 2013. Transcriptional response of honey bee larvae infected with the bacterial pathogen Paenibacillus larvae. PLoS One 8, e65424.

Dermauw, W., Leeuwen, Van, Feyereisen, R, T., 2020. Diversity and evolution of the P450 family in arthropods. Insect Biochem. Mol. Biol. 127, 103490.

EFSA, 2019. Peer review of the pesticide risk assessment of the active substance thiacloprid. EFSA J. 17, e05595.

EFSA, 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance flupyradifurone. EFSA J. 13, 4020.

EU, 2015. COMMISSION IMPLEMENTING REGULATION (EU) 2015/ 2084 - of 18 November 2015 - approving the active substance flupyradifurone, in accordance with Regulation (EC) No 1107/ 2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending the Annex to Commission Implementing Regulation (EU) No 540/ 2011 302, 89–93.

EU, 2013. Commission Implementing Regulation (EU) No 485/2013 of 24 May 2013 amending Implementing Regulation (EU) No 540/2011, as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds treated with plant protection products containing those active substancesText with EEA relevance. Off. J. Eur. Union 139, 12–27.

Feyereisen, R., 2011. Arthropod CYPomes illustrate the tempo and mode in P450 evolution. Biochim. Biophys. Acta 1814, 19–28.

Fowler, S., Zhang, H., 2008. In vitro evaluation of reversible and irreversible cytochrome P450 inhibition: current status on methodologies and their utility for predicting drug-drug interactions. AAPS J 10, 410–424.

Godfray, H.C.J., Blacquière, T., Field, L.M., Hails, R.S., Petrokofsky, G., Potts, S.G., Raine, N.E., Vanbergen, A.J., McLean, A.R., 2014. A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proc. R. Soc. B Biol. Sci. 281, 20140558.

Gong, Y., Diao, Q., 2017. Current knowledge of detoxification mechanisms of xenobiotic in honey bees. Ecotoxicology 26, 1–12.

Goulson, D., 2013. REVIEW: an overview of the environmental risks posed by neonicotinoid insecticides. J. Appl. Ecol. 50, 977–987.

Haas, J., Nauen, R., 2021. Pesticide risk assessment at the molecular level using honey bee cytochrome P450 enzymes: a complementary approach. Enviorn. Int.

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

Hayward, A., Beadle, K., Singh, K.S., Exeler, N., Zaworra, M., Almanza, M.-T., Nikolakis, A., Garside, C., Glaubitz, J., Bass, C., Nauen, R., 2019. The leafcutter bee, Megachile rotundata, is more sensitive to N -cyanoamidine neonicotinoid and butenolide insecticides than other managed bees. Nat. Ecol. Evol. 3, 1521–1524.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.

Hesselbach, H., Scheiner, R., 2019. The novel pesticide flupyradifurone (Sivanto) affects honeybee motor abilities. Ecotoxicology 28, 354–366.

Hesselbach, H., Scheiner, R., 2018. Effects of the novel pesticide flupyradifurone (Sivanto) on honeybee taste and cognition. Sci. Rep. 8, 4954.

Hesselbach, H., Seeger, J., Schilcher, F., Ankenbrand, M., Scheiner, R., 2020. Chronic exposure to the pesticide flupyradifurone can lead to premature onset of foraging in honeybees Apis mellifera. J. Appl. Ecol. 57, 609–618.

Hladik, M.L., Main, A.R., Goulson, D., 2018. Environmental risks and challenges associated with neonicotinoid insecticides. Environ. Sci. Technol. 52, 3329–3335.

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

Janmohamed, A., Thaunsukon, P., Shephard, E.A., Phillips, I.R., 2006. Expression of recombinant flavin-containing monooxygenases in a baculovirus/insect cell system. In: Phillips, I.R., Shephard, E.A. (Eds.), Cytochrome P450 Protocols, Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 39–59.

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

Jeschke, P., Nauen, R., Beck, M.E., 2013. Nicotinic acetylcholine receptor agonists: a milestone for modern crop protection. Angew. Chem. Int. Ed. 52, 9464–9485.

Jeschke, P., Nauen, R., Gutbrod, O., Beck, M.E., Matthiesen, S., Haas, M., Velten, R., 2015. Flupyradifurone (Sivanto™) and its novel butenolide pharmacophore: structural considerations☆. Pestic. Biochem. Physiol. 121, 31–38.

Jeschke, P., Nauen, R., Schindler, M., Elbert, A., 2011. Overview of the status and global strategy for neonicotinoids. J. Agric. Food Chem. 59 (7), 2897–2908.

Johnson, R.M., Wen, Z., Schuler, M.A., Berenbaum, M.R., 2006. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. J. Econ. Entoml. 99, 1046–1050.

Johnson, R.M., Dahlgren, L., Siegfried, B.D., Ellis, M.D., 2013. Acaricide, fungicide and drug interactions in honey bees (Apis mellifera). PLoS ONE 8, e54092.

Johnson, R.M., 2015. Honey bee toxicology. Annu. Rev. Entomol. 60, 415–434.

Kablau, A., Eckert, J.H., Pistorius, J., Sharbati, S., Einspanier, R., 2020. Effects of selected insecticidal substances on mRNA transcriptome in larvae of Apis mellifera. Pestic. Biochem. Physiol. 170, 104703.

Kosaka, M., Kosugi, Y., Hirabayashi, H., 2017. Risk assessment using cytochrome P450 time-dependent inhibition assays at single time and concentration in the early stage of drug discovery. J. Pharm. Sci. 106, 2839–2846.

López-Osorio, F., Wurm, Y., 2020. Healthy pollinators: evaluating pesticides with molecular medicine approaches. Trends Ecol. Evol. 35, 380–383.
Lourenço, A.P., Mackert, A., dos Santos Cristino, A., Simões, Z.L.P., 2008. Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. Apidologie 39, 372–385.
Lundin, O., Rundlöf, M., Smith, H.G., Fries, I., Bommarco, R., 2015. Neonicotinoid insecticides and their impacts on bees: a systematic review of research approaches and identification of knowledge gaps. PLoS One 10, e0136928.
Manjon, C., Troczka, B.J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G., Singh, K.S., Zimmer, C.T., Homem, R.A., Lueke, B., Reid, R., Kor, L., Kohler, M., Benting, J., Williamson, M.S., Davies, T.G.E., Field, L.M., Bass, C., Nauen, R., 2018. Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides. Curr. Biol. 28 (1137-1143.e5).
Mao, Schuler, M.A., Berenbaum, M.R., 2015. Task-related differential expression of four cytochrome P450 genes in honeybee appendages. Insect Mol. Biol. 24, 582–588.
Mao, W., Schuler, M.A., Berenbaum, M.R., 2011. CYP9Q-mediated detoxification of acaricides in the honey bee (Apis mellifera). Proc. Natl. Acad. Sci. USA 108, 12657–12662.
McLeman, A., Troczka, B.J., Homem, R.A., Duarte, A., Zimmer, C., Garrood, W.T., Pym, A., Beadle, K., Reid, R.J., Douris, V., Vontas, J., Davies, T.G.E., ffrench Constant, R., Nauen, R., Bass, C., 2020. Fly-Tox: A panel of transgenic flies expressing pest and pollinator cytochrome P450s. Pestic. Biochem. Physiol. 169, 104674.
Nauen, R., Ebbinghaus-Kintscher, U., Schmuck, R., 2001. Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in Apis mellifera (Hymenoptera: Apidae). Pest Manag. Sci. 57, 577–586.
Nauen, R., Jeschke, P., Velten, R., Beck, M.E., Ebbinghaus-Kintscher, U., Thielert, W., Wölfel, K., Haas, M., Kunz, K., Raupach, G., 2015. Flupyradifurone: a brief profile of a new butenolide insecticide. Pest Manag. Sci. 71, 850–862.
Nauen, R., Zimmer, C.T., Vontas, J., 2021. Heterologous expression of insect P450 enzymes that metabolize xenobiotics. Current Opinion in Insect Science 43, 78–84.
OECD, 1998a. Test No. 213: Honeybees, Acute Oral Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD.
OECD, 1998b. Test No. 214: Honeybees, Acute Contact Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD.
Pagano, M., Stara, A., Aliko, V., Faggio, C., 2020. Impact of neonicotinoids to aquatic invertebrates—in vitro studies on mytilus galloprovincialis: a review. J. Mar. Sci. Eng. 8, 801. https://doi.org/10.3390/jmse8100801.
Panini, M., Manicardi, G.C., Moores, G.D., Mazzoni, E., 2016. An overview of the main pathways of metabolic resistance in insects. Invertebr. Surviv. J. 13, 326–335.
Pilling, E.D., Bromleychallenor, K.A.C., Walker, C.H., Jepson, P.C., 1995. Mechanism of synergism between the pyrethroid insecticide λ-cyhalothrin and the imidazole fungicide prochloraz, in the honeybee (Apis mellifera L.). Pestic. Biochem. Physiol. 51, 1–11.
Pilling, E.D., Jepson, P.C., 1993. Synergism between EBI fungicides and a pyrethroid insecticide in the honeybee (*Apis mellifera*). Pestic. Sci. 39, 293–297.
Potts, S.G., Imperatriz-Fonseca, V., Ngo, H.T., Aizen, M.A., Biesmeijer, J.C., Breeze, T.D., Dicks, L.V., Garibaldi, L.A., Hill, R., Settele, J., Vanbergen, A.J., 2016. Safeguarding pollinators and their values to human well-being. Nature 540, 220–229.
Rane, R.V., Ghodke, A.B., Hoffmann, A.A., Edwards, O.R., Walsh, T.K., Oakeshott, J.G., 2019. Detoxifying enzyme complements and host use phenotypes in 160 insect species. Curr. Opin. Insect Sci. Insect Genom. Dev. Regul. 31, 131–138.
Reid, R.J., Troczka, B.J., Kor, L., Randall, E., Williamson, M.S., Field, L.M., Nauen, R., Bass, C., Davies, T.G.E., 2020. Assessing the acute toxicity of insecticides to the buff-tailed bumblebee (Bombus terrestris audax). Pestic. Biochem. Physiol. 166, 104562.
Robinson, A., Hesketh, H., Lahive, E., Horton, A.A., Svendsen, C., Rortais, A., Dorne, J.L., Baas, J., Heard, M.S., Spurgeon, D.J., 2017. Comparing bee species responses to chemical mixtures: Common response patterns? PLoS ONE 12, e0176289.
Roditakis, E., Stavrakaki, M., Grispou, M., Achimastou, A., Waetermeulen, X.V., Nauen, R., Tsagkarakou, A., 2017. Flupyradifurone effectively manages whitefly Bemisia tabaci MED (Hemiptera: Aleyrodidae) and tomato yellow leaf curl virus in tomato. Pest Manag. Sci. 73, 1574–1584.
Sakata, K., Aoki, K., Chang, C.-F., Sakurai, A., Tamura, S., Murakoshi, S., 1978. Stemospironine, a new insecticidal alkaloid of Stemona japonica Miq. isolation, structural determination and activity. Agric. Biol. Chem. 42, 457–463.
Schmuck, R., 2001. Ecotoxicological profile of the insecticide thiacloprid. Pflanzenschutz Nachr. Bayer 54, 161–184 (English Edition).
Schmuck, R., Stadler, T., Schmidt, H.W., 2003. Field relevance of a synergistic effect observed in the laboratory between an EBI fungicide and a chloronicotinyl insecticide in the honeybee. Pest Manag. Sci. 59, 279–286.
Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M.T., Tosi, S., Bogo, G., Teper, D., Porrini, C., Molowny-Horase, R., Bosch, J., 2017. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. Pest Manag. Sci. 73, 1236–1243.
Soloway, S.B., Henry, A.C., Kollmeyer, W.D., Padgett, W.M., Powell, J.E., Roman, S.A., Tieman, C.H., Corey, R.A., Horne, C.A., 1978. Nitromethylene heterocycles as insecticides. In: Shankland, D.L., Hollingworth, R.M., Smyth, T. (Eds.), Pesticide and Venom Neurotoxicity. Springer US, Boston, MA, pp. 153–158.
Sparks, T.C., Crossthwaite, A.J., Nauen, R., Banba, S., Cordova, D., Earley, F., Ebbinghaus-Kintscher, U., Fujioka, S., Hirao, A., Karmon, D., Kennedy, R., Nakao, T., Popham, H.J.R., Salgado, V., Watson, G.B., Wedel, B.J., Wessels, F.J., 2020. Insecticides, biologics and nematicides: updates to IRAC's mode of action classification - a tool for resistance management. Pestic. Biochem. Physiol. 167, 104587.
Stara, A., Pagano, M., Capillo, G., Fabrello, J., Sandova, M., Albano, M., Zuskova, E., Velisek, J., Matozzo, V., Faggio, C., 2020a. Acute effects of neonicotinoid insecticides on Mytilus galloprovincialis: a case study with the active compound thiacloprid and the commercial formulation calypso 480 SC. Ecotoxicol. Environ. Saf. 203, 110980 https://doi.org/10.1016/j.ecoenv.2020.110980.
Stara, A., Pagano, M., Capillo, G., Fabrello, J., Sandova, M., Vazzana, I., Zuskova, E., Velisek, J., Matozzo, V., Faggio, C., 2020b. Assessing the effects of neonicotinoid insecticide on the bivalve mollusc Mytilus galloprovincialis. Sci. Total Environ. 700, 134914 https://doi.org/10.1016/j.scitotenv.2019.134914.
Stokstad, E., 2019. Mite fight. Science 365, 310–313.
Suchail, S., Guez, D., Belzunces, L.P., 2001. Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in Apis mellifera. Environ. Toxicol. Chem. 20, 2482–2486.
Suchail, S., De Sousa, G., Rahmani, R., Belzunces, L.P., 2004. In vivo distribution and metabolization of [14C]-imidacloprid in different compartments of Apis mellifera L. Pest Manage. Sci. 60, 1056–1062.
Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of synergism in honeybees (Apis mellifera) of exposure to neonicotinoids and sprayed fungicides in crops. Apidologie 45, 545–553.
Tong, L., Nieh, J.C., Tosi, S., 2019. Combined nutritional stress and a new systemic pesticide (flupyradifurone, Sivanto®) reduce bee survival, food consumption, flight success, and thermoregulation. Chemosphere 237, 124408.
Tosi, S., Nieh, J.C., 2019. Lethal and sublethal synergistic effects of a new systemic pesticide, flupyradifurone (Sivanto ®), on honeybees. Proc. R. Soc. B 286, 20190433. https://doi.org/10.1098/rspb.2019.0433.
Troczka, B.J., Homem, R.A., Reid, R., Beadle, K., Kohler, M., Zaworra, M., Field, L.M., Williamson, M.S., Nauen, R., Bass, C., Davies, T.G.E., 2019. Identification and functional characterisation of a novel N-cyanoamidine neonicotinoid metabolising cytochrome P450, CYP9Q6, from the buff-tailed bumblebee Bombus terrestris. Insect Biochem. Mol. Biol. 111, 103171.
US EPA, O., 2014. Pollinator Risk Assessment Guidance [WWW Document]. US EPA. URL ⟨https://www.epa.gov/pollinator-protection/pollinator-risk-assessment-guidance⟩ (Accessed 11.20.20).
Vannette, R.L., Mohamed, A., Johnson, B.R., 2015. Forager bees (Apis mellifera) highly express immune and detoxification genes in tissues associated with nectar processing. Sci. Rep. 5, 16224.
Wernecke, A., Frommberger, M., Forster, R., Pistorius, J., 2019. Letale Auswirkungen verschiedener Tankmischungen aus Insektiziden, Fungiziden und Düngemitteln auf Honigbienen unter Labor-, Halbfreiland- und Freilandbedingungen. J. Consum. Prot. Food Saf. 14, 239–249.
Wieczorek, P., Frąckowiak, P., Obrępalska-Stęplowska, A., 2020. Evaluation of the expression stability of reference genes in Apis mellifera under pyrethroid treatment. Scientific Reports 10, 16140.
Wu, Y.-Y., Pasberg, P., Diao, Q.-Y., Nieh, J.C., 2021. Flupyradifurone reduces nectar consumption and foraging but does not alter honey bee recruitment dancing. Ecotoxicol. Environ. Saf. 207, 111268.
Zaworra, M., Koehler, H., Schneider, J., Lagojda, A., Nauen, R., 2019. Pharmacokinetics of Three Neonicotinoid Insecticides upon Contact Exposure in the Western Honey Bee, *Apis mellifera*. Chem. Res. Toxicol. 32, 35–37.