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Bee pollinators and pesticides

a toxicogenomics approach to illuminate the basis for selectivity and synergism

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Abstract

Bees are the most important pollinators in many ecosystems including agroecosystems. Reports of declining bee populations in abundance and diversity are therefore alarming. Many factors are involved in the decline of bees and insects in general. Insecticides and especially neonicotinoid insecticides have been at the center of attention for the last decade. While thorough pollinator risk assessment schemes exist in most countries which have helped to reduce acute bee poisoning events by insecticides, several areas with substantial knowledge gaps have been identified in course of the scientific debate over the impact of pesticides on bees. These areas include for example the toxicity of pesticide mixtures, the appropriateness of the honey bee (*Apis mellifera*) as a surrogate species for other bee species or the general toxicokinetic behavior of pesticides in bees. Some of these questions are difficult to answer with classic methodologies but can be addressed with the help of molecular and biochemical approaches within the field of toxicogenomics.

(Honey) bees possess an evolutionary adapted detoxification system against many diverse xenobiotics. This detoxification system is also capable to soften and mitigate the effects of insecticides. Especially cytochrome P450 enzymes have been shown to be an integral part of the honey bee's defense system against several insecticidal chemotypes. Here, the knowledge of this important enzyme family in bees is expanded and leveraged to improve the evaluation of pesticides regarding their bee safety profile.

In chapter 2 a fluorescence-based, high-throughput *in-vitro* assay is described to assess the risk of synergistic interaction between mixture partners due to the inhibition of important P450s enzymes – the most frequent reason for synergistic interactions observed in bees. In chapter 3 the molecular determinants of the reduced bee toxicity of the butenolide insecticide flupyradifurone were investigated in detail revealing the importance of the cytochrome P450 isoforms CYP6AQ1, CYP9Q2 and CYP9Q3 for its detoxification. In chapter 4 the involvement of these enzymes in chlorantraniliprole metabolism was investigated fostering the role of CYP9Q2+3 in the detoxification of various chemical classes. Considering those findings the appearance of orthologs of these essential determinants of insecticide selectivity is investigated across 75 bee species and by recombinant expression of 26 CYP9Q-related genes from 20 bee species the functional similarity was validated (chapter 5).

Together, these results contribute significantly to the understanding of insecticide toxicology in bees, help to understand insecticide selectivity issues, may complement current risk assessment procedures for the evaluation of pesticide safety and can support the development of novel, next-generation insecticides with a further improved environmental profile.

Zusammenfassung

Bienen sind die wichtigsten Bestäuber in vielen Agrarökosystemen. Berichte über abnehmende Bienenpopulationen, sowohl bezüglich der Anzahl als auch der Vielfalt, sind daher besorgniserregend. Viele Faktoren spielen eine Rolle bei der Abnahme von Bienen- und Insektenvielfalt im Allgemeinen. Insektizide und insbesondere Neonicotinoide waren während der letzten Dekade im Zentrum der Aufmerksamkeit. Obwohl in den meisten Ländern Rahmenwerke für die Risikoabschätzung der Wirkung von Pflanzenschutzmittel auf Bestäuber existieren, wurden im Laufe der wissenschaftlichen Debatte über die Auswirkungen von Pflanzenschutzmitteln auf Bienen bestimmte Bereiche identifiziert, in denen es substanzielle Wissenslücken gibt. Diese Bereiche beinhalten zum Beispiel die Toxizität von Pestizid-Mischungen, die Eignung der Honigbiene (*Apis mellifera*) als Stellvertreter für andere Bienenarten oder dem generellen, toxikokinetischen Verhalten von Pflanzenschutzmitteln in Bienen. Manche dieser Fragen lassen sich nur schwer mit klassischen Methoden beantworten, aber können mithilfe von molekularen und biochemischen Ansätzen aus dem Feld der Toxikogenomik adressiert werden. (Honig)-bienen besitzen ein evolutionär angepasstes Entgiftungssystem, welches mit vielfältigen Fremdstoffen umgehen kann. Dieses Entgiftungssystem ist auch in der Lage die Effekte von Insektiziden abzuschwächen und abzumildern. Insbesondere cytochrome P450 Enzyme sind integraler Bestandteil dieses Verteidigungssystem gegenüber verschiedenen insektiziden Chemotypen. Im Rahmen dieser Arbeit wird das Wissen über diese wichtige Enzymfamilie erweitert und genutzt, um die Evaluierung von Pestiziden bezüglich ihres Bienensicherheits-Profils zu verbessern. In Kapitel 2 wird ein fluoreszenz-basierter, Hochdurchsatz-*in-vitro*-Assay beschrieben, um das Risiko synergistischer Interaktion zwischen Mischungspartnern aufgrund der Inhibierung wichtiger P450 Enzyme einschätzen zu können – der häufigste beschriebene Grund für beobachtete synergistische Effekte in Bienen. In Kapitel 3 werden die molekularen Bestimmungsfaktoren für die geringe Bienentoxizität des Butenolid-Insektizid Flupyradifurone im Detail untersucht und die Bedeutung der P450-Isoformen CYP6AQ1, CYP9Q2 und CYP9Q3 für die Entgiftung herausgearbeitet. In Kapitel 4 wird die Beteiligung dieser Enzyme für den Metabolismus von Chlorantraniliprole untersucht und die Involvierung von CYP9Q2 und CYP9Q3 in der Entgiftung verschiedener chemischer Klasse bestätigt. Aufgrund dieser Erkenntnisse wird das Vorhandensein orthologer Gene dieser essentiellen Bestimmungsfaktoren der Insektizid-Selektivität in 75 Bienenarten untersucht und mithilfe von rekombinanter Expression von 26 CYP9Q-verwandten Gene von 20 Arten die funktionelle Ähnlichkeit validiert (Kapitel 5). Zusammen steuern die Ergebnisse einen signifikanten Teil zum Verständnis von Insektizid-Toxikologie in Bienen bei, helfen Selektivitätsprobleme zu verstehen, ergänzen möglicherweise die momentanen Risikobewertungs-Verfahren von Pflanzenschutzmitteln und können die Entwicklung neuer Insektizide mit verbessertem Umweltprofil unterstützen.

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Table of contents

List of abbreviations

Å	Ångström
a.i.	active ingredient
ABC	ATP-binding cassette
ACh	acetylcholine
AChBP	acetylcholine-binding protein
ACT	acetamiprid
ADME	absorption, distribution, metabolism, excretion
ADP	adenosinediphosphate
ANOVA	analysis of variance
ATP	adenosinetriphosphate
BFC	7-benzyloxy-4-(trifluoromethyl)-coumarin
BOMFC	7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin
CA	concentration addition
Ca^{2+}	calcium ion
CaM	calmodulin
CCD	colony collapse disorder
CCE	carboxy / cholinesterase
CI	confidence interval
CICR	calcium-induced calcium release
CPR	chlorantraniliprole
CRISPR	clustered regularly interspaced short palindromic repeats
CsoI	core solenoid domain
CTD	C-terminal domain
Da	Dalton
DDT	dichlordiphenyltrichloroethane
DHPR	dihydropyridine receptor
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DTT	dithiotreitol
e.g.	exempli gratia

EC	European Comission
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EIL	economic injury level
EU	European Union
EPA	Environmental Protection Agency
ESI	electro-spray ionization
ET	economic threshold
ETR	exposure toxicity ratio
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
Fe^{II}	ferrous iron
Fe^{III}	ferric iron
FLB	flubendiamide
FLV	τ-fluvalinate
FMN	flavomononucleotide
FPF	flupyradifurone
FPF-AA	FPF-acetic acid
FPF-AF	FPF-4-[(2,2-difluoroethyl)amino]-furanone
FPF-DFEA	FPF-difluoroethanamine
FPF-OH	FPF-hydroxy
g	gram
GM	genetically modified
GMO	genetically modified organism
GSH	glutathione
GST	glutathione S-transferase
ha	hectare
HC	7-hydroxy-4-(trifluoromethyl) coumarin
Hit	new molecular starting point
HPLC	high pressure liquid chromatography

HQ	hazard quotient
HSD	honest significant difference
Hsp70	heatshock protein 70
HTS	high-throughput screening
i.e.	id est (that is)
IA	independent action
IC	inhibitory concentration
IMD	imidacloprid
IMD-OH	imidacloprid-hydroxy
IPM	integrated pest management
IRAC	Insecticide Resistance Action Committee
K^+	potassium ion
K_m	Michaelis-Menten constant
LC	lethal concentration
LD	lethal dose
M	molar
Mg^{2+}	magnesium ion
MoA	mode of action
MoI	multiplicity of infection
mRNA	messenger RNA
MS	mass spectrometry
Mya	million years ago
Na^+	sodium ion
nAChR	nicotinic acetylcholine receptor
NADPH	nicotinamide adenine dinucleotide phosphate
NBD	nucleotide binding domain
OECD	Organisation for Economic Co-operation and Development
OP	organophosphate
P450	cytochrome P450 monooxygenase
PCR	polymerase chain reaction

PDB	protein database
PEC	predicted environmental concentration
P_i	inorganic phosphate
PPP	plant protection product
PRC	prochloraz
PRP	propiconazole
PRT	prothioconazole
pVSD	pseudo voltage sensor domain
R&D	research & development
RH	relative humidity
RNA	ribonucleic acid
RNAi	RNA interference
qPCR	quantitative polymerase chain reaction
RyR	ryanodine receptor
SeqAPASS	Sequence Alignment to Predict Across Species Susceptibility
SOICR	spontaneous store-overload induced Ca^{2+} release
SR	sarcoplasmic reticulum
SR	synergistic ratio
TaF	thumb and forefingers domain
TCP	thiacloprid
TMD	transmembrane domain
TMX	thiamethoxame
UAS	upstream activating sequence
UDP	uridine diphosphate
UGT	UDP-glycosyltransferase
UK	United Kingdom
UPLC	ultra-performance liquid chromatography
US	United States
VGSC	voltage-gated sodium channel
V_{max}	rate of reaction when the enzyme is fully saturated with substrate

Chapter 1 – Introduction

Agriculture is arguably one of the oldest industries in the world. But today it faces challenges like maybe never before in its more than 10,000-year-old history. Feeding an ever-increasing world population is complicated enough. To achieve this goal on a decreasing area of land without overexploitation of natural resources is a huge task. Even more so as climate change is expected to further decrease available farmland and to heavily interfere with food production. In the last decades achievements of the green revolution, i.e. plant breeding, synthetic fertilizers, advanced cultivation and harvesting techniques, irrigation and chemical crop protection have allowed for a huge increase of productivity per area arable land. However, many of those technologies have reached their limits and novel technological advancements such as genetically modified organisms (GMOs) are not adopted everywhere for various reasons. Additionally, synthetic fertilizers and chemical crop protection compounds are increasingly under scrutiny for potential ecological side effects jeopardizing the sustainability of the current global food production system.

One of the greater concerns are the side effects of pesticides on beneficial insects, including bees as important pollinators. Discussions about the negative impact of agricultural sprays on bees go way back at least to the 1920s when incidents of honey bee poisoning by lead arsenate have been reported (Carreck, 2017). The topic got once again reignited by the phenomena of increased colony losses due to the mysterious disappearance of worker bees from their hives also referred to as colony collapse disorder (CCD) (vanEngelsdorp et al., 2009) and incidents of bee poisoning by insecticides used as seed treatment due to inappropriate application / sowing techniques in the 2000s (Pistorius et al., 2008). Since then, insecticides have moved to the center of public attention regarding (honey) bee health issues and initiated a revision of bee pollinator pesticide risk assessment schemes in the US and Europe (EFSA, 2013; US EPA, 2014).

Advanced data requirements have led to the loss of many available insecticidal solutions to farmers, especially in Europe (Keulemans et al., 2019), including the complete ban of neonicotinoid seed treatments for outdoor uses (EU, 2013, 2018). Often, uncertainty rather than unequivocal scientific evidence have led to use restrictions or withdrawals and research areas were defined where regulatory bodies saw significant knowledge gaps which needed to be addressed (EFSA, 2014b). This included, amongst others, toxicokinetic behavior of xenobiotics in bees, interaction effects between pesticides or the suitability of the honey bee as a surrogate species for the diverse clade of bees.

Some of these open questions are difficult to address solely with conventional methodology. Fortunately, we live in an era of unparalleled progress in the field of genomics and molecular biology converging with bioinformatics and data science. Combined with conventional

methodology, molecular approaches can provide valuable insights into pollinator physiology and their interaction with pesticides (López-Osorio & Wurm, 2020). Progress in this area can help with the safety evaluation of insecticides and support the design of novel, more bee-compatible chemotypes.

The focus of this thesis is the molecular dissection of the detoxification system of (honey) bees, with special reference to the cytochrome P450 gene family, and its role in insecticide selectivity. In this chapter, background, general aspects, and principles necessary for the comprehension of the complex topic are introduced.

1.1 Bees

If you ask someone to imagine a bee, most people will think of honey bees (*Apis mellifera*) or bumblebees (*Bombus spp.*). However, bees are a remarkably diverse group of insects with many different shapes and traits. But what exactly are bees? What defines them? And why are they so important for many ecosystems including agroecosystems?

Bees are part of the Aculeata – a group of Hymenoptera whose shared feature is the conversion of the ovipositor into a sting which includes wasps, ants and bees (Michener, 2007). More narrowly, they can be placed as part of the Apoidea alongside four families of hunting wasps (Heterogynaidae, Ampulicidae, Sphecidae and Crabronidae) (Danforth et al., 2019). Most recent insights into the origin of bees suggest that bees are descendants of a small group of thrips-hunting wasps (Ammoplanina) (Sann et al., 2018).

Like all insects, adult bees have three body parts (head, thorax, abdomen) and three pairs of legs. The first abdominal segment is fused to the thorax and the remainder is narrowly connected leading to a typical “wasp waist”-like body shape. Compared to wasps, bees are usually more robust, covered with feathered hair and tend to have a longer proboscis (Figure 1A) (Michener, 2007). They are holometabolous insects with a haplodiploid sex determination system as most Hymenoptera. Fertilized eggs develop into females and unfertilized ones into males. Fertilization can be controlled by the female for each egg separately as she has a life-time supply of sperm cells stored in her spermatheca after mating. During oviposition, females may release some of the sperm cells to fertilize the egg (Michener, 2007). Larvae are soft, white, and legless. In many species, adult activity is restricted to a short period in the year while a large amount of time is spent in diapause – either as adult or larvae (Danforth et al., 2019). Other bees such as the honey bee are active for several months or even year-round depending on the geographical region (Winston, 1991).

While their closest relatives rely on arthropod prey as food source, bees have switched to a vegetarian lifestyle collecting pollen as protein source for their offspring mixed with nectar and sometimes floral oils (Murray et al., 2018). Although pollinivory was found to be necessary but not sufficient for the diversification of bees, it is definitely a driver of their success (Murray et al., 2018). Since their origin (estimated ~125 million years ago (Mya)) they have diversified into

seven families, 28 subfamilies, 529 genera and over 20.000 species (five times as many as mammals) (Danforth et al., 2019).

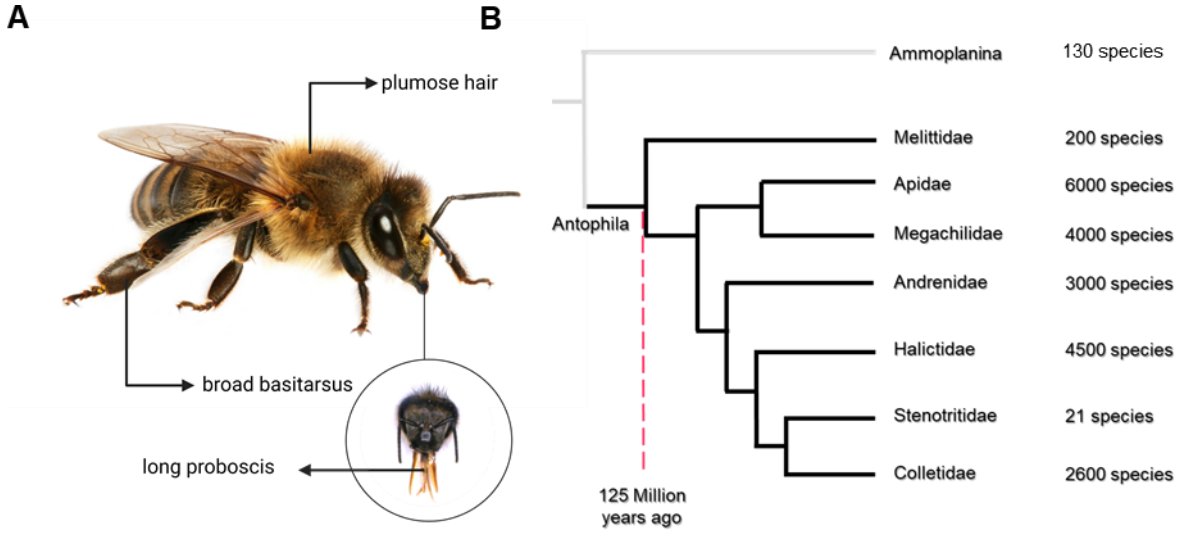


Figure 1: The bee basics. (A) Distinct morphological characteristics which differentiate bees from their closest relatives according to Michener et al. (2007). (B) Phylogenetic relationship of the seven major bee families and the number of described species in each family. Depicted in grey are the thrips-hunting wasps (Ammoplanina) from which bees most probably derived from (Sann et al., 2018).

While historically controversial, the utilization of DNA sequencing strongly supports the view that Melittidae are the basal sister family to all other bee families comprising approximately 200 species (Figure 1B) (Branstetter et al., 2017; Michez et al., 2009). Their origin is assumed to be the African continent, although they occur in many temperate, xeric and Mediterranean climate regions (Michener, 1979). The only family which is even smaller is the Stenotritidae with 21 species from two genera restricted to Australia. Stenotritidae diverged from the Colletidae approximately 92 Mya (Almeida et al., 2012). Colletid bees are a larger group with 2600 described species and were often considered the basal group of bees owing to their tongue (glossa) which is similar to that of crabronid wasps (Danforth et al., 2019). However, molecular analysis showed that this is a derived trait related to a unique feature of Colletidae – brood-cell lining with a polyester material – and that Colletidae are a rather young family (Danforth et al., 2006; Hefetz et al., 1979). Closest relatives of Colletidae and Stenotritidae are the Halicticidae, the second largest family (~ 4500 described species). Halictid bees are assumed to be 75 to 96 million years old and are distributed worldwide (Cardinal & Danforth, 2013). One prominent member is the only ground-nesting, solitary bee species ever used as managed pollinator for crop pollination – the alkali bee *Nomia melanderi* (Cane, 2008). Andrenidae (~3000 species) is the last family of the so-called “short-tongued” bees – a historical classification based on the length of the bee’s mouthparts. Andrenidae are found worldwide except in Australia with the core area being xeric regions of America and the Palearctic (Danforth et al., 2019). Apidae (6000 species, largest family) and Megachilidae (around 4000 species, third largest family) form the “long-tongued” bees. Megachilidae are found on all five major continents in a diversity

of habitats ranging from rain forests to deserts (Danforth et al., 2019). Two of the most intensively used managed solitary bee species are members of the Megachilidae: *Megachile rotundata,* used for alfalfa pollination in North America (Pitts-Singer & Cane, 2011) and *Osmia lignaria* (alongside other *Osmia* spp.), used in orchards for pollination of a diversity of trees such as almonds (Bosch et al., 2000). Apidae is the family with the most prominent member – the honey bee. Its relevance for agriculture cannot be overstated and it can be considered as livestock due to the huge industry that has developed worldwide based on its pollination services and other products (Lee et al., 2019). Bees and their lifestyles are as diverse as their number might suggest. In fact, the honey bee cannot be considered as an ordinary representative of its kind. This is largely because the honey bee has advanced and perfected a behavior that has developed repeatedly in bees: social behavior, i.e. the division of labor and cooperativity among mates of the same nest which comprises several generations at the same time leading to the formation of colonies with many thousand bees in the case of honey bees (Cardinal & Danforth, 2011; Michener, 1969).

Unlike the honey bee most bees live a solitary lifestyle. This means each female is responsible for its own nest construction, provision of food for her offspring and nest protection (Wcislo & Tierney, 2009). Intermediate lifestyles are also common where unrelated bees share a nest but do not cooperate regarding brood care (communal lifestyle) or show signs of cooperative breeding (quasi-social lifestyle) (Wcislo & Fewell, 2017). Social behavior can be further divided into primitively eusocial and advanced eusocial behavior depending on the lifetime of a colony (one-year vs. perennial) and morphological distinction of reproductively active females and worker females (body size difference only vs. more distinct morphological changes) (Danforth et al., 2019). A completely different behavior comprises parasitic bees which either lay their eggs in foreign brood cells after killing the host egg (cleptoparasitic or “cuckoo” bees) or replace the queen of a social bee host and use the workforce to rear their own offspring (social parasites) (Danforth et al., 2019).

Another trait where bees show a remarkable diversity is their nesting behavior. Most bee species are ground-nesting which is presumed to be the ancestral state. All Melittidae and Andrenidae as well as most Halictidae and Colletidae are ground nesters (Michener, 2007). But even within ground nesters there is enormous variation among bees in regard to preference for soil substrate and texture, as well as nest architecture (reviewed by Antoine & Forrest, 2021). Others (especially Apidae and Megachilidae) build their nest above-ground using a wide array of preexisting cavities, excavate nests in wood by themselves or construct freestanding nests (Danforth et al., 2019; Michener, 2007).

One trait that is shared by all bees is the collection of floral resources to feed themselves and their offspring. However, the collection behavior differs substantially between bee species. Some bees have specialized on a distinct plant family or even a single genus for the collection

of pollen which is called oligolecty (Robertson, 1925). Polylectic bees, on the other hand, collect pollen from a wide variety of plant species and families. Dietary specialization should be viewed as a continuum with many intermediates between strict oligolecty and broad polylecty (Danforth et al., 2019). Notably, oligolectic bees also tend to visit flowers from a broader range of plants for the collection of nectar (Robertson, 1925). The collection of floral resources, especially pollen, is linked to one of the most valuable ecosystem services provided by wild animals and the reason why bees are so important to nature but also to mankind: pollination.

1.1.1 Pollination and its contribution to agriculture

Seed bearing plants, i.e. plants which produce seeds, comprise gymnosperms and angiosperms, and arose around 325 Mya (Magallón et al., 2015). For 200 million years gymnosperms, which are mainly wind-pollinated, dominated the terrestrial ecosystems. Oldest fossils of flowering plants (angiosperms) date back to the early cretaceous (~135 Mya) with the exact origin still unresolved (but estimated around 150 Mya (Smith et al., 2010)). Today, angiosperms comprise more than 350,000 species, are found in almost every habitat and are the most diverse group of land plants (Li et al., 2019; The Plant List, 2021). This remarkable development can be traced back to apparent rapid radiations and diversification of lineages in the mid-cretaceous shortly after their appearance (~100 ± 30 Mya) – an event that even puzzled Darwin who called it an “abominable mystery” (Soltis et al., 2019). Pollination by insects is generally considered to be one of the key drivers of this tremendous diversification (van der Niet & Johnson, 2012). In an elegant study with rapid cycling *Brassica rapa,* Gervasi and Schiestl could show adaptive evolution of flower characteristics driven by different pollinators in only eleven generations supporting the notion of pollination as a major driver of angiosperm diversification (Gervasi & Schiestl, 2017).

Bees are the single most important pollinating taxon today (Ollerton, 2017). However, ancient angiosperms were pollinator generalists with beetles (assumably the original pollinators), flies, thrips, and moths as significant pollen vehicles (Gottsberger, 2015; Takhtajan, 1980). Nonetheless, it has not gone unnoticed to the careful reader that the diversification of angiosperms overlaps with the origin and diversification of bees. In fact, it is estimated that bees originated concurrently with eudicots, which make up 75 % of angiosperm species today (Cardinal & Danforth, 2013). This includes all major lineages with clear affinities for bee pollination leading to the assumption that the rise of angiosperms is linked to general insect pollination but the success of eudicots in particular is linked to pollination services provided by bees (Danforth et al., 2019).

Plant pollination by bees is a co-evolved relationship. It can be viewed as mutualism as both sides win – the plants disperse their pollen efficiently for sexual reproduction and bees collect food for their offspring in the process. The reality is more nuanced and might be best described

as “balanced mutual exploitation” as bees and plants are actually competing for pollen (Westerkamp, 1996). Plants attract pollinators via floral rewards which differ in their production cost. Nectar is the primary reward and is basically a composition of water and sugar mixed with low concentrations of amino acids, small proteins and secondary plant metabolites (Nicolson & Thornburg, 2007). Pollen is a more costly floral reward due to its often high protein content (Roulston & Cane, 2000). Bees are one of the few taxa which need large quantities of pollen not just to feed themselves, but also their offspring. Therefore, plants have evolved mechanisms to protect and restrict access to their floral rewards while bees have evolved morphological traits and behaviors to overcome these mechanisms (Thorp, 2000). Such adaptations further underline the intimate, co-dependent relationship of bees and flowering plants.

Quantification of importance of bees for pollination services in natural ecosystems is difficult but is most probably invaluable (Ollerton, 2017; Potts et al., 2010). For agroecosystems, the importance and value of pollination have been estimated. Approximately 75% of the most important crops worldwide are at least partially dependent on animal pollination, but these crops only account for 35% of the global production volume (Klein et al., 2007). This discrepancy is explained by the fact that the world’s most important staple crops (rice, wheat, corn) are wind pollinated. However, animal pollination-dependence is positively correlated with micronutrient content of crops (Eilers et al., 2011) indicating that production volume alone is not a good proxy for the value of animal pollination for agriculture. In a bioeconomic analysis, global animal pollination value was estimated at €153 billion or 9.5% of the total agricultural production for human consumption in the year 2005 (Gallai et al., 2009). As the cultivation of pollinator-dependent crops is steadily increasing (Aizen et al., 2019), this estimation is most probably conservative today.

Rader et al. have investigated the relative importance of bees compared to other insect pollinators across crop plants (Rader et al., 2016). They found that bees are responsible for 61% of visits to crop flowers confirming the outstanding role of bees on crop pollination and yield considering that not each visitor is also a pollinator and bees are considered as highly effective pollinators (Garibaldi et al., 2013; Hoehn et al., 2008; Klatt et al., 2014; Rader et al., 2016).

Bees are the single most important pollinating guild and thus contribute significantly to agricultural production. In a scenario of immediate loss of all pollinators, the current demand for fruits, vegetables and stimulant crops would no longer be met with several other crop categories facing drastic production declines (Gallai et al., 2009). While such a scenario is highly unrealistic, reports of bee declines around the globe are still worrying (Biesmeijer et al., 2006; Burkle et al., 2013; Cameron et al., 2011; Pauw, 2007). It is not only a serious threat to biodiversity but also a direct threat to food security and agricultural productivity.

1.2 Insect decline and its drivers

The decline in bee pollinators is part of an overall decline of animals in the Anthropocene (Dirzo et al., 2014). For insects, the topic received increased attention after the publication of a study reporting dramatic losses of more than 75% flying insect biomass over three decades in protected areas in Germany (Hallmann et al., 2017). While there is general consensus that an overall trend of insect biodiversity loss around the world exists, its extent cannot be fully grasped since long-term monitoring data are often lacking (Bell et al., 2020; McDermott, 2021; Wagner, 2020). Additionally, not all insect taxa are declining everywhere, but the picture is more heterogenous. For example, moth biodiversity and abundance in Europe is generally decreasing, however with marked differences among and within regions (Wagner, Fox, et al., 2021). Some studies report no general decline of insect abundance over a longer period, but high interannual variability due to extreme weather events (Marquis et al., 2019; Wagner, Fox, et al., 2021). Even if the overall trend of a taxonomic group is decreasing, it might be that single species within this group are thriving as shown by a study of the Dutch moth fauna (Groenendijk & Ellis, 2011). It appears that the extent of insect decline in abundance and diversity is highly region-, taxon- and species-specific.

For bees, it is important to differentiate between managed bee species and wild bee species. While Europe and North America experience a loss of honey bee colonies (by far the most important pollinator in agriculture) (Potts et al., 2010), global stocks are actually increasing (Aizen & Harder, 2009). Honey bees are exposed to a diversity of stressors impacting their health consequently leading to increased annual colony losses (Kulhanek et al., 2017). Nevertheless, the discrepancy between the world's increase and the industrialized nations' decrease in the long term can be mostly explained by socioeconomic and political factors (Smith et al., 2013). Therefore, it is difficult to compare managed pollinators with wild bee species. Wild bee species are less well studied, and most data is available for bumblebees indicating a general decline in the studied regions (Biesmeijer et al., 2006; Cameron et al., 2011; Grixti et al., 2009). A study from Illinois reported a 50% loss of bee species over a 120-year period (Burkle et al., 2013) while another study found extensive (bee) pollinator biodiversity losses in three European countries before 1990 which slowed down or reversed for certain taxa afterwards (Carvalheiro et al., 2013). Generally, it can be assumed that drivers impacting other insect taxa also impact wild bees, albeit with potentially different relative importance (Goulson et al., 2015).

Not surprisingly, the potential drivers of insect decline, and their relative importance are not easily understood and determined. Unambiguously, there is no single cause solely responsible for insect decline. In fact, the only thing that is certain is that there are multiple factors with temporal and spatial variations and interaction among them (Figure 2) (Wagner, Grames, et al., 2021). In the following sections some of the most relevant drivers and their potential impact

on bees will be introduced. It must be noted that it is not a comprehensive review of all potential drivers and that length of description does not reflect relative importance but rather relevance for this thesis.

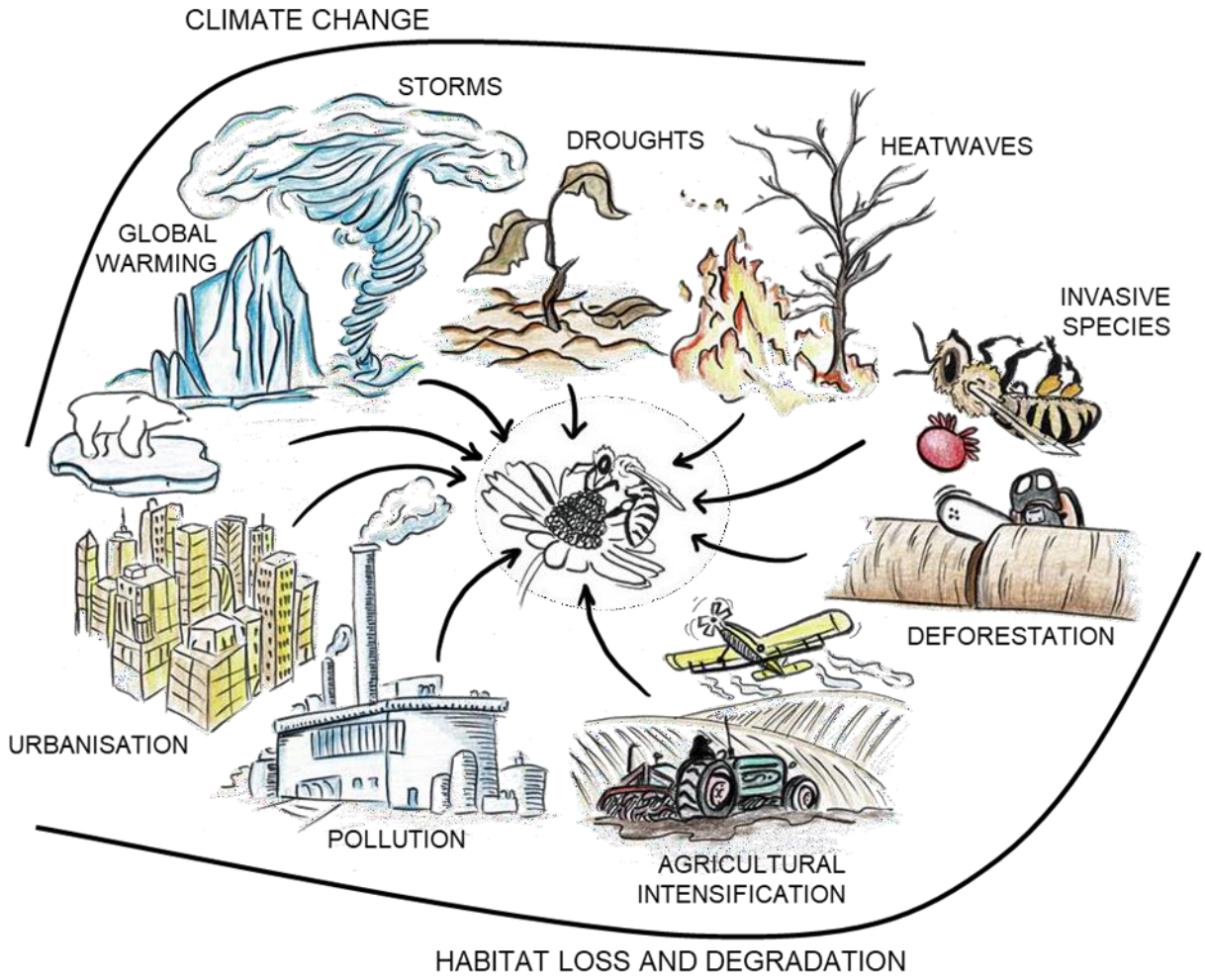


Figure 2: Global drivers of insect and pollinator declines. Many of the drivers are attributable to the two meta trends climate change and habitat loss / degradation. The drawing is inspired by Wagner, Grames et al. (2021). Original artwork by Elena Beck.

One factor which has possibly the most extensive impact on insect communities today is ***climate change*** as it is geographically omnipresent and likely to interact with all other factors (Halsch et al., 2021). However, this also complicates linking climate change directly to changes in insect communities. Studies are complex because climate change is not a one-dimensional factor – it can mean shifts in limits (minima and maxima), shift of average conditions, or higher variability of weather factors which must be interpreted in a temporal and spatial context (Halsch et al., 2021). Literature on direct response of insects to climate change is therefore still scarce and mostly focused on agricultural pests and butterflies in the northern hemisphere (Boggs, 2016). As evident by a study investigating populations trends of different taxonomic groups from the 1970s to 2011 in Great Britain, there are likely more and less climate-vulnerable species and taxa (Martay et al., 2017). Moth populations declined 1.4% annually over the observation period (48% of this decline could be attributed to climate change), but aphid populations

increased annually by 0.7% (62.7% could be accounted for by climate change) (Martay et al., 2017). Probably, cold-adapted species are more vulnerable while southern, warm-adapted species thrive as seen for moth species in Great Britain (Fox et al., 2014). Cold-adapted bee species such as bumblebees are therefore negatively affected by increasing temperatures, partially explaining their widespread declines across Europe and North America (Rasmont et al., 2015; Soroye et al., 2020). On the other side of the spectrum models predict range expansions for arid-adapted bees (Dew et al., 2019). Temperature increases might also interact with physiological properties. Bees overwintering as adults and early-season bees are more affected by increasing temperatures due to elevated energy demand (Fründ et al., 2013). A more bee-specific threat of climate change is a potential asynchrony between flowering and bee emergence (Memmott et al., 2007). Specialist bees are expected to be at greater risk, although it seems that even oligolectic bees are buffered against asynchrony by the flexibility in their choice of interaction partners (Benadi et al., 2014; Willmer, 2012).

Climate change can also facilitate the introduction of ***invasive species*** due to range expansions following changing climate conditions. Global trade and globalization in general are even bigger factors for this driving force of insect decline. One of the most prominent examples of invasive species is the introduction of the varroa mite (*Varroa destructor*) into western honey bee (*Apis mellifera*) hives. The mite's natural host is the eastern honey bee (*Apis cerana*). Transfer from its natural host to the honey bee probably occurred in Japan and has spread across the globe with few exceptions (e.g. Australia) (de Guzman et al., 1997; Roberts et al., 2017). Unlike the eastern honey bee, the western honey bee is not adapted to the infestation by the parasitic mite, which feeds primarily on the bee's fat body and acts as a virus vector (Moore et al., 2014; Ramsey et al., 2019). This has allowed for the rapid spread of mites so that today almost no honey bee colony is free of varroa mites which is considered the most important single driver of honey bee colony losses (Boecking & Genersch, 2008; Rosenkranz et al., 2010).

Sometimes bees are victim and perpetrator at the same time. The buff-tailed bumblebee (*Bombus terrestris*) is traded globally as an excellent pollinator of many crops (Velthuis & Doorn, 2006). Unfortunately, this can have negative implications on native (bumble)bee populations, e.g. for *Bombus dahlbomii* which got replaced rapidly within a 5-year period in Patagonia (Morales et al., 2013). In fact, *B. terrestris* is considered an invasive species outside its native range (temperate Eurasia) (Dafni et al., 2010).

In the last three centuries, the area of wild habitats on ice-free land with no or only minor human disturbance has decreased from almost 95% to less than 50% (Ellis et al., 2010). Not surprisingly, ***habitat degradation and fragmentation*** have been major factors for insect declines and continue to be so (Wagner, 2020).

Different areas contribute to habitat loss (e.g. urban development, pollution, deforestation, land use change including mining, logging, agriculture etc.) which are partly intertwined.

For example, tropical forests are a hub of (insect) biodiversity and ***deforestation*** is one of the biggest threats to the entomofauna (Stork, 2018; Wagner, 2020). Much of the tropical forest area gets converted to agricultural land linking deforestation directly to agriculture (Curtis et al., 2018). For bees, deforestation in tropical regions is a minor factor relative to other insect groups as bees show a species distribution curve with an unusual bimodal latitudinal richness gradient meaning there is highest bee diversity in xeric-temperate regions and low species richness in tropical areas (Orr et al., 2021).

This in turn also means that species richness is highest in many regions where conditions are perfect for large-scale agricultural production. Bees are therefore impacted by ***agricultural intensification***. Agricultural intensification comprises several practices which may affect insects and bees negatively. The expansion of agricultural land has led to a decrease in native habitat, e.g. prairie land in North America which got converted to row crop production such as maize (Koh et al., 2016). ***Loss of native habitat*** is directly related to ***loss of floral resources*** in space and time. Especially solitary bee species with a limited foraging range and a narrow host range are at risk to suffer from food shortages, but also polylectic bees suffer from the lack of resource diversity in monoculture systems (Belsky & Joshi, 2019; Koh et al., 2016). Also nesting strategies may get disturbed by agriculture. Ground-nesting bees, for example, are significantly affected by increased tillage (Williams et al., 2010). Considerable input of fertilizers and pesticides are further characteristics of agricultural intensification. ***Increased nitrogen levels*** can lead to a loss of plant biodiversity which is followed by a loss of insect richness (Bobbink et al., 2010; Dise et al., 2011). A similar effect is expected from ***increased herbicide input***, especially in combination with herbicide-tolerant genetically modified (GM) crops limiting the availability of in-field floral resources from weeds (Wagner, 2020).

An intense, often polarized debate is lead about the role of ***insecticides*** in insect and pollinator declines (Cressey, 2017). Especially neonicotinoid insecticides are in the crossfire as they are the most widely used insecticide class, broadly active - meaning toxic also for non-target insects - and relatively stable combined with high water solubility (Jeschke et al., 2019). These latter properties make them suitable for seed dressing applications, where the seed is coated with the active ingredient which is distributed throughout the plant after germination resulting in systemic protection. Seed treatment applications developed from a niche market in 1990 (before the neonicotinoid commercialization) to a significant part of the overall market in 2008 dominated by neonicotinoids (Jeschke et al., 2013). Water solubility and resulting systemic properties are, however, also the reason for environmental concerns. Neonicotinoids can appear in pollen and nectar after being used as seed treatment or may leach into water systems potentially impacting terrestrial and aquatic non-target invertebrate taxa (Goulson, 2013).

Concerning bees, the impact of neonicotinoids has been intensively studied (Godfray et al., 2014, 2015). Even after more than ten years of intense research, there is no consensus about the extent of negative impacts on bees. Undoubtedly, some neonicotinoids are intrinsically highly toxic to bees. At high enough concentrations they will harm bees and other insects which explains the incidents of acute bee poisoning (Pistorius et al., 2008). Usually, environmental concentrations are much lower so that acute poisoning is seldomly observed. However, also sub-lethal doses were found to negatively affect bees, for example, by reducing foraging activity, affecting orientation, or inhibiting reproduction and development (Gill & Raine, 2014; Henry et al., 2012; Schneider et al., 2012; Whitehorn et al., 2012). Critics of those studies raised the point that used concentrations were still higher than those encountered in the field and direct oral feeding of dosed diet is not comparable to the exposure via seed-treated crops. Indeed, studies placing colonies near treated fields instead of dosing bees directly found no significant effects of treatment (Cutler et al., 2014; Cutler & Scott-Dupree, 2007; Pilling et al.,2013; Thompson et al., 2013). Here, critic was raised that the study designs were insufficient to detect statistically significant effects due to low level of replication or other flaws. Illustrative for the ambiguous scientific evidence is the different conclusions regulatory bodies have drawn. While the EU has cancelled the outdoor use of three neonicotinoid insecticides, in many other regions of the world, including the US, regulatory bodies have acknowledged potential risks to pollinators but judged that there is no unacceptable risk of neonicotinoids for bees when implementing certain mitigation measures (EU, 2013, 2018; US EPA, 2020b, 2020a). While neonicotinoids have attracted most attention, also other insecticide classes have been investigated and similar concerns have been expressed (Kadala et al., 2019, 2020; Li et al.,2021; Siviter et al., 2020; Siviter & Muth, 2020; Tosi et al., 2021). Likewise, the combination of pesticides (especially fungicides and insecticides) has been proposed as a significant risk to pollinators by exhibiting increased mixture toxicity (Fisher et al., 2017; Tosi & Nieh, 2019; Wernecke et al., 2019).
Certainly, pesticides can pose an additional stress factor to bees and insects in general. Their overall impact on insect declines is still not fully understood. Unlike other factors, however, it would be theoretically possible to stop their usage and introduction into the environment. Why this is, at least without adequate replacement, not a realistic scenario becomes evident when factoring in the benefits of pesticides.

1.3 Pest management in modern agriculture

Since the first cropping season of agriculture, growers had to take care of biotic stressors threatening their crop by competing for resources (e.g. weeds) or by directly lowering quality and quantity of the potential yield (e.g. plant pathogens and insect pests). The intensification of agriculture in the last century has contributed substantially to increased food production, but the burden of harmful organisms remained or even increased as low genetic diversity related

to monocultures can facilitate pest outbreaks (Andow, 1991; Matson et al., 1997). Several studies have tried to quantify potential yield losses associated with weeds, diseases, and animal pests on key crops in different world regions. In 1967 Cramer estimated - based on limited available data - that 35% of potential yield is lost due to biotic factors (14% due to insects, 12% due to fungal diseases and 9% due to weeds) (Cramer, 1967). Looking at some of the most important crops individually, Oerke & Dehne estimated actual losses between 26% for sugar beet and 40% for rice with weeds being the most significant cause (Oerke & Dehne, 2004). These estimations are supported by an expert-based assessment of the situation in five major world crops (wheat, maize, potato, rice and soybean) which found a similar range of yield losses due to pests and pathogens (from 17.2% for potato and up to 30% for rice) (Savary et al., 2019). Notably, these estimations are made under the assumption that crop protection measures are implemented. In a scenario without any protective actions, losses could range from 40% up to 62% (Keulemans et al., 2019). This demonstrates the necessity of pest management solutions to reduce yield losses on the farm level which translates to reduction of economic losses and ultimately to increases in food security on a global scale.

More than 60 years ago, the principle of integrated pest management (IPM) has been introduced and since then has been propagated as the ideal scenario for pest management around the world (Dent, 2000; Stern et al., 1959). Its focus and toolbox have changed over the years, but it can be viewed as a holistic approach with three main stages – avoidance of pest outbreaks, monitoring of pest occurrence / crop status and control measures if economic thresholds are reached (Figure 3).

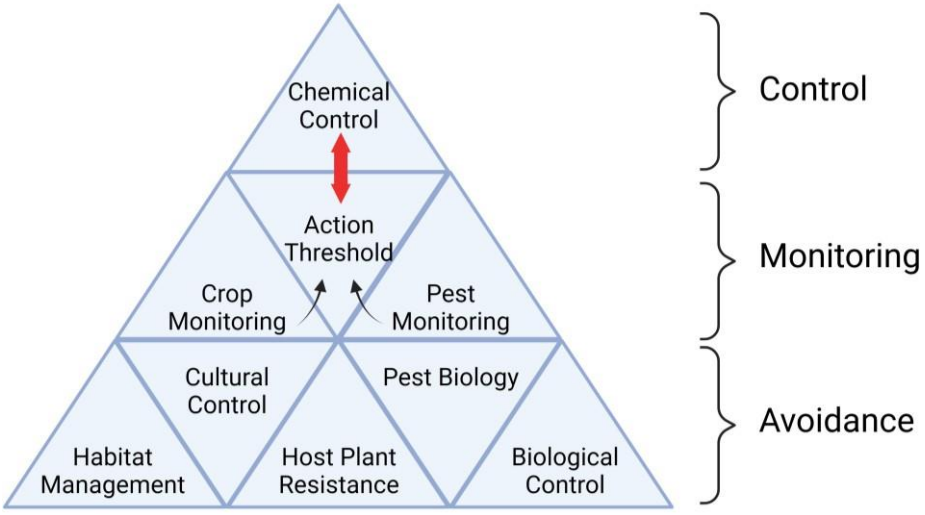


Figure 3: The classical framework of integrated pest management (IPM) illustrated as a pyramid with three main stages. The foundation of IPM is avoidance of pest outbreaks above economic thresholds which is monitored carefully. Only if action thresholds are reached, chemical control options are deemed necessary. Created with biorender.com.

Considering insect pest management this means that strategies to avoid a significant damage to the crop should be considered by the grower throughout the entire year.

This can include cultural control measures (e.g. crop rotation), habitat management to enhance natural opponents or reduce pest occurrence, consideration of insect resistant crop varieties, considering the biology of pest species to disrupt plant-pest synchronization, or biological control measures (e.g. pheromones or deployment of predators) (Koul et al., 2004). Monitoring forms the second stage of IPM strategies. The economic threshold (ET) and the economic injury level (EIL) are the central elements of this stage. The EIL is defined as "*the lowest population density that will cause economic damage"* while the ET is "*the density at which control measures should be determined to prevent an increasing pest population from reaching the EIL*" (Stern et al., 1959). EIL and ET must be determined experimentally for each pest in each crop, can vary under different environmental conditions, different cultivars, or different commodity prices and is therefore a dynamic value with many variables (Higley & Pedigo, 1993; Stern et al., 1959). Once determined, monitoring of pest populations is key to the ET concept. As crops are not equally susceptible in each development stage or might be stressed due to abiotic factors, monitoring of the crop status is equally important. Only if the ET has been reached, further control options (i.e. chemical crop protection) are deemed necessary and justified.

In agricultural practice, chemical crop protection is a much more essential portion of pest management strategies, than the IPM pyramid might suggest. This has many reasons. For example, not all crops necessary for an ideal crop rotation are profitable everywhere and are therefore not implemented by growers (Peltonen-Sainio et al., 2016). The efficacy of insect-resistant cultivars has remained limited so far except for certain GM crops which are not accessible to farmers everywhere (De Vos & VanDoorn, 2013). Biological control options are often more costly, more difficult to use and / or less effective (Matyjaszczyk, 2019). Additionally, most economic thresholds were established decades ago and are outdated considering novel varieties, cropping systems and commodity prices (Hokkanen, 2015). Since also predictions and modelling approaches of pest outbreaks are not as precise as needed for a targeted action, preventive control measures are often deployed. Besides, preventive crop protection solutions such as seed treatments may appear counter-intuitive to the IPM strategy, but also have the potential to reduce foliar insecticide applications (Perry & Moschini, 2020) and are appreciated by farmers as a risk insurance tool for early season pest outbreaks (Grout et al., 2020).

To conclude, synthetic insecticides are and will remain for the foreseeable future one of the most important crop protection tools for many crops in many regions. They contribute significantly to the reduction of yield losses alongside several other benefits (Aktar et al., 2009). However, to justify their use, the risk for potential negative effects on humans and the environment must be kept at a minimum. Therefore, the risk of crop protection compounds for several organism groups – including bees - is assessed before their commercialization.

1.4 Bee risk assessment of plant protection products in the EU

The goal of environmental risk assessment of a plant protection product (PPP) is to evaluate if it can be used as intended without exhibiting an unacceptable effect on the environment including non-target species such as bees (EU, 2009). First question is always whether the organism of interest is potentially exposed to the product. If exposure is deemed unlikely, there is no risk, and no further action is needed. Considering the applications of PPPs, exposure to bees is however likely (Figure 4) and usually requires further assessment.

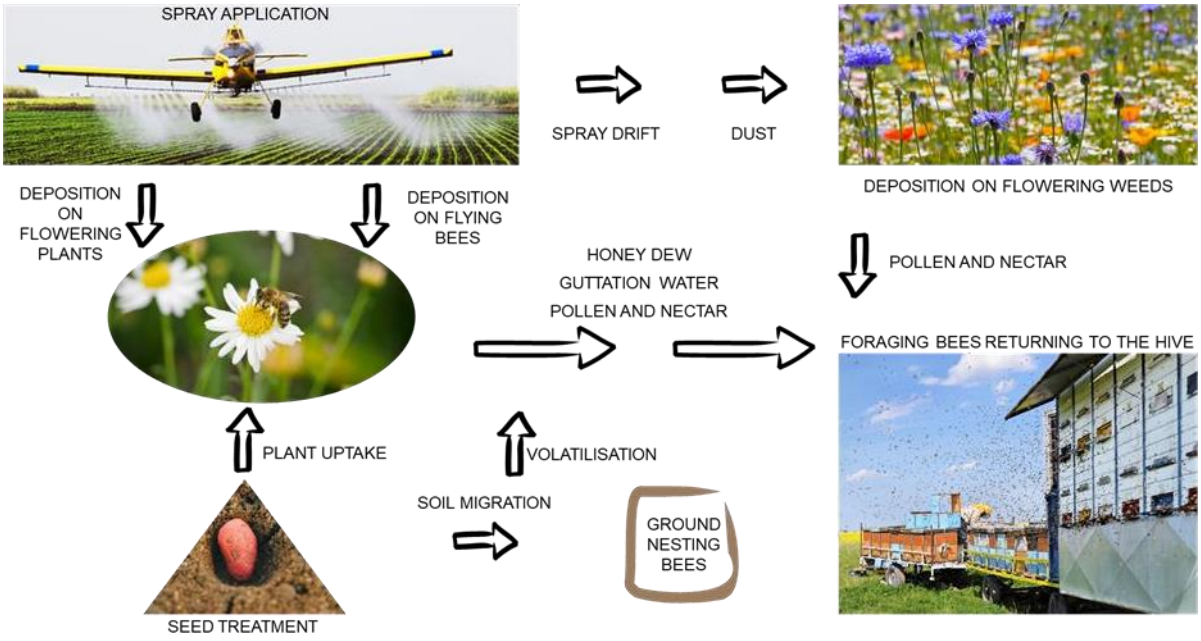


Figure 4: An overview over the main exposure routes for bees following spray application or seed treatment application with plant protection products. Next to deposition on foraging bees, residues in pollen, nectar, guttation water and honey dew may be relevant. Not just the crop, but also adjacent flowering strips are considered for exposure due to spray drift or dust. The soil migration of compounds after seed treatment is especially relevant for ground-nesting bees. Adapted from (EFSA, 2014b).

Now it is crucial to determine what exactly needs to be protected and what effect is considered unacceptable (EFSA, 2012). For that, a specific protection goal must be defined which has been proposed by the European Food Safety Authority (EFSA) for bees in their guidance document. There are three primary protection goals formulated: 1. Pollination services 2. Hive product production (for honey bees only) 3. Conservation of bee biodiversity (EFSA, 2013). As these are abstract targets which are difficult to measure directly, a proxy has been established to measure the effects of PPPs on bees: the colony size of honey bees (EFSA, 2013). A reduction in colony size of <7% was set as the threshold for a negligible effect, which was recently revised and raised to <10% (EFSA, 2013, 2021). The revision was necessary as colony sizes can vary significantly due to other reasons throughout the year and it was (and still is) debated whether an effect <7% or 10% can be detected statistically within a realistic study design. This is one of the reasons why the bee guidance document from 2013 has never been endorsed by most of the member states in the European Union and several countries rely on previous guidelines for bee risk assessment (EC, 2002; EPPO, 2010).

Here, the honey bee colony survival and development is the protection goal with no concrete thresholds determined (Alix et al., 2009). Subsequently, the risk is evaluated in a tiered process with a laboratory-based, cost-effective first tier to identify PPPs with negligible risk to bees and higher tier studies under semi field (tier II) or field (tier III) conditions to further assess PPPs which failed the tier I screen. To understand the procedure, it is essential to explain how the term risk is defined in this context. The risk of a PPP is a function of its hazard (i.e. the intrinsic toxicity of the compound) and its exposure (i.e. which route of exposure, how long and at which quantities is the organism exposed to the compound).

In tier I studies the hazard is evaluated according to official guidelines for several exposure scenarios – acute contact toxicity (OECD, 1998b), acute oral toxicity (OECD, 1998a) and chronic oral toxicity (OECD, 2019) are all evaluated for adult worker bees and additionally larval toxicity can be evaluated via single or repeated exposure (OECD, 2013, 2014). Toxicity is expressed as LD_{50} or LC_{50} value which is defined as the dose / concentration of a compound which is lethal for 50% of test organisms. Those values are then related to the predicted environmental concentration (PEC) to calculate trigger values for higher-tier studies. The hazard quotient (HQ) is the relevant trigger value for contact toxicity. It is defined as the quotient of the application rate in g / ha and the LD_{50}. For oral exposure the exposure toxicity ratio (ETR) has been proposed as a novel trigger value which is the ratio between the amount of residues that may be ingested by an adult bee in one day and the LC_{50} value (EFSA, 2012). Values under a certain threshold are considered acceptable and the risk is considered as low. Higher values warrant further evaluation.

There are different options how to continue the risk assessment depending on which trigger value has been exceeded and which use pattern is pursued. Risk mitigation measures (e.g., use restrictions) can be considered, the exposure assessment can be refined by replacing conservative default residue values with compound specific values, or higher tier studies can be conducted under semi-field or field conditions which evaluate the effects under increasingly field-realistic scenarios. Once the assessment is finalized the risk is categorized and the product is registered accordingly.

Similar bee risk assessment schemes are in place in most countries around the world (e.g.: Australian Pesticides and Veterinary Medicines Authority, 2017; US EPA, 2014). Thorough bee pesticide risk assessment has contributed to the continuously low number of reported bee poisoning reports in countries such as the UK (Carreck & Ratnieks, 2014). Nonetheless, regulatory authorities and other stakeholders have identified some areas where knowledge gaps exist. This includes sub-lethal effects, the risk of PPPs for non-*Apis* bees or potential interaction effects between pesticides. Knowledge gaps were identified during the controversial debate over the impact of neonicotinoids on bees (see Chapter 1.2).

Sub-lethal effects have been proposed to be the reason for potential long-term effects on bees. However, it is difficult to link sub-lethal effects observed on individual bees (mostly studied under laboratory conditions or with non-realistic dose exposure) to significant impairment and effects on the colony level (Godfray et al., 2014, 2015). The question whether or not non-*Apis* bees are sufficiently protected by a risk assessment scheme depending on the honey bee as a surrogate species gained traction after a large-scale field study reported no significant effects of oilseed rape coated with the insecticides clothianidin + *β*-cyfluthrin on honey bee colonies but significant effects on bumblebee (*Bombus terrestris*) and red mason bee (*Osmia bicornis)* populations (Rundlöf et al., 2015). The co-occurrence of multiple chemicals in bee hives (Mullin et al., 2010) has sparked investigations of potential synergistic mixture toxicity (Johnson et al., 2013; Wade et al., 2019).

Regulatory bodies and other stakeholders have reacted to these findings. At the moment, a factor of 10 is employed on honey bee endpoints to account for uncertainties regarding other bee species (EFSA, 2013) and the development of first methods to assess the effects of pesticides on other managed bee species (e.g. bumblebees) is already well advanced (OECD, 2017a, 2017b). Similarly, a guideline for assessing the homing flight ability as a first attempt to assess sub-lethal effects has been recently published (OECD, 2021) and methodologies to assess the risk of multiple chemicals have been proposed (EFSA, 2014a, 2019). These methodologies mostly rely on *in-vivo* tests sometimes combined with simplistic models to predict adverse effects. While this is a valid approach, it also has its limitations. For example, only for a very limited number of bees it is possible to design laboratory toxicity studies not to mention higher tier studies. For mixture toxicity, it is only feasible to test a very limited number of combinations in *in-vivo* studies. The guideline on homing flight success is performed under a huge amount of additional stress for individual bees (captured multiple times, starved, immobilized, glued with a tag) (OECD, 2021). Thus, the significance of the test system to capture sub-lethal effects attributable to pesticide exposure alone remains questionable.

All these areas, however, can profit from a detailed mechanistic understanding of the interaction between PPPs and the organism. Detailed toxicological studies, especially those at the molecular level, have the potential to explain the basis of synergistic interactions, chronic / sub-lethal effects or species-dependent sensitivity differences and could complement the *in-vivo* studies outlined above.

1.5 Insect toxicology

One of the most common definitions of toxicology is as follows: "*Toxicology is that branch of medical science that deals with the nature, properties, effects and the detection of poisons. It is, therefore, the science of poisons*" (DuBois & Geiling, 1959). It illustrates that the origin of toxicology was centered on humans as part of medical sciences. It was only until the appearance of Rachel Carson's famous book *Silent Spring* in 1962 that the discipline environmental toxicology has emerged and broadened the view on all forms of biological systems (Matsumura, 1985). It can be defined as the study of the "*incidental exposure of plants and animals, including humans, to pollutant chemicals and unnatural environmental stresses*" (Laws, 2013). Notably, before the advent of environmental toxicology a lot of progress on insect toxicology has already been made, owing to the emerging problem of insecticide resistance in insect species threatening the successful control of vector-borne diseases and effective crop protection in agriculture (Brown, 1958; Georghiou, 1972).

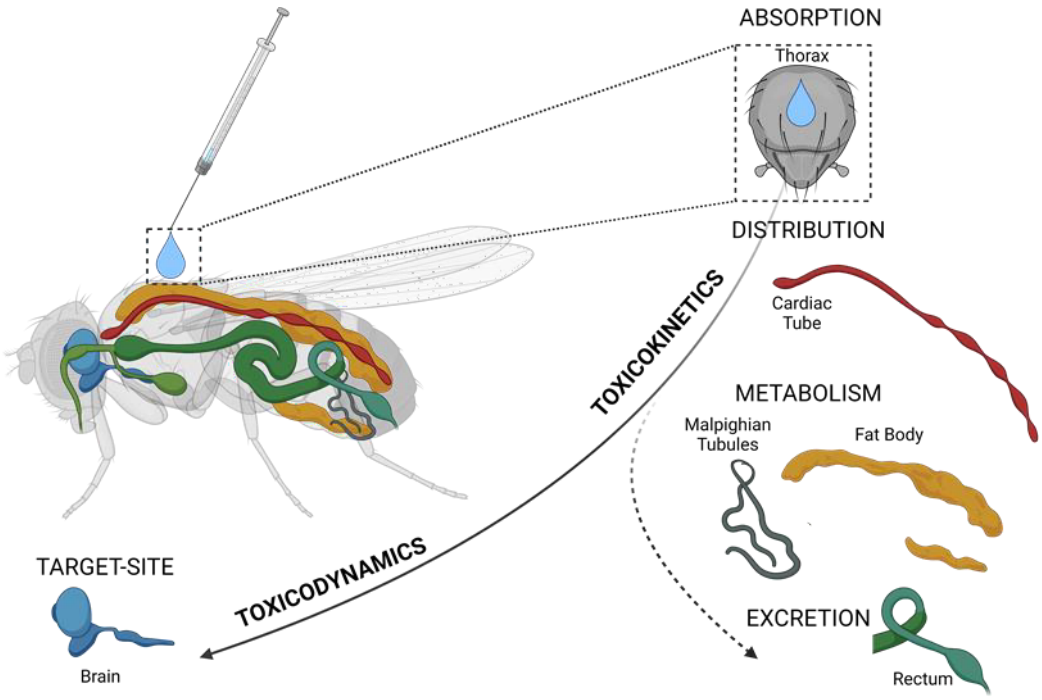


Figure 5: Simplified sketch of the processes involved in insecticide toxicology. After contact exposure on the thorax, the compound is absorbed mainly over the cuticle. It is distributed within the insect via the hemolymph with the cardiac tube as important driver. Upon distribution the compound may be subject to metabolism. The Malpighian tubules, midgut and fat body of insects are organs with high metabolic capacity. Metabolism may lead to enhanced water solubility and finally to rectal excretion of the compound. The remaining compound will reach its target (often neuronal targets in the brain) and exert its biological effect. Created with biorender.com.

While the organism of study may differ, the general aspects of toxicology are always the same. The most important principle is that the toxicity of any substance is always dose related. At a high enough concentration, most chemicals become toxic. In classic toxicology, toxicity is usually evaluated with *in-vivo* dose-response studies and expressed as LD_{50} values. Depending on the organism and the compound under investigation, the test design can vary.

Different application techniques (topical, oral, injection, residual, etc.) can influence toxicity and can be adapted to the specific need. Similarly, duration of exposure time can be varied. Acute effects are usually observed within hours or a few days, while chronic effects may be evaluated over extended periods.

To put the results obtained from such studies into perspective and to understand differences in sensitivity between species, detailed knowledge about the interaction of the chemical and organism is needed. This interaction can be categorized in different phases (Figure 5). Upon exposure, the chemical is taken up, distributed, transformed, and finally excreted by the organism. These processes are summarized under the term toxicokinetics. Within the organism, the chemical interacts with a target site exerting its biological effect (toxicodynamics). Essential for the study of toxicodynamics is the knowledge of the mode of action. With the progress in genomics, a relatively novel field of research has emerged, which combines toxicology with genomics: toxicogenomics. These concepts are explained in more detail with reference to insecticides and insects in the following sections.

1.5.1 Toxicodynamics and insecticide mode of action

The basis for the understanding of insecticide toxicodynamics is their mode of action (MoA). Detailed studies on the MoA of insecticides are conducted during research and development and are gathered and published in the MoA classification scheme of the Insecticide Resistance Action Committee (IRAC) (Sparks & Nauen, 2015). The insecticides with the highest market share target the nerve and muscle system of insects (Sparks et al., 2020). Many important insecticides today target ion channels (e.g. voltage-gated sodium channels, nicotinic acetylcholine receptors, ryanodine receptors), while the enzyme acetylcholinesterase is the main target of early insecticide classes introduced in the 1940s and 50s (organophosphates and carbamates). Other relevant insecticide classes interfere with growth regulation (e.g. ecdysone receptor agonists), energy metabolism (e.g. mitochondrial electron transport inhibitors) or midgut membranes (e.g. *Bacillus thuringiensis*). For some insecticides the mode of action remains unknown or uncertain (Sparks et al., 2020). Two of the most important insecticide targets and their modulators are introduced below as they are highly relevant for this thesis.

1.5.1.1 Nicotinic acetylcholine receptors and their modulators

The nicotinic acetylcholine receptor (nAChR) belongs to the pentameric cys-loop ligand-gated ion channels found in animals (Dent, 2010). It is a transmembrane protein located in the postsynaptic membrane and neuromuscular junctions and involved in electrochemical signal transduction (Changeux & Paas, 2009). Binding of acetylcholine (ACh), the principal excitatory transmitter for rapid neurotransmission in insects (Casida & Durkin, 2013), leads to a conformational change allowing permeability of mono- and divalent cations (mainly Na^+, K^+ and Ca^{2+} under biological conditions) (Figure 6A) (Adams et al., 1980; Dani, 2015).

Pentamers can be formed by the same subunit (homo-pentamers) or different subunits (hetero-pentamers) (Dani, 2015) with hetero-pentamers assumed to be the predominant form, particularly in insects (Matsuda et al., 2020). Subunits are divided in α-, β-, γ-, δ-, ε-subunits based on the presence (α-subunits) or absence (non-α-subunits) of two adjacent cysteines in their N-terminal sequence (Fasoli & Gotti, 2015; Jones & Sattelle, 2010; Millar & Gotti, 2009). Each subunit has a large N-terminus, four transmembrane domains (with TM2 lining the pore) and an intracellular loop (Figure 6B) (Albuquerque et al., 2009). In mammals 17 subunits assembling to different compositions have been identified (Ho et al., 2020) with α4β2 heteromers and α7 homomers as the predominant forms in the brain (Dani, 2015). Insects have between 10-12 nAChR α- and β-subunit genes and are devoid of vertebrate γ-, δ-, and ε-subunits (Crossthwaite et al., 2017). However, how they assemble to form functional nAChRs remains elusive, hampered by the fact that functional expression of insect nAChR has been unsuccessful until recently (Ihara et al., 2020). Additionally, posttranslational regulation may increase the potential combinations considerably (Jones & Sattelle, 2010).

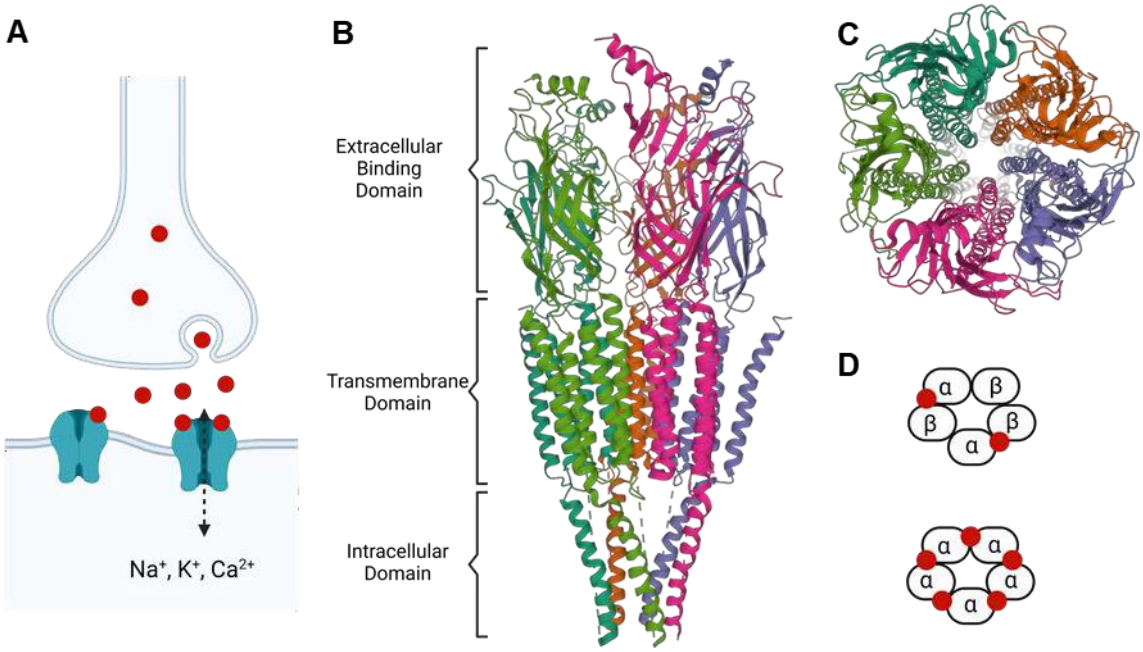


Figure 6: Nicotinic acetylcholine receptors (nAChR). (A) Scheme of their function regulating a cation ion channel in the postsynaptic membrane. Channel opens upon binding of acetylcholine illustrated as red circles. (B) The pentameric structure of a functional nAChR with three domains per subunit. Each subunit is illustrated in a different color. (C) Top view of the nAChR with the channel pore in the center. (D) Illustration of a hetero - and homo-pentameric nAChR which differ in the number of ligand binding sites. Created with biorender.com.

The ligand (ACh) binding site is located in the extracellular N-terminal domain at the interface between two adjacent subunits (Bartos et al., 2009). One principal subunit (α-type) contributes three loops (A to C) while the other complementary subunit (α- or non-α-type) contributes additional loops (loops D to F) to the binding pocket (Changeux & Taly, 2008). Thus, homo-pentameric receptors possess five orthosteric binding sites, while hetero-pentameric only have two (or three for e.g. mammalian $(\alpha4)_3(\beta2)_2$ receptors) (Figure 6D) (Changeux & Taly, 2008; Fasoli & Gotti, 2015).

nAChRs are among the most important targets, addressed by insecticides (and insecticidal peptides) of IRAC groups 4 (competitive modulators), 5 (allosteric modulators, site I), 14 (channel blockers) and 32 (allosteric modulators, site II) (Figure 7) (Sparks et al., 2020). Competitive modulators are by far the most important group and reversibly bind to the orthosteric site of nAChRs causing transient cation influx resulting in the generation of action potentials (Jeschke et al., 2013). Neonicotinoids (group 4A) have been and continue to be the most important subgroup in terms of market share (Sparks et al., 2019). Their discovery began with the nitromethylene nithiazine, but commercial success started with the discovery and market introduction of imidacloprid (IMD) in the early 1990s (Bai et al., 1991; Casida, 2018). Its compelling characteristics (e.g. high selectivity for insect nAChRs resulting in high insecticidal efficacy combined with low bird and mammalian toxicity) sparked the development of many other neonicotinoids. Structurally, they can be divided into noncyclic compounds and compounds with five- or six-membered ring systems. Their pharmacophore can be described as [-N-C(E)=X-Y] with [=X-Y] being an electron-withdrawing group (X: N(H) or CH; Y: NO_2 or CN) and E being NH, NMe, sulfur or methyl (Jeschke et al., 2019).

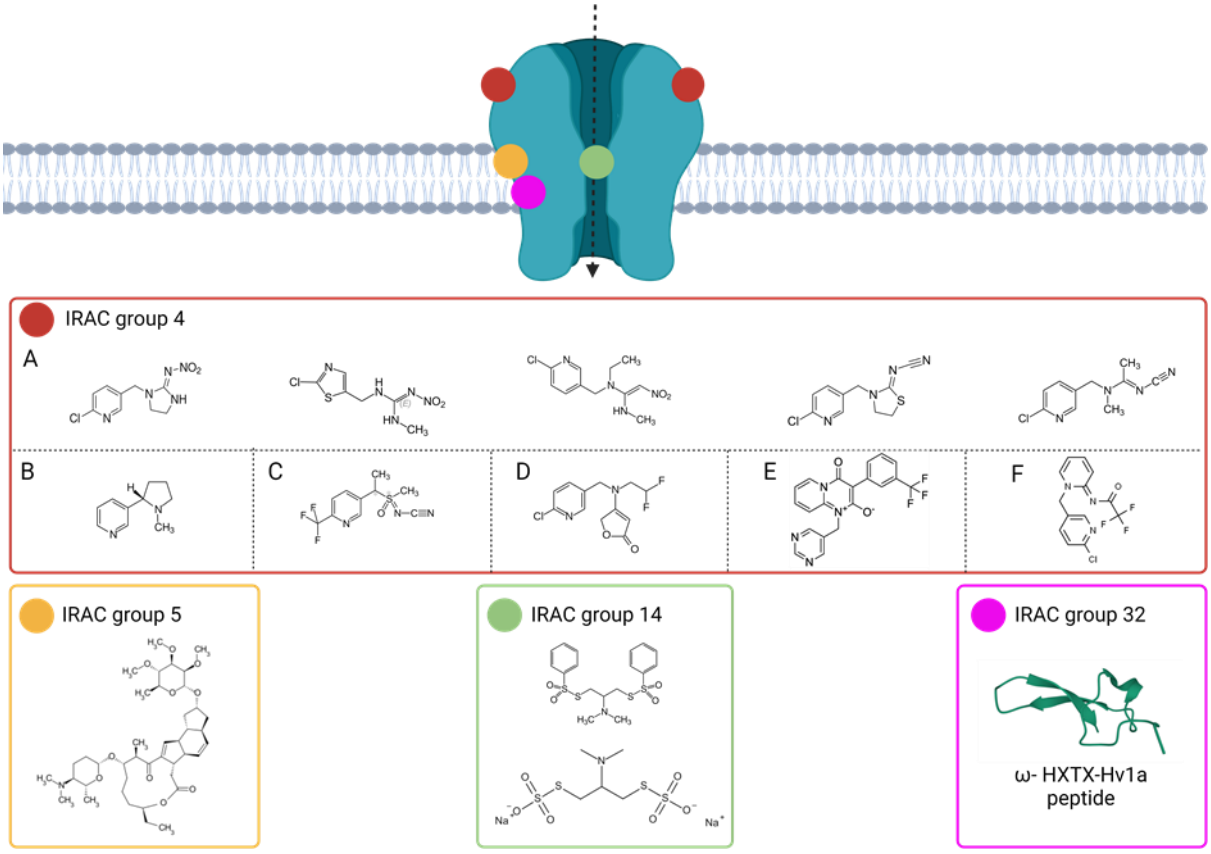


Figure 7: Different insecticides targeting the nicotinic acetylcholine receptor (nAChR) classified by the IRAC mode of action scheme. Color of the circles illustrates the binding site of the different groups. The red circle represents the orthosteric binding site of the natural ligand acetylcholine. IRAC group 4 comprises competitive modulators with neonicotinoids (subgroup A) represented by imidacloprid, clothianidin, nitenpyram, thiacloprid and acetamiprid (from left to right). Subgroup B are nicotinoids represented by nicotine. Subgroup C: Sulfoximines (sulfoxaflor). Subgroup D: Butenolides (flupyradifurone). Subgroup E: Mesoionics (triflumezopyrim). Subgroup F: Pyridylidenes (flupyrimin). IRAC group 5: allosteric modulators represented by spinosad. Group 14: nAChR channel blockers represented by bensultap and thiosultap-sodium. Group 32: allosteric modulators at a distinct binding site comprising neuroactive peptides. Created with biorender.com.

Neonicotinoids are especially effective against homopteran pests (i.e. aphids, leafhoppers, planthoppers, thrips and whiteflies) with secondary activity against a range of coleopteran, dipteran or lepidopteran pest species (Elbert et al., 1998). They can be used with versatile application methods (e.g. foliar treatment, in soil drench / dripping systems, or trunk / bud injections) but their incredible success is based on their application as seed treatment owing to their high water solubility leading to systemic distribution in the plant (Elbert et al., 2008). This quality is influenced by the pharmacophore explaining why especially IMD, clothianidin and thiamethoxam are used as seed treatments (although soil stability is also a considerable factor) (Elbert et al., 2008; Jeschke et al., 2019).

Unlike neonicotinoids, nicotinoids (group 4B), with its most prominent representative (-)-nicotine, are more potent on vertebrate than on insect nAChRs resulting in high vertebrate toxicity (Matsuda et al., 2020). These differences can be explained by the charge of their pharmacophore. While the protonated nitrogen of the pyrrolidine ring of nicotine is positively charged, neonicotinoids possess a negatively charged tip at their respective pharmacophore interacting with positively charged amino acid residues solely found in insect nAChRs (Matsuda et al., 2005; Tomizawa et al., 2003).

More recently, novel compounds binding to the orthosteric site have been introduced such as the sulfoximine sulfoxaflor (group 4C), the butenolide flupyradifurone (group 4D), the mesoionic triflumezopyrim (group 4E) and the pyridylidene flupyrimin (group 4F). They are chemically distinct from neonicotinoid insecticides, have overlapping binding sites with IMD as evident by radioligand displacement studies and a similar biological spectrum as neonicotinoids (Cordova et al., 2016; Nauen et al., 2015; Onozaki et al., 2017; Zhu et al., 2011). Notably, triflumezopyrim and flupyrimin, unlike the other compounds, are not agonists but rather antagonists of nAChRs occupying the ligand site without opening the channel (Cordova et al., 2016; Onozaki et al., 2017).

1.5.1.2 Ryanodine receptors and their modulators

Ryanodine receptors (RyR) are regulatory channels of calcium release located in the endo- / sarcoplasmic reticulum (SR) membrane (Smith et al., 1988) and are involved in excitation-contraction coupling of muscles (Pessah et al., 1985) and other Ca^{2+}-dependent signaling mechanisms (Fill & Copello, 2002). Upon stimulation, RyRs release Ca^{2+} rapidly from the SR lumen increasing [Ca^{2+}] from 0.1 µM to > 0.1 mM (Figure 8A) (Baylor et al., 1983). In mammals, three isoforms were identified with different tissue preference (Hakamata et al., 1992; Otsu et al., 1990; Takeshima et al., 1989). RyR1 is primarily expressed in skeletal muscles, RyR2 in cardiac muscle tissue and RyR3 in brain tissue, albeit they are found in many other tissues (Lanner et al., 2010). Insects only have one RyR gene (first identified in *Drosophila melanogaster*) sharing around 45% sequence identity with the mammalian counterparts (Takeshima et al., 1994).

For insect RyR there is no high-resolution cryo-EM structure available as it is the case for mammalian RyRs with diverse ligands (Ma et al., 2020; Ogawa et al., 2021). However, crystal structures of specific domains as well as homology modelling suggest that the overall structure is similar (Lin et al., 2018, 2020; Xu & Yuchi, 2019; Zhou, Ma, et al., 2020; Zhou, Wang, et al., 2020). RyRs are homotetramers (~550 kDa per monomer) and form a mushroom-like shape with a large, cytoplasmic cap and a transmembrane stalk (Figure 8B+C) (Petegem, 2012). The large, cytosolic N-terminal region forms the cap, while a C-terminal region includes six transmembrane domains surrounding the channel pore. Overall RyRs are highly modular with 20 individual domains (Lin et al., 2020). Skeletal muscle RyRs in vertebrates are associated with and directly regulated by dihydropyridine receptors (DHPRs), also known as $Ca_v1.1$ voltage-gated Ca^{2+} channels, which after depolarization do not only allow the influx of Ca^{2+}, but open RyR directly via protein-protein interaction leading to Ca^{2+} release and muscle contraction (depolarization induced Ca^{2+} release (DICR)) (Franzini-Armstrong et al., 1999; Zalk et al., 2007). Not all RyRs in skeletal muscles are directly linked to DHPRs but rather to each other by the accessory protein FKBP12, thereby exhibiting simultaneous opening / closing – a mechanism called coupled gating (Marx et al., 1998).

Notably, cardiac muscle RyR2 does not have the same direct link with DHPRs and preparations from arthropod muscles indicate that insects resemble vertebrate RyR2 in this regard (Takekura & Franzini-Armstrong, 2002; Zalk et al., 2007). Here, Ca^{2+} influx through DHPRs induces the release of more Ca^{2+} in a process called calcium-induced calcium release (CICR) (Endo, 2009). Ca^{2+} influences open probability of the RyR in a bell-shaped form with low concentrations (µM range) leading to increased open probability while high cytoplasmic Ca^{2+} concentrations (mM range) inhibit channel opening (Bezprozvanny et al., 1991). Additionally, high [Ca^{2+}] in the sarcoplasmic reticulum can lead to spontaneous channel opening and Ca^{2+} release (spontaneous store-overload induced Ca^{2+} release (SOICR)) (Chen et al., 2014; Palade et al., 1983).

RyRs also have several other endogenous ligands including Mg^{2+}, ATP and calmodulin (CaM) which influence channel activation (Brillantes et al., 1994; Fabiato, 1983; Georges et al., 2016; Meissner & Henderson, 1987). Eponymous for the RyR is, however, the exogenous ligand ryanodine – an alkaloid first described in the tropical shrub *Ryania speciosa* (Rogers et al., 1948). Ryanodine binds to the receptor in its activated state locking it in a partially open state causing a permanent leakage of Ca^{2+} while high concentrations inhibit Ca^{2+} release (Fessenden et al., 2001; Smith et al., 1988). Owing to its insecticidal efficacy it has been used as an insecticide especially in organic farming in the US, however due to its high mammalian toxicity it is not registered anymore (Pessah et al., 1985; US EPA, 1999).

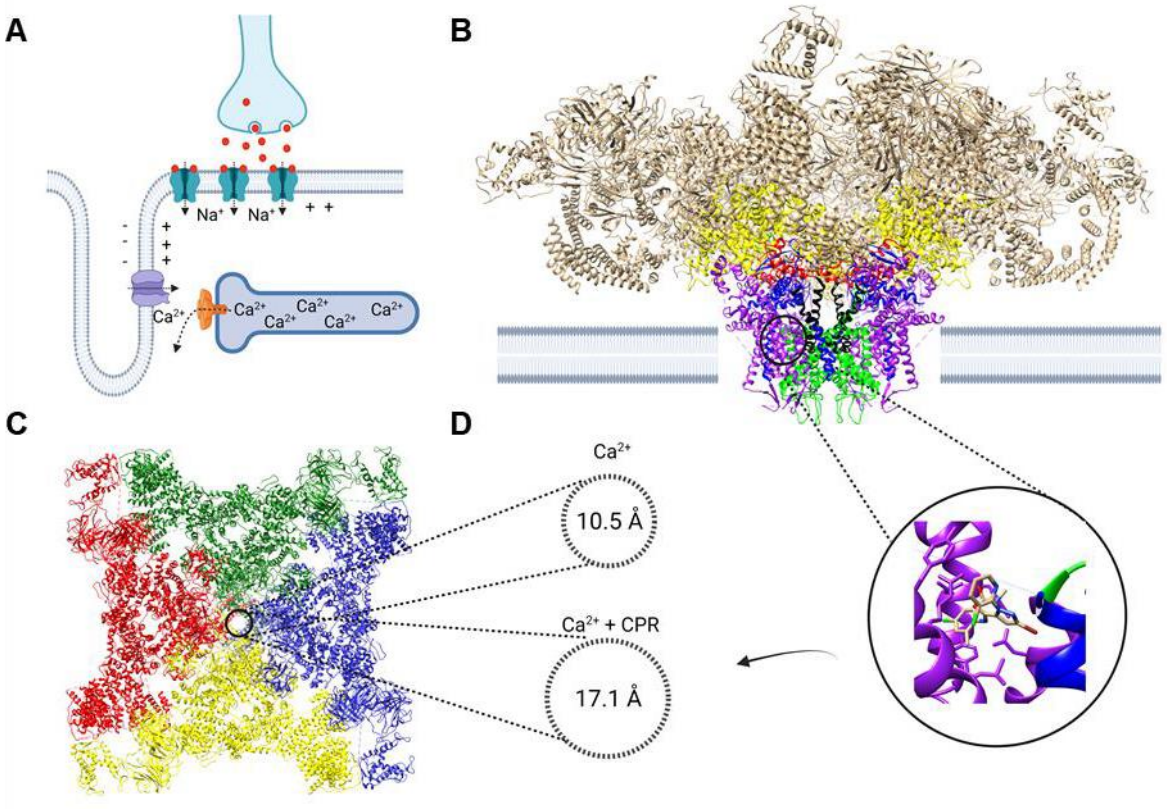


Figure 8: Ryanodine receptors. (A) Scheme of ryanodine receptor (RyR, orange) function. After reception of an action potential the dihydropyridine receptor (purple) allows calcium influx which in turn triggers the opening of the ryanodine receptor localized in the sarcoplasmic membrane leading to Ca^{2+} efflux from the sarcoplasmic lumen. The process is called Ca^{2+}-induced-calcium-release. (B) Structure of rabbit RyR1 in the open state in complex with chlorantraniliprole (CPR; PDB: 6M2W) embedded in the membrane. Important domains for ligand binding are colored – core solenoid domain (csol, yellow); thumb and forefingers domain (TaF, blue); pseudo voltage sensor domain (pVSD, purple), C-terminal domain (CTD, red) channel pore domain (green), cytosolic extension of channel pore domain (black). (C) Top view of the rabbit RyR1 homotetramer with each subunit colored differently. (D) Zoom in on the CPR binding pocket in the transmembrane region with contact residues in the pVSD. CPR preferentially binds to the open state and induces additional widening of the pore compared to the open state under influence of Ca^{2+} alone (Ma et al. 2020). Created with biorender.com.

The insect RyR is also the target of IRAC group 28 insecticides (ryanodine receptor modulators, Figure 9). The commonly called diamides (based on their shared structural feature of two amide bonds) have rapidly gained market share (12% as of 2020) since their introduction and are now one of the most important chemical classes (Sparks et al., 2020). Binding to the RyR induces calcium release from the SR disrupting calcium homeostasis leading to typical symptoms such as feeding cessation, paralysis, muscle contractions and eventually death. The development of the phthalic acid diamide flubendiamide (FLB) marked the beginning of the era (Ebbinghaus-Kintscher et al., 2006; Tohnishi et al., 2005) quickly followed by the discovery of anthranilic diamides such as chlorantraniliprole (CPR) (Lahm et al., 2005, 2007). They exhibit high activity especially against lepidopteran pest species combined with low mammalian toxicity indicated by selective binding to insect ryanodine receptors (Cordova et al., 2006; Ebbinghaus-Kintscher et al., 2006).

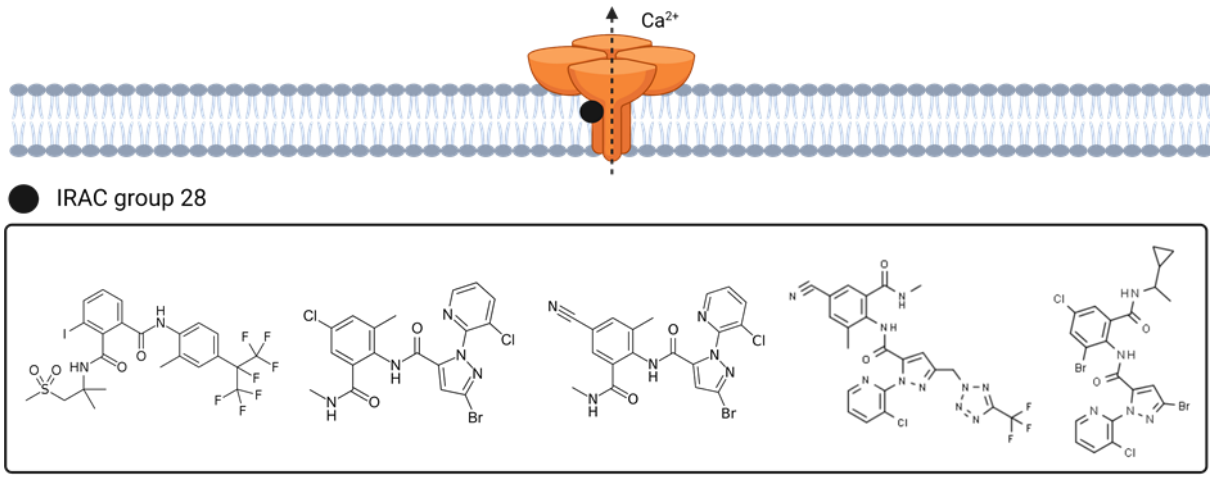


Figure 9: Ryanodine receptor modulators classified in IRAC group 28. Flubendiamide (left) represents the phthalic acid subtype. Other representatives are anthranilic diamides: chlorantraniliprole, cyantraniliprole, tetraniliprole and cyclaniliprole (from left to right). Created with biorender.com.

The diamide binding site at the rabbit RyR1 has been structurally resolved using cryo-EM (Ma et al., 2020). Upon binding near the interface between the transmembrane region and the cytoplasmic csol domain, chlorantraniliprole induces a widening of the pore accompanied by conformational changes of the cytosolic cap (Figure 8D) (Ma et al., 2020). The findings confirmed previous assumptions of the binding site derived from resistance-conferring mutations in insect RyRs (Nauen & Steinbach, 2016) and computational modelling (Lin et al., 2020; Sindhu et al., 2017). It also explains species-specific differences in the binding site leading to differential toxicity between mammals and insects, but also within different insect orders (Qi et al., 2014; Qi & Casida, 2013).

1.5.1.3. Insecticide selectivity influenced by toxicodynamics

How differential interaction with the target site influences toxicity, is best illustrated by concrete examples. Neonicotinoids were introduced in the 1990s and had one significant advantage over many previous insecticide classes - they are significantly less toxic to mammals than to insects (Jeschke et al., 2013). This is in stark contrast to nicotinoids which are more toxic for mammals (Yamamoto, 1999) raising the question why structurally similar compounds acting at the same target behave differently. Toxicity in mice is well correlated with binding and agonistic action at the $\alpha_4\beta_2$ nAChR indicating that selectivity is driven by toxicodynamic differences and differential insect / mammalian nAChR receptor properties (Tomizawa et al., 2001; Tomizawa & Casida, 2003). Nicotinoids are characterized by a protonated state under physiological conditions (Tomizawa, 1994). On the other hand, neonicotinoids have a strong electronegative tip (NO_2 or CN) forming a planar layer with the substituted guanidine / amidine moiety (Kagabu & Matsuno, 1997). *N*-unsubstituted imine derivatives of imidacloprid and thiacloprid (i.e. no electronegative tip, but instead an iminium cation) bind with high affinity to mammalian rather than insect nAChRs, strongly suggesting that this moiety is responsible for nAChR selectivity (Tomizawa et al., 2000).

Exploiting crystal structures from mollusc acetylcholine-binding proteins (AChBPs) – structural surrogates for the binding domains of nAChRs – confirmed the differential binding poses of neonicotinoids and nicotinoids at the orthosteric site (Talley et al., 2008; Tomizawa & Casida, 2009). With the help of computational modeling approaches, the ligand-receptor interaction has been further elucidated. Binding mode hypothesis suggests three major interactions of neonicotinoids with insect nAChRs. First, the pyridine nitrogen atom forms a H-bond to the backbone of loop D (β-subunit) for imidacloprid and nicotine alike. Second, an aromatic residue cluster stabilizes the N-methylpyrrolidine / N-(nitroimino)imidazolidine moiety of nicotine or imidacloprid, respectively. Third, a charged arginine in loop D interacts tightly with the N-nitroimino group of imidacloprid, while a weak, repulsive force is predicted between this residue and the protonated nitrogen atom in nicotine (Beck et al., 2015, 2021). Functional validation for the importance of the loop D arginine is provided by the occurrence of neonicotinoid-resistant aphid strains possessing a vertebrate-like arginine / threonine substitution at this position rendering neonicotinoid binding less effective (Bass et al., 2011; Hirata et al., 2017). In conclusion, neonicotinoids provide an excellent example of how toxicodynamics can influence toxicity and how the knowledge of the molecular basis can help to understand insecticide selectivity between mammals and insects. Toxicodynamic differences are also (partially) responsible for insect order specific differences in toxicity observed for ryanodine receptor modulators (especially flubendiamide). Flubendiamide is particularly effective against lepidopteran species, while it lacks activity against a range of beneficial insects from other orders (Tohnishi et al., 2005). Radioligand binding experiments with thoracic muscle preparations show a lack of a high affinity flubendiamide binding site in the house fly *Musca domestica* and the honey bee suggesting differential binding properties between lepidopteran species and other insect orders (Qi et al., 2014; Qi & Casida, 2013). Support is provided by resistance mutations in lepidopteran pests, where an isoleucine / methionine substitution in the proposed binding region leads to reduced efficacy (Nauen & Steinbach, 2016). In other insect orders a methionine at this position is conserved leading to reduced binding and thus reduced efficacy of flubendiamide in non-lepidopteran insect species (Douris et al., 2017).

Of course, target interaction is not the only factor influencing toxicity. It is important to realize that an interplay of different factors is responsible for the observed toxicity. In the above outlined examples, it needs to be considered that nicotine toxicity in insects might also be influenced by its weak transport across the nerve sheath membrane in its protonated state (Tomizawa, 1994) or extensive metabolic degradation as observed in honey bees (du Rand, Human, et al., 2017; du Rand, Pirk, et al., 2017). Similarly, for flubendiamide its physiochemical properties (e.g. high lipophilicity) may reduce bioavailability and thus reduce its efficacy especially against sucking pests (Jeanguenat, 2013). Such questions are addressed in toxicokinetic studies.

1.5.2 Toxicokinetics

Toxicokinetics comprises all processes of a chemical within an organism unrelated to target binding and can be divided in the phases: absorption, distribution, metabolism, and excretion. These four categories are often referred to as ADME. There are various routes of insecticide entry in insects depending on the application technique and exposure scenario. Most neuroactive insecticides are contact poisons, therefore cuticle uptake is an important route of entry as its surface is a large proportion of the entire body surface area (Matsumura, 1985). In some cases, the insecticide does not primarily penetrate directly through the cuticle, but instead the uptake takes place over the tracheal system as demonstrated for pyrethrum (Roy & Ghosh, 1944) or over the intersegmental membranes as observed with dichlorodiphenyltrichloroethane (DDT) (Quraishi & Poonawalla, 1969). Exposed sensory organs such as antennae or tarsi may also be especially vulnerable to insecticide uptake (Hayes & Liu, 1947; Matsumura, 1985). With the introduction of systemic insecticides, ingestion has become an increasingly important mode of entry. The digestive system of insects is basically a hollow tube from mouth to anus which can be subdivided into foregut, midgut, and hindgut. The midgut is generally thought to be the place of major insecticide penetration and is composed of a single cell, thick epithelial layer (Denecke et al., 2018). Experimental studies investigating the exact mechanisms of insecticidal uptake are scarce, but penetration is thought to happen via three main routes: 1. Transcellular diffusion across membranes 2. Paracellular diffusion via septate junctions 3. Active transport via transporter proteins (Denecke et al., 2018).

Notably, uptake efficiency is dependent on the compound but may also vary between insect species (Camp & Arthur, 1967). Influencing factors are cuticle composition, solvent effects and physiochemical properties (Lipinski's rule of 5) (Lipinski et al., 1997; Yu, 2011). Once taken up, it is generally accepted that distribution within the body is happening via the hemolymph, although there is some evidence of lateral transport within the integument (cuticle + epidermis) after contact exposure (Gerolt, 1970). Within the insect body, insecticides encounter a potent detoxification system evolutionary adapted to numerous naturally occurring xenobiotics (Heckel, 2014). Metabolism can be divided into three phases – 1. Functionalization, 2. Conjugation, 3. Elimination / Export (Amezian et al., 2021). In phase I, lipophilic compounds are converted into more polar metabolites which often decreases biological activity and sometimes allows direct excretion. Phase I reactions are often rate-limiting with respect to acute toxicity (Yu, 2011). In phase II, products from phase I metabolism or the parent compounds are conjugated with sugars, glutathione, or other endogenous molecules to further increase water solubility. Phase 3 comprises the transport processes from xenobiotics or their phase I + II metabolites out of the cell with the help of transporter proteins (Kennedy & Tierney, 2013; Van Leeuwen & Dermauw, 2016). In each phase major enzyme classes and families are involved. Some of the major enzyme families are briefly introduced.

1.5.2.1 Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases (P450s) originated in prokaryotes and are ubiquitous in living organisms including animals with only few exceptions (Werck-Reichhart & Feyereisen, 2000). P450s are heme-thiolate enzymes and named after their common feature: an absorbance peak near 450 nm of their Fe^{II}-CO complex (Omura & Sato, 1964). With the progress in genome sequencing, the number of discovered P450s is steadily increasing with one million identified sequences expected soon (Nelson, 2018). This called for a common nomenclature. Based on amino acid sequence identity, phylogenetic relationship , and gene organization, P450 genes are named with the following rules: *CYP* is the root symbol followed by a number for family affiliation (>40% amino acid sequence identity), a letter for subfamily (>55% sequence identity) and a running number as gene identifier (example: CYP9Q3) (Nelson, 2006; Nelson et al., 1996). Similarly, CYP clans / clades are defined as clusters of CYP families with a common ancestor and designated with a number. Arthropod CYPomes range from 23 P450s (*Aculops lycopersici*) to 261 (*Sinella curviseta*) and are assembled from members of six clans (CYP2, CYP3, CYP4, CYP20, CYP16 and mitochondrial). CYP20 and CYP16 are not found in most insects (Dermauw et al., 2020). Members of the mitochondrial clan are assumed to be located on the inner membrane of mitochondria, however classification within this clan should not be taken as a definitive evidence of subcellular localization (Dermauw et al., 2020). The insect clan 2 is usually small and stable and comprises many genes involved in physiological functions while clan 3 and 4 show large variations in gene numbers (Feyereisen, 2019). Especially clan 3 genes have been implicated in xenobiotic metabolism, while the insect clan 4 has been studied less intensively with few exceptions (Dermauw et al., 2020; Feyereisen, 2006).

The sequence diversity of P450 enzymes is extremely high. There is no ultimately conserved residue in all P450s, although the cysteine ligand to the heme is conserved in all members containing a heme prosthetic group (Feyereisen, 2019; Sezutsu et al., 2013). Despite that, the overall, three-dimensional structure is highly conserved, especially in elements surrounding the heme center (Figure 10) (Poulos & Johnson, 2015). Insect P450s contain five well-conserved motifs: WxxxR located in helix C, GxE/DTT/S (helix I), ExLR (helix K), PxxFxPE/DRF (after helix K') and PFxxGxRxCxG/A (containing the conserved C, preceding helix L) (Feyereisen, 2019). Eukaryotic P450s are generally membrane-bound and most are incorporated in the endoplasmic reticulum by their hydrophobic N-terminal sequence (~30 amino acid residues) (Sakaguchi et al., 1987). Some P450s, however, are located on the inner membrane of mitochondria and their transport is driven by a N-terminal peptide extension (Omura & Ito, 1991).

On top of those conserved features, P450 contain six variable regions involved in substrate recognition (substrate recognition sites - SRS) (Gotoh, 1992).

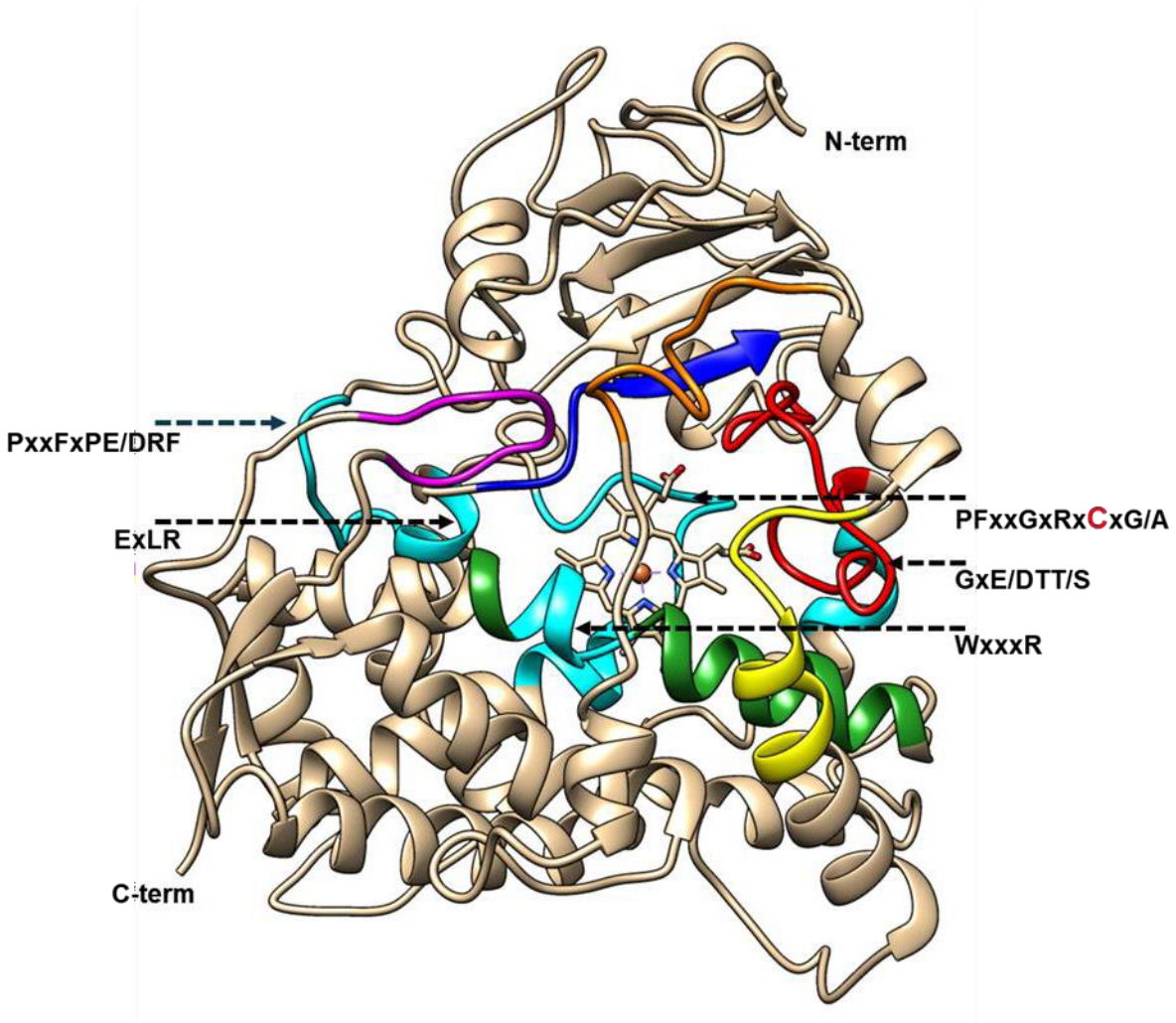


Figure 10: Homology model of *Apis mellifera* CYP9Q3 based on human CYP3A4 (PDB: 4D6Z). Conserved insect P450 motifs are colored in cyan and highlighted with arrows (Feyereisen 2019). The highly conserved cysteine bound to the heme is highlighted in red. SRS are colored individually: 1 – red; 2 – orange, 3 – yellow, 4 – green, 5 - blue, 6 – magenta (Gotoh et al. 1992, Mao et al. 2011). The heme prosthetic group is in the center.

P450 enzymes catalyze a diversity of reactions by activating molecular oxygen and transferring one of the oxygen atoms into their substrate. The principal mechanism is a multi-step process (Figure 11): First, a substrate is bound to the enzyme. Then, the ferric cytochrome P450 is reduced to its ferrous form by an electron received from a redox partner. Molecular oxygen is bound to the ferrous heme and with a second reduction step and a protonation, a Fe^{III}-hydroperoxy complex is formed. Further protonation and heterolytic cleavage of the O-O bond leads to an iron-oxo intermediate and H_2O production. Finally, the oxygen from the iron-oxo species is transferred to the bound substrate and the product is dissociated (Ortiz de Montellano, 2005).

This reaction type is referred to as monooxygenase or mixed-function oxidase reaction and is commonly described by the simple stoichiometry with RH as the substrate:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+ \quad (1)$$

Oxygen atom transfer is not the only catalytic function. P450 can catalyze a range of other reactions including reductions, oxidations, desaturations, dehydrations and many more (Guengerich, 2001). As described above, P450s need a redox partner to complete their reaction cycle. Cytochrome P450 reductase is obligatory for microsomal P450 function (Crankshaw et al., 1981). It is a four-domain diflavoprotein (flavomononucleotide (FMN)- binding, connecting domain, flavin adenine dinucleotide (FAD)- and nicotinamide adenine dinucleotide phosphate (NADPH) - binding domains) (Wang et al., 1997). It accepts a hydride ion (one proton and two electrons) from NADPH and donates the electrons sequentially to P450 enzymes (Feyereisen, 2019). The pathway of electron transfer is NADPH -> FAD -> FMN -> P450 (Muratalie v et al., 2004). A second redox partner for some P450s is cytochrome b5 which may donate both electrons, only the second electron or functions as an allosteric modulator of P450s without acting as a redox partner (Porter, 2002).

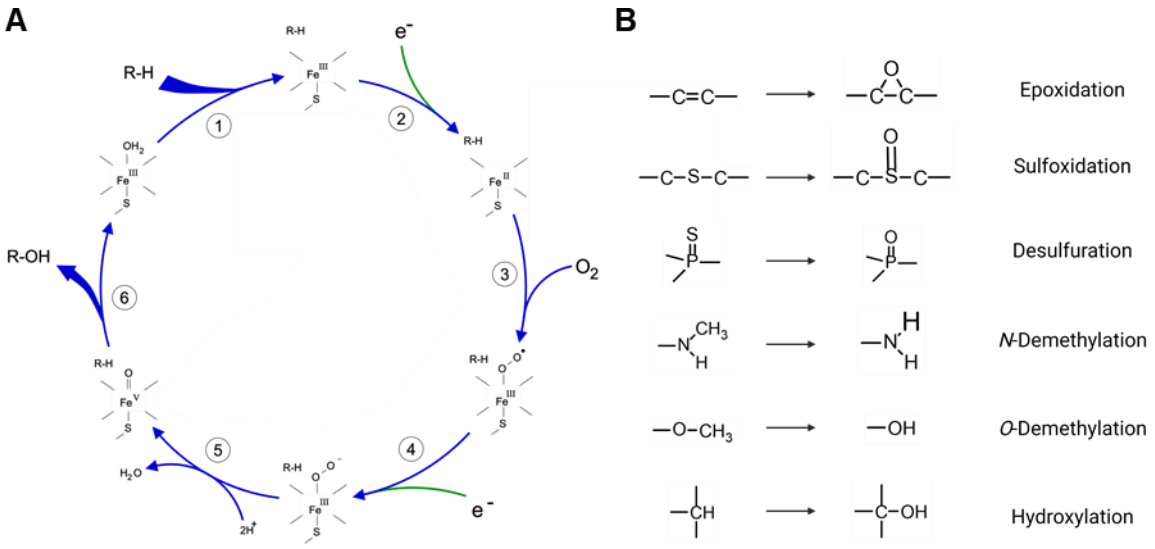


Figure 11: Catalytic cycle of cytochrome P450s. (A) The six steps of the general cytochrome P450 reaction mechanism: 1: Substrate binding to the heme 2. Reduction of the ferric prosthetic group with the help of a donated electron 3. Binding of molecular oxygen to the ferrous heme 4. With a second reduction step a ferric-peroxo intermediate is formed which is 5. protonated and the O-O bond is cleaved under the formation of H_20. 6. The oxygen from the iron-oxo species is transferred to the substrate and the product dissociates. Adapted from: Richfield, David. "Medical gallery of David Richfield 2014". Wikiversity Journal of Medicine **1** (2). (B) Six reactions often involved in insecticide metabolism: Epoxidation, sulfoxidation and desulfuration may lead to activation rather than detoxification of insecticides (Yu et al., 2011).

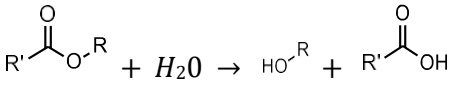
P450 enzymes take over a diversity of functions in endogenous physiological processes as well as in the metabolism of exogenous substrates. In insects, P450s are involved in the biosynthesis of ecdysteroids (molting hormones) and long-chain hydrocarbons as well as in the metabolism of farnesoids, pheromones, fatty acids and lipids (Feyereisen, 2019).

They are intensively studied, however, for their role in the phase I metabolism of xenobiotics – natural plant toxins and insecticides alike (Li et al., 2007).

Among the vast array of possible reactions, six oxidative mechanisms are often involved in insecticide metabolism (Figure 11) (Yu, 2011). Notably, not all of them lead to detoxification but sometimes also to activation. The classic example is P=S ester organophosphate insecticides such as parathion where oxidative desulfuration leads to paraoxon (P=O ester), which binds with much higher affinity to the molecular target acetylcholinesterase (Nakatsugawa & Dahm, 1965).

1.5.2.2 Carboxylesterases

Esterase is a common term for enzymes that hydrolyze ester bonds (Wheelock et al., 2005). Carboxylesterases hydrolyze esters of carboxylic acids and are involved in phase I metabolism of several insecticide classes:



(2)

They belong to the alpha / beta hydrolase fold protein superfamily which comprises several functionally different enzymes (Lenfant et al., 2013; Ollis et al., 1992). A universal classification system is currently not available. An early nomenclature divided esterases based on their interaction with the organophosphate (OP) paraoxon in A- (hydrolyze OPs), B- (inhibited by OPs) , and C-type (no interaction with OPs) esterases (Aldridge, 1953a, 1953b; Bergmann et al., 1957). Another classification groups the enzymes in A- and B-esterases based on their hydrolysis preference towards the model substrates α- and β-naphthyl acetate (Georghiou et al., 1980). Most comprehensively is the classification by phylogeny. Phylogeny divides insect carboxy / cholinesterase (CCE) in three major classes correlating with function: esterases with neuro / developmental functions, secreted enzymes with hormones / pheromones among the substrates and intracellular enzymes with detoxification function (Claudianos et al., 2006; Oakeshott et al., 2005). The three classes can be further divided in several clades (clades A – N).

Catalytically active members of this enzyme class have the same reaction mechanism based on a catalytic triad of residues (often: serine, histidine, aspartic acid) (Montella et al., 2012). Hydrolysis happens in two steps. The oxygen of the serine residue attacks on the carbonyl carbon of the substrate displacing the alcohol product. Then, water displaces the serine residue and releases the acid product of the reaction. Both reactions are of nucleophilic nature.The serine is highly nucleophilic thanks to its interaction with a histidine residue which is brought in the right position by an acidic residue – hence catalytic triad (Oakeshott et al., 2005). Carboxylesterases can mediate insecticide resistance by two distinct mechanisms described in detail for the green peach aphid *Myzus persicae.* The esterases E4 and FE4 are highly overexpressed in resistant strains, accounting for up to 1% of the total protein content (Devonshire & Moores, 1982). Despite conferring strong resistance, the esterases hydrolyze organophosphate and carbamate

insecticide only slowly, indicating that sequestration is the major driver of resistance while metabolic detoxification (i.e. hydrolysis) is only a contributing factor (Devonshire, 1989). Sequestration is facilitated by the strong affinity of those insecticide classes to esterases (their MoA is the inhibition of acetylcholinesterase). Carboxylesterases have also been implicated in pyrethroid resistance. Here, increased hydrolytic activity is the major mechanism behind the reduced sensitivity (Oakeshott et al., 2005).

1.5.2.3 Glutathione S-transferases

Glutathione S-transferases (GSTs) are an important enzyme family in aerobic organisms involved in phase II metabolism of xenobiotics, but also in oxidative stress response and other metabolic and signaling pathway functions (Ketterman et al., 2011). There are cytosolic as well as microsomal members in the family, which are structurally unrelated, although sharing overlapping substrate specificity (Hayes et al., 2005). The cytosolic members are most often involved in insecticide metabolism (Enayati et al., 2005) and are therefore further described here. Cytosolic GSTs consist of two domains: The *N*-terminal domain containing the glutathione (GSH) binding site is part of the thioredoxin superfamily fold, while the C-terminal domain is an all α-helical domain with a unique fold involved in substrate recognition (Armstrong, 1997). GSTs are homo- or heterodimers in their active form, with the dimer interface providing an additional noncatalytic site for ligand binding (Hayes et al., 2005). Insects possess several cytosolic GSTs ranging from 8 (*A. mellifera*) to 37 (*Drosophila melanogaster*) (Oakeshott et al., 2010). Insect GSTs are divided into six classes (delta, epsilon, omega, theta, zeta and sigma) based on amino acid sequence identity (Tu & Akgül, 2005). The general reaction catalyzed by GSTs can be simplified as follows:

$$GSH + RX \rightarrow GSR + HX \quad (3)$$

with GSH being glutathione (the tri-peptide cofactor bound to the enzyme) and RX being an electrophilic substrate (Armstrong, 1997). Conjugation of reduced glutathione increases the water solubility facilitating the excretion of the xenobiotic (Ioannides, 2001). In addition to generating GS-conjugates, GSTs may detoxify compounds by direct metabolism as seen for DDT-dehydrochlorination (Clark & Shamaan, 1984). Sequestration of xenobiotics at a non-catalytic binding site as well as alleviation of oxidative stress by reduction of reactive oxygen species are further mechanisms contributing to xenobiotic detoxification and increased tolerance. GSTs have been implicated in insecticide detoxification in many species for many compounds from diverse chemical classes (Pavlidi et al., 2018).

1.5.2.4 Uridine diphosphate (UDP)-glycosyltransferases

UDP-glycosyltransferases (UGTs) comprise another enzyme superfamily ubiquitous in living organisms and involved in phase II metabolism of xenobiotics and endogenous compounds. They have been intensively studied in vertebrates (Bock, 2003), but have also been implicated in the metabolism of phytochemicals in insects (Després et al., 2007).

Unlike vertebrate UGTs, insect UGTs preferentially use UDP-glucose instead of UDP-glucuronic acid (Morello & Repetto, 1979). Except for the different sugar donor, the general characteristics are similar. UGTs are membrane-bound enzymes located in the endoplasmic reticulum facing the lumen with a short C-terminal end on the cytoplasmic side (Magdalou et al., 2010). The *N*-terminal domain harbors the substrate binding domain and is highly variable, while the *C*-terminal domain comprises the sugar donor binding domain and the transmembrane anchor and is significantly more conserved (Miley et al., 2007). High variability in the *N*-terminal domain contributes to the significant diversity in functional groups and substrates which can be conjugated by the enzyme family (Hu et al., 2019). UGTs exist as homo- or hetero-oligomers which adds even more diversity to their catalytic capacity (Fujiwara et al., 2016). The general mechanism can be described as a transfer of a sugar moiety from an activated donor to a suitable, nucleophilic acceptor group (Testa & Krämer, 2008). A nomenclature has been implemented based upon amino acid identity (>40% same family indicated by number, >60% same subfamily indicated by capital letter) (Mackenzie et al., 1997). In insects, a considerable number of UGTs has been identified in all orders ranging from 12 in *A. mellifera* to 58 in the pea aphid *Acyrthosiphon pisum* (Ahn et al., 2012). Despite that, studies on insect UGTs and their role in insecticide detoxification are scarce and often limited to the investigation of expression in different strains or upon insecticide exposure (Li et al., 2018; Luque & O'Reilly, 2002; Pedra et al., 2004).

1.5.2.5 ATP-binding cassette (ABC) transporters

ABC transporters are the most prominent protein family involved in phase III metabolism. They translocate all kinds of molecules across cell membranes and are therefore involved in many physiological processes including protection from (cyto-)toxins. In prokaryotes ABC-transporters mediate uptake and efflux of molecules, while in eukaryotes efflux transporters are predominant (Higgins, 1992).

They consist of four domains – two transmembrane domains (TMD) embedded in the membrane and two nucleotide binding domains (NBD) facing the cytosol. NBDs are structurally conserved with some common sequence motifs while TMDs are rather variable reflecting the chemical diversity of the translocated substrates (Rees et al., 2009). The four domains may be fused together in one polypeptide chain which is called a "full-length transporter". Common is also the formation of "half-size transporters" where one TMD and NBD are fused together and form a protein. The functional complex is then a homo- or heterodimer of two half-size transporters (Biemans-Oldehinkel et al., 2006; Wu et al., 2019).

ABC-transporters require ATP to translocate molecules across lipid membranes. The ATP-switch model is the preferred mechanistic model: The transport cycle is initiated by substrate binding to the TMDs causing an affinity increase of the NBDs for ATP binding, leading to a conformational change of the whole transporter. The substrate binding site is now exposed to the extracellular space and its binding affinity is lowered resulting in substrate release . Then, ATP is hydrolyzed, and the release of P_i and ADP restores the basal configuration of the transporter (Higgins & Linton, 2004). A classification system exists based on sequence homology of the NBD domains and is divided into eight families (ABCA to ABCH) (Dean & Dean, 2001). Arthropods possess members in each of the subfamilies with a total number ranging from 34 in the green orchard bee *Euglossa dilemma* to 132 in the springtail *Folsomia candida* (Denecke et al., 2021). Members of the -B, -C, -G, -F, and -H families have at least circumstantially been implicated with resistance phenotypes towards various chemical classes, but functional studies and characterization of specific candidates are often lacking (Denecke et al., 2021; Dermauw & Van Leeuwen, 2014).

1.5.2.6 Insecticide selectivity influenced by toxicokinetics

Toxicokinetic characteristics have a huge impact on insecticidal efficacy and inter-species sensitivity differences, which becomes evident by many studies on the mechanisms of insecticide resistance. Resistant insect strains have been shown to exhibit reduced penetration of insecticides through the cuticle (Forgash et al., 1962) or the nervous system (Telford & Matsumura, 1971). In most of these cases, however, reduced penetration is a contributing factor to enhanced metabolism (Matsumura, 1985; Yu, 2011). Enhanced detoxification capacity is one of the most prevalent resistance mechanisms across insect orders and can be mediated by enzymes in any of the three phases as outlined above (Li et al., 2007; Van Leeuwen & Dermauw, 2016).

Toxicokinetic behavior is also often fundamental for bee selectivity of insecticidal compounds. *N*-cyanoamidine neonicotinoids such as thiacloprid are more than 300-fold less toxic than *N*-nitroguanidine compounds (e.g imidacloprid) after contact exposure. While receptor binding affinity is similar, this differential toxicity can be traced back to reduced penetration and rapid metabolism of *N*-cyanoamidine compounds in bees (Iwasa et al., 2004; Zaworra et al., 2019) .A similar case is *τ*-fluvalinate – a pyrethroid insecticide acting on the voltage-gated sodium channel (VGSC) – which is practically non-toxic to bees and used as an in-hive acaricide to control the varroa mite. While the honey bee's VGSC is even slightly more susceptible to τ-fluvalinate than the mite's counterpart (Gosselin-Badaroudine & Chahine, 2017), metabolic detoxification explains the lower intrinsic toxicity in honey bees (Johnson et al., 2006).

While cytochrome P450s were expected to confer differential toxicity in those cases, the definitive identification of the responsible drivers was only achieved in pioneer bee toxicogenomics studies (Manjon et al., 2018; Mao et al., 2011).

1.5.3 Toxicogenomics

Toxicogenomics is a subdiscipline of toxicology and combines toxicology with novel technologies within the -omics area (genomics, transcriptomics, proteomics, metabolomics). In its broadest sense it employs molecular approaches to elucidate and confirm toxicological findings at the molecular level. Classic toxicology mostly relies on *in-vivo* studies and the study of tissue homogenates to investigate target site interactions or the involvement of detoxification enzymes pharmacologically. While this is a highly effective approach, it lacks the resolution to identify specific isoforms of enzymes or different receptor compositions. As described above many enzymes involved in xenobiotic detoxification are members of large superfamilies and insecticide target receptors can be combinations of different subunits. The decryption of entire genomes facilitated studies on single genes and gene families to identify key components defining the interaction between xenobiotics and organisms. Comparisons can be drawn between different genomes to identify differences between species (Claudianos et al., 2006; Perry et al., 2021) or within populations of the same species (Calla et al., 2021). Studies of the transcriptome can be employed, for example to identify differentially expressed genes between susceptible and resistant insects or between developmental stages (Oppenheim et al., 2015). Proteomic and metabolomic studies, although executed in fewer numbers, have similar potential for insect toxicology (Ardalani et al., 2021; du Rand, Human, et al., 2017). These approaches are complemented by tools to functionally validate the importance of identified genes. Today the functional expression of detoxification enzymes (especially cytochrome P450s) is a routine technique and has significantly improved the knowledge on mechanisms of insecticide resistance and selectivity (Nauen et al., 2021, 2022). Another tool is the fruit fly *D. melanogaster.* Owing to its character as a model organism for molecular biology and genetics (Roberts, 2006), it has become an invaluable (reverse genetic) resource to introduce and study genes and mutations implicated in insecticide toxicology (Douris et al., 2020; McLeman et al., 2020; Tasman et al., 2021). One of its many advantages is the possibility to study a single element (gene or mutation) in isolation in an otherwise identical genetic background. Other approaches which are increasingly used also in non-model organisms include RNA interference (RNAi) to silence genes of interest and characterize the emerging phenotype, or clustered regularly interspaced short palindromic repeats (CRISPR) for precise genome modifications (Homem & Davies, 2018).

The higher resolution of toxicogenomic studies can be exploited to improve the understanding of both - toxicodynamic and toxicokinetic behavior. In an effort to understand differences in imidacloprid sensitivity (>30x fold) between different earthworm species, Short et al. (2021) offered a compelling example. Differential toxicity could not be explained by toxicokinetic behavior as compound accumulation within the body did not correlate with toxicity.

Similarly, comparison of nAChR subunits did not predict differential affinity as ligand binding domains were highly similar. The reason for differential toxicity was found to be the expression of high affinity non-synaptic AChBPs in less sensitive species effectively lowering the amount of imidacloprid binding to nAChRs and therefore reducing toxicity by off-target binding (Short et al., 2021). This illustrates how toxicogenomics can explain even complicated cases on a molecular basis where classical toxicology reaches its limits.

Indeed, toxicogenomics investigations have also contributed significantly to elucidate the mechanisms of differential toxicity of insecticides between bees and pest species which are often (but not always) based on differential toxicokinetic behavior (Iwasa et al., 2004; Johnson et al., 2006). The previous assumption that the honey bee is generally more sensitive to insecticides than other insects due to a reduced detoxification gene inventory (Claudianos et al., 2006) did not hold up (Hardstone & Scott, 2010). However, significant differences in sensitivity towards individual compounds of the same chemical class of insecticides can be found. Insecticides inhibiting acetylcholinesterase have an extreme broad range of acute topical bee toxicity (LD_{50}: 0.018 – 31.2 µg / bee), as do pyrethroids (LD_{50} 0.017 – 20 µg / bee) and neonicotinoids (LD_{50}: 0.004 – 14.6 µg / bee) (Johnson, 2015). In a landmark study, Manjon et al. investigated the differential toxicity of neonicotinoid subtypes in honey bees and bumblebees by classic toxicological studies combined with functional expression of individual P450s, the use of transgenic *Drosophila* expressing bee P450s and expression and localization studies of genes of interest (Manjon et al., 2018). One of the key findings showed that enzymes of a single P450 subfamily (CYP9Q) account for the rapid metabolism of *N*- cyanoamidine neonicotinoids while they exhibit only minor activity against *N*-nitroguanidine neonicotinoids explaining the >300-fold difference in toxicity between neonicotinoid subgroups. Previous studies already implicated the same subfamily in the detoxification of important acaricides used against the varroa mite (Mao et al., 2011), thus suggesting a fundamental role of the CYP9Q subfamily in xenobiotic detoxification. Subsequent studies in the field of comparative genomics identified functional orthologs in other managed bee pollinators (*B. terrestris* and *O. bicornis)* conferring a similar degree of tolerance towards selected compounds (Beadle et al., 2019; Troczka et al., 2019) while the lack of such orthologs in the alfalfa leafcutting bee *Megachile rotundata* is linked to a high sensitivity towards the same insecticides (Hayward et al., 2019). These findings demonstrate that phase I metabolism and P450 enzymes in particular play a key role in bee toxicology justifying further investigation of this prominent enzyme family.

1.6 Aims and objectives

Aim of this project is the advancement of molecular approaches as additional tools for the assessment of potential pesticide risks in non-target species with special reference to bee pollinators. A strong focus is on honey bees as the most important managed bee pollinator in agriculture and regulatory considerations on bee safety. Emphasis is spent on cytochrome P450 enzymes as main contributors to insecticide selectivity mediating phase I metabolism of xenobiotics, including insecticides.

In **chapter 2** a fluorescence-based *in-vitro* assay is described allowing the high-throughput screening of substances interacting with (honey bee) cytochrome P450 enzymes. The assay is shown to reliably predict synergistic interaction between azole fungicides and insecticides by correlating the strength of P450-inhibition *in-vitro* to synergistic mixture toxicity observed *in-vivo*. Such an assay could be easily adapted to screen thousands of substances for potential P450 inhibition – the most common and field-relevant mechanism of synergism between pesticides described in bees. Furthermore, the assay has a potential to predict insecticide metabolism by P450s, thus it could potentially facilitate insecticide discovery and help to develop bee-safe insecticides.

In **chapter 3** classic toxicology is combined with toxicogenomics approaches to characterize the bee safety profile of flupyradifurone – a recently introduced butenolide insecticide acting on the nAChR in a similar fashion like neonicotinoids. Considered bee safe owing to its low acute toxicity, the drivers behind its bee safety are resolved at the molecular level fostering the mechanistic understanding of its selectivity.

In **chapter 4** the importance of P450-mediated metabolism for diamide insecticide detoxification in honey bees is assessed. Simultaneously, the assay described in chapter 2 is applied in a practical setting assessing the risk of synergistic potential between chlorantraniliprole and frequently applied fungicides in Californian almond orchards – an intensively managed agricultural crop with a detailed record list of applied pesticides and a high dependence on honey bees as pollinators.

In **chapter 5** toxicology is merged with phylogeny to assess whether genes responsible for insecticide selectivity identified in honey bees are evolutionary conserved. Functional validation of conserved detoxification mechanisms across bee species and subsequent identification of nodes in the (bee) tree of life, where similar insecticide sensitivity can be expected, addresses concerns regarding the appropriateness of the honey bee as a surrogate species in pesticide risk assessment.

Finally, in **chapter 6** the results are put into the broader context and discussed regarding their implications on (honey) bee toxicology, pesticide risk assessment and potential applications for the development of next-generation insecticides.

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Chapter 2
Pesticide risk assessment at the molecular level using honey bee cytochrome P450 enzymes: A complementary approach

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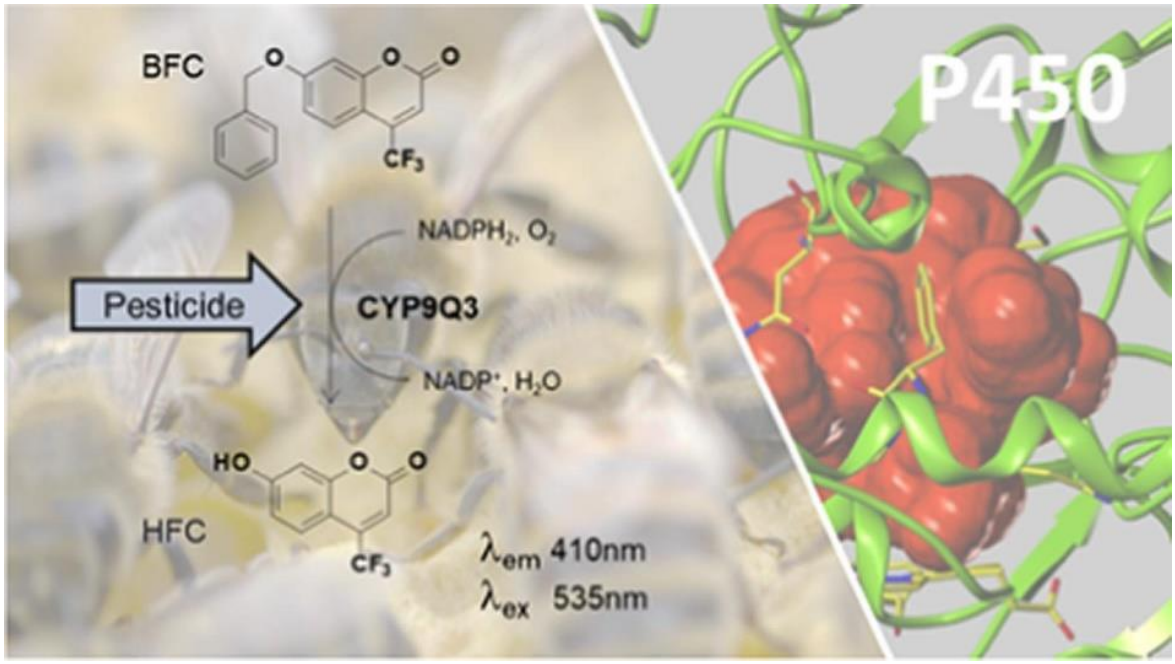

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Pesticide risk assessment at the molecular level using honey bee cytochrome P450 enzymes: A complementary approach

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ABSTRACT

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

1. Introduction

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

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

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regulatory requirement in a tiered risk assessment approach (OECD, 1998a, 1998b; US EPA, 2014). Whereas, cyanoimine neonicotinoids such as thiacloprid and acetamiprid are considered practically non-toxic to honey bees based on standardized acute toxicity tests (Casida, 2018; Iwasa et al., 2004); the classification "practically non-toxic" is based on LD_{50}-values ≥1 μg/bee according to EPA guidance for assessing pesticide risks to bees (US EPA, 2014). Neurotoxic insecticides including neonicotinoids are also known to be able to induce sublethal effects in bee pollinators with implications for e.g. cognitive functions, olfaction, gustation, orientation and foraging behavior (Belzunces et al., 2012; Blacquière et al., 2012).

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

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

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

2. Materials and methods

2.1. Chemicals

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

2.2. Honey bees and insect cells

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

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

2.3. Recombinant expression of honey bee P450s

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

2.4. Enzyme kinetics and honey bee cytochrome P450 inhibition assays

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

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

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

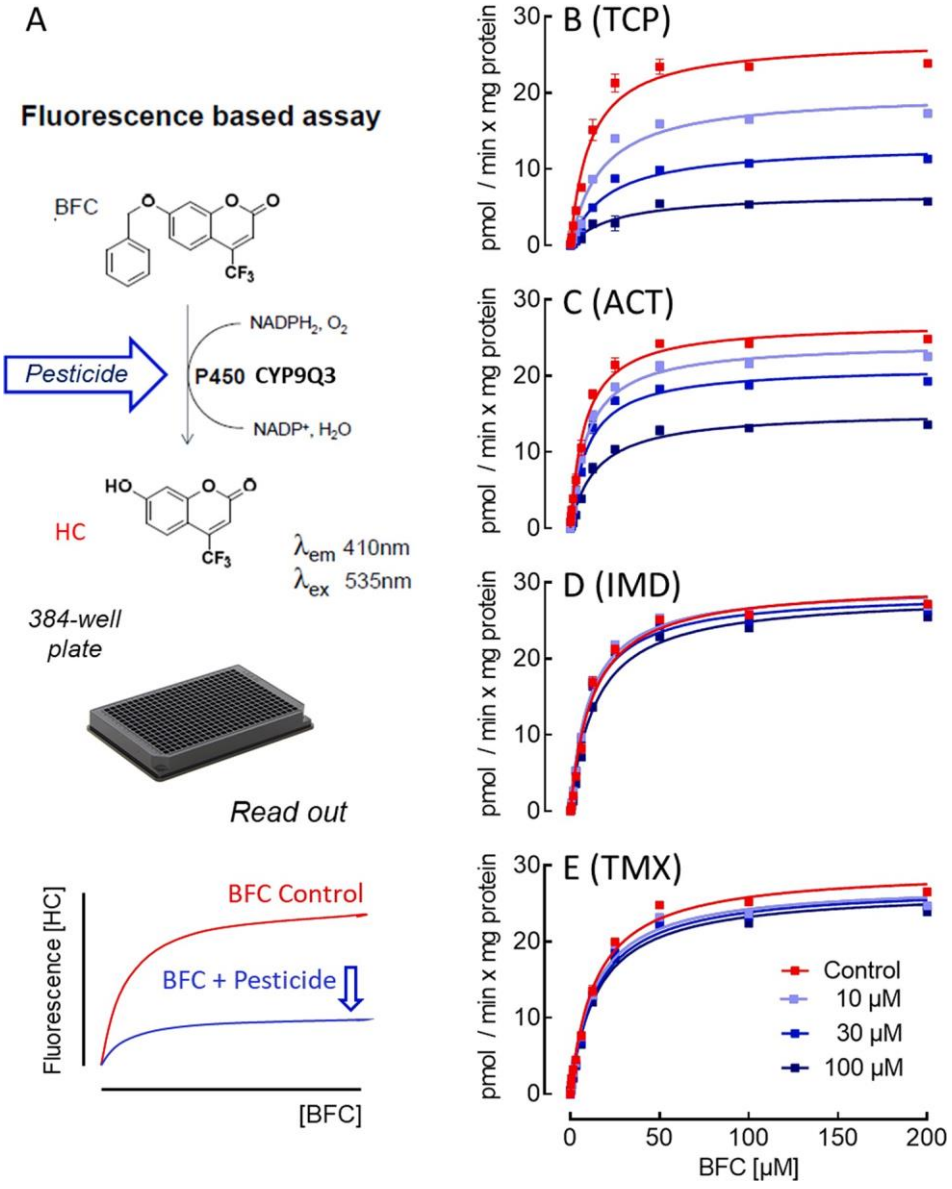

Fig. 1. Basic principle (A) of the fluorescence-based screening assay measuring the pesticide mediated inhibition of 7-hydroxy-4-(trifluoromethyl)coumarin (HC) formation by recombinantly expressed honey bee CYP9Q3 incubated with different concentrations of (B) thiacloprid (TCP), (C) acetamiprid (ACT),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

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

2.5. Honey bee acute contact toxicity and synergism studies

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

2.6. Correlation analysis

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

3. Results and discussion

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

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

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

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

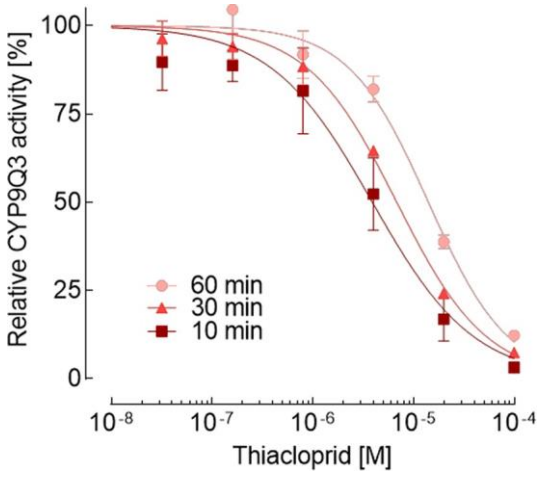

Fig. 2. Increase in IC_{50}-values for thiacloprid at different elapsed time intervals when incubated with recombinantly expressed honey bee CYP9Q3 suggesting thiacloprid depletion by metabolism. The inhibition of 7-hydroxy-4-(trifluoromethyl)coumarin generation was measured by using the probe substrate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

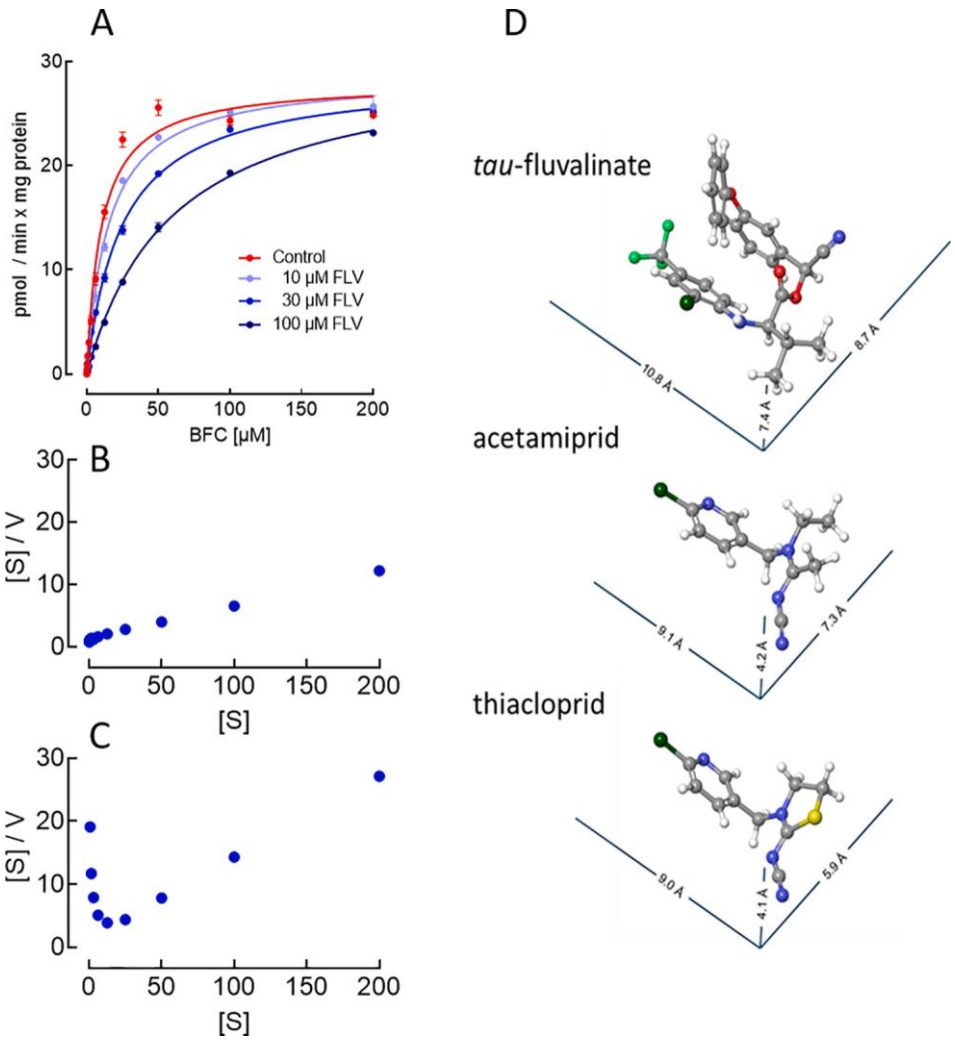

Fig. 3. Fluorescence-based inhibition assay measuring the pesticide mediated inhibition of 7-hydroxy-4-(trifluoromethyl)coumarin (HC) formation by recombinantly expressed honey bee CYP9Q3 incubated with different concentrations of (A) *tau*-fluvalinate (FLV). Linearization of the Michaelis-Menten equation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

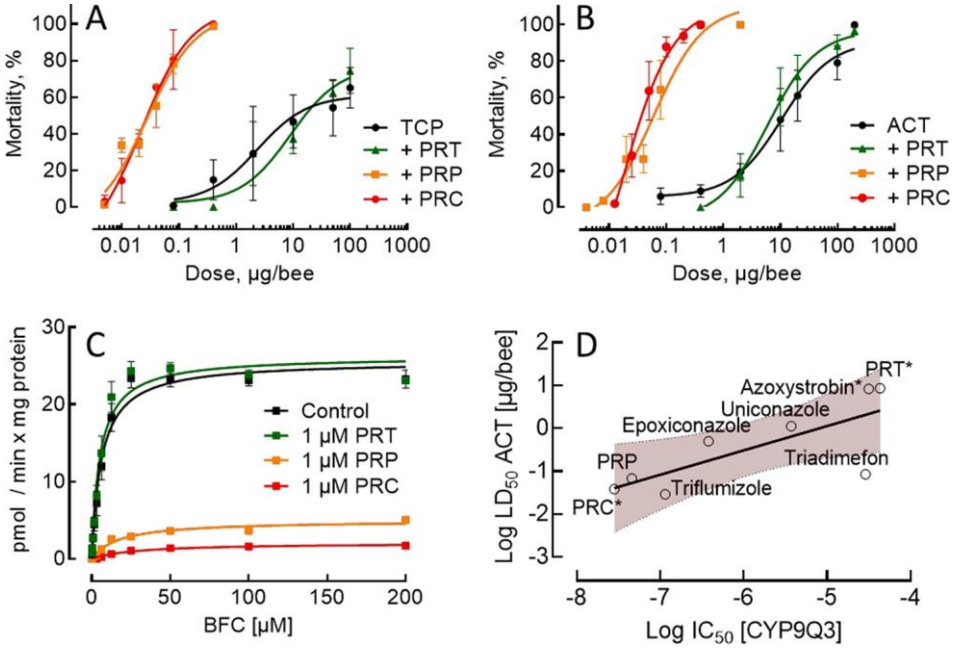

Fig. 4. Dose-response relationship and synergism of (A) thiacloprid (TCP) and (B) acetamiprid (ACT) toxicity when topically applied to honey bees either alone or pre-treated with the azole fungicides prothioconazole (PRT), propiconazole (PRP) and prochloraz (PRC). Data are mean values ± SEM (n = 2–4).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

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

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

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

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

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

4. Conclusion

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

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

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

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

pollinator is of benefit in the regulatory landscape as recently suggested (López-Osorio and Wurm, 2020), but we are also aware it won't completely replace higher tier laboratory and field testing.

CRediT authorship contribution statement

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

Declaration of Competing Interest

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

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Appendix A. Supplementary material

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

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Chapter 3

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

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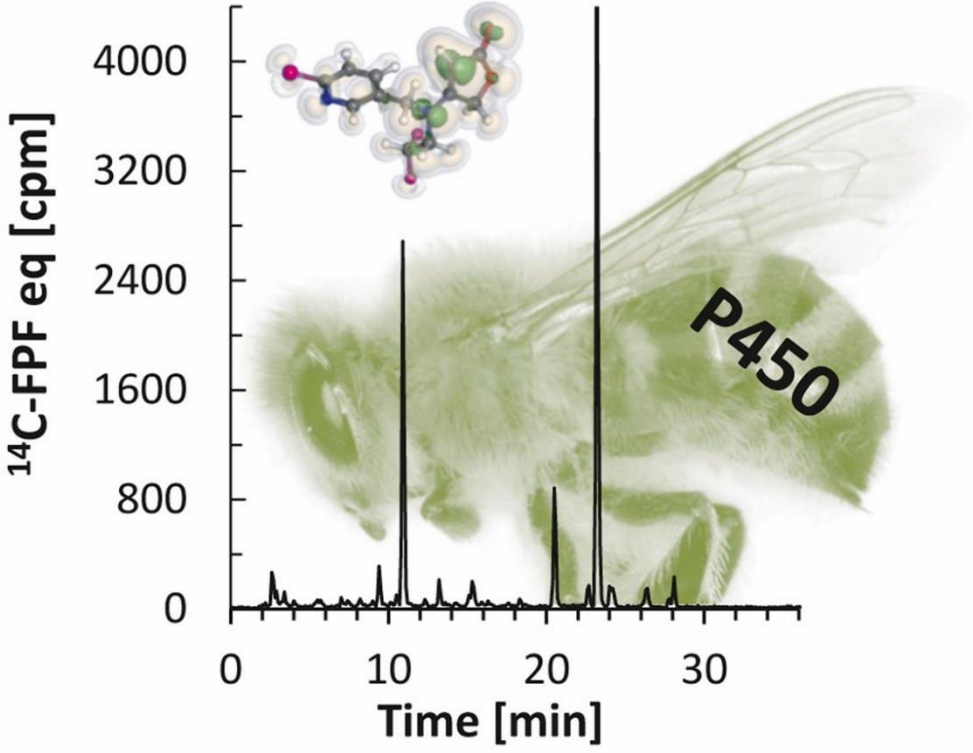

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A toxicogenomics approach reveals characteristics supporting the honey bee (*Apis mellifera* L.) safety profile of the butenolide insecticide flupyradifurone

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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.

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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, Ger- many). 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 μMand 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

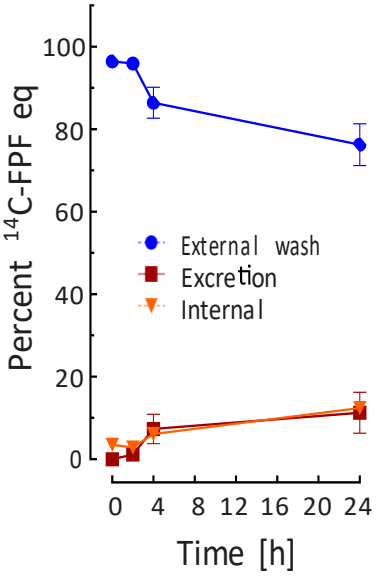

Fig. 1. Uptake and distribution of [^{14}C]-FPF-equivalents expressed as percent recovered radioactivity at different elapsed time intervals after topical application of honey bee adults with [^{14}C]-FPF (900 ng). Data are mean values ± SEM (n = 3). (For interpretation of the references to colour in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

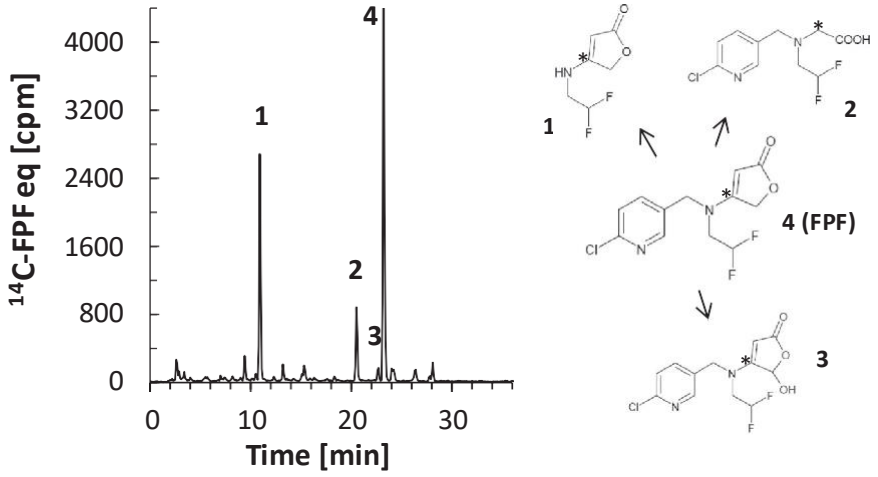

Fig. 2. HPLC radiohistogram of a sample extracted from honey bees topically treated with 900 ng [^{14}C]-FPF 24 h prior to extraction for qualitative metabolite profiling using ESI-MS. Reference standards allowed the identification of three metabolites: (1) FPF-(difluoroethyl)amino-furanone (FPF-AF),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

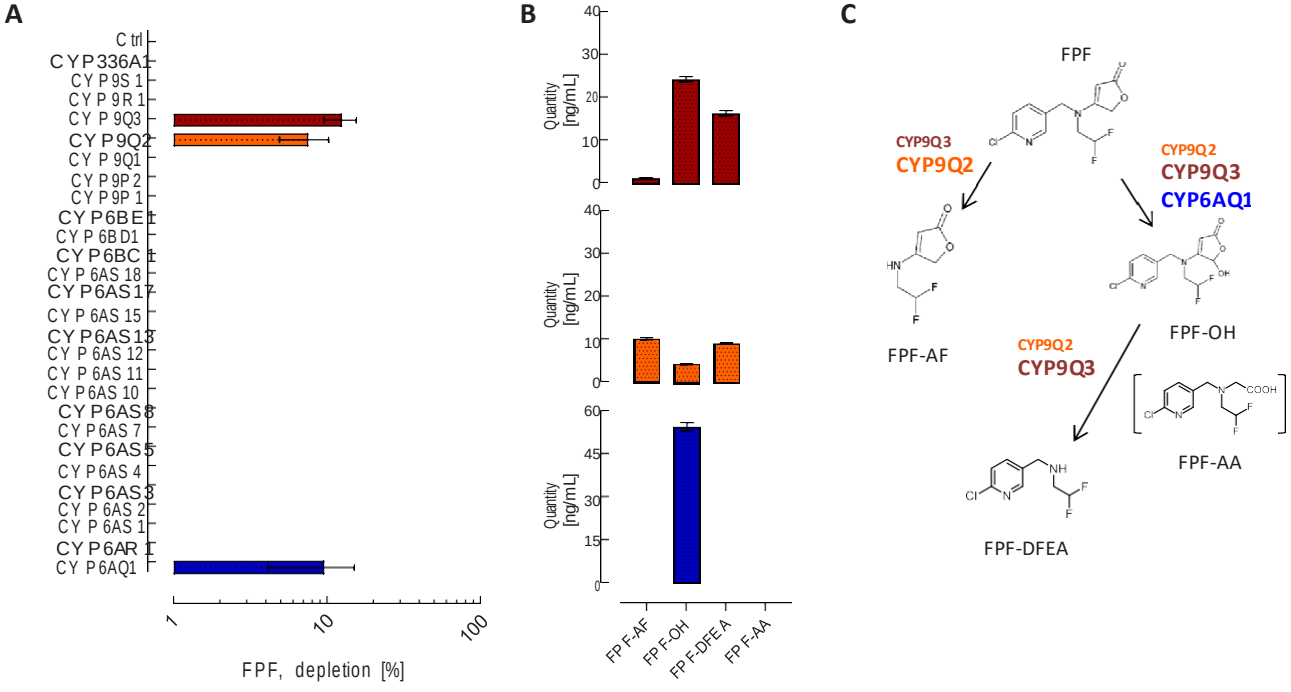

Fig. 3. (A) Flupyradifurone (FPF) depletion by 27 recombinantly expressed cytochrome P450s of the honey bee CYP3 clade measured by UPLC-MS/MS analysis and (B) quantity of metabolites detected after the incubation of FPF with recombinantly expressed honey bee CYP9Q3 (top), CYP9Q2 (center) and CYP6AQ1 (bottom). Data are mean values ± SD (n = 3). FPF-(difluoroethyl)amino-furanone (FPF-AF), FPF-5´hydroxy (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-chloropyridinylmethylamine 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

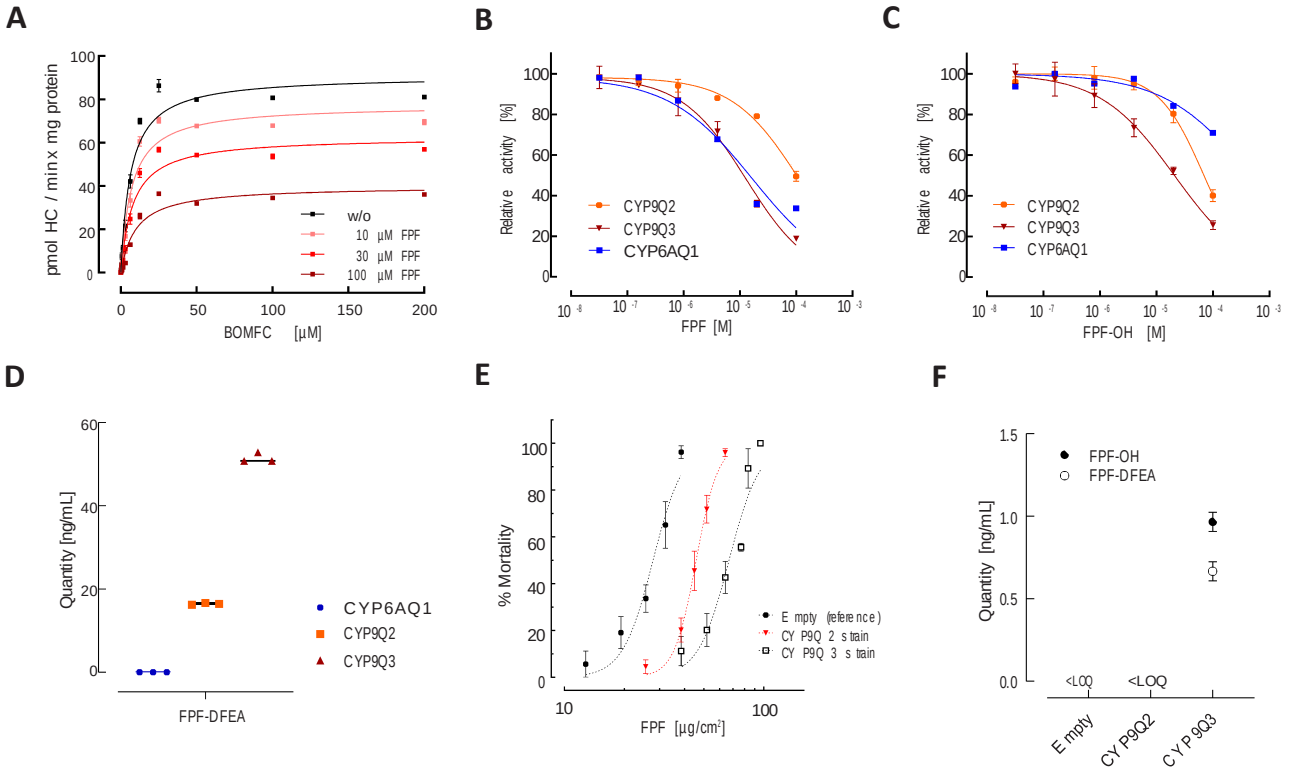

Fig. 4. (A) Steady-state kinetics of 7-hydroxy-4-(trifluoromethyl)coumarin (HC) formation by CYP9Q3 using BOMFC as a substrate in the presence of flupyradifurone (FPF). (B) Effect on P450-mediated BOMFC metabolism by increasing concentrations of FPF and (C) FPF-OH; data are mean values ± SD (n = 4). (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.

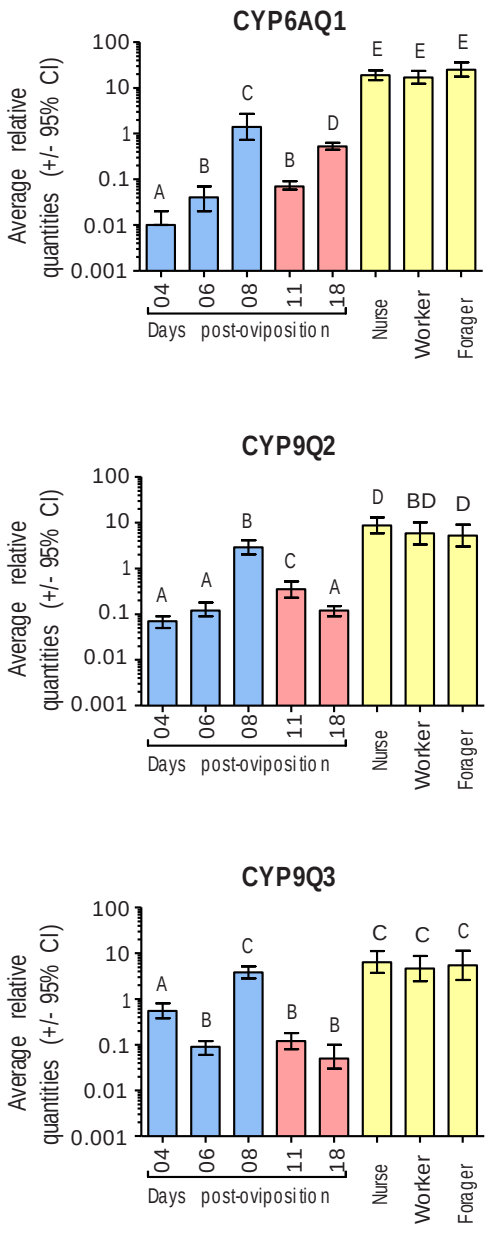

Fig. 5. Relative expression measured by quantitative PCR of honey bee P450 genes involved in the oxidative detoxification of flupyradifurone. Significant differences ($p < 0.05$) in expression between life-stages are denoted by different letters above bars as determined by one-way ANOVA with *post hoc* 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

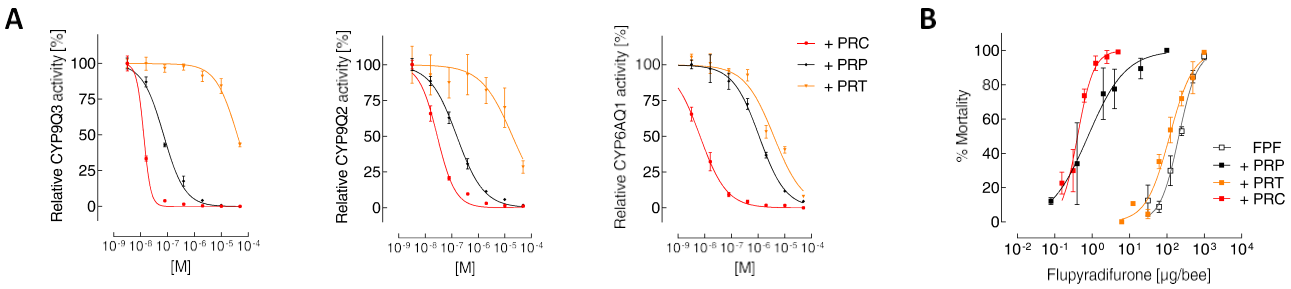

Fig. 6. (A) Inhibition of recombinantly expressed CYP9Q3, CYP9Q2 and CYP6AQ1 by three azole fungicides using BOMFC as a substrate. Data are mean values ± SD (n = 4). (B) Dose-response relationship and synergism of flupyradifurone (FPF) when topically applied to honey bees either alone or pre-treated 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 [fur- anone-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).

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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.

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Chapter 4
A mechanism-based approach unveils metabolic routes potentially mediating chlorantraniliprole synergism in honey bees *Apis mellifera* L. by azole fungicides

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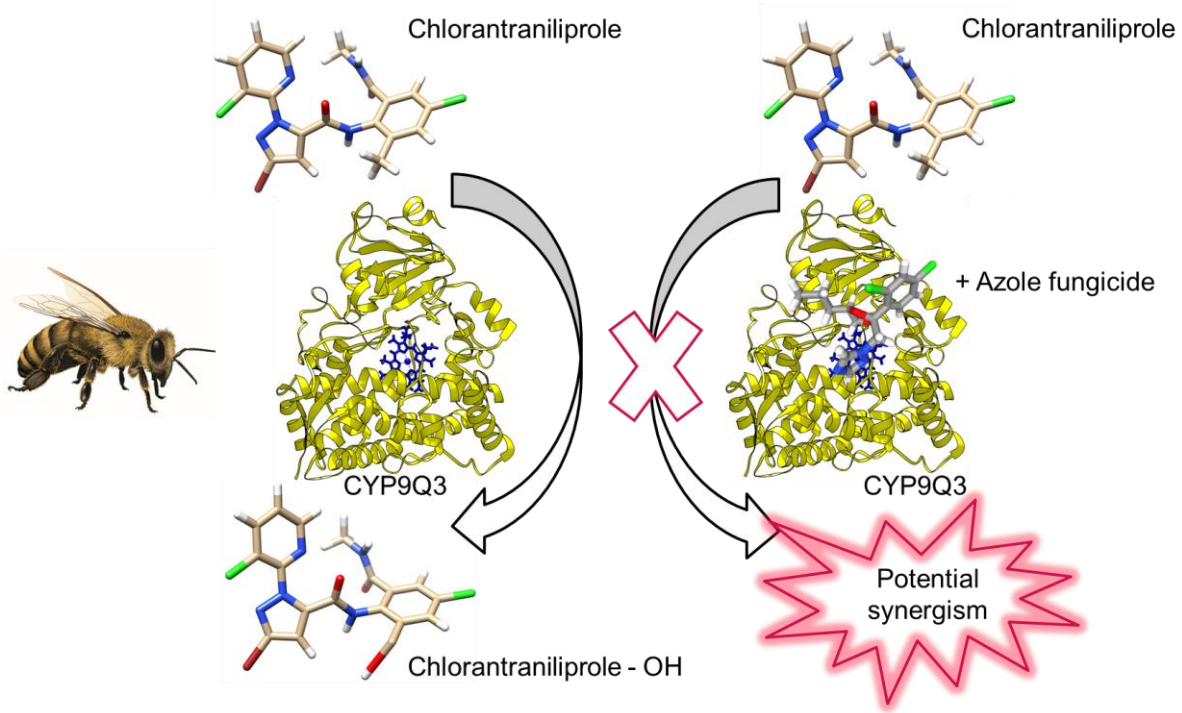

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A mechanism-based approach unveils metabolic routes potentially mediating chlorantraniliprole synergism in honey bees, *Apis mellifera* L., by azole fungicides

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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.

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

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

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be divided into two structurally different subtypes: phthalic (e.g. flubendiamide) and anthranilic (e.g. chlorantraniliprole)[6,7] 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[9,23,24] 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., radioligand binding studies revealed a higher selectivity for FLB over anthranilic 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-benzyloxymethoxy-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, Kremsmünster, 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 ddH_20 containing 0.1% (w/v) Triton X-100 before dispersing the insecticide over the diet surface. Mortality was scored after 48 h and LC_{50} values calculated by probit analysis using PoloPlus 2.0 (LeOra Software, Petaluma, CA, USA).

2.3 Bee acute contact toxicity and synergism study

Apis 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

(Bio-Rad, CA, USA) and bovine serum albumin (BSA) as a reference.

The inhibitory potential (IC_{50} 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 K_m value (CYP9Q3: 6.5 µM, CYP9Q2 15 µM, CYP6AQ1: 20 µM).[29,33] 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 $\otimes_{em}$ 510 nm (20 nm band width) while excited at $\otimes_{ex}$ 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 & Nauen[29] 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 IC_{50}-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 (eluent A) and water/0.25% formic acid (eluent 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 Metabolynx software (Waters Corporation, MA, USA).

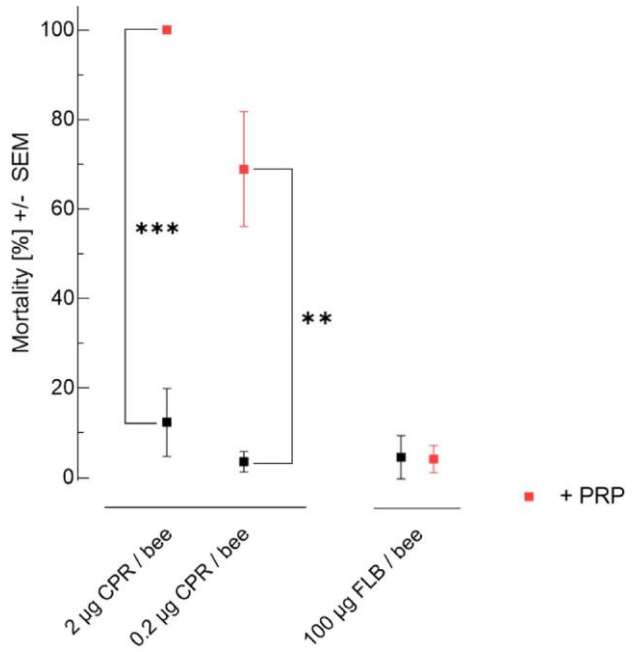

Figure 1. Synergism of diamide acute contact toxicity by propiconazole in laboratory bioassays. Acute contact toxicity to honey bees of chlorantraniliprole (CPR) and flubendiamide (FLB), respectively, without and with prior application of propiconazole (PRP). PRP was applied at 10 µg/bee 1 h prior to insecticide treatment (n = 3). Error bars display standard error of mean (SEM). Asterisks mark significant differences between treatment groups (unpaired t-test, $P < 0.01$).

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 Vina[37] embedded in UCSF Chimera software (v1.14, UCSF, CA, USA). Input comprised the created CPY9Q3 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/bee and 0.2 µg/bee CPR alone resulted in mortality of <15% (in accordance with the reported LD_{50} of >4 µg/bee[5]), honey bee pretreatment with 10 µg/bee 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/bee.

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Table 1. Inhibitory potential (IC_{50}-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

	CYP9Q3		CYP9Q2		CYP6AQ1	
Diamide	IC_{50} [μM]	95% CI†	IC_{50} [μM]	95% CI†	IC_{50} [μM]	95% CI†
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[31,33] are also involved in diamide metabolism we used a recently published fluorescent probe (BOMFC) assay[29] 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, CPR clearly affected the O-debenzylation of BOMFC by CYP9Q3 as demonstrated by an IC_{50} value of 17.7 μM (CI_{95}: 15.28–19.76) (Fig. 2(A), Table 1). Subsequent Michaelis–Menten saturation kinetics revealed that fluorescent product (7-hydroxy coumarin) formation was inhibited by increasing concentrations of CPR suggesting a non-competitive inhibition pattern for CYP9Q2 (unchanged K_m value for BOMFC and decreasing V_{max} value of 7-hydroxy coumarin formation). Whereas a mixed-type inhibition of 7-hydroxy coumarin formation by CPR was obtained for CYP9Q3, characterized by significantly increased K_m values for BOMFC and decreasing V_{max} values (Fig. 2(B), Table S1). FLB on the other hand did not show any obvious interaction with BOMFC binding in fluorescence assays with the expressed P450s, even at the highest concentration (100 μM) tested.

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 bioassays 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 (LC_{50} value 155 ppm (CI_{95}: 126–191)) compared to control flies (LC_{50} of 22.2 ppm (CI_{95}: 19.7–24.9)) (Fig. 3(A)). In contrast, transgenic flies expressing CYP9Q3 were only 1.2-fold less susceptible to FLB compared to control flies, confirming a minor (if any) role of CYP9Q3 in FLB metabolism (Fig. 3(B)). The calculated LC_{50} value of FLB against control flies was 147 ppm (CI_{95}: 136–156), and the obtained LC_{50} value for flies expressing CYP9Q3 was similar, i.e. 179 ppm (CI_{95}: 166–193) (Table 2). Transgenic flies expressing CYP9Q2 under the control of the Hsp70 promotor followed the same trend with a marked decrease in sensitivity against CPR, but not FLB (Table 2).

3.4 Molecular docking and metabolite search

Encouraged by the lines of evidence obtained for the involvement of CYP9Q3 in CPR metabolism, we generated a homology model of CYP9Q3 allowing us to conduct CPR docking studies supporting the identification of CPR metabolites resulting from oxidative metabolism by CYP9Q3. Energetically most favorable docking poses positioned the anthraniloyl moiety of CPR with either the methylphenyl or the *N*-methyl carbon at less than 3.5 Å to the heme iron center (Fig. 4(A) and (B); Fig. S2). In both cases the conformation is coordinated by the bromine of the pyrazole moiety which is near amino acid residues M119 and F121

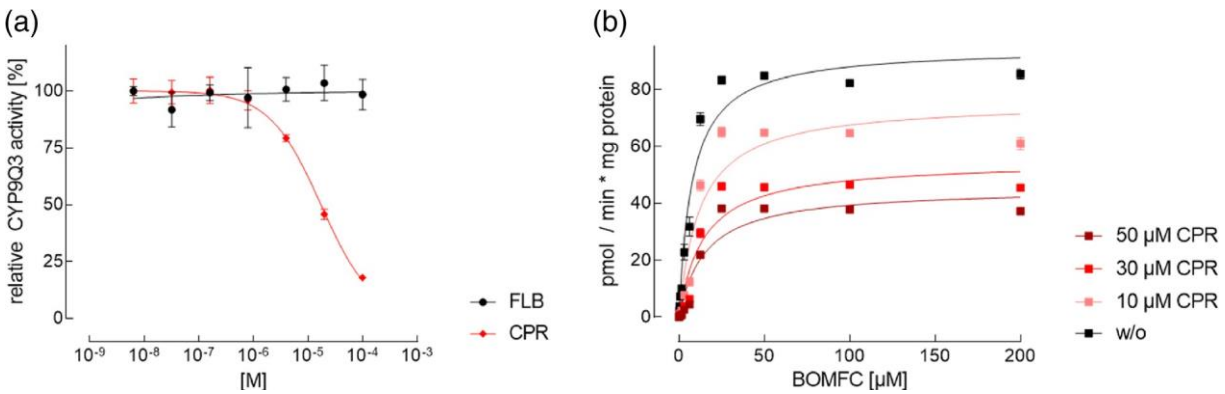

Figure 2. Effect of diamides on coumarin fluorescent probe metabolism by CYP9Q3. (A) Inhibitory potential of the diamide insecticides flubendiamide (FLB) and chlorantraniliprole (CPR) against functionally expressed honey bee CYP9Q3 using a fluorescence-based assay with 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (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†	95% CI‡	Chi^2 (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]
† Resistance ratio: LD_{50} of transgenic strain divided by the LD_{50} of reference strain (Empty × Hsp70).
‡ Confidence Interval 95%.

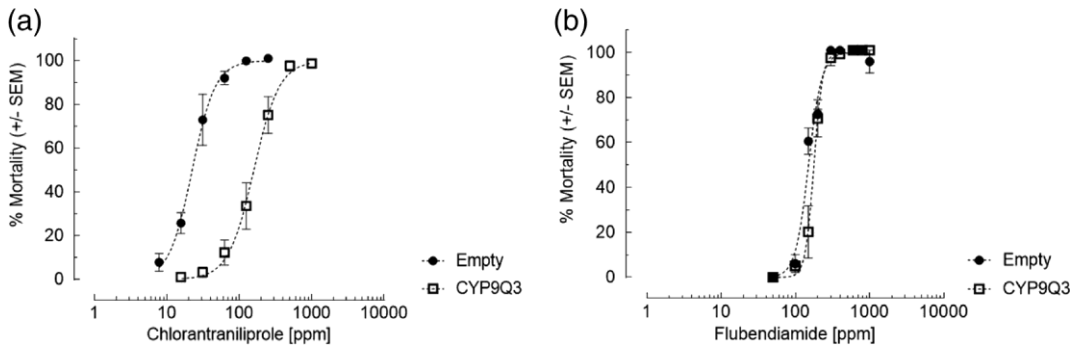

Figure 3. Diamide toxicity against Drosophila lines in diet overlay bioassays. Toxicity of (A) chlorantraniliprole and (B) flubendiamide against transgenic *Drosophila* adults ectopically expressing honey bee CYP9Q3 and a control strain (Empty) with the same genetic background. Data are mean values ±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

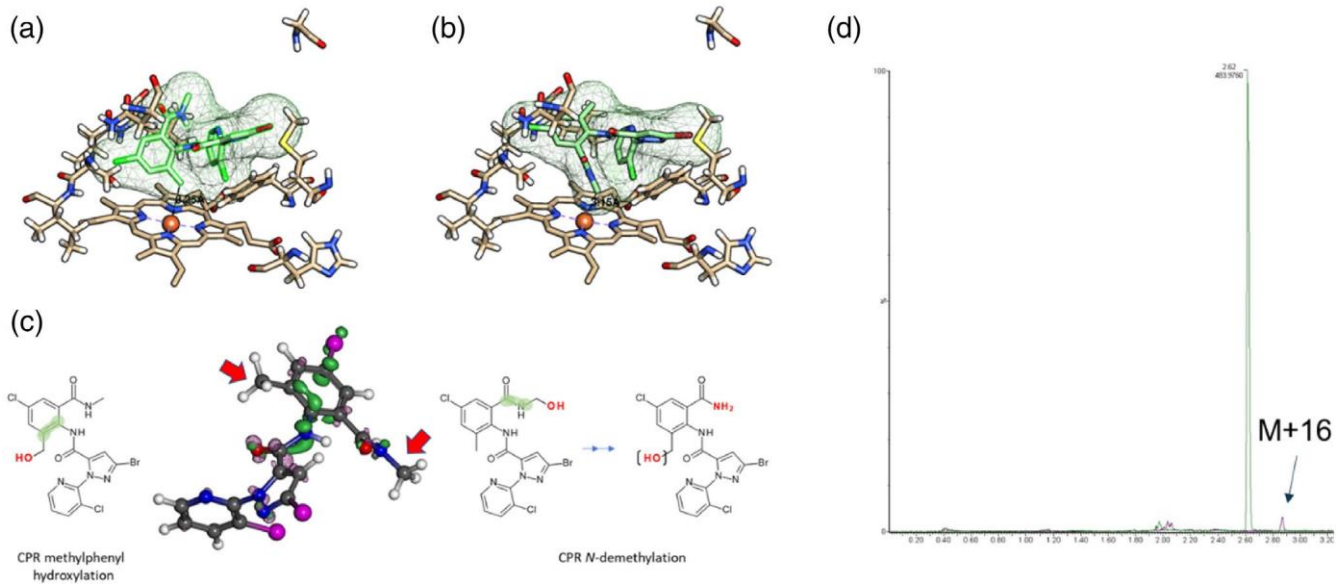

Figure 4. Computational modelling and chlorantraniliprole (CPR) metabolism by CYP9Q3. (A) Most favorable docking poses of CPR in the catalytic pocket of a CYP9Q3 homology model based on human CYP3A4 with methylphenyl carbon and (B) the *N*-methyl carbon of the anthraniloyl moiety oriented towards the 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*.

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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://calpip.cdpr.ca.gov/main.cfm). All azole fungicides tested were strong inhibitors of CYP9Q2 and CYP9Q3, respectively, with IC_{50} values in the nanomolar range, thus suggesting potential to synergize insecticides detoxified by these enzymes (Table 3). The lowest IC_{50} 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 carboxamide boscalid (IC_{50}: 4.13 μM) and the strobilurin pyraclostrobin (IC_{50}: 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

Californian 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 vivo*,[31,33] 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.[33] Another P450, CYP6AQ1, recently shown to hydroxylate

Table 3. Inhibitory potential (IC_{50}-values) of commonly applied fungicides (e.g. in Californian almond orchards[27]) against honey bee P450 enzymes CYP9Q2 and CYP9Q3 using a fluorescence-based assay with 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) as a probe

		CYP9Q3				CYP9Q2			
Fungicide	Class	IC_{50} [μM]	95% CI†	Hill slope	Adj. R^2	IC_{50} [μM]	95% CI†	Hill slope	Adj. R^2
Propiconazole*	Triazole	0.072	0.065–0.081	−1.10	0.99	0.151	0.136–0.169	−0.88	0.99
Difenoconazole	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
Cyprodinil	Anilino-Pyrimidine	>10	—	—	—	>10	—	—	—
Iprodione	Dicarboximide	>10	—	—	—	>10	—	—	—
Boscalid	Carboxamide	4.13	3–5.76	−1.05	0.90	>10	—	—	—
Pyraclostrobin	Strobilurin	7.84	6.04–10.2	−0.56	0.93	>10	—	—	—
Chlorothalonil	Chloronitrile	>10	—	—	—	>10	—	—	—

The values for propiconazole (marked with *) were taken from Haas *et al.* (2021)[33]
† 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,[42] 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.[31,33,44] 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 CYP9Q3 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 diamide binding, which differs slightly between benzenedicarboxamide-type diamides such as FLB and anthranilic diamides like CPR.[11,45] 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 CPR[23] and stronger resistance towards FLB when the I4790M mutation was introduced in a susceptible *P. xylostella* strain.[24] Honey bee RyR were shown to be much less sensitive to FLB than CPR,[20] 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,[5] 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.[48–50] 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.[29,31] A number of fungicide-insecticide combinations have been shown to be synergistic, not just in laboratory bioassays,[30] 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] 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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Chapter 5
Phylogenomic tracking and functional validation of anevolutionary conserved cytochrome P450-based mechanism of detoxification in bees

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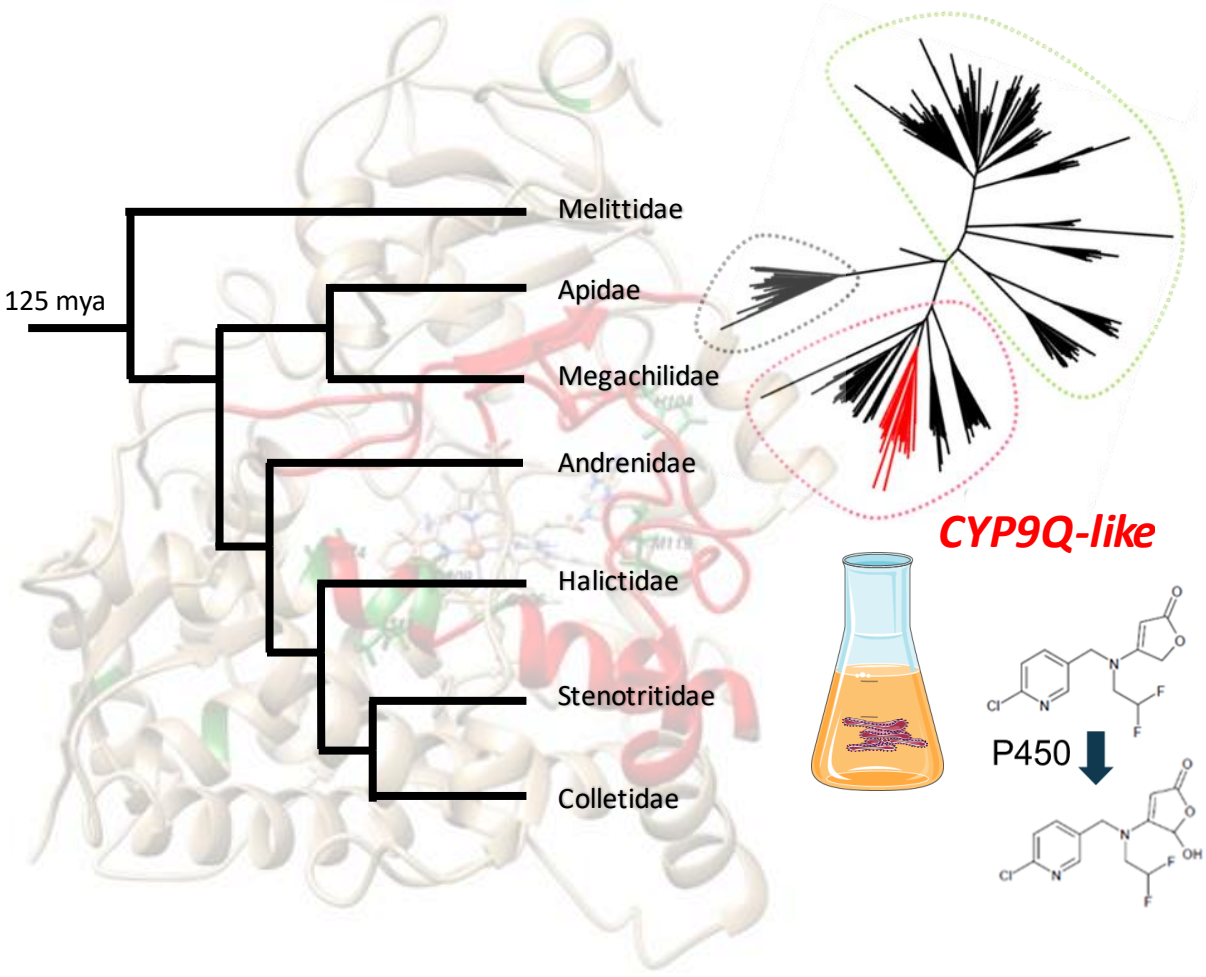

The content of this chapter has been submitted for publication. The published version may slightly differ from this manuscript.

Abstract

The regulatory process for assessing the risks of pesticides to bees relies heavily on the use of the honey bee, *Apis mellifera* as a model for other bee species. However, the validity of using *A. mellifera* as a surrogate for other *Apis* and non-*Apis* bees in pesticide risk assessment has been questioned. Relating to this, recent work on *A. mellifera* has shown that specific P450 enzymes belonging to the CYP9Q subfamily are critically important molecular determinants of insecticide sensitivity in this species. However, the extent to which the presence of functional orthologs of these P450 enzymes is conserved across the diversity of bees is unclear. Here we used a phylogenomic approach to identify >100 putative CYP9Q functional orthologs across 75 bee species encompassing all major bee families. Functional analysis of 26 P450s from 20 representative bee species revealed that P450-mediated detoxification of certain systemic insecticides, including the neonicotinoid thiacloprid and the butenolide flupyradifurone, is conserved across all major bee pollinator families. However, our analyses also reveal that *CYP9Q*-related genes are not universal to all bee species, with some Megachilidae species lacking such genes. Thus, our results reveal an evolutionary conserved capacity to metabolize certain insecticides across all major bee families, while identifying a small number of bee species where this function may have been lost. Furthermore, they illustrate the potential of a toxicogenomic approach to inform pesticide risk assessment for non-managed bee species by predicting the capability of bee pollinator species to break down synthetic insecticides.

Significance Statement

Bee pollinator pesticide risk assessment is a regulatory requirement for pesticide registration and is largely based on experimental data collected for surrogate species such as the western honey bee. Insecticide sensitivity in honey bees has recently been linked to the detoxification enzyme CYP9Q3, a honey bee cytochrome P450 with the capacity to detoxify certain insecticides such as the butenolide flupyradifurone and the neonicotinoid thiacloprid. Here we analyzed genomic data for 75 bee species and demonstrated by the recombinant expression of 26 CYP9Q3 putative functional orthologs that this detoxification principle is an evolutionary conserved mechanism across bee families. Our toxicogenomics approach has the potential to inform pesticide risk assessment for non-managed bee species that are not accessible for acute toxicity testing.

Introduction

Pollination is essential for most flowering plants and is functionally integral to the stability of ecosystems, including agroecosystems (1). While the staple crops responsible for the majority of human calory intake are wind-pollinated, an estimated 75% of globally produced crops benefit from animal pollination (2), especially crops providing important micronutrients (3). Pollination is an ecosystem service carried out by a diverse range of animals, but insects, particularly bees, are widely recognized as the most important taxa and as such are considered vital to the maintenance of high agricultural productivity (2, 4).

Bee species are not exempt from the declines in insect diversity and abundance reported in many regions of the world over the last decades (5–8). There are many drivers and stressors behind these losses which have been described as “death by a thousand cuts” (9). One such driver is agricultural intensification, with the associated use of pesticides, especially insecticides, although the relative importance of individual stressors is still under debate (9, 10). Pesticide regulation includes a thorough risk assessment for bees, which, due to its economic importance, worldwide abundance, and accessibility, is largely reliant on the use of the domesticated honey bee, *Apis mellifera,* as a surrogate for other *Apis* and non-*Apis* bee species. Although bees are monophyletic, they are a highly diverse clade of insects comprising more than 20,000 known species, with broad differences in ecology and life history traits, and, as such, the appropriateness of using the honey bee as a surrogate species is a matter of intensive debate (11). A recent publication applying a trait-based vulnerability analysis, across 10 bee species, concluded that, based on a lower reproductive potential and higher likelihood of exposure, certain solitary bees may be more at risk from pesticides than the honey bee (12). Apart from exposure, the intrinsic toxicity of pesticides is another important determinant for their safe use. Assessment of the toxicity of insecticides used for sustainable pest control against several non-target arthropods is conducted as part of existing regulatory requirements. This aims to identify any side-effects of insecticides on beneficial insects, such as bees, and, where possible, minimize their impact by ensuring appropriate label recommendations (e.g., application timing). Adverse intrinsic effects of pesticides on honey bees are well studied and under constant review and, where necessary, appropriate measures to alleviate risks are taken in order to avoid adverse effects to bee pollinators whilst facilitating sustainable pest control for growers (13–15).

Systemic insecticides acting on nicotinic acetylcholine receptors (nAChR), such as neonicotinoids, are widely used to control highly destructive agricultural and horticultural pests (16, 17). However, concerns have been expressed about their environmental and ecotoxicological risks, including a potential role in bee pollinator declines (15, 18). In 2013, the European Commission (EC) first suspended the use of clothianidin, thiamethoxam and imidacloprid in bee-attractive crops (19). Subsequently, in 2018, the EC prohibited all outdoor uses due to the high level of risk to bee pollinators and amended the conditions of approval to

restrict uses to only crops grown within permanent greenhouses. However, not all systemic insecticides binding to the orthosteric site of insect nAChRs are equally toxic to honey bees, with some assessed as practically non-toxic, according to standard regulatory measures such as acute oral and contact toxicity bioassays (20). For example, based on their low acute toxicity to honey bees, the *N*-cyanoamidine neonicotinoids (21, 22) and the butenolide flupyradifurone (23) are considered 'bee safe'. Surprisingly, these insecticides bind to the nAChRs of pest insects and honey bees with similar nanomolar affinity. Despite this, they are orders of magnitude more toxic *in vivo* to pest insects (24, 25). Recent toxicogenomic studies of managed bee pollinators have shed light on this paradox by demonstrating that cytochrome P450 enzymes from the CYP9 family act as key molecular determinants of insecticide selectivity in these bee species by providing protection to certain insecticides from multiple different classes, including *N*-cyanoamidine neonicotinoids (24–29). More specifically, in the honey bee (24) and bumblebee, *Bombus terrestris* (29), P450 enzymes from the CYP9Q subfamily have been shown to efficiently metabolize *N*-cyanoamidine neonicotinoids but not *N*-nitroguanidine compounds, explaining the profound differences in toxicity of the two neonicotinoid chemotypes to these bee species (24-26). Whereas, in the red mason bee, *Osmia bicornis*, alternative, but closely related P450 enzymes, belonging to the CYP9BU subfamily, perform a similar function (27). The importance of the CYP9Q / CYP9BU (CYP9Q-related) P450 subfamily in the detoxification of certain insecticidal chemotypes has been recently demonstrated by studies using the alfalfa leafcutting bee, *Megachile rotundata,* which lacks functional orthologs of such genes. This species was found to be up to 2,500-fold more sensitive to *N*-cyanoamidine neonicotinoids than honey bees in acute contact toxicity bioassays (30). Toxicogenomic investigations revealed that the increased sensitivity is correlated with the absence of *CYP9Q*-related genes in the genome of this species, resulting in a lack of detoxification capacity (30).

The recent findings on the role of P450s in defining the sensitivity of managed bee pollinators to insecticides lead to a number of important questions on the potential importance of these enzymes across the wide diversity of bee species. These include: 1) What is the level of evolutionary conservation of this important P450 subfamily in bees? 2) Do CYP9Q-related P450s, from a broad range of bee species, have the conserved capacity to detoxify certain insecticides? 3) By providing insight into key molecular determinants of bee sensitivity to insecticides, can a toxicogenomics approach be leveraged to inform the pesticide risk assessment for non-managed and solitary bee species? Given the importance of these questions for a more holistic approach to bee pollinator pesticide risk assessment, we recruited the entirety of available public genomic and transcriptomic resources on bees (as of 2021) – covering 75 bee species from all the major bee families – to assemble and / or curate the respective CYP9 family P450 gene inventory. Phylogenetic analyses were recruited to identify potential *CYP9Q*-related P450s genes and 26 of these from 20 representative bee species

were selected for recombinant expression and subsequent biochemical characterization of their capacity to metabolize six coumarin model substrates, two neonicotinoids and the butenolide flupyradifurone.

Results

Phylogenetic analyses of CYP3 clan P450s reveals a distinct branch of *CYP9Q*-related sequences across bee families.

The public databases for genomic and transcriptomic information were interrogated for data on bee species (Anthophila) (Table S1). We retrieved assemblies from 75 species covering all bee families, except Stenotritidae where no sequence information was available. Stenotritidae is the smallest bee family comprising approximately twenty species, in two genera, all of which are restricted to Australia (31). The other bee families were represented by 12 Megachilidae, 6 Andrenidae, 3 Colletidae, 10 Halictidae, 3 Melittidae and 41 Apidae species. There is a bias in the available sequence information towards the Apidae, which is by far the largest and most well-studied of the bee families and includes honey bees (*Apis* spp.) and bumblebees (*Bombus* spp.).

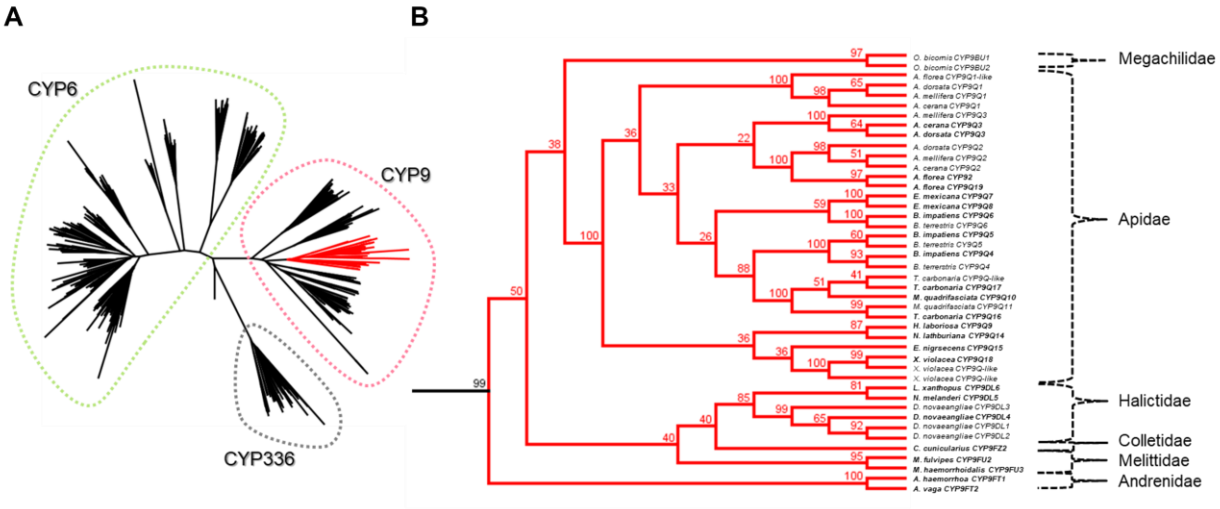

Figure 1. Phylogenetic relationship between CYP3 clan P450 genes from 24 bee species including all major Anthophila families. (A) Phylogenetic tree of bee CYP3 clan P450 genes seperated into three families, CYP6 (green), CYP336 (grey) and CYP9 (red). Apidae subfamily CYP9Q P450s including *Apis mellifera* CYP9Q1-3 are highlighted in red. (B) Detailed view of the CYP9Q branch with member of each of the six major bee families present. Node numbers represent bootstrap support values (%; 100 replicates). Candidate P450s selected for functional expression and characterization are highlighted in bold.

To establish the broader gene repertoire of the CYP3 clan of P450s across bee families, we selected a subset of 24 representative species with sufficient genomic information to use in phylogenetic analyses (Table S2). In all 579 CYP3 clan P450 sequences were identified with the resulting maximum likelihood tree revealing three distinct gene families: CYP336, CYP6 and CYP9 (Figure 1A). The CYP9 family separates into five major subfamilies comprising *CYP9DN, CYP9R*, *CYP9S*, *CYP9P* and *CYP9Q*-related genes. The sequences that form the *CYP9Q*-related clade include genes from all six bee families, with each family possessing a different lineage: *CYP9BU* for Megachilidae, *CYP9Q* for Apidae, *CYP9DL* for Halictidae, *CYP9FZ* for

Colletidae, *CYP9FU* for Melittidae and *CYP9FT* for Andrenidae (Figure 1B). With the exception of *CYP9DN1*, which had two exons and appears on a separate scaffold, the CYP9 genes are intronless and organised in a cluster at one locus in the genome. To examine the organization of the *CYP9* family and, in particular, the distribution of *CYP9Q*-related genes in more depth, phylogenetic analyses of the sequences from the main *CYP9* locus of 75 bee species were performed using Bayesian inference (Figure 2). The majority of the nodes in the topology had strong posterior probability support (>80%), and despite the fact that the support values drop for some of the deeper nodes in the tree, none were lower than 52%. Within the Megachilidae, *CYP9BU*-related genes do not appear to be universal, with 6 out of the 12 species without a sequence that clustered as *CYP9BU*-related, suggesting the loss of this subfamily in some Megachilidae species (Table S1). With the exception of 5 out of the remaining 63 species, *CYP9Q*-related genes are ubiquitous across the other bee families. However, in this case the 5 species 'missing' full length *CYP9Q*-related genes had partial sequences, suggesting their absence is a result of incomplete assemblies of transcriptomic data.

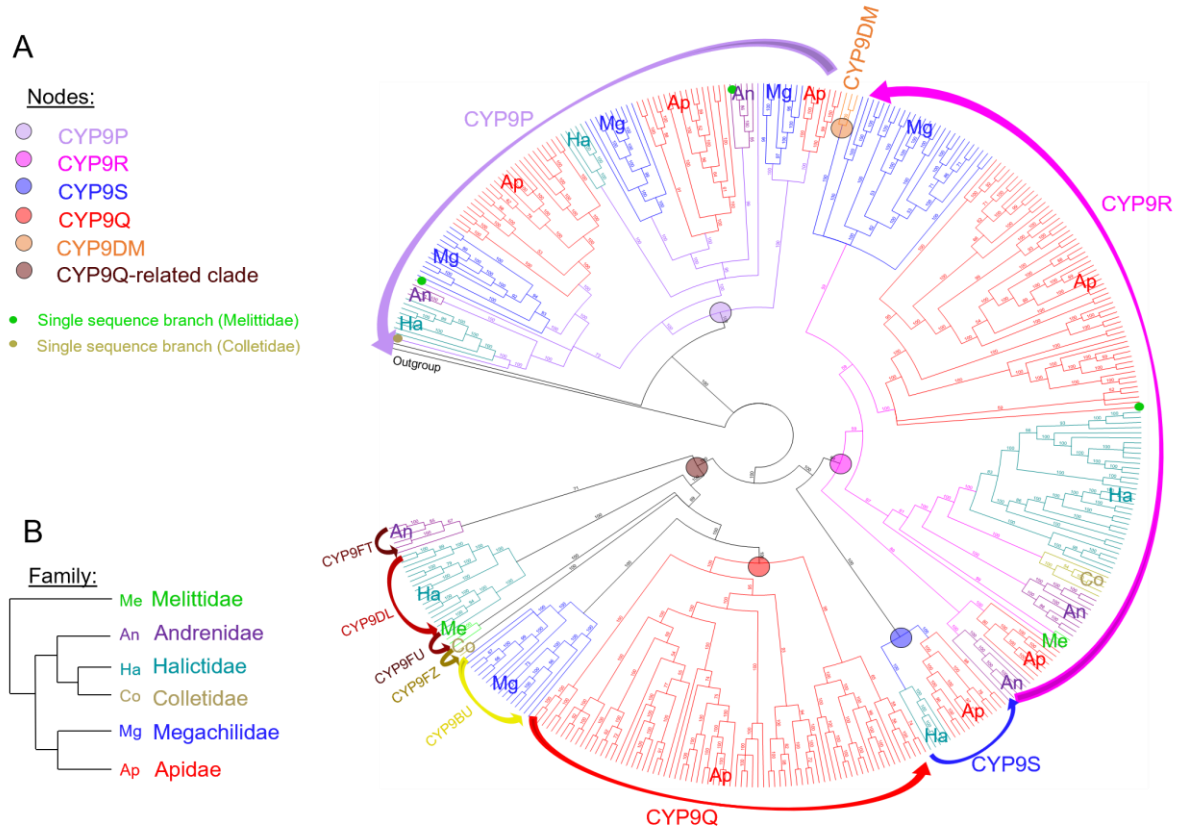

Figure 2. Phylogenetic tree based on the assembly and annotation of the CYP9 family of 75 bee species. CYP9 P450s seperate into several subfamilies: CYP9P (purple), CYP9S (blue) and CYP9R (pink) and CYP9Q (red). CYP9Q-related genes are further clustered into CYP9BU (yellow) and CYP9DL(-like) (orange). The small CYP9DM subfamily is specific for certain *Megachile* species.

Analysis of gene synteny reveals conserved genomic architecture of the *CYP9* locus across bee families.

The extent to which gene order and content is conserved between species (microsynteny) can provide a useful complement to sequence-based phylogenetic trees in inferring the shared ancestry of groups of genes. Six species, with good quality genomic assemblies, were selected as exemplars of 4 bee families (Apidae, Megachilidae, Halictidae and Colletidae). Scaffolds from each assembly containing the *CYP9* locus were investigated for evidence of microsynteny (Figure 3).

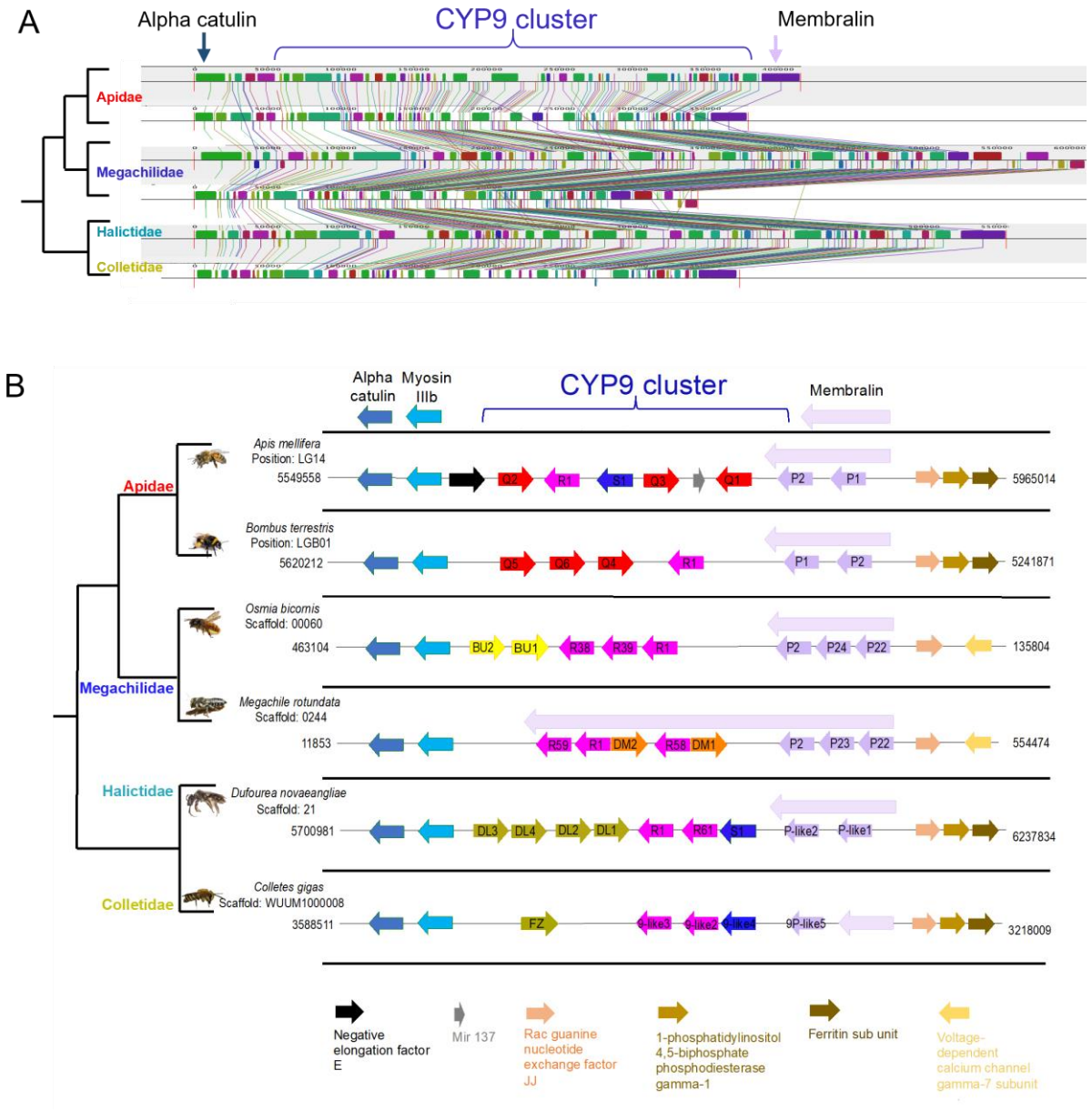

Figure 3. (A): Locally collinear blocks (LCB) identified at the CYP9 loci among six bee species across four families. Each coloured shape is a region without rearrangement of homologous backbone sequence (a collinear block). Lines between sequences trace orthologous LCBs through the genomes. Figure generated by the Mauve rearrangement viewer (Darling et al., 2004) **(B):** Schematic representation of the syntenic relationship at the CYP9 loci in six bee species across four families. Arrows represent syntenic genes. CYP9 genes are coloured by lineage and the arrows denote reading frame (not drawn to scale).

CYP9Q-related genes were found to be highly uniform in regard to their genomic position and orientation and are found in a cluster with *CYP9R* and *CYP9P* genes as part of the larger *CYP9* locus. The *CYP9* locus is framed by the same genes in all species, with *membralin* on the one side, in association with *CYP9P* genes, and *myosin IIIb* and *alpha-catulin* on the other, in association with *CYP9Q*-related genes. With the exception of *CYP9Q1* in the honey bee, *CYP9Q*-related genes show conserved gene orientation across the bee families. *Colletes gigas* was found to have the fewest CYP9 genes, (5 in total with only a single *CYP9Q*-related gene), and *Dufourea novaeangliae* the most (9 in total with 4 *CYP9Q*-related genes). It appears likely that the *CYP9* cluster emerged through tandem duplication and inversion events of an ancestral *CYP9* sequence, with additional duplication events and divergence of sequences occurring following the separation of the bee families. In *M. rotundata CYP9DM*s are substituents for *CYP9BU*s in terms of genomic position and transcriptional direction. However, from a phylogenetic perspective the *CYP9DMs* appear distant to the *CYP9Q*-related sequences, the topology of the tree placing them as a sister group to the *CYP9R* subfamily (Figure 2)

Functional expression of CYP9Q-type P450s from different bee species across families reveals a similar metabolic profile for coumarin substrates.

To gain insight into the substrate profile of CYP9Q-type P450s identified by phylogenetic and syntenic analysis we selected a representative panel of 26 P450 genes from 20 different bee species for heterologous expression *in vitro* and examined their capacity to metabolize model coumarin substrates (Table S3, S4). We excluded the two Megachilidae managed pollinators, *O. bicornis* and *M. rotundata,* in these analyses because P450s of these species have previously been investigated in detail for their capacity to metabolize xenobiotics, including *N*-cyano neonicotinoids and flupyradifurone (28, 30, 32). All selected candidate P450s share between 44 % and 88 % predicted protein sequence identity with *A. mellifera* CYP9Q3 and possess the common P450 consensus and signature motifs (Figure S1+S2). They show conservation of the helix C WxxxR, helix K Ex[LM]R consensus sequences and the heme binding domain signature motif FXXGXRXCXG, whereas minor divergence was detected for a few P450s in the consensus sequences of the helix I motif Gx[ED][TS][VI] and the PERF motif PxxFxP[ED]RF (Figure S2).

Using a baculovirus-mediated expression system in insect (*Trichoplusia ni*) cells, we were able to successfully express 25 of the 26 P450 enzymes and demonstrate their capacity to metabolize at least one fluorescent coumarin model substrate (Figure 4, Table S5). Carbon monoxide (CO)-difference spectra exhibited a 450 nm peak for almost all the enzymes, allowing P450 quantification covering a range from 3 to 123 pmol per mg microsomal protein (Table S6). Despite rather low expression levels of some P450s, such as *Andrena haemorrhoa* CYP9FT1 and *Apis cerana* CYP9Q3, we detected significant activity against some coumarins, so included them in follow-up experiments with insecticides (Table S6).

Only *Bombus impatiens* CYP9Q6 could not be successfully expressed. This was substantiated by the absence of a 450 nm peak in the CO-difference spectra and a lack of activity against the entire range of coumarins tested. We therefore excluded this P450 from additional experiments (Table S5, S6). However, *B. impatiens* CYP9Q5 was expressed and confirmed to be active.

In general, CYP9Q-related enzymes across bee species showed a similar preference for coumarin substrates. A stronger affinity was observed for fluorinated coumarins than non-fluorinated analogs, and bulkier O-arylated coumarins were metabolized more effectively than O-alkylated coumarins. In keeping with earlier results for honey bee CYP9Q3, the highest enzyme activity across the CYP9Q-related P450s was detected against 7-benzyloxymethoxy-4-trifluoromethyl coumarin (BOMFC) followed by 7-benzyloxy-4-trifluoromethyl coumarin (BFC) (24). A pair-wise comparison revealed that coumarin substrate profiles between most recombinantly expressed P450 were highly correlated, with the exception of *Apis dorsata* CYP9Q3, *A. haemorrhoa* CYP9FT1 and *Macropis fulvipes* CYP9FU2 (Figure S3).

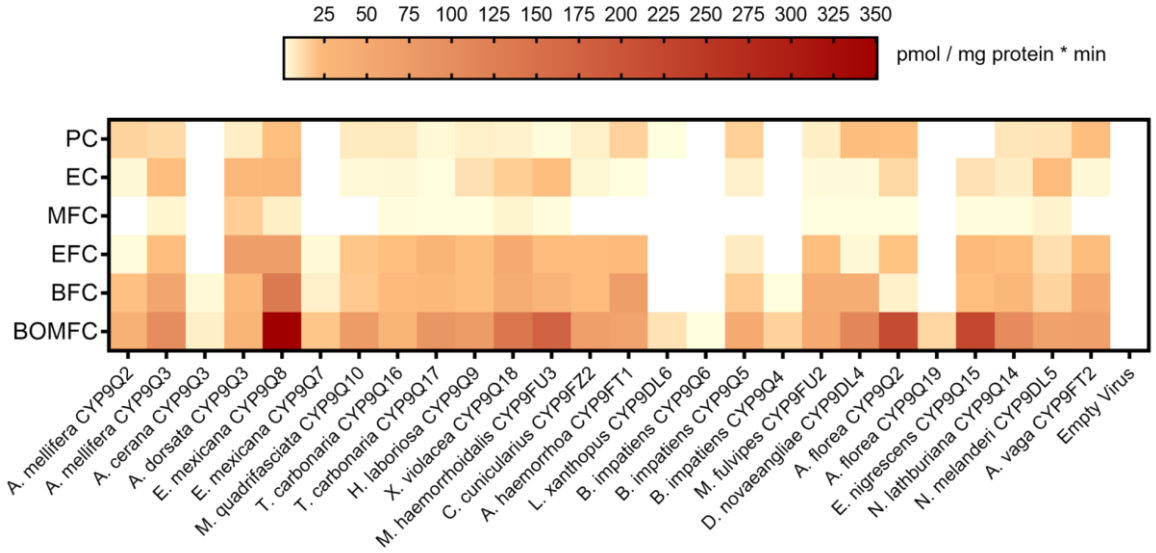

Figure 4. Heat map displaying the coumarin substrate profile of 26 recombinantly expressed CYP9Q related P450s from 20 different bee species. Metabolism of selected coumarin model substrates resulting in fluorescent 7-hydroxy-4-(trifluoromethyl) coumarin (BOMFC, BFC, EFC, MFC) and 7-hydroxy coumarin (EC, PC) respectively. Data are mean values (n = 4). Abbreviations: BFC, 7-benzyloxy-4-trifluoromethyl coumarin; BOMFC 7-benzyloxymethoxy-4-trifluoromethyl coumarin; EC, 7-ethoxy coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; PC, 7-pentoxy coumarin. A table including all calculated values in detail is found in the supplement (Table S5).

Following the identification of model coumarin substrates for the analysed CYP9Q-related enzymes we employed a fluorescent probe assay that was recently described for honey bee CYP9Q2 and CYP9Q3, to screen for the interaction between BOMFC and insecticides, measuring competitive / non-competitive insecticide-mediated inhibition of BOMFC degradation (33). We selected a single bee P450 to represent the major bee families, with the exception of Megachilidae for the reasons outlined above, i.e., *Xylocopa violacea* (Apidae) CYP9Q18, *Melitta haemorrhoidalis* (Melittidae) CYP9FU3, *Colletes cunicularius* (Colletidae) CYP9FZ2, *Dufourea novaeangliae* (Halictidae) CYP9DL4 and *Andrena vaga* (Andrenidae) CYP9FT2.

It has recently been demonstrated that the butenolide insecticide flupyradifurone (FPF) binds to honey bee CYP9Q2 and CYP9Q3 and interferes with BOMFC degradation (25). Here we found that these findings can be extended to P450s from CYP9Q-related subfamilies present in other bee families. FPF non-competitively inhibited BOMFC metabolism catalyzed by the five tested enzymes, thus indicating enzyme – FPF interaction (Figure S4, Table S7). Notably, Hanes-Woolf plots of Michaelis Menten kinetics data of all five enzymes suggested heterotropic interaction between FPF and BOMFC (Figure S4), possibly indicating allosteric behavior and the presence of multiple binding sites similar to previous findings with honey bee CYP9Qs (33).

Phylogeny correlates with functional conservation of CYP9Q-related insecticide metabolism despite sequence diversity.

Following the indirect confirmation of an interaction between FPF and various CYP9Q-related representative P450s we used UPLC-MS/MS to investigate insecticide metabolism in more detail. First, we asked whether, in common with honey bee CYP9Q3 (24), the CYP9Q-related enzymes expressed in this study have the ability to metabolize the *N*-cyanoamidine neonicotinoid thiacloprid (TCP) with high efficiency, in comparison to the *N*-nitroguanidine neonicotinoid imidacloprid (IMD). We observed that 22 out of the 25 recombinantly expressed CYP9Q-related P450s showed significant depletion of thiacloprid compared to controls (-NAPDH), ranging from 511 pmol per mg protein (SD: ± 214; *A. vaga* CYP9FT2) to 9092 pmol per mg protein (SD: ± 66; *A. mellifera* CYP9Q3), with an average depletion across all analyzed P450s of 3357 pmol per mg protein (Figure 5).

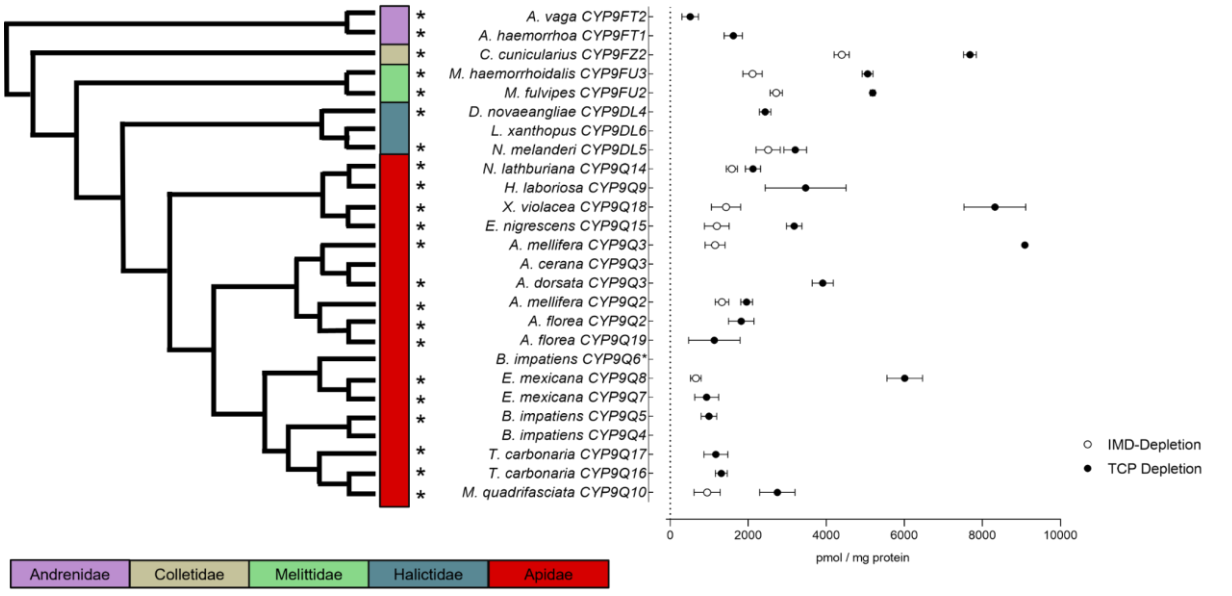

Figure 5. Thiacloprid (TCP) and imidacloprid (IMD) depletion by 26 recombinantly expressed bee P450s. The phylogenetic relationship displayed is based on maximum likelihood tree covering only the 26 P450s analyzed (branch length does not mirror actual distances). *Bombus impatiens CYP9Q6** is excluded from the analysis due to failed expression. Insecticide depletion was measured after 2h by UPLC-MS/MS and expressed in pmol/mg protein. Data are mean values ± SD (n = 3). Missing data points indicate non-significant neonicotinoid depletion compared to controls without NADPH ($p > 0.05$, unpaired t-test). Asteriks indicate significant differences between IMD and TCP depletion ($p < 0.05$, unpaired t-test).

In contrast, only 11 out of 25 enzymes exhibited significant IMD depletion compared to controls void of the cofactor NADPH. Furthermore, for all CYP9Q-related P450s, TCP depletion was significantly greater than IMD depletion (unpaired t-test, $p < 0.05$). Hydroxy-imidacloprid (IMD-OH) was the main metabolite identified accounting for most of the IMD depletion. Notably, for TCP some of the enzymes showed a significant gap between hydroxy-thiacloprid (TCP-OH) quantity and TCP depletion, indicating the formation of additional metabolites not covered in our analysis (Table S8).

As recently demonstrated, honey bee CYP9Q2 and CYP9Q3 are essential for the oxidative metabolism of FPF by two metabolic pathways leading to the acutely non-toxic metabolites FPF-4-[(2,2- difluoroethyl)amino]-furanone (FPF-AF), FPF-hydroxy (FPF-OH) and FPF-difluoroethanamine (FPF-DFEA) (25). Here, our results demonstrated that FPF metabolite formation was remarkably uniform among all the P450s tested, suggesting conserved FPF detoxification pathways across bee species (Figure 6). Hydroxylation and subsequent degradation of the furanone moiety is the preferred oxidative metabolic fate confirmed for all tested bee P450s, resulting in FPF-DFEA as the major metabolite followed by FPF-OH and FPF-AF. Average FPF depletion was 1227 pmol per mg protein ranging from 447 (SD: ± 198; *A. cerana* CYP9Q3) to 3086 pmol per mg protein (SD: ± 238; *X. violacea* CYP9Q18).

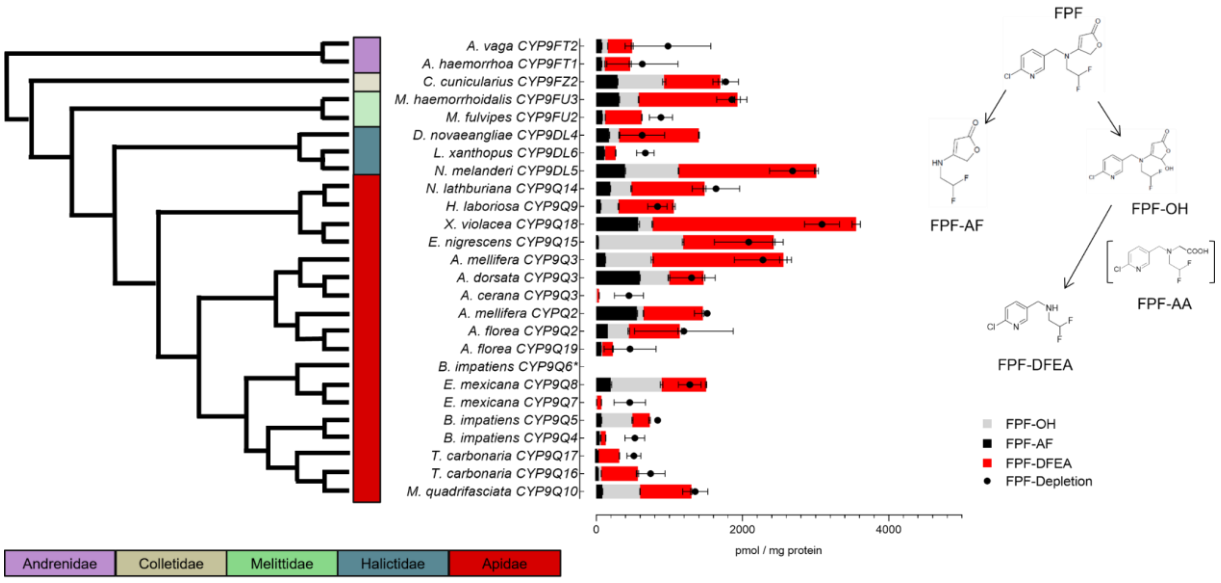

Figure 6. Flupyradifurone (FPF) metabolism and depletion by 26 recombinantly expressed bee P450s. Metabolites identified were flupyradifurone-hydroxy (FPF-OH), flupyradifurone-difluorethanamine (FPF-DFEA) and flupyradifurone-difluoroethyl-furanone (FPF-AF). The phylogenetic relationship displayed is based on maximum likelihood tree covering only the protein sequences of the 26 P450s analyzed (branch length does not mirror actual distances). Insecticide depletion was measured after 2h by UPLC-MS/MS and expressed in pmol / mg protein. Data are mean values ± SD (n = 3). *Bombus impatiens CYP9Q6** is excluded from the analysis due to failed expression. The metabolic fate of FPF is based on Haas et al. (2021).

Discussion

The *CYP9Q* P450 subfamily of honey bees matches many characteristics of gene subfamilies involved in environmental response and xenobiotic detoxification. Its members are expressed in tissues and organs involved in the detoxification process (24, 34), and across bee life stages that are exposed to xenobiotics (25, 34). Furthermore, the apparent broad substrate specificity of its members is linked to potential multiple substrate binding sites (33), which is a known feature of xenobiotic-metabolizing P450s (35, 36). Finally, CYP9Q3 has been shown to be a key molecular determinant of insecticide selectivity in *A. mellifera* and, remarkably, is capable of metabolizing different insecticidal chemotypes belonging to five different insecticide classes including pyrethroids, neonicotinoids, organophosphates, diamides and butenolides (24–27).

Beyond honey bees, related genes, such as CYP9Q4/5/6 and CYP9BU1/2 in bumblebees and red mason bees, respectively, have been shown to have a similar profile and capacity to detoxify insecticides (24, 28, 29). Taken together, these findings suggest that, just as in humans, where the P450s CYP3A4 and CYP2D6 are together responsible for the metabolism of > 50% of clinically used drugs (37), a handful of key P450s in bees may be important metabolizers of natural and synthetic environmental xenobiotics. However, recent work has demonstrated that not all bee species have CYP9Q-related P450s, with the managed pollinator *M. rotundata* lacking such enzymes. This was found to have profound implications for the sensitivity of *M. rotundata* to insecticides, with this species displaying >2,500-fold greater sensitivity to the neonicotinoid thiacloprid than other managed bee pollinators. Given these findings, it is imperative to understand which species of bees have P450 enzymes that provide protection against certain insecticides, and which do not.

In this study we addressed this important knowledge gap. Our analysis of bee genomic resources, covering 75 species, revealed the presence of *CYP9Q* functional orthologs in species across all the major bee families. However, our analyses also provide further evidence that *CYP9Q*-related genes are not universal to all bee species, with 6 out of the 12 species within the Megachilidae lacking a sequence that clustered as *CYP9Q*-related, suggesting the loss of this subfamily in certain Megachilidae species. Furthermore, syntenic analyses of the *CYP9* locus across bee families highlighted that in the Megachilidae family, *M. rotundata* has *CYP9DM* genes rather than the known insecticide-degrading *CYP9BU* genes found in *O. bicornis*. The increased sensitivity of *M. rotundata* to certain insecticides suggests that *CYP9DM* genes have lost the capability to detoxify such compounds due to divergent evolution (30). Thus, further analysis of Megachilidae species is warranted to understand the extent to which individual Megachile species lack *CYP9Q*/*CYP9BU*-related genes and the implications of this for their sensitivity to insecticides.

As previously shown for honey bees (38), *CYP9Q*-related genes are part of a bigger CYP9 cluster across bee families which appears to have arisen before the evolutionary separation of bee families.

The presence of gene clusters is a common feature of P450 genes, and is often observed for genes involved in environmental response and xenobiotic detoxification, such as those P450s found in the arthropod CYP3 clan (39, 40). The CYP9 cluster in bees also shows characteristics that are typical for environmental response genes: duplication events and rapid rates of evolution (39). *CYP9Q*-related genes show recent duplication events in some but not all species with up to five genes in *Friesomelitta varia*. Rapid rates of evolution (i.e., sequence diversity) are especially high for *CYP9Q*-related genes, effectively leading to the annotation of a distinct CYP9Q-related subfamily for each bee family (< 55% sequence identity). Therefore, it is difficult to identify them as functional orthologs based on sequence identity alone. However, our phylogenetic, syntenic and functional analyses has provided compelling evidence for an evolutionary conserved role of such genes (41).

Specifically, our data revealed a conserved functional role of CYP9Q-related enzymes in insecticide metabolism across more than 20 different bee species, including important stingless Apidae species such as *Melipona quadrifasciata* (native to Brazil) and *Tetragonula carbonaria* (endemic to Australia), and important Halictidae species such as *Nomia melanderi* (an alkali bee native to the U.S.). In all cases, our results reveal a common capacity to degrade thiacloprid and to sequentially metabolize flupyradifurone.

The observed preference for thiacloprid over imidacloprid and the conserved sequential oxidative metabolism of flupyradifurone across CYP9Q-related enzymes of all bee species investigated provides clear evidence for functional conservation in terms of insecticide detoxification capacity. Similar conservation of xenobiotic metabolism within an insect P450 subfamily has been previously reported for the CYP6AE, CYP6B and CYP9A subfamilies in the cotton bollworm, *Helicoverpa armigera* (42, 43), while the only reported examples for insect P450 families with functionally validated detoxification capacity across related species, to the best of our knowledge, are CYP6B orthologs in Papilionidae (swallowtail butterflies) (44), and CYP9A orthologs in some noctuid pests (43, 45). However, studies in Papilionidae dealt with a rather narrow phylogenetic range investigating the differences in furanocoumarin metabolism between closely related species from the same genus (44). Here, we demonstrate a case of conserved detoxification capacity across bee families which diverged more than 100 million years ago.

CYP9Qs are among the largest subfamilies in the CYP3 clan of bees after the CYP6AS subfamily, with up to five members in bee species (46) (Table S1). In lepidopteran species, diversification at the P450 subfamily level is an indicator of host range expansion and thus specialization (47). In bees, the expansion of the CYP6AS family has been linked to the transition from carnivory to florivory and eusocial resin-collecting behavior, but no such pattern is evident for the CYP9Q subfamily (46). This is consistent with a recent study where no relationship between the P450 repertoire and bee ecology was identified (48).

Similarly, no obvious pattern emerges between bee families, life history traits or dietary spectrum and the capacity of CYP9Q-related enzymes to metabolize the tested compounds in the present study. CYP9Q enzymes have been shown to metabolize the flavonol quercetin at lower rates than members of the CYP6AS family (26), leading Johnson *et al*. to suggest that CYP9Qs might have a broader substrate profile than CYP6AS enzymes, which are optimized for flavonoid defense in honey bees (46). The results provided in this study provide additional evidence that CYP9Q-related enzymes are key components of the response against diverse xenobiotics rather than specialized enzymes that provide defense to a single ecology-related type of chemistry.

The approach used in this study demonstrates the power of phylogenomic and syntenic analysis of genomic data to identify bee P450 genes that are putative functional orthologs of known insecticide metabolizers. With the exponential increase in genomic data being generated for insects, including bees, this approach has immense potential to inform pesticide risk assessment and avoid negative bee-pesticide interactions. Specifically, we envisage these approaches have utility to: i) explore the appropriateness of surrogate bee species in current risk assessment frameworks, ii) inform decisions on which bee species should be prioritized for toxicity testing, iii) predict and avoid negative outcomes of pesticide use on bees, and iv) facilitate the rational design of future insecticides. We briefly expand on these points below.

Currently a handful of managed bee species are used as a proxy for other species in pesticide risk assessment. By significantly advancing our understanding of the extent to which these model species are accurate representatives of non-model species, the research generated in this study will aid in the development of robust risk assessment frameworks. Our data illustrates the promise of leveraging phylogenetic and syntenic approaches to predict acute bee toxicity to pesticides from genomic data, and we envisage that this approach could be used as a component of a Tier 0 molecularly informed risk assessment tool. Such an approach would have parallels with molecular medicine approaches used to characterize P450-drug interactions in the pharmaceutical industry that provide important insight into organismal physiology and health (49). To employ such a phylogenetically inspired approach some requirements must be met.

First, the molecular determinants of insecticide selectivity in the surrogate species, for example, CYP9Q2 and CYP9Q3 metabolizing thiacloprid and flupyradifurone in *A. mellifera*, must be known. Available genomic data can then be screened to identify potential functionally orthologous genes in related species. In this study we show that none of the 41 Apidae species with sufficient genomic information available and investigated here lacks CYP9Q-related genes. This suggests, assuming functional conservation of CYP9Q-related P450s, that all these Apidae species have the potential to detoxify these compounds and may therefore show surrogate species (*A. mellifera*) sensitivity and selectivity.

Subsequent functional validation of selected candidate P450s following recombinant expression would help to increase the confidence in their conserved capacity to detoxify certain insecticide chemotypes. Such as shown in this study for the detoxification of thiacloprid and flupyradifurone by CYP9Q-related P450s of a diverse range of bee species belonging to the families Apidae, Halictidae, Colletidae, Andrenidae and Mellitidae. Further investigation of the acute toxicity of these insecticides for those species with and without CYP9Q-related P450s will provide further data on the robustness of the predictions made using this approach. While a phylogenomic approach to predicting bee sensitivity to insecticides will not replace conventional toxicity trials , such an approach will significantly aid decisions on which species should be prioritized for toxicity testing, while also informing pesticide risk assessment in bees not readily accessible for acute toxicity testing.

Finally, as functional characterization of bee P450s expands, the data obtained has the potential to inform tools that assess protein sequence similarity across taxonomic groups of species as a means to predict their relative intrinsic susceptibility to a chemical of interest. An example of this is the US Environmental Protection Agency Sequence Alignment to Predict Across Species Susceptibility tool (SeqAPASS v6.0; https://seqapass.epa.gov/seqapass/) which extrapolates chemical toxicity knowledge across species through the evaluation of conserved protein sequence and structure (50).

A toxicogenomic approach can also be used to predict and avoid negative outcomes of pesticide use. Specifically, understanding which bees have P450s that can metabolize certain insecticides allows rational strategies to be developed for the deployment of these products that avoid or minimize exposure to species that lack these protective enzymes. Furthermore, the panel of recombinant P450s developed in this study can be used to screen existing pesticides to identify and avoid harmful synergistic pesticide-pesticide interactions that inhibit these enzymes. The utility of this approach has been recently demonstrated using recombinant honey bee CYP9Q3 to explain the synergistic effects between insecticides and fungicides observed at the phenotypic level (33).

Finally, the tools generated here can be used in the development of next-generation bee-safe insecticides. Specifically, the recombinant P450 panels provide a filtering tool to examine the metabolic liability of future lead compounds to understand it they are likely to be rapidly metabolized by bees. This is of high value as live bioassays on bees are expensive and time-consuming to perform, and it is only possible to screen honeybees and solitary species for a few months of the year. In contrast, recombinant P450 panels are inexpensive and rapid to use, and can be employed year-round.

In conclusion our results reveal an evolutionary conserved capacity of CYP9Q-related P450s to metabolize certain insecticides across all major bee families and illustrate the promise of a toxicogenomics approach in informing bee pollinator pesticide risk assessment for non-managed bee species.

Material and Methods

Chemicals

Flupyradifurone (FPF), FPF-4-[(2,2- difluoroethyl)amino]-furanone (FPF-AF), FPF- acetic acid (FPF-AA), FPF-difluoroethanamine (FPF-DFEA), FPF-hydroxy (FPF-OH), thiacloprid (TCP), thiacloprid-hydroxy (TCP-OH), imidacloprid (IMD) and imidacloprid-hydroxy (IMD-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. (Riga, Latvia) with a purity of 95 %. 7-pentoxy-coumarin (PC) was synthesized in- house (Bayer AG, Monheim, Germany). Other coumarin model substrates and products (7-ethoxy-coumarin (EC), 7-ethoxy-4-(trifluoromethyl)-coumarin (EFC), 7- methoxy-4-(trifluoromethyl)-coumarin (MFC), 7-benzyloxy-4-(trifluoromethyl)- coumarin (BFC)), 7- hydroxy-coumarin (HC), 7-hydroxy-4- trifluoromethylcoumarin (HFC)) were of analytical grade and purchased at Sigma-Aldrich. β-Nicotinamide adenine dinucleotide 20-phosphate (NADPH) reduced tetrasodium salt hydrate (CAS: 2646-71-1 anhydrous, purity ≥ 93%) was also obtained from Sigma-Aldrich. Unless otherwise mentioned all other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

Bioinformatic and phylogenetic analysis

Genomic and transcriptomic assemblies from bee species (Apoidea (bees); taxid: 34735) were retrieved from the NCBI database (Table S1). Cytochrome P450 sequences were identified querying proteins for the conserved cytochrome P450 domain (Pfam: PF00067) using InterProScan (51) and Blast2GO (52). Clade 3 P450s from 24 species were selected and used for generation of a maximum likelihood tree using PhyML (53) with JTT as the substitution model and 100 bootstraps. Protein sequences were aligned using MUSCLE (54) built in Geneious (v10.2.6, Biomatters, New Zealand).

To generate the CYP9 phylogeny the nucleotide sequences for *A. mellifera* CYP9Q3 (XM_006562300.3), CYP9P1 (XM_006562302.3) and CYP9R1 (XM_026445177.1) were used as the query sequences in a BLASTN search through the assembly of the genome or transcriptome of each bee species to find CYP9 homologs. A TBLASTN translated protein similarity search was also performed using the same query sequences. All resulting hit tables were downloaded. Scaffolds containing CYP9 P450s were downloaded as a GenBank (full) file and imported into Geneious. Unannotated CYP9 sequences were found using the 'find in document' tool and the BLASTN alignment results. CYP9 sequences for each bee species were translated and inspected for the presence of conserved motifs. Partial sequences and those that contained stop codons were removed. The resulting CYP9 protein sequences were aligned with the outgroup sequence CYP9AG4 from *Nasonia vitripennis* (NP_001166010.1) in Geneious using MUSCLE (default settings).

MEGAX (55) was used to find the best-fit model of amino acid substitution, using a maximum likelihood fit of 56 different models. Parameters including substitution model, proportion of invariable sites and rate variation were calculated. The substitution model with the lowest Bayesian Information Criterion (BIC) score was selected for use in phylogeny estimation. The alignment was used to generate phylogeny using Bayesian inference (56) [Substitution model: LG+G (57); Chain length: 1,100,000; Subsampling frequency:200; Burn-in length: 100,000; Heated chains: 4; Heated chain temperature: 0.2].

Analysis of synteny was conducted as follows: Genomic sequences containing CYP9 sequences were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/) for *A. mellifera* (DH4 linkage group LG14, Amel_HAv3.1 WGS), *B. terrestris* (LG B01, Bter_1.0 WGS), *M. rotundata* (MROT_1.0: scf_0244), *O. bicornis* (Obicornis_v3: scf00060), *D. novaeangliae* (ASM127255v1: scaffold21) and *C. gigas* (ASM1312311v1: WUUM01000008). Synteny analysis between these scaffolds (macro-synteny) was performed using Mauve (multiple alignment of conserved genomic sequence with rearrangements) v2.4.0 (58, 59). This allowed for order and orientation of segments to be displayed and all locally collinear blocks (LCBs) to be outlined. The region ~200 Kbp upstream and downstream of the CYP9 genes was examined in more detail for micro-synteny. The region containing the CYP9 cluster in *A. mellifera* was used as the reference, and the annotated scaffolds were examined manually, and flanking genes noted. For a region to be considered as showing micro-synteny the minimum requirement was the conservation of two neighbouring homologs with no more than five unrelated genes in the intervening DNA. *C. gigas* genome was unannotated. To identify the flanking genes a BLAST database was created from the scaffold (ASM1312311v1: WUUM01000008) and flanking genes from *D. novaeangliae* used as query sequences in a discontiguous BLASTN search. First and last exons were identified, and genes annotated in Geneious.

Functional expression and metabolism assays

Functional expression of recombinant P450 proteins (Table S4) was conducted in High-5 insect cells as previously described (24, 25). All P450s were co-expressed with *A. mellifera* NADPH-dependent cytochrome P450 reductase (CPR) (Accession No.: XP_006569769.1).

Activity of isolated membrane fractions were tested using six fluorescent coumarin model substrates. Assays were performed in flat-back, black 384-well microplates with 50 µL total reaction volume and 4 technical replicates. Assay conditions and fluorescence readout were as recently described (60) with slight modifications: BOMFC was tested at 50 µM final concentration and microsomal preparations isolated from High 5 cells infected with an empty plasmid bacmid served as negative control. Fluorescent probe kinetic assays were done exactly as described previously (33). Relative fluorescent units (RFUs) were converted into pmol HC/HFC by generating a standard curve of the fluorescent products.

Carbon monoxide difference spectra of recombinantly expressed proteins were determined as previously described (61) using a Specord 200 Plus Spectrophotometer (Analytik Jena, Jena, Germany).

For insecticide depletion and metabolite quantification, incubation assays and subsequent UPLC-MS/MS analysis was performed exactly as described before (24, 25). Briefly, 80 µg microsomal protein were incubated in 100 µL reaction volume with 10 µM FPF, TCP or IMD in the presence of NADPH regeneration 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 hours at 30 °C. Controls included replicates without regeneration system and incubation of microsomal preparations isolated from insect cells infected with an empty baculovirus. 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, TCP, IMD and their metabolites were measured in positive ion mode. The peak integrals were calibrated externally against a standard calibration curve. For ion transitions and the linear range for quantification see Table S9. Recovery rates of parent compound in -NADPH samples were normally close to 100%. Obtained concentrations were converted into pmol parent / metabolite per mg microsomal protein. An unpaired t-test ($p < 0.05$) was used to determine if parent compound concentrations in +NADPH samples were significantly different from -NADPH controls. Parent depletion was calculated by subtracting the values from +NADPH samples from the average of -NADPH replicates.

Data analysis and visualization

Unless otherwise stated all data was analysed and visualized using GraphPad Prism (v9.1.0, GraphPad Software Inc., CA, USA). Data from Michaelis-Menten kinetics experiments were analyzed for competitive, non-competitive and mixed-type inhibition by non-linear regression assuming Michaelis-Menten kinetics.

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Chapter 6 - General Discussion

Modern agriculture faces a dilemma. It is expected to provide nutritious, healthy, and diverse food to all humans at any time not to mention other demands such as fuel and fiber. Meanwhile it is in the spotlight for its negative impact on biodiversity and climate. Especially in some parts of Europe, public opinion is driven by an idealization of "organic farming" which is perceived as farming in harmony with nature while "conventional farming" practices with the input of synthetic fertilizers and pesticides are marked unsustainable and novel technologies are often confronted with skepticism. Undoubtedly, the focus in agriculture has been on increasing productivity without always prioritizing sustainability in the past. Nonetheless, solely relying on farming practices without input of synthetic fertilizers and crop protection is neither realistic nor desirable as food supply at the global scale would be at risk (at least with the current state of technology). It may even be a risk for biodiversity conversation due to necessary range expansions that accompany reduced yields (Connor, 2013; Goulding et al., 2009; Kirchmann et al., 2008). A recent assessment of the farm to fork and biodiversity strategy which was recently launched by the EU commission expects huge negative economic impacts of the proposed 50% reduction of pesticide and synthetic fertilizer use (Bremmer et al., 2021). Production could decline up to 30% in some crops, prices would increase substantially, imports would increase while farm income would decrease. For perennial crops it is questioned if the strategies may benefit sustainability goals or are counterproductive.

Thus, the goal should be to combine the best of two worlds and reach a state of sustainable intensification with high and ideally increasing yields per area land without jeopardizing the environment and over-exploiting natural resources (Pretty et al., 2018; Rockström et al., 2017). For the management of insect pests, this means the advancement of IPM components other than chemical control, but also the improvement of synthetic insecticides considering their environmental profile. One key for this is the compatibility of insecticide use with pollinators such as bees. This may be achieved by further reducing the risk of exposure, and advances - for example in the field of precision agriculture - are promising in this regard (Dammer & Adamek, 2012; Finger et al., 2019). Another approach is to further reduce the toxicity of insecticides for beneficial insects such as bee pollinators. To achieve this, the knowledge of insecticide toxicology must be further advanced to identify potential determinants driving differential toxicity between pest insects and beneficial insects. The current study adds to the understanding of toxicological processes in honey bees regarding different, important insecticide classes and broadens the scope towards the entity of bees. In the following, the status quo regarding bee toxicology of insecticides is discussed and areas of interest for further studies are pointed out. Additionally, the utility of the obtained knowledge and the developed toolbox is discussed in the context of pesticide evaluation during risk assessment and for guiding the research and development of novel, bee-compatible insecticides.

6.1 Bee toxicology

Herbivorous insects have been exposed to chemical agents produced as plant defense mechanisms throughout evolution (Whittaker & Feeny, 1971). Already decades ago, it was recognized that one adaptation to overcome allelochemicals by herbivorous insects is an increased activity of detoxification enzymes (Krieger et al., 1971). This has led to the broadly accepted assumption that herbivorous insects are pre-adapted to the exposure of xenobiotics including insecticides which are often derived from natural compounds (Alyokhin & Chen, 2017; Rosenheim et al., 1996). Indeed, a lot of the mechanisms to overcome plant defense chemicals and mechanisms that lead to insecticide resistance are based on the same principles: reduced target site affinity and increased detoxification / sequestration capacity (Heckel, 2014; Heidel-Fischer & Vogel, 2015).

Bees qualify as herbivores as they feed only on plant material. However, they differ from other herbivores in their diet choice: they exclusively feed on pollen and nectar instead of vegetative tissues or plant sap throughout their entire life cycle (Danforth et al., 2019; Wäckers et al., 2007). Although toxic compounds in nectar and pollen can be frequently found (Adler, 2000), it is assumed that mutualistic co-evolution between plants and pollinators has led to reduced concentrations of herbivore-deterrent substances in bee-relevant matrices compared to vegetative tissues attacked by other herbivores (Cook et al., 2013). The identification of a reduced number of genes in major detoxification gene families in the honey bee (Claudianos et al., 2006) as well as in other bee species (Xu et al., 2013), seemed to support concerns that bees lack the preadaptation potential of other herbivores and the metabolic capacity to deal with insecticides and are therefore disproportionately affected by their use.

While these concerns are still replicated by many researchers, the evidence clearly suggests otherwise. (Honey) bees are well adapted to toxic compounds found in their environment and the molecular machinery responsible for this adaptation also leads to enhanced tolerance towards certain insecticides (Johnson, 2015).

Especially cytochrome P450s have been shown to be an integral part of the honey bee's response to plant secondary metabolites such as flavonoids (Mao et al., 2009), and alkaloids (du Rand et al., 2017) as well as to fungal-derived mycotoxins (Niu et al., 2011) and to diverse insecticide classes ranging from neonicotinoids (Iwasa et al., 2004) over pyrethroids to organophosphates (Johnson et al., 2013). The current study contributes to these findings by confirming P450-mediated phase I metabolism as an essential part of the honey bee's tolerance towards the butenolide insecticide flupyradifurone (chapter three) and the diamide insecticide chlorantraniliprole (chapter four). More specifically, it gradually becomes apparent that one P450 subfamily – CYP9Q – is catalytically active against a broad range of insecticidal chemotypes.

Kinetic studies conducted in chapter two suggest a promiscuous catalytic site and possibly multiple binding sites as key drivers behind this catalytic promiscuity. These are remarkable parallels to the human CYP3A4 which is responsible for the metabolism of the majority of administered pharmaceutical drugs (Guengerich, 1999). While CYP3A4 has been intensively studied regarding mechanisms and structural features behind multiple substrate binding and substrate diversity (Sevrioukova & Poulos, 2013), the lack of an available 3D crystal structure hampers a similar investigation of any insect P450. Detailed examination of the structural features of CYP9Q enzymes could further elucidate their potential substrate diversity and shed light on multiple substrate binding sites and substrate interaction.

CYP9Q-related detoxification is linked to high tolerance of selected compounds observed *in-vivo* (Haas, Glaubitz, et al., 2021; Haas, Zaworra, et al., 2021; Manjon et al., 2018; Mao et al., 2011). Next to the general ability to metabolize these substrates, this also means that CYP9Q enzymes are abundant at significant levels within the relevant tissues. One mechanism that insects often recruit upon exposure to naturally occurring xenobiotics is enhanced transcription of detoxification genes (substrate-induction) (Vandenhole et al., 2021). Indeed, some P450s in honey bees have been shown to be more abundant after consumption of honey, propolis or pollen (Johnson et al., 2012). It was shown that specific pollen ingredients such as p-coumaric acid induce detoxification genes from diverse enzyme families including P450s which leads to enhanced acaricide metabolism (Mao et al., 2013). Since then, it has been repeatedly shown that dietary phytochemicals enhance pesticide tolerance most probably by enhancing detoxification capacity via transcriptional upregulation of relevant genes (Ardalani et al., 2021; Johnson et al., 2012; Liao et al., 2020; Mitton et al., 2020). Induction by insecticides is less well understood. CYP9Q3 is induced by the pyrethroid insecticide τ-fluvalinate (Mao et al., 2011), but after exposure to thiacloprid only P450s incapable of thiacloprid metabolism were found upregulated (Alptekin et al., 2016). Transcriptional upregulation after sub-lethal exposure to an insecticide must therefore be viewed with caution and is no absolute proof for metabolic involvement of the induced candidates. To better understand the link, advanced knowledge of the underlying mechanisms such as transcription factors and regulatory pathways is needed to fully grasp the varying response to different xenobiotics at the mRNA level (Amezian et al., 2021).

Generally, analysis of expression patterns (with or without an inducing agent) is a promising way to identify candidate genes involved in xenobiotic detoxification. CYP9Q enzymes have been shown to be constitutively expressed and especially abundant in adult worker bees which are most likely to encounter diverse xenobiotics during foraging, and in tissues related to detoxification such as the Malpighian tubules (Haas, Zaworra, et al., 2021; Manjon et al., 2018; Mao et al., 2015).

A more general investigation of gene expression in specific tissues (e.g. Malpighian tubules, fat body, antennae, and legs) or across developmental stages may identify genes with specific expression patterns related to environmental response which could then be functionally characterized with the available toolbox.

Simultaneously other enzyme classes involved in environmental response could be investigated to identify enzymes from one or different superfamilies which act in concert to overcome the adverse effects of xenobiotics. That is to say, aside from cytochrome P450s the knowledge of the contribution of other enzymes to insecticide metabolism in bees is still scarce and often limited to studies reporting increases or decreases of enzyme activity after insecticide exposure (Li et al., 2017; Milone et al., 2020; Nielsen et al., 2000; Rouibi et al., 2016). Synergist studies suggest a minor role of carboxylesterases in pyrethroid metabolism (Johnson et al., 2006) and one candidate gene (GB10854) has been found upregulated after *p*-coumaric acid and coumaphos treatment (Mao et al., 2013; Schmehl et al., 2014). Functional characterization of specific esterase genes in bees is lacking as are detailed studies on enzymes involved in phase II or phase III metabolism. One GST enzyme from the sigma class was characterized in more detail showing transcriptional upregulation upon exposure to various xenobiotics, high expression in the fat body and removal of H_2O_2 (Yan et al., 2013). Two UGTs were shown to be upregulated after *p*-coumaric acid treatment in the western honeybee (Mao et al., 2013) while one candidate enzyme was found to be involved in oxidative stress response in the eastern honey bee *Apis cerana* (Cui et al., 2020). ABC-transporters as essential phase III protein family were implicated in insecticide tolerance by synergist studies with the inhibitor verapamil (Hawthorne & Dively, 2011). However, own experiments have shown that verapamil is also a potent CYP9Q3-inhibitor (similar to CYP3A4 (Wang et al., 2004)) and that observed synergism between verapamil and tested insecticides must be interpreted with caution (unpublished data).

To conclude, cytochrome P450s comprise the enzyme family which is best studied in honey bee detoxification systems and is involved in the metabolism and detoxification of various insecticide classes. For other enzymes the data is much more limited and warrants further investigations regarding their role in insecticide toxicology. Nonetheless, the view that cytochrome P450s are the most important single enzyme class involved in insecticide detoxification in honey bees is unlikely to change. With the CYP6AS and CYP9Q families as well as CYP6AQ1 many CYP3 clan enzymes have already been implicated in the metabolism of exogenous compounds (Haas, Glaubitz, et al., 2021; Haas, Zaworra, et al., 2021; Manjon et al., 2018; Mao et al., 2009, 2011). Those studies were conducted using the reference sequences from the official genome project. No investigation has been focused on intra- or inter-population sequence diversity and if potential allelic variations or copy number variations may influence the catalytic capacity.

Such a study would be highly interesting to investigate differences in insecticide sensitivity within and between populations (Rinkevich et al., 2015) and could contribute to the identification of key structural features in enzymes responsible for the catalytic activity.

Most of the research of insecticide toxicology in bees is based on the western honey bee (*A. mellifera*). Other bee species have been investigated less thoroughly but for some managed bee pollinators (especially *Bombus spp., Nomia melanderi, Megachile rotundata*) a surprisingly yielding database exists (Devillers & Pham-Delegue, 2002). First studies date back to the late 1940s and investigated the effect of DDT dust or residues on several bee species with the overall finding of similar toxicity between species with solitary bee species being slightly more tolerant (Bohart & Lieberman, 1949; Linsley et al., 1950; Way & Synge, 1948). The conduction of dose-response studies under laboratory conditions made comparisons easier. In one of the first studies which tested the contact toxicity of 15 different insecticides (with the focus on organophosphates) on the honey bee, the alfalfa leafcutting bee and the alkali bee, the authors concluded that *M. rotundata* had the highest tolerance to most compounds tested but also the highest variation while the honey bee was the most susceptible species (Torchio, 1973). Johansen et al. found that *M. rotundata* and *N. melanderi* are inherently more tolerant to selected insecticides than the honey bee (Johansen et al., 1983). Follow-up studies confirmed the picture of a comparable toxicity with a trend for slightly higher tolerance of solitary bees when investigating pyrethroids (Mayer, 1990; Mayer et al., 1990; Tasei et al., 1988) or carbamates (Johansen et al., 1984). Concordantly, the stingless bee *Trigona spinipes* exhibits a similar contact toxicity profile compared to the honey bee across thirteen insecticides tested (organophosphates, organochlorides, carbamates and pyrethroids) (Macieira & Hebling-Beraldo, 1989). Overall, the assumption that the honey bee is a rather sensitive bee species across all chemical classes was confirmed by a meta-analysis of all publicly available LD_{50} values showing a significantly increased sensitivity of any bee in comparison to the honey bee (i.e. LD_{50} more than 10x lower) in only eight of 150 cases (5.6%) (Arena & Sgolastra, 2014).

Considering this background, it was unexpected that *M. rotundata* has been recently shown to be significantly more susceptible than other bee species to no less than five compounds: the neonicotinoids thiacloprid and acetamiprid, the pyrethroid τ-fluvalinate, the organophosphate coumaphos and the butenolide flupyradifurone (Hayward, 2021; Hayward et al., 2019). Unexpected, but thanks to advances in the molecular understanding of insecticide selectivity not inexplicable. All these insecticides are readily metabolized by the CYP9Q-subfamily in honey bees (Haas, Zaworra, et al., 2021; Manjon et al., 2018; Mao et al.,2011). Consequently, the identified lack of functional orthologs in the alfalfa leafcutting bee is a compelling explanation at the molecular level for its higher sensitivity (Hayward et al., 2019). In chapter five this unique finding was leveraged to track molecular determinants of insecticide selectivity across bee families.

The first major finding: CYP9Q-related genes can be found ubiquitously in bee families except for Megachilidae where some tribes or genera apparently lost related genes. Related genes are not lost due to a random event in some Megachilids but phylogenetic and syntenic analysis suggest a divergent evolution leading to CYP9DM genes instead of CYP9BU, which were shown to be functional orthologs of CYP9Q in *O. bicornis* as they metabolize certain insecticides (Beadle et al., 2019). The reasons for this divergence remain elusive. What differentiates CYP9BU-bearing Megachilids from those with CYP9DM genes? Could it be that it is connected to the leaf-cutting behavior which is unique to some genera within the Megachilidae? Leaf-cutting is an intimate interaction with vegetative tissues of plants which is not shared by any other bee group and could significantly change the composition and quantity of exogenous compounds encountered by the bee leading to adaptations in environmental response genes such as the CYP9 family. However, more high-quality sequence information is needed for the Megachilidae to follow up on this hypothesis, and with the increasing use of genome sequencing this can be investigated further.

The case of *M. rotundata* also confirms that low insecticide sensitivity observed in honey bees cannot *a priori* be extrapolated to all other bee species. Likewise, it shows that sensitivity must be investigated compound-wise and high sensitivity to one compound does not necessarily mean high sensitivity to all compounds. This is supported by the high variability in toxicity which can be found within the same chemical class (Arena & Sgolastra, 2014; Reid et al., 2020). Concurrently there are cases where insecticides are less toxic to other bee species compared to the honey bee. Trichlorfon's reduced toxicity to *M. rotundata* was proposed to be due to differential pH values of the body fluids leading to a faster breakdown of trichlorfon in *M. rotundata* (Ahmad & Johansen, 1973). Other than that, the biochemical basis for increased tolerance of other bee species is largely unexplored, although there are some interesting cases. DDT and mevinphos are 10x less toxic for *M. rotundata* than for *A. mellifera* (Torchio, 1973). Some bee species are even known to actively collect huge amounts of DDT without apparent adverse effects (Roberts et al., 1982). *N. melanderi* is considerably less susceptibleto fipronil than the honey bee (Mayer & Lunden, 1999). Deltamethrin is significantly less toxic to the bumblebee *B. terrestris* (Reid et al., 2020). All these cases are potential initial starting points for further studies investigating the drivers of differential toxicity between bee species. This study provides a streamlined design for such investigations: Identification of potential drivers via classic toxicological studies (toxicokinetics and -dynamics) followed by a functional characterization of potential molecular determinants using the described toxicogenomics toolbox including phylogenomic tracking in bees or beyond.

This leads over to the second major finding of chapter five: Despite high sequence diversity, related CYP9Q-like enzymes across bee families are also functional orthologs regarding insecticide detoxification which is a significant contribution to the field of insect P450 evolution – a complex topic which is thoroughly investigated to understand this enzyme superfamily and its role in endogenous and exogenous metabolism (Dermauw et al., 2020; Feyereisen, 2006, 2011). While their structural fold is highly similar in all organisms, P450s are characterized by an extreme variation in amino acid sequence (Feyereisen, 2019; Sezutsu et al., 2013). Generally accepted is the notion that P450s involved in exogenous substrate metabolism are especially variable regarding their amino acid sequence which is supported by a lack of clear 1:1 orthologs in clades associated with environmental response (such as the CYP3 clan) (Dermauw et al., 2020). A second characteristic are "CYPome blooms" which are formed after gene duplication followed by non-, neo-, or sub-functionalization of a P450 leading to lineage-specific expansions (Feyereisen, 2011; Thomas, 2007). Both characteristics are matched by the CYP9Q family in bees (Johnson et al., 2018). Together with the functional validation of xenobiotic detoxification (chapter five) there is strong evidence that CYP9 is a model environmental response family in bees. As outlined in chapter five CYP9Q-related genes are part of a bigger CYP9 cluster located on the same chromosome. Tracking of this genetic block within other Hymenopteran species would allow to elucidate the origin of this cluster and functional validation studies could determine when the insecticide detoxification capacity has emerged. Answering the question whether the metabolic capacity towards certain insecticides is exclusive to bees or extends into other hymenopteran lineages would allow to further characterize species selectivity within this extremely important insect order with many beneficial insects beyond bees while simultaneously improving our understanding of P450 evolution (Peters et al., 2017).

Most often differential toxicity of commercial insecticides is based on toxicokinetic differences. There are only few examples such as flubendiamide where toxicodynamics is the critical parameter leading to low bee toxicity (Qi & Casida, 2013). The exact residues leading to differential flubendiamide binding are unresolved highlighting one of the major limitations: the lack of high-resolution studies regarding insecticide-target interaction across many insect species. Perhaps advances in computational predictions of structures will generate progress in this complex topic by delivering reliable models of target proteins across a broad phylogenetic range to allow species-specific analysis (Jumper et al., 2021). However, most target sites are highly conserved among insects especially within the ligand binding region limiting the potential for differential interaction with the target site between insect species (Casida & Durkin, 2013).

Another aspect which deserves further attention going forward is the understanding of sub-lethal effects of insecticides on bees. Chemical concentrations which induce no apparent mortality in the experimental population are defined as sub-lethal and may exert such effects in different ways.

Studies have investigated the effects of pesticides on bee physiology, development, longevity, immunity, fecundity, and behavior (Desneux et al., 2007). Many attempts have been undertaken to link such effects to molecular or biochemical endpoints (Di Noi et al., 2021). Studies have tried to link a sub-lethal effect to changes in single or few biomarker genes / enzymes deemed important in the specific case. Often used markergenes are involved in neurological functions (e.g. acetylcholinesterase), detoxification (e.g. P450s), immunity (e.g. alkaline phosphatase), development (e.g. vitellogenin) or oxidative stress (e.g. superoxide dismutase). A comprehensive investigation of regulatory pathways and potential systemic disturbances is often lacking. Many studies have analyzed the transcriptomic response to insecticide exposure trying to identify differently expressed genes between treatment groups (Fent, Schmid, & Christen, 2020; Fent, Schmid, Hettich, et al., 2020; Gao et al., 2020; Kablau et al., 2020; Li et al., 2019). However, without further characterization how this differential expression manifests in a specific phenotype, such studies lack informative value as transcriptional response after the reception of a stimulus is not a definitive proof of a severely disturbed system. Instead, a multi-faceted approach including transcriptomics, proteomics and metabolomics would be necessary to resolve sub-lethal effects at the molecular level systemically. Generally, it can be stated that sub-lethal effects at the molecular level need to be translated to the organism level and subsequently to field-realistic scenarios to determine how sub-lethal effects affect insect population parameters such as honey bee colony size.

6.2 The utility of bee toxicogenomics for pesticide risk assessment

The obtained knowledge and refined toolbox can complement and support the risk assessment of pesticides. As outlined in chapter one, risk is basically dependent on two variables: hazard (i.e. toxicity) and exposure. Studies conducted during this project focused on toxicity and its determinants which is why hazard is the main variable discussed here.

Several areas of concern regarding underestimated risks for bees have been formulated (see chapter one), mixture toxicity being one of them. Mixtures can be intentionally composed, for example in plant protection products with multiple active ingredients. Or they may be unintentional / coincidental, for example after sequential application of PPPs on the same field / area, or combined residues of PPPs and medications used as in-hive treatment against diseases (More et al., 2019). Models predicting mixture toxicity are based on two theoretical principles. Chemicals exerting their effects via the same or similar mode of action would additively increase the effect of each other and the combined effect is predicted from dose addition of the chemicals (called concentration addition (CA)).

Chemicals that are not assumed to interact with each other in any way are expected to exert their effects independently and the combined effect would be based on response addition of the single effects (independent action (IA)) (Berenbaum, 1981; Cedergreen et al., 2008).
If the observed effects deviate from these predictions the interaction is defined as synergistic (enhanced effect) or antagonistic (reduced effect). Synergistic interactions are quite rare. In a systematic literature review considering environmental toxicology in only 7% of all cases synergistic interaction between pesticides was reported (Cedergreen, 2014). Interestingly, 95% of those cases included cholinesterase inhibitors or azole fungicides (Cedergreen, 2014). Both compound classes are designed to inhibit enzymes from families involved in metabolism. Cholinesterase inhibitors such as organophosphates or carbamates are designed to inhibit acetylcholinesterase but may also inhibit other carboxylesterases with high affinity (Devonshire, 1977). Similarly, azole fungicides are designed to inhibit fungal CYP51 (Lamb etal., 1999), but can also inhibit cytochrome P450s involved in detoxification with high affinity (see chapter two). This demonstrates that interference with the metabolic clearance of one of the mixture partners by the other is the most common mechanism behind synergistic interaction. For bees, P450 inhibition is the most frequently reported driver of synergism between pesticides (Carnesecchi et al., 2019). In light of those findings, the relevance and elegance of the developed screening assay for mixture toxicity based on P450 inhibition (chapter 2) is obvious (Haas & Nauen, 2021). It allows to rapidly classify mixture partners regarding their inhibitory potential towards important P450s and *in-vitro* inhibition correlates well with *in-vivo* synergism indicating reliability. Similar approaches have been explored in environmental toxicology before with limited success (Gottardi & Cedergreen, 2019). What sets the here presented approach apart from previous attempts is the use of a single, recombinantly expressed P450 isoform instead of a blend of many P450s present *in-vivo* or in microsomal preparations. While studies investigating many different P450s at once may be physiologically more relevant, a selective probe substrate specific for the P450 involved in detoxification is needed to correlate inhibition of fluorescence activity to synergistic potential. This is often a limiting factor which is why studies on recombinantly expressed P450s can be a valuable alternative to increase prediction accuracy, highlighting once again the utility of the identification of essential detoxification enzymes / isoforms. Indeed, probing of recombinantly expressed single P450 isoforms is a common approach also in human pharmacology to investigate potential drug-drug interactions (Ong et al., 2013). If used correctly, the assay could serve as a high-throughput screening system to filter candidates where synergism based on interference with P450-mediated clearance may be possible or even likely. This would result in a significant reduction of potential mixtures which would be subject to further investigations in *in-vivo* studies and higher tier risk assessment steps to see whether synergistic potential is confirmed *in-vivo* and remains under more field-relevant exposure scenarios.

Notably, other bee species differ in their sensitivity to specific pesticide mixtures (Alkassab et al., 2020; Reid et al., 2020). Further investigations whether *in-vitro* inhibitory potential towards P450s correlates well with observed synergism in those species will elucidate if isoforms are differentially susceptible to inhibition by selected compounds or if observed differences stem from other toxicokinetic parameters such as reduced uptake or increased biotransformation of the azole fungicides themselves (Rösch et al., 2016).

As outlined in section 6.1 different bee species can vary in their sensitivity to insecticides. Observed changes in bee diversity and species composition in agricultural landscapes (Burkle et al., 2013; Carvalheiro et al., 2013; Grab et al., 2019; Mathiasson & Rehan, 2020) have raised the question if a pesticide risk assessment scheme relying on the honey bee *A. mellifera* as a surrogate species for bee pollinators is sufficiently protective of other bee species. Although the available data suggest that it is - especially when accounting for differences in body size - the amount of data is too scarce for a definitive answer to this question and exceptions exist (Pamminger, 2021; Thompson & Pamminger, 2019). It is difficult to assess toxicity *in-vivo* over a broad phylogenetic range. Most bee species are inaccessible for laboratory testing due to temporal restrictions (e.g. bees with only a few weeks of foraging activity) and quantitative restrictions (e.g. rare, solitary bee species) or the inability to survive under lab conditions. A certain degree of extrapolation is therefore inevitable and remains a widely accepted concept in (ecotoxicological) pesticide risk assessment.

With increasing genomic information and knowledge of the toxicogenomic basis, it becomes possible to compare and extrapolate from phylogeny and genomic information rather than from *in-vivo* testing. In chapter five a suggestion for a general procedure is outlined. Key is once again the identification of elements responsible for the observed toxicity in a surrogate species such as the honey bee. If toxicity is significantly influenced by genetics (i.e. genes, their products and potential allelic variation) it is possible to track those elements over a broad range of related species to explore similar elements in the genomic inventory of species which are supposed to be covered by the surrogate. What are the challenges? The identification of molecular determinants of toxicity is not trivial and needs to be determined compound by compound. It must be ensured that all major factors have been identified. Then, phylogenetic proximity does not necessarily mean similar function which is why functional validation is essential. Similarly, *in-vivo* data for at least a handful of species across the phylogeny would be ideal to increase confidence. But if all those requirements are fulfilled it creates tremendous opportunities to predict toxicity *in-silico* from genomic data based on molecular / biochemical evidence, syntenic analysis and phylogeny allowing for a more comprehensive comparison.

Other attempts to predict and extrapolate toxicity *in-silico* have resulted in applications such as the SeqAPASS tool (LaLone et al., 2016).

Originally developed to predict species sensitivity based on protein sequence comparison of the molecular target of chemicals (i.e. based on toxicodynamic differences), its potential to predict differences in metabolic capacity of cytochrome P450s was explored in the course of this project (unpublished data).

The bottom line: Prediction accuracy is not high enough (yet). This is not completely surprising. Unlike many molecular targets such as ion channels and receptors, which are highly conserved especially in the ligand-binding domains and rather rigid in their conformation since they are designed to have only one or few natural ligands, detoxification enzymes are designed to deal with a variety of substrates. To put this into perspective: humans are assumed to be exposed to 1-3 million xenobiotics during their lifetime (Idle & Gonzalez, 2007). The human genome comprises 57 P450 isoforms. Even under the assumption that P450s play a key role only in the minority of xenobiotic encounters, they are vastly outnumbered (and not all P450s are involved in xenobiotic metabolism). Thus, xenobiotic metabolizing cytochrome P450s are characterized by a high degree of flexibility combined with broad, often overlapping substrate specificity and reaction diversity. Identification of key residues or structural features which influence reaction parameters is therefore challenging. Nonetheless, there are examples where even single amino acid substitutions in insect P450s lead to changes in metabolic capacity or efficacy (Ibrahim et al., 2015; Schuler & Berenbaum, 2013; Zimmer et al., 2018). Further investigations to explore which features drive CYP9Q- mediated metabolism would improve prediction power of *in-silico* approaches to predict sensitivity differences based on CYP9Q-mediated detoxification. Ideally, an approach to co-crystallize AmCYP9Q3 in complex with TCP and FPF would shed light on specific insecticide-enzyme interaction. More generally, any insect P450 crystal structure would increase our understanding and facilitate homology modeling and subsequent docking studies or molecular dynamic simulations to better understand the complex binding interactions involved in P450-mediated metabolism of xenobiotics in insects. Until we can explain the molecular mechanisms of insecticide detoxification with high resolution at the level of substrate-enzyme interaction, the combination of genomic investigation and functional validation outlined in chapter five is an elegant way to inform pesticide risk assessment on the appropriateness of surrogate species and identify phylogenetic nodes where further investigations may be warranted (in this case: the Megachilidae family).

Included in the pesticide risk assessment scheme for bees is also an assessment of metabolite toxicity of plant protection products (EFSA, 2013). Relevant are metabolites that may occur in pollen and nectar exceeding concentrations of 0.01 mg / kg. This is a challenge as many metabolites formed *in planta* cannot be easily synthesized at all or in amounts necessary to conduct *in-vivo* toxicity studies. However, it is sufficient to convincingly show that metabolites have lost their toxophore (“a structural feature or moiety that gives the toxic property” (EFSA, 2013)) which renders additional testing obsolete.

Thus, molecular insights into the mode of action and toxicodynamic properties of insecticides can be used to avoid laborious, expensive synthesis procedures and subsequent *in-vivo* testing. In cases with a well characterized target such as the nicotinic acetylcholine receptor it may be sufficient to conduct *in-silico* docking studies to confirm a lack of binding potential.

A recent study has investigated in detail the different binding interactions of neonicotinoids and second generation competitive nAChR modulators using a double-mutated, insect-like acetylcholine-binding protein from the marine snail *Lymnaea stagnalis* (Montgomery et al., 2022). Combined with sophisticated computational methodologies to investigate and quantify binding interactions (Beck et al., 2021), a high level of reliability can be expected when quantifying the potential of metabolites to bind to the target site without the need to synthesize any compound. With less well characterized targets or in unambiguous cases only microgram amounts of the metabolite would be enough to conduct *in-vitro* binding studies to check if the metabolite retained binding potential to the target. One example of binding studies and their applicability to assess metabolite binding is described in chapter three. For most developed insecticides the target site has been identified during the R&D process including available *in-vitro* assays.

An emerging technology to complement current ecological pesticide risk assessment is environmental modelling. Depending on the model their purpose can be to demonstrate, understand, or predict real-life effects of a certain event on an environmental system (Grimm et al., 2020). Translated to pesticide risk assessment for honey bees this means that they can potentially aid by linking generated study results to the specific protection goals formulated (e.g. honey bee colony size), to demonstrate that results from studies conducted in different tiers during risk assessment are in agreement or to extrapolate effects to different environmental and exposure profiles. Several models have been developed or are currently under development and considered for honey bee risk assessment such as the BEEHAVE model (Becher et al., 2014), the APISRAM model (S. More et al., 2021) or the VarroaPop + Pesticide model (Minucci et al., 2021). Concordantly, first models for non-*Apis* bees are under development (Becher et al., 2018; Gegear et al., 2021). Naturally, if models are considered for decision making or support, trust and confidence in them must be high and they need to be thoroughly validated (Pilkey & Pilkey-Jarvis, 2007). Given the complexity of the topic, this is easier said than done. Most promising models have a modular design where modules from different relevant areas are combined to achieve the most realistic outcome (Roeben et al., 2020). This includes a toxicological module which can be used to provide data regarding toxicokinetic and toxicodynamic characteristics and other relevant properties of the investigated substance. Exploring how data generated using the molecular and toxicological toolbox developed here can inform models, can help with the accuracy of environmental models, and can increase confidence for decision making.

For that, it is important to know what kind of data is helpful and how the module can be adapted to make the best use of existing data. The integration and combination of those two areas has great potential to further advance risk assessment.

These are four examples of how methodologies and knowledge obtained from toxicogenomic studies may benefit different areas of interest within the pesticide risk assessment for bee pollinators directly and there are certainly many more to think of. Lastly however, I would like to highlight an indirect benefit of studies which explain the molecular basis and mechanisms behind observed effects in toxicological studies: They can increase the trust decision makers have in the data and booster the confidence in a decision. Pesticide risk assessment is always connected to uncertainty. It is seldomly possible to reach an absolute level of certainty that no risk exists for each and every scenario. Decision makers are therefore used to deal with uncertainty which is increased by a plurality of studies from many stakeholders sometimes contradicting each other. Unfortunately, we live in a time where decision makers are threatened if they come to a decision which seems unacceptable for others (Lehmann, 2021). Under these circumstances it is only logical that decisions are reached with increasing precaution. This is of course not a bad thing per se but if it exceeds disproportionately, it can seriously hamper progress (one example is the handling of gene editing techniques for plant breeding in the EU). Studies investigating not only what the effects of a pesticide are but also how the effects can be explained mechanistically can help to reduce uncertainties, strengthen the trust in a decision and ultimately contribute to a framework guided – above all - by science.

6.3 The utility of bee toxicogenomics for insecticide development

Among many available solutions, synthetic insecticides have remained the principal mechanism to combat insect pests in many agricultural settings (Swale, 2019). The spread of insecticide resistance is a major driver of investments into the development of novel insecticides with new modes of action or other resistance-breaking properties (Sparks & Nauen, 2015). Similarly, regulatory requirements for improved environmental and toxicological profiles further accelerates the need for new compounds. Advanced data requirements or more conservative interpretation of existing data have led to the loss of 75% of available pesticides in the EU with more to follow during upcoming re-registration processes (Corsi & Lamberth, 2015). While this is an opportunity to meet market demand with adapted products, it is also a challenge as advanced requirements increase development times and add to the already high costs necessary for agrochemical discovery (~$300 million per compound, 55 % increase compared to 2000) (Sparks & Lorsbach, 2017). To maximize the return of investment it is therefore more important than ever to consider registrability of compounds already in early development phases (Corsi & Lamberth, 2015). This includes the optimization of compounds towards a more favorable ecotoxicological and environmental safety profile.

While this comprises a variety of species and other factors, this section here is focused on the honey bee as a surrogate for bee pollinators. The discovery of a crop protection product begins with the identification of new molecular starting points (Hits) which exhibit basal activity against test species (Shelton & Lahm, 2015).

This is for example achieved by high-throughput screening (HTS) of huge chemical libraries. Screening input can be random but to increase success rates it is usually hypothesis-driven, inspired by natural products, patent observations, predictions by algorithms and cheminformatics, or just by intuition of chemists. Virtual screening is employed to filter and evaluate compounds against pre-defined criteria which allows the exploration of a huge chemical space without the need to synthesize compounds (Jeschke et al., 2012). *In-vitro* screening is employed to identify candidates which exhibit activity against known molecular targets. However, specific requirements regarding delivery and pharmacokinetics of insecticides often hamper the successful translation of *in-vitro* to *in-vivo* activity in insects (Sparks et al., 2019; Swale, 2019). Thus, the backbone for the discovery of new agrochemical Hits is high-throughput *in-vivo* screening of relevant model pest species. Continuous improvement over the years enabled the rapid, automated screening of thousands of compounds each year against a number of model pest species with only small amounts of substances needed (Jeschke et al., 2012; Tietjen et al., 2005).

Once a Hit has been identified, the next step is the generation of a lead compound which involves the profiling of many analogs with a diversity of approaches and tools with the aim to sharpen and optimize the biological profile (Loso et al., 2017). Only if all characteristics of a compound are satisfactory, a lead compound is found and will eventually result in a commercial product. It is this iterative process coupled with feedback loops that also allows to characterize the (eco-)toxicological profile of a candidate and identify analogs which are less toxic to beneficial insects such as bees (Shelton & Lahm, 2015).

Considering honey bees, the ideal system would be a high-throughput *in-vivo* screening system allowing the rapid assessment of acute toxicity via the oral and / or contact exposure route. This is, however, difficult to develop for various reasons. First, honey bees are seasonal insects in temperate climates restricting possible *in-vivo* testing to a few months (roughly from April to September). Second, their handling in the laboratory is complicated. They are social insects meaning that isolation of an individual is an additional stressor potentially leading to increased mortality. Honey bees are also known for trophallaxis (food transfer between individuals) which complicates the determination of the actual received dose if bees are kept in cohorts (Korst & Velthuis, 1982). Moreover, individuals of one test group do not die independently of each other when kept in one cage (Moncharmont et al., 2003). These factors contribute to high variability of honey bees in response to insecticides making a certain degree of replication necessary to obtain meaningful, reliable data.

This, in turn substantially increases the amount of substance needed which is a limiting factor at this stage of the discovery process. Third, their ability to fly (and sting) makes automated handling even more complicated especially considering that honey bees are adversely affected by prolonged or repeated anesthesia with CO_2 or cold temperature (Ebadi et al., 1980; Gooley & Gooley, 2021).

All these hurdles are not absolute and may be overcome in the future to increase throughput in *in-vivo* bee testing. Alternative and complementary approaches are needed either way to generate additional data and increase optimization success. Insights generated in this study may help to address critical points in this attempt.

An alternative for *in-vivo* screening of bees could be the *in-vivo* screening of *D. melanogaster* carrying important honey bee genes involved in insecticide toxicokinetics. On this way the impact of single genes of the honey bee's detoxification system can be investigated one by one in an otherwise identical genetic background. The proof of concept is illustrated in chapters three and four. The advantages are obvious. *D. melanogaster* can be tested year-round and is easy to maintain in huge numbers. Their handling in the laboratory is much more convenient and standardized bioassays exist. Although the GAL4 / UAS system used in this project is not designed for HTS, the well characterized model system *D. melanogaster* offers other systems to introduce transgenes which are better suited for this application (McGuire et al., 2004). One limitation of this approach is that investigated compounds must be active against *D. melanogaster* which is not always given since compound activity is usually tested against relevant pest species from other insect orders (often Lepidoptera or Hemiptera).

Another drawback is the different biology of flies and bees which could lead to difficulties by the translation of the results to bees. For example, several enzymes from different families may act together and form assemblies facilitating channeling of metabolic intermediates which could substantially differ between insect species (Laursen et al., 2016). Especially the assessment of phase II metabolism enzymes could prove difficult as they often process phase I metabolites which potentially differ between flies and bees. For all enzymes which may interact directly with the compound such as P450s, esterases (both phase I), or ABC-transporters (phase III) the screening of a transgenic *Drosophila* library with the most essential honey bee genes involved in insecticide metabolism presents a promising addition to *in-vivo* screening of honey bees. Insights and future studies outlined in section 6.1 will guide the decision which genes to include in such a library.

P450s have the greatest potential as determinants of insecticide tolerance in honey bees based on toxicokinetics, because phase I metabolism is often rate limiting regarding toxicity(Yu, 2011). *In-vitro* screening of the P450 honey bee library described in chapter three is therefore an obvious strategy to assess potential metabolism of novel compounds. Highest throughput offers the fluorescence depletion assay (Haas & Nauen, 2021).

Next to its high throughput, it is cheap, automatable and requires no specialized equipment. One drawback is the lack of a suitable model substrate for all P450s which impedes the investigation of the entire library. While it reliably detects the interaction between a P450 and a compound, a reduction in fluorescence signal does not necessarily suggest metabolism of the test compound. Allosteric interactions or occupation of the catalytic center without efficient metabolism may lead to false positive hits. To reduce the risk for false positives, investigation of metabolism via HPLC-MS/MS or similar systems may be better suited. Increased accuracy comes with the price of higher costs and lower throughput. In both cases the translation of *in-vitro* metabolism to reduced toxicity *in-vivo* will be complicated since metabolites may be as toxic as the parent compound.

Such problems are also inherent to the extrapolation of *in-vitro* screening of target sites to *in-vivo* activity in pest species (Sparks et al., 2019). As briefly touched in section 6.1 our understanding of species-specific binding of insecticides to target sites is limited. To identify compounds which show differential toxicity based on differences in toxicodynamic properties simultaneous screening of molecular targets from beneficial insects and pest species could lead to the identification of compounds which may be differentially active at the target site. Although our understanding of the specific binding interactions is limited, HTS *in-vitro* screening may identify candidates which bind differentially. Of course, such testing can only be done with known target sites limiting the scope to known targets where suitable assays are available.

The outlined approach would be an example of innovation driven by evolution: taking an established system (insecticide discovery pipeline) and adapt and optimize it to new needs (enhanced environmental profile) wherever necessary. Another strategy where (toxico)genomic insights have huge potential would be innovation by revolution: completely new thinking of how pest control is achieved. The design of the traditional discovery process described above is centered around the identification of acute toxicity by ideally fast-acting mode of actions with a broad activity spectrum. Such modes of action are preferred as they are reliable, easy to handle for the farmer, and the fast onset of control successfully prevents any feeding damage. Especially important is the fast action for the control of sucking pests which are often vectors of viral or bacterial diseases. Broad activity is desired as development costs do not justify investments into compounds targeting only a very narrow range of pest species. Several factors limit the number of biochemical targets addressable by commercial insecticides (Casida & Quistad, 1997). However, it is proposed that a significant number of potential targets is still unexplored (Swale, 2019). With the explosion of genomic information of arthropods gathered by initiatives worldwide (Childers et al., 2021; i5K Consortium, 2013) it may be possible to identify targets which are specific for pest species / groups but still allow for rapid feeding cessation and thus efficient control.

Genome-wide comparisons to find potential targets shared only by relevant pest species and not by beneficials such as bees would be an ideal solution to unite effective pest control with high selectivity towards non-pest insects. In summary, insights into the species-specific determinants of toxicity can be leveraged to invent novel solutions for pest control in agriculture which have less impact on the environment. Established systems may be adapted or completely new approaches may arise based on the continuous improvement of molecular determinants of toxicity. While this study adds knowledge to the field of molecular entomology and toxicology it has only scratched the surface of what will be possible in the future.

Embracing innovation in this field in combination with other promising technologies will aid to make agriculture resilient to the challenges it encounters and contribute to a future where crop protection and sustainability goals are not opponents but are truly integrated.

6.4 References

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Appendix A supporting data (Chapter 2)

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

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Supporting data TABLES

Table S1. Steady-state kinetics data for BFC metabolism (resulting in 7-hydroxy-4-(trifluoromethyl)coumarin (HC)) by CYP9Q3 co-incubated with increasing concentrations of different neonicotinoid insecticides and *tau*- fluvalinate. The inhibition type is based on kinetic characteristics of reversible inhibition models according to Fowler and Zhang[5].

BFC + insecticide (Inhibition type)	K_m [µM]	95% CI[a]	V_{max} pmol HC / min * mg protein	95% CI[a]	Adjusted R^2
Thiacloprid (mixed)					
w/o	11.1	9.27 to 13.0	26.9	25.7 to 28.2	0.98
10 µM	17.6	13.6 to 21.5	20.0	18.7 to 21.3	0.97
30 µM	20.6	16.3 to 25.0	13.2	12.3 to 14.0	0.97
100 µM	24.3	15.5 to 33.0	6.8	6.02 to 7.59	0.92
Acetamiprid(mixed)					
w/o	8.12	7.23 to 9.00	26.9	26.2 to 27.7	0.99
10 µM	9.56	8.65 to 10.5	24.4	23.8 to 25.0	0.99
30 µM	9.71	8.42 to 11.0	21.2	20.5 to 21.9	0.99
100 µM	14.4	12.2 to 16.6	15.4	14.7 to 16.0	0.98
Imidacloprid(none)					
w/o	12.5	10.8 to 14.2	29.9	28.8 to 31.0	0.99
10 µM	10.9	9.70 to 12.1	29.5	28.7 to 30.4	0.99
30 µM	11.2	9.85 to 12.5	28.7	27.8 to 29.6	0.99
100 µM	14.2	12.0 to 16.5	28.4	27.1 to 29.6	0.98
Thiamethoxam(none)					
w/o	14.1	12.6 to 15.6	29.5	28.6 to 30.4	0.99
10 µM	13.6	12.0 to 15.1	27.6	26.7 to 28.4	0.99
30 µM	14.7	13.2 to 16.2	27.3	26.5 to 28.1	0.99
100 µM	15.1	13.3 to 16.9	26.8	25.9 to 27.7	0.99
Tau-fluvalinate(competitive)					
w/o	10.2	8.9 to 11.81	28.1	26.9 to 29.2	0.98
10 µM	15.3	14.2 to 16.5	28.7	28.0 to 29.3	0.99
30 µM	25.0	23.6 to 26.4	28.7	28.2 to 29.2	0.99
100 µM	59.8	57.5 to 62.3	30.4	29.9 to 30.9	0.99

[a] Confidence Interval, 95%

Table S2. Synergistic effects of fungicide pretreatment on acute contact toxicity of acetamiprid and thiacloprid against honey bees. All tested fungicides were not acutely toxic at the applied dose of 10 µg / bee.

Insecticide +synergist	LD_{50} 48h [µg/bee]	95% CI[a]	Slope	SR[b]	95% CI[a]	Chi² (df)	Neonicotinoid doses tested [µg/bee]
Acetamiprid							
Alone	8.81	4.4-15.2	1.15	-	-	9.63 (5)	0.08; 0.04; 2; 10; 20; 100; 200
+Propiconazole	0.057	0.037-0.080	2.90	151	104-220	9.21 (5)	0.004; 0.008; 0.02; 0.04; 0.08; 0.4; 2
+Prothioconazole	8.57	4.53-14.4	1.35	1.03	0.67-1.58	7.44 (4)	0.4; 2; 10; 20; 100; 200
+Prochloraz	0.038	0.03-0.046	2.19	214	158-341	3.60 (4)	0.0125; 0.025; 0.05; 0.1; 0.2; 0.4
Thiacloprid							
Alone	21.5	6.42-73.4	0.56	-	-	4.85 (4)	0.08; 0.4; 2; 10; 50; 100
+Propiconazole	0.031	0.023-0.043	2.01	686	332-1420	6.79 (4)	0.005; 0.01; 0.02; 0.04; 0.08; 0.4
+Prothioconazole	19.5	9.02-45	0.93	1.10	0.5-2.43	8.67 (4)	0.08; 0.4; 2; 10; 50; 100
+Prochloraz	0.029	0.024-0.034	2.30	752	362-1560	2.59 (4)	0.005; 0.01; 0.02; 0.04; 0.8; 0.4

[a] Confidence Interval, 95% [b] Synergistic Ratio (LD_{50} Insecticide divided by LD_{50} Insecticide + Fungicide)

Table S3. Steady-state kinetics data for BFC metabolism (resulting in 7-hydroxy-4-(trifluoromethyl)coumarin) by CYP9Q3 co-incubated with three different azole fungicides at 1µM.

BFC + fungicide	K_m [µM]	95% CI[a]	V_{max} pmol HC / min * mg protein	95% CI[a]	Adjusted R^2
w/o	6.21	5.14 to 7.29	25.6	21.0 to 22.6	0.98
1 µM Prochloraz	30.1	22.2 to 38.0	2.06	1.91 to 2.29	0.96
1 µM Propiconazole	17.6	13.6 to 21.7	4.95	4.72 to 5.40	0.96
1 µM Prothioconazole	5.26	4.25 to 6.27	26.2	25.1 to 27.4	0.97

[a] Confidence Interval, 95%

Supporting data FIGURES

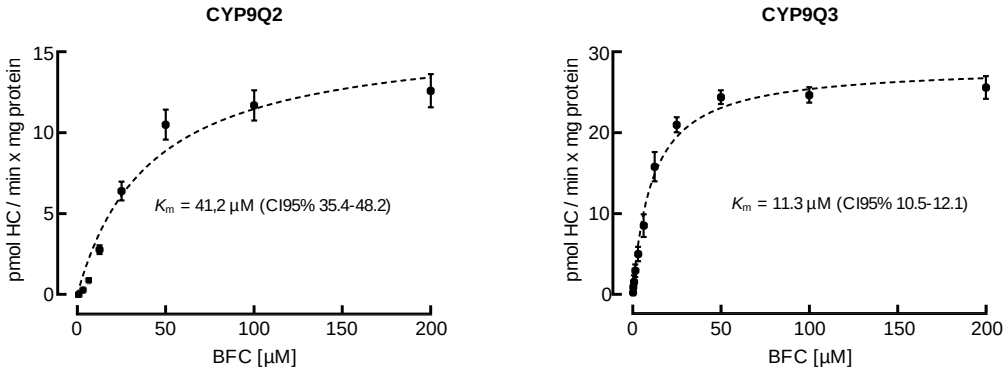

Figure S1. Michaelis-Menten kinetics of BFC O-dearylation leading to 7-hydroxy-4-(trifluoromethyl)coumarin (HC) by recombinantly expressed honey bee CYP9Q2 and CYP9Q3 analysed by non-linear regression. Data points are mean values ± SEM (n = 4). CYP9Q2 does apparently not strictly follow single substrate binding kinetics with BFC, but sigmoidicity of velocity, suggesting positive cooperativity possibly mediated by two-substrate binding sites. For the sake of simplicity, we consider the *K*m value given for BFC as an apparent value, as we have refrained from using more complex kinetic models.

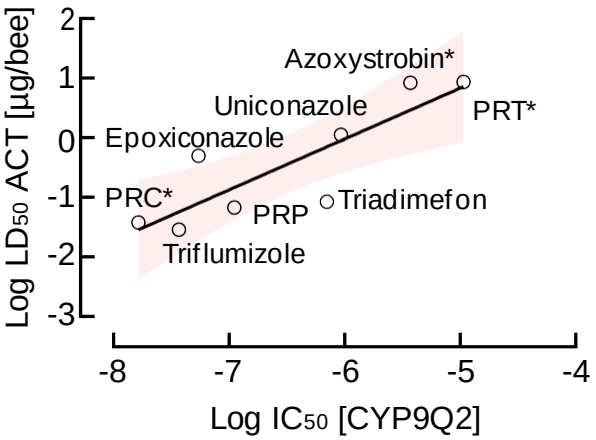

Figure S2. Pearson correlation analyses (r = 0.85) between *in vitro* IC50 values and *in vivo* LD50 values obtained from CYP9Q2 fungicide inhibition assays and honey bee acute contact bioassays with acetamiprid in combination with fungicides, respectively. LD50-values for acetamiprid in combination with fungicides were taken from Iwasa et al.[3] or generated in this study (those marked with an asterisk). The obtained correlation suggests a strong relationship between the inhibition of CYP9Q2 by the tested fungicides and their potential to synergize acetamiprid toxicity in honey bee contact bioassays.

Appendix B supporting data (Chapter 3)
A toxicogenomics approach reveals characteristics supporting the honey bee (*Apis mellifera* L.) safety profile of the butenolide insecticide flupyradifurone

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

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Table S1. Honey bee clade 3 cytochrome P450 genes used in this study

Species	Gene ID	Accession number
Apis mellifera	CYP9Q1	XP_006562364
Apis mellifera	CYP9Q2	XP_392000
Apis mellifera	CYP9Q3	XP_006562363
Apis mellifera	CYP9R1	GB16803
Apis mellifera	CYP9S1	XP_016771487
Apis mellifera	CYP336A1	XP_001119981
Apis mellifera	CYP9P1	XP_006562365
Apis mellifera	CYP9P2	GB19055
Apis mellifera	CYP6AQ1	NP_001191991
Apis mellifera	CYP6AR1	XP_623362
Apis mellifera	CYP6AS1	GB16899
Apis mellifera	CYP6AS2	GB19197
Apis mellifera	CYP6AS3	GB15681
Apis mellifera	CYP6AS4	XP_395671
Apis mellifera	CYP6AS5	DQ232888
Apis mellifera	CYP6AS7	XP_006565064
Apis mellifera	CYP6AS8	XP_006565076
Apis mellifera	CYP6AS10	XP_016771320
Apis mellifera	CYP6AS11	XP_016771191
Apis mellifera	CYP6AS12	XP_397347
Apis mellifera	CYP6AS13	GB17831
Apis mellifera	CYP6AS15	XP_623595
Apis mellifera	CYP6AS17	XP_006565063
Apis mellifera	CYP6AS18	XP_006565063
Apis mellifera	CYP6BC1	XP_016766476
Apis mellifera	CYP6BD1	XP_006564499
Apis mellifera	CYP6BE1	XP_624795

Table S2. Primer pairs used for qPCR

Name	Sequence	Gene ID	Source
1419_CYP6AQ1 F	5'-CGGTGGATTCGAAACATCCT-3'	408383	This study
1420_CYP6AQ1 R	5'-AGAGCCTCGTGAATCTCAGT-3'		
AmCyp9Q3 F1	5'-GATGTGCGTCGAGAGTTTCC-3'	408453	Manjon et al. 2018
AmCyp9Q3 R1	5'-CTGTCCGGGTCGAATTTGTC-3'		
AmCyp9Q2 F1	5'-ATGGAAGGAGCACAGGAACA-3'	408452	Manjon et al. 2018
AmCyp9Q2 R1	5'-ACGTCGTTGGTGTATCTGGT-3'		
AmCyp9Q1 F1	5'-GGAGGAGGGGAAGAGAGGTA -3'	410492	Manjon et al. 2018
AmCyp9Q1 R1	5'-CCTCCTGAAGCCTCTGTTGA-3'		
1519_Tbp_F1	5'-TTGGTTTCATTAGCTGCACAA-3'	410004	Lourenco et al. 2008
1520_Tbp_R1	5'-ACTGCGGGAGTCAAATCTTC-3'		
1007_Ubi_F1	5'-ACAGCTGGAAGATGGTCGTACAC-3'	409675	Cornman et al. 2013
1008_Ubi_R1	5'-CGAAGTACAAGGTGAAGAGTCGATTC-3'		

Table S3. *Drosophila melanogaster* strains used in this study.

Strain	Genotype
CYP9Q2	y w M(eGFP, vas-int, dmRFP)ZH-2A; P{w[+mC]=UAS-Cyp9Q2}attp40
CYP9Q3	y w M(eGFP, vas-int, dmRFP)ZH-2A; P{w[+mC]=UAS-Cyp9Q3}attp40
Empty pUAST	y w M(eGFP, vas-int, dmRFP)ZH-2A; P{w[+mC]=UAS}attp40
Hsp70-GAL4	w[*]; P{w[+mC]=GAL4-Hsp70.PB}89-2-1

Table S4. Steady-state kinetics data for BOMFC metabolism (resulting in 7-hydroxy-4- (trifluoromethyl)coumarin (HC)) by the respective honey bee P450 co-incubated with increasing concentrations of flupyradifurone (FPF). The inhibition type is based on kinetic characteristics of reversible inhibition models according to Fowler and Zhang (*The AAPS Journal* **10** (2008) 410-424).

P450 ± [FPF] (Inhibition type)	K_m [µM]	95% CI[a]	V_{max} pmol HC / min * mg protein	95% CI[a]	Adjusted R^2
CYP9Q3					
(non-competitive)					
w/o	6.41	5.24 to 7.83	90.9	86.2 to 95.7	0.96
10 µM	7.01	5.69 to 8.61	77.3	73.2 to 81.7	0.96
30 µM	7.95	6.40 to 9.85	62.7	59.1 to 66.4	0.95
100 µM	9.86	7.79 to 12.5	39.9	37.3 to 42.6	0.95
CYP9Q2					
(non-competitive)					
w/o	14.4	10.9 to 18.9	70.1	64.4 to 76.2	0.93
10 µM	15.1	10.9 to 20.8	65.9	59.6 to 72.7	0.91
30 µM	15.7	11.7 to 21.1	64.2	58.5 to 70.4	0.92
100 µM	17.4	12.7 to 23.8	50.6	45.7 to 55.9	0.91
CYP6AQ1					
(non-competitive)					
w/o	20.6	17.1 to 24.8	7.53	7.09 to 7.99	0.97
10 µM	25.9	20.3 to 33.0	6.65	6.11 to 7.25	0.95
30 µM	30.2	24.3 to 37.7	5.48	5.07 to 5.92	0.96
100 µM	26.0	20.7 to 32.7	3.14	2.91 to 3.39	0.96

[a] 95% Confidence Intervals

Table S5. Inhibition of 7-hydroxy-4-(trifluoromethyl)coumarin formation by flupyradifurone (FPF) and its metabolite FPF-OH in a fluorescence assay using BOMFC as a substrate with recombinantly expressed honey bee CYP9Q3, CYP9Q2 and CYP6AQ1.

Compound	CYP9Q3 pIC_{50} [M][a]	95% CI[b]	CYP9Q2 pIC_{50} [M]	95% CI	CYP6AQ1 pIC_{50} [M]	95% CI
FPF	4.89	4.81 - 4.97	3.95	3.87 – 4.01	4.77	4.60 – 4.93
FPF-OH	4.68	4.59 – 4.77	4.16	4.10 – 4.21	<3.5	-

[a] pIC_{50} : negative $\log_{10}$ value of the concentration affecting (inhibiting) enzyme activity by 50%
[b] 95% Confidence Intervals

Table S6. Flupyradifurone toxicity against adult females of transgenic *Drosophila melanogaster* strains expressing honey bee CYP9Q2 and CYP9Q3, respectively, in comparison to a reference control strain with the same genetic background. Expression was driven by the GAL4/UAS system using the GAL4-Hsp70 driver line(McLeman et al., 2020).

Crossing	LD_{50} 72h [µg cm^{-2}]	95% CI	Slope	RR[a]	95% CI[a]	Chi² (df)
Empty x Hsp70	26.5	20.1 – 36.0	6.3	-	-	19.8 (3)
CYP9Q2 x Hsp70	45.3	39.8 – 51.7	8.7	1.7	1.6 – 1.8	9.1 (3)
CYP9Q3 x Hsp70	65.7	55.3 – 81.6	6.82	2.5	2.3 – 2.7	21.8 (4)

[a] Resistance ratio: LD_{50} of transgenic strain divided by the LD_{50} of reference strain (Empty x Hsp70)

Table S7. Inhibition potential (pIC_{50} values) of different azole fungicides against recombinantly expressed honey bee P450s CYP9Q3, CYP9Q2 and CYP6AQ1 using BOMFC as a substrate in a fluorescence-based assay.

Fungicide	CYP9Q3 pIC_{50} [M][a]	95% CI[b]	CYP9Q2 pIC_{50} [M]	95% CI	CYP6AQ1 pIC_{50} [M]	95% CI
Propiconazole	7.14	7.09 – 7.19	6.80	6.75 – 6.85	5.96	5.89 – 6.02
Prothioconazole	4.40	4.33 – 4.46	4.72	4.45 – 4.91	5.41	5.29 – 5.52
Prochloraz	7.89	7.80 – 7.92	7.54	7.49 – 7.60	8.17	8.16 – 8.22

[a] pIC_{50} : negative $\log_{10}$ value of the concentration affecting (inhibiting) enzyme activity by 50%
[b] 95% Confidence Intervals

Table S8. Synergistic effects of azole fungicide pre-treatment on acute contact toxicity of flupyradifurone against honey bees. All tested fungicides were non-toxic at the applied dose of 10 µg bee^{-1}.

Insecticide + synergist	LD_{50} 48h [µg/bee]	95% CI	Slope	SR[a]	Chi² (df)
Flupyradifurone					
Alone	>100	-	-	-	-
+Propiconazole	0.89	0.37 - 1.71	1.16	>112	9.1 (4)
+Prothioconazole	86.8	62.0 – 115	1.48	>1.15	8.3 (6)
+Prochloraz	0.41	0.254 - 0.577	2.52	>243	9.2 (4)

[a] Synergistic ratio: LD_{50} FPF divided by the LD_{50} of FPF + azole

Figure S1. (A) Chemical structure of flupyradifurone with the novel butenolide pharmacophore highlighted in red. (B) Isosurface of the electrostatic potential of flupyradifurone showing the electronegative nature of the butenolide pharmacophore providing selectivity to insect nAChRs. (C) Isosurface of the Fukui functions predicting sites of oxidative attack by an electrophile (green, solid; isolevel 0.05 au), as calculated from DFT (Density functional theory) electron densities (Beck, 2005).

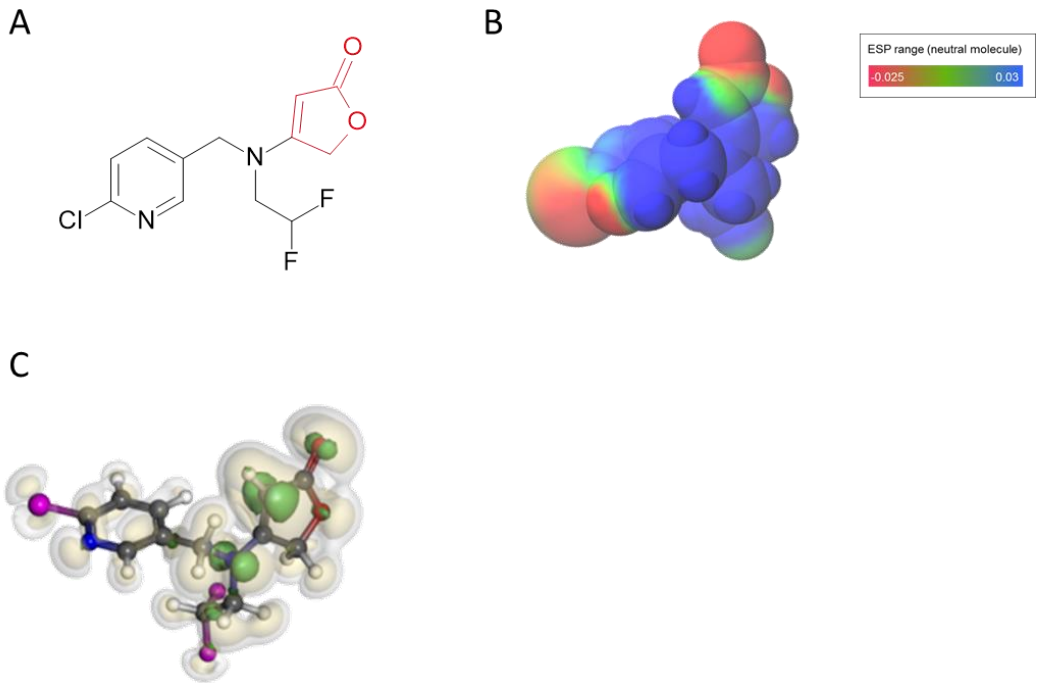


Figure S2. Honey bee cage used to feed worker bees according to OECD oral acute toxicity testing guideline 213 and described under Materials and Methods.

Figure S3. Mass spectrum of flupyradifurone (FPF).

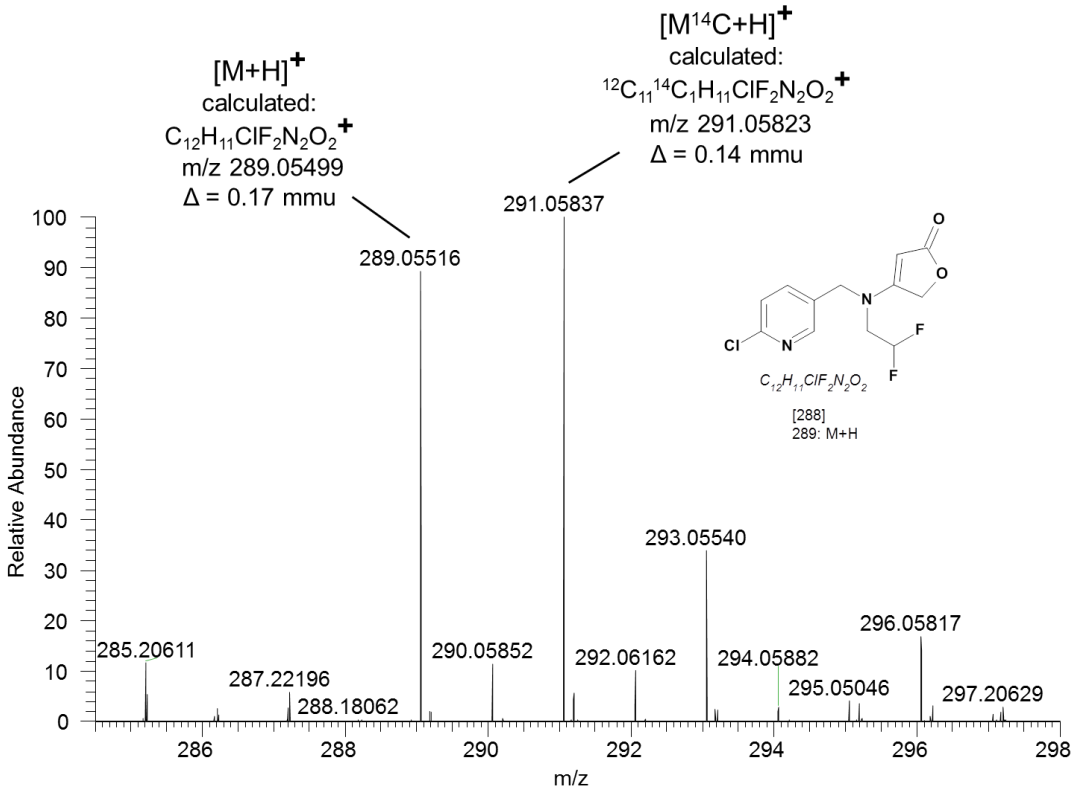


Figure S4. Mass spectrum of hydroxylated flupyradifurone (FPF-OH).

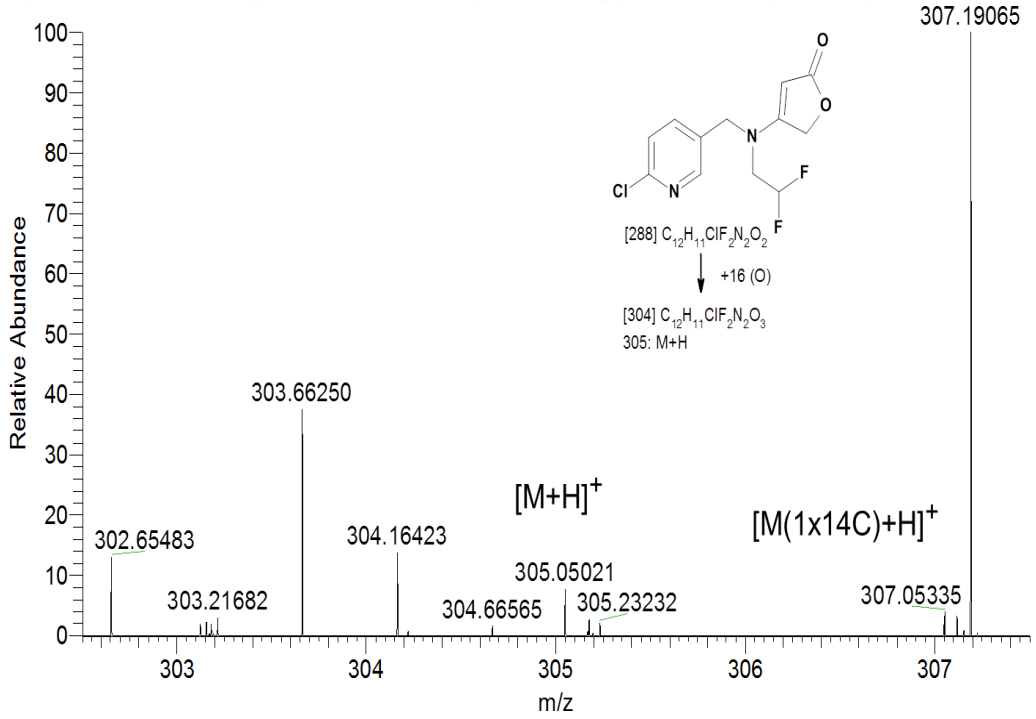


Figure S5. Mass spectrum of flupyradifurone(2,2-difluoroethyl)amino-furanone (FPF-AF).

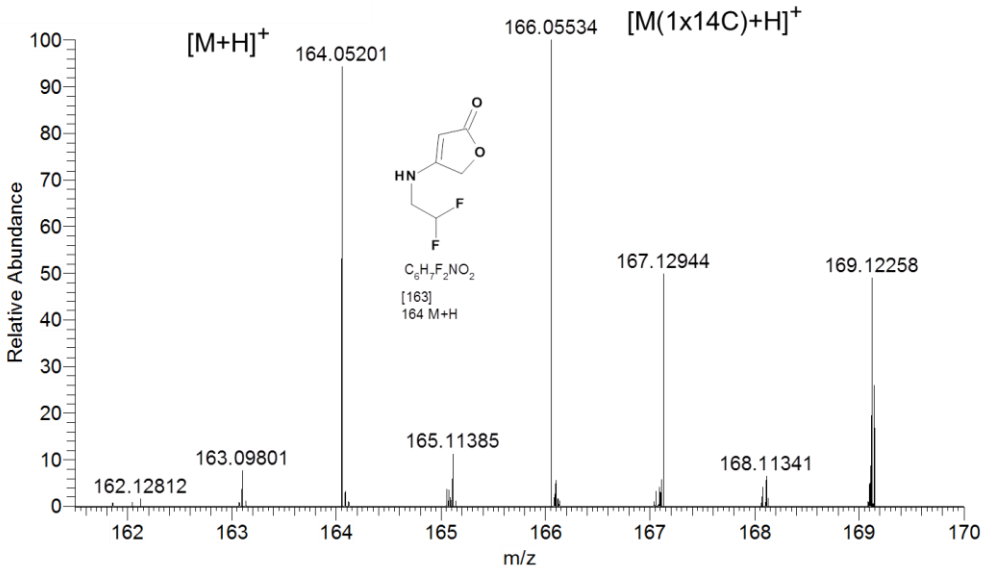


Figure S6. Mass spectrum of flupyradifuroneacetic acid (FPF-AA).

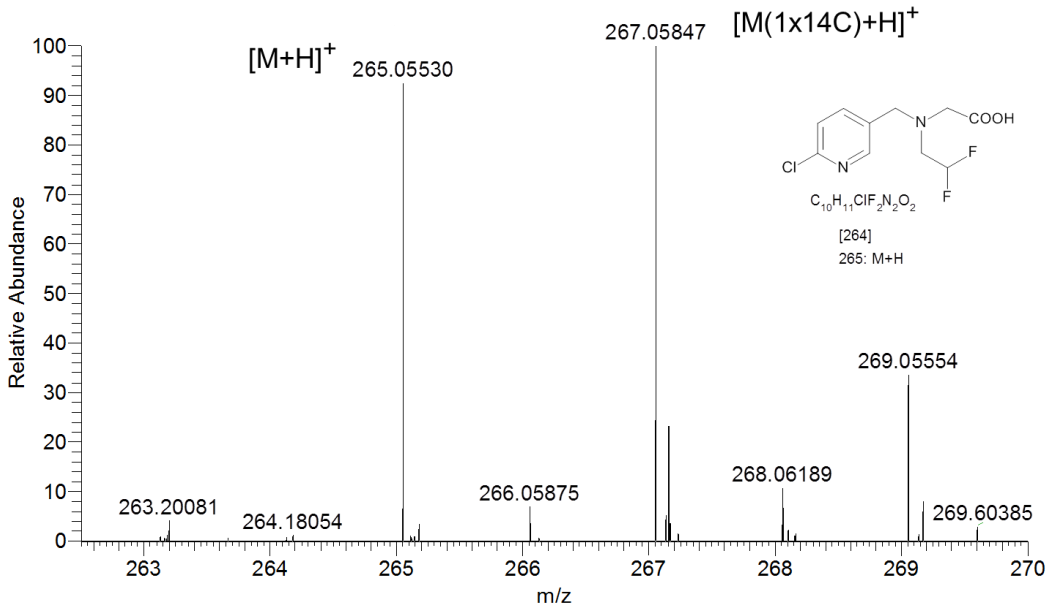


Figure S7. Michaelis-Menten kinetics of BOMFC metabolism leading to 7-hydroxy-4-(trifluoromethyl)coumarin (HC)) by recombinantly expressed honey bee CYP9Q2, CYP9Q3 and CYP6AQ1 analyzed by non-linear regression. Data points are mean values ± SEM (n = 4). CYP9Q2 does apparently not strictly follow single substrate binding kinetics with BOMFC, but sigmoidicity of velocity, suggesting positive cooperativity possibly mediated by two-substrate binding sites. For the sake of simplicity, we consider the Km value given for BFC as an apparent value, as we have refrained from using more complex kinetic models.

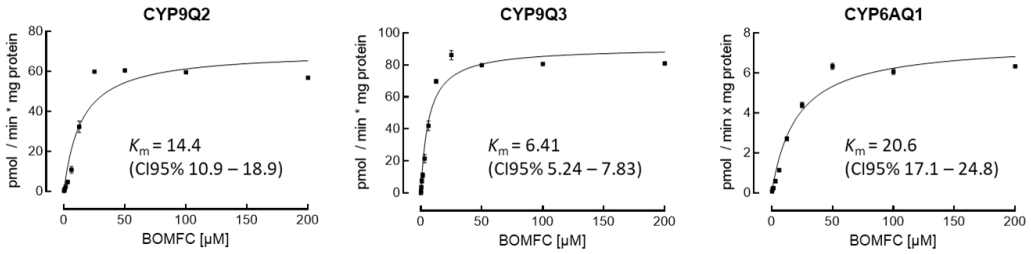
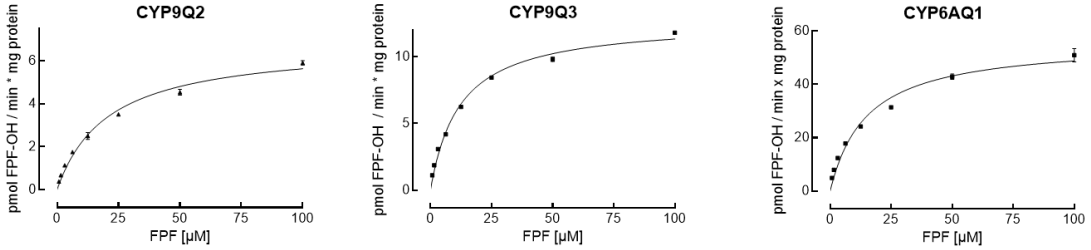


Figure S8. Michaelis-Menten kinetics of flupyradifurone (FPF) hydroxylation by recombinantly expressed honey bee CYP9Q2, CYP9Q3 and CYP6AQ1 analyzed by non-linear regression. Data are mean values ± SEM (n = 3).



K_m = 20.8 µM (CI95% 17.1 – 25.2)
V_{max} = 6.80 (CI95% 6.35 – 7.31)

K_m = 12.0 µM (CI95% 10.4 - 13.9)
V_{max} = 12.7 (CI95% 12.2 – 13.3)

K_m = 15.0 µM (CI95% 11.6 – 19.3)
V_{max} = 56.1 (CI95% 51.7 – 61.0)

Appendix C Supporting data (chapter 4)

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

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[*] corresponding author

Table S1. Steady-state kinetics data for BOMFC metabolism (resulting in 7-hydroxy-4- (trifluoromethyl)coumarin (HC)) by CYP9Q2 and CYP9Q3 co-incubated with increasing concentrations of chlorantraniliprole. T he inhibition type is based on kinetic characteristicsof reversible inhibition models.

BOMFC + insecticide (Inhibition type)	K_m [µM]	95% CI[a]	V_{max} pmol HC[a] / min *mg protein	95% CI[b]	Adjusted R^2
CYP9Q3					
Chlorantraniliprole (mixed)					
w/o	7.8	6.39 to 9.49	94.8	89.9 to 99.9	0.96
10 µM	12.2	9.25 to 16.2	75.7	69.5 to 82.5	0.93
30 µM	14.3	10.8 to 18.8	54.8	50.3 to 59.6	0.93
50 µM	15.3	11.5 to 20.4	45.3	41.4 to 49.5	0.92
CYP9Q2					
Chlorantraniliprole (non-competitive)					
w/o	13.9	10.1 to 19.1	81.4	73.8 to 89.6	0.93
10 µM	15.7	11.6 to 21.4	77.0	69.6 to 85.0	0.91
30 µM	16.7	12.4 to 22.4	63.9	58.2 to 70.1	0.92
50 µM	18.1	13.3 to 24.6	56.3	51.0 to 62.1	0.91

[a] 7-hydroxy-4-trifluoromethyl-coumarin, [b] 95% confidence intervals

Figure S1. Effect of diamides on coumarin fluorescent probe metabolism by CYP9Q2 and CYP6AQ1. (A) Inhibitory potential of the diamide insecticides flubendiamide (FLB) and chlorantraniliprole (CPR) against honey bee CYP9Q2 and (B) CYP6AQ1 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. Data are mean values ± SD (n = 4).

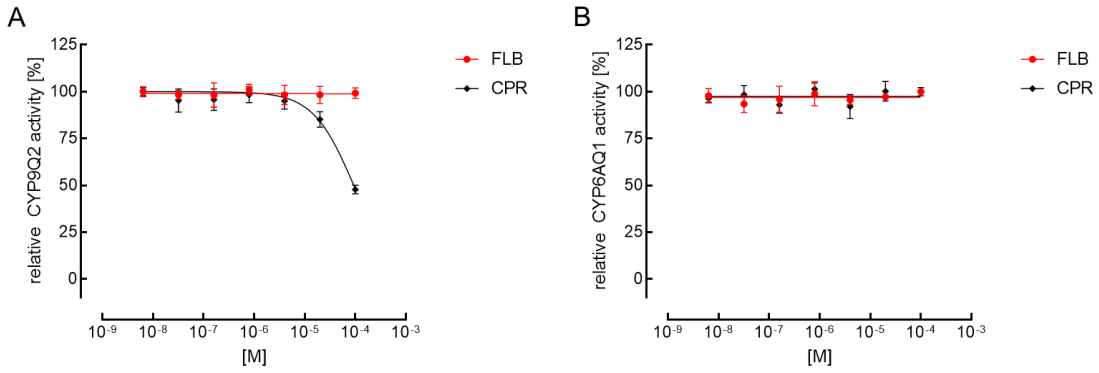


Figure S2. AutoDock Vina docking scores of (A) chlorantraniliprole and (B) flubendiamide at the catalytic center of a CYP9Q3 homology model based on human CYP3A4. Score is defined as –ΔG with ΔG being the predicted binding energy in kcal / mol. RMSD is defined as the square root of the mean of the squared distances between the matched atoms. (C) Flubendiamide's poor fit in the catalytic pocket (score 14.8) indicated by the abundant predicted contacts / clashes with CYP9Q3 residues (green lines).

A

S	Score	RMSD l.b.▲	RMSD u.b.
V	-3.4	0.0	0.0
V	-2.6	1.397	2.757
V	-2.5	1.492	1.583
V	-0.9	3.422	6.673
V	-1.0	3.635	6.151

B

S	Score	RMSD l.b.	RMSD u.b.
V	14.8	0.0	0.0
V	15.1	1.889	2.936
V	15.5	2.153	4.376
V	15.5	2.096	3.749
V	15.9	1.354	1.987

C

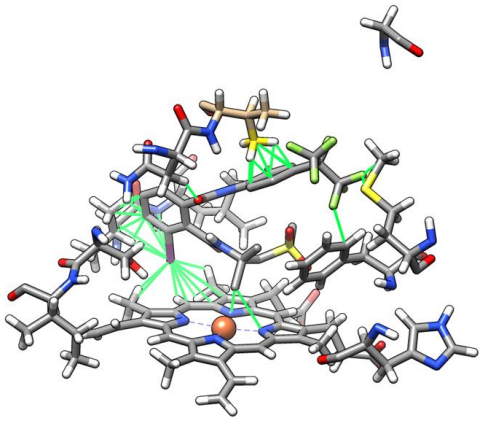
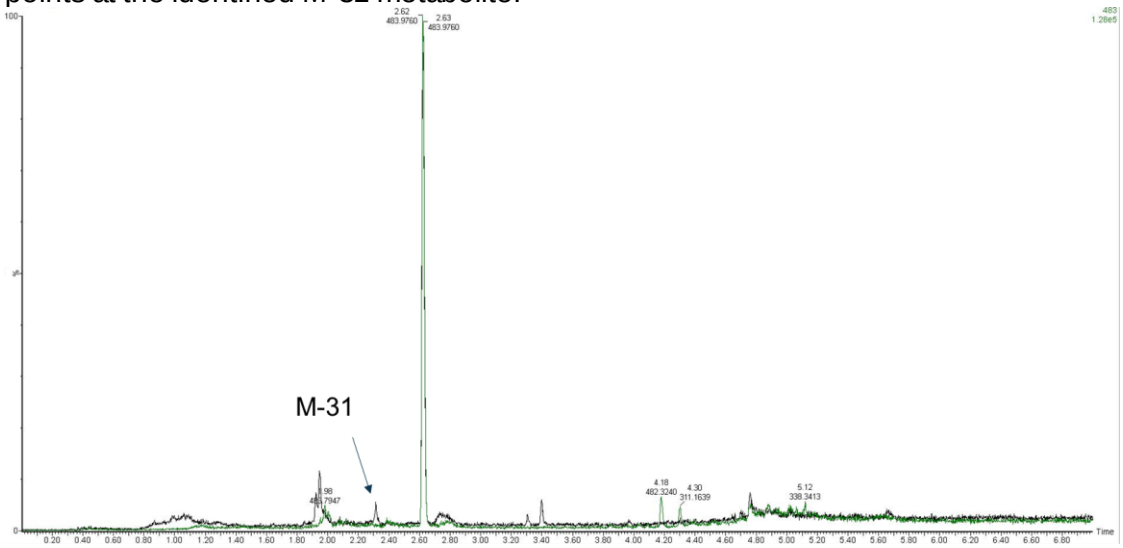


Figure S3. HPLC-MS of a sample obtained from the incubation of chlorantraniliprole (CPR) with functionally expressed honey bee CYP9Q2. The arrow points at the identified M-31 metabolite.



Appendix D Supporting Data (Chapter 5)

Phylogenomic tracking and functional validation of an evolutionary conserved cytochrome P450-based mechanism of detoxification in bees

Julian Haas[1,a,c], Angela Hayward[1,b], Benjamin Buer[c], Frank Maiwald[c], Birgit Nebelsiek[c], Johannes Glaubitz[c], Chris Bass[b,*] and Ralf Nauen[c,*]

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[1] Authors contributed equally
[*] corresponding author

Table S1. Sequence information obtained for 75 bee species (WGS = Whole Genome Sequencing; TSA = Trancsriptome Shotgun Assembly). N/F = not found due to incomplete assembly.

Species name	Family	CYP9Q-like genes	Assembly type	Assembly accession
Ammobates syriacus	Apidae	1	TSA	GBMX00000000
Andrena cineraria	Andrenidae	N/F	TSA	GHFW00000000
Andrena fulva	Andrenidae	N/F	TSA	GHFR00000000
Andrena haemorrhoa	Andrenidae	1	TSA	GHFU00000000
Andrena vaga	Andrenidae	1	TSA	GBLF00000000
Anthidium manicatum	Megachilidae	N/F	TSA	GBOJ00000000
Apis cerana	Apidae	3	WGS	JANR01000000
Apis dorsata	Apidae	3	WGS	AUPE01000000
Apis florea	Apidae	3	WGS	AEKZ01000000
Apis mellifera	Apidae	3	WGS	QIUM02000000
Bomubs bifarius	Apidae	3	WGS	JAAQOX01
Bombus campestris	Apidae	3	WGS	GCA 905332975.1
Bombus cryptarum	Apidae	3	TSA	GHGD01000000
Bombus impatiens	Apidae	3	WGS	AEQM02000000
Bombus pascuorum	Apidae	3	WGS	GHFY01000000
Bombus pyrosoma	Apidae	N/F	WGS	GGGP01000000
Bombus rupestris	Apidae	3	TSA	GBQF01000000
Bombus terrestris	Apidae	3	WGS	AELG01000000
Bombus vancouverensis	Apidae	3	WGS	JAAQRE01
Bombus vosnesenkii	Apidae	3	WGS	JAAQVK01
Camptopoeum sacrum	Andrenidae	3	TSA	GBOI01000000
Ceratina australensis	Apidae	2	WGS	SHCU01000000
Ceratina calcarata	Apidae	4	WGS	LSNX01000000
Ceratina chalybea	Apidae	2	TSA	GBPU01000000
Chelostoma florisomne	Megachilidae	1	TSA	GBPN01000000
Coelioxys conoidea	Megachilidae	N/F	TSA	GBPZ01000000
Colletes cunicularius	Colletidae	1	TSA	GBUJ01000000
Colletes gigas	Colletidae	1	WGS	WUUM01
Dasypoda hirtipes	Melittidae	1	TSA	GBMP01000000
Dioxys cincta	Megachilidae	1	TSA	GBPP01000000
Dufourea dentiventris	Halictidae	2	TSA	GBTY01000000
Dufourea novaeangliae	Halictidae	4	WGS	LGHO01000000
Epeolus variegatus	Apidae	2	TSA	GBPW01000000
Eucera nigrescens	Apidae	1	TSA	GBPG01000000
Eucera plumigera	Apidae	1	TSA	GBLY01000000
Eucera syriaca	Apidae	1	TSA	GBLZ01000000
Eufriesea mexicana	Apidae	2	WGS	LLKC01000000
Euglossa cordata	Apidae	1	TSA	SRX040737
Euglossa dilemma	Apidae	2	WGS	NIJG01000000
Eulaema nigrita	Apidae	N/F	TSA	SRX040736
Friesomelitta varia	Apidae	5	WGS	WNWW01
Habropoda laboriosa	Apidae	1	WGS	LHQN01000000
Halictus quadricinctus	Halictidae	2	TSA	GBQP01000000
Heriades truncorum	Megachilidae	2	TSA	GBQK01000000
Heterotrigona itama	Apidae	1	TSA	ERX4231413
Hylaeus variegatus	Colletidae	1	TSA	GBPS01000000
Lasioglossum albipes	Halictidae	2	WGS	ANOB01000000
Lasioglossum xanthopus	Halictidae	1	TSA	GBPT01000000
Lepidotrigona ventralis	Apidae	2	WGS	NIPQ01000000
Lithurgus chrysurus	Megachilidae	N/F	TSA	GBMJ01000000
Macropis fulvipes	Melittidae	1	TSA	GBNX01000000
Megachile rotundata	Megachilidae	0	WGS	AFJA01000000
Megachile willughbiella	Megachilidae	0	TSA	GBQN01000000
Megalopta genalis	Halictidae	4	WGS	GELL01000000
Melipona quadrifasciata	Apidae	2	WGS	LIRP01000000
Melitta haemorrhoidalis	Melittidae	1	TSA	GBVK01000000
Nomada lathburiana	Apidae	1	TSA	GBLA01000000
Nomia diversipes	Halictidae	1	TSA	GBWP00000000.1
Nomia melanderi	Halictidae	1	WGS	REGV01000000
Osmia bicornis	Megachilidae	2	WGS	MPJT01000000
Osmia cornuta	Megachilidae	2	TSA	GHFP01000000
Osmia lignaria	Megachilidae	2	WGS	JAAOZW01
Panurgus dentipes	Andrenidae	1	TSA	GBME01000000
Sphecodes albilabris	Halictidae	N/F	TSA	GBKZ01000000
Stelis punctulatissima	Megachilidae	N/F	TSA	GBWV01000000
Systropha curvicornis	Halictidae	1	TSA	GBWF01000000
Tetragonula carbonaria	Apidae	2	WGS	GBTL01000000
Tetragonula clypearis	Apidae	1	WGS	WIUT01
Tetragonula davenporti	Apidae	2	WGS	WIUW01
Tetragonula hockingsi	Apidae	3	WGS	WIUV01
Tetragonula mellipes	Apidae	4	WGS	WINE01
Tetralonia malvae	Apidae	1	TSA	GBNI00000000.1
Tetraloniella nigriceps	Apidaea	1	TSA	SRX642804
Thyreus orbatus	Apidae	1	TSA	GBLR01000000
Xylocopa violacea	Apidae	1	TSA	GBUM01000000

Table S2. Bee species included in the bee phylogenetic tree of clade 3 of cytochrome P450s.

Species name	Family	CYP336	CYP6	CYP9	CYP9Q-like genes	Assembly type	Assembly accession
Andrena fulva	Andrenidae	4	10	10	ND	TSA	GHFR00000000
Andrena haemorrhoa	Andrenidae	3	9	8	1	TSA	GHFU00000000
Andrena vaga	Andrenidae	3	7	5	1	TSA	GBLF00000000
Apis cerana	Apidae	1	18	7	3	WGS	JANR01000000
Apis dorsata	Apidae	1	17	6	3	WGS	AUPE01000000
Apis florea	Apidae	1	17	5	3	WGS	AEKZ01000000
Apis mellifera	Apidae	1	22	7	3	WGS	QIUM02000000
Bombus impatiens	Apidae	2	23	5	3	WGS	AEQM02000000
Bombus terrestris	Apidae	4	21	7	3	WGS	AELG01000000
Colletes cunicularius	Colletidae	4	14	7	1	TSA	GBUJ01000000
Dufourea novaeangliae	Halictidae	4	17	7	4	WGS	LGHO01000000
Eucera nigrescens	Apidae	1	6	3	1	TSA	GBPG01000000
Eufriesea mexicana	Apidae	2	19	8	2	WGS	LLKC01000000
Habropoda laboriosa	Apidae	1	14	5	1	WGS	LHQN01000000
Lasioglossum xanthopus	Halictidae	1	15	9	1	TSA	GBPT01000000
Macropis fulvipes	Melittidae	1	6	5	1	TSA	GBNX01000000
Megachile rotundata	Megachilidae	3	20	7	0	WGS	AFJA01000000
Melipona quadrifasciata	Apidae	6	24	9	2	WGS	LIRP01000000
Melitta haemorrhoidalis	Melittidae	1	8	5	1	TSA	GBVK01000000
Nomada lathburiana	Apidae	1	10	5	1	TSA	GBLA01000000
Nomia melanderi	Halictidae	1	12	12	1	WGS	REGV01000000
Osmia bicornis	Megachilidae	3	21	9	2	WGS	MPJT01000000
Tetragonula carbonaria	Apidae	1	19	8	2	WGS	GBTL01000000
Xylocopa violacea	Apidae	2	11	6	1	TSA	GBUM01000000

Table S3. Bee species from which candidate P450s were functionally expressed and some of their life traits.

Species	Family	Lifestyle	Diet preference	Nesting Behavior
Apis mellifera	Apidae	eusocial	Polylectic	Cavity nesters above-ground
Apis cerana	Apidae	eusocial	Polylectic	Cavity nesters above-ground
Apis dorsata	Apidae	eusocial	Polylectic	Cavity nesters above-ground
Apis florea	Apidae	eusocial	Polylectic	Open nesting above-ground
Eufriesea mexicana	Apidae	Putatively social	Unknown	Unknown
Melipona quadrifasciata	Apidae	eusocial	Polylectic	Cavity nesters above-ground
Tetragonula carbonaria	Apidae	eusocial	Polylectic	Cavity nesters above-ground
Habropoda laboriosa	Apidae	Solitary	Oligolectic	Soil excavators
Xylocopa violacea	Apidae	Solitary	Polylectic	Wood excavators
Melitta haemorrhoidalis	Mellitidae	Solitary	Oligolectic	Soil excavators
Colletes cunicularius	Colletidae	Solitary	Oligolectic	Soil excavators
Andrena haemorrhoa	Andrenidae	Solitary	Polylectic	Soil excavators
Lasioglossum xanthopus	Halictidae	Solitary	Polylectic	Soil excavators
Bombus impatiens	Apidae	Social	Polylectic	Cavity nesters below-ground
Macropis fulvipes	Mellitidae	Solitary	Oligolectic	Soil excavators
Dufourea novaeangliae	Halictidae	Solitary	Oligolectic	Soil excavators
Eucera nigrescens	Apidae	Solitary	Oligolectic	Soil excavators
Nomada lathburiana	Apidae	Cuckoo	Polylectic (nectar)	-
Nomia melanderi	Apidae	Solitary	Polylectic	Soil excavators
Andrena vaga	Andrenidae	Solitary	Polylectic	Soil excavators

Table S4. CYP3 clan P450 genes used in this study.

Species	Gene Name	Accession number	Nucleotide Sequence	Length (AA[a])	Molecular Weight (kDa)*	Isoelectric Point*
Apis mellifera	CYP9Q2	XP_392000		532	60.952	8.52
Apis mellifera	CYP9Q3	XP_006562363		517	58.891	8.58
Apis cerana	CYP9Q3	XP_016922294.2		517	59.246	8.83
Apis dorsata	CYP9Q3	XP_006613022.1		515	58.932	8.32
Apis florea	CYPQ2	XP_012347837.2		528	60.968	8.07
Apis florea	CYP9Q19	XP_031775226.1		531	60.772	8.21
Eutriesea mexicana	CYP9Q8	XP_017758640.1		531	60.989	8.17
Eufriesea mexicana	CYP9Q7	XP_017758639.1		521	59.989	8.23
Melipona quadrifasciata	CYP9Q10	KOX69484.1		520	59.464	8.83
Tetragonula carbonaria	CYP9Q17		GBTL01077204.1	522	59.219	8.45
Tetragonula carbonaria	CYP9Q16		GBTL01078920.1	519	59.509	8.43
Habropoda laboriosa	CYP9Q9	XP_017794730.1		529	60.432	9.32
Xylocopa violacea	CYP9Q18		GBUM01016761.1	526	59.996	9.16
Melitta haemorrhoidalis	CYP9FU3		GBVK01019397.1	524	59.638	8.44
Colletes cunicularius	CYP9FZ2		GBUJ01004521.1	523	59.269	8.38
Andrena haemorrhoa	CYP9FT1		GHGA01007590.1	522	58.986	8,45
Lasioglossum xanthopus	CYP9DL6		GBPT01021710.1	522	59.783	7.96
Bombus impatiens	CYP9Q6	XP_033174303.1		525	60.288	8.29
Bombus impatiens	CYP9Q5	XP_003486050.1		525	59.799	7.97
Bombus impatiens	CYP9Q4	XP_033174303.1		518	59.551	9.11
Macropis fulvipes	CYP9FU2		GBNX01015534.1	511	58.229	9.00
Dufourea novaeangliae	CYP9DL4	XP_015439019.1		522	59.543	8.36
Eucera nigrescens	CYP9Q15		GBPG01002643.1	524	60.289	9.12
Nomada lathburiana	CYP9Q14		GBLA01022698.1	513	58.820	8.18
Nomia melanderi	CYP9DL5	XP_031837097.1		522	59.818	8.21
Andrena vaga	CYP9FT2		GBLF01017912.1	513	58.464	9.18

[a] AA = amino acids
* Calculated by Geneious v10.2.3 (Biomatters Ltd., New Zealand)

Table S5. Coumarin activity of selected CYP9Q-like bee P450s. Values are expressed as pmol product / mg protein * min (n = 4).

	BOMFC		**BFC**		**EFC**		**MFC**		**EC**		**PC**	
P450	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
AmCYP9Q2	38.92	1.43	9.71	0.32	1.40	0.16	0.41	0.02	1.88	0.21	7.16	0.43
AmCYP9Q3	100.38	3.92	54.43	1.11	11.89	0.33	2.41	0.07	12.25	0.37	6.24	0.89
AcCYP9Q3	3.21	0.57	1.72	0.06	0.97	0.03	0.42	0.02	0.68	0.04	0.34	0.07
AdCYP9Q3	29.39	4.52	22.15	1.30	69.01	13.79	7.91	0.34	22.88	1.24	3.40	0.20
EmCYP9Q8	350.54	22.70	135.74	1.73	69.47	10.01	3.28	0.13	27.72	0.61	11.49	1.16
EmCYP9Q7	9.00	0.18	3.12	0.13	1.83	0.11	0.40	0.02	0.59	0.05	0.62	0.06
MqCYP9Q10	73.91	3.12	8.58	0.16	9.07	1.72	0.60	0.05	1.76	0.26	4.00	0.09
TcCYP9Q16	29.21	4.29	17.29	1.11	11.41	0.33	1.32	0.21	1.86	0.07	4.01	0.03
TcCYP9Q17	83.43	4.76	22.62	1.24	31.07	1.32	1.03	0.08	1.19	0.22	1.73	0.09
HlCYP9Q9	73.08	5.38	12.11	0.74	12.39	0.16	1.08	0.05	5.29	0.11	3.07	0.16
XvCYP9Q18	137.93	3.81	42.49	0.79	47.35	1.72	2.49	0.02	7.95	0.34	2.61	0.23
MhCYP9FU3	173.60	4.66	28.20	2.82	15.37	0.37	1.36	0.04	12.36	0.67	1.37	0.06
CcCYP9FZ2	66.99	1.16	15.42	1.23	16.24	0.92	0.84	0.07	2.18	0.12	3.05	0.20
AhCYPFT1	60.52	1.72	68.55	1.93	20.05	0.71	0.87	0.08	1.10	0.04	7.32	0.30
LxCYP9DL6	4.95	0.15	-0.01	0.03	0.58	0.02	0.25	0.01	0.98	0.06	1.07	0.08
BiCYP9Q6	1.09	0.07	0.62	0.12	0.40	0.02	0.30	0.03	0.22	0.04	0.26	0.06
BiCYP9Q5	48.67	1.06	8.12	0.78	3.76	0.06	0.44	0.04	2.88	0.23	7.80	0.19
BiCYP9Q4	7.30	0.25	1.03	0.05	0.73	0.02	0.21	0.01	0.64	0.05	0.60	0.03
MfCYP9FU2	49.00	1.40	42.85	2.49	13.13	0.62	1.17	0.06	1.50	0.10	3.42	0.28
DnCYP9DL4	110.03	0.45	43.32	2.55	1.94	0.05	1.24	0.08	1.64	0.08	13.64	1.51
AfCYP9Q2	216.37	5.32	2.99	0.18	9.67	0.83	1.13	0.04	6.44	0.27	11.68	0.91
AfCYP9Q19	6.88	0.19	-0.19	0.04	0.34	0.02	0.31	0.02	0.45	0.07	0.43	0.04
EnCYP9Q15	224.51	5.31	11.64	0.42	19.89	2.19	1.29	0.04	5.19	0.17	0.46	0.04
NlCYP9Q14	103.12	3.72	25.68	0.87	13.02	0.90	1.37	0.07	3.58	0.10	4.53	0.12
NmCYP9DL5	62.01	2.09	7.24	0.18	5.52	0.55	2.63	0.06	15.37	1.84	4.82	0.10
AvCYP9FT2	63.60	1.06	48.70	1.08	14.18	1.27	0.89	0.07	1.86	0.11	11.86	0.75
Empty Virus	0.83	0.04	0.05	0.06	0.15	0.03	0.26	0.07	0.06	0.03	0.21	0.01

Table S6. pmol P450 content per mg protein determined by CO-difference spectra according to Guengerich et al. (2009).

Species	Gene Name	pmol per mg protein
Apis mellifera	CYP9Q2	115.30
Apis mellifera	CYP9Q3	32.10
Apis cerana	CYP9Q3	3.11
Apis dorsata	CYP9Q3	20.74
Apis florea	CYPQ2	77.15
Apis florea	CYP9Q19	33.89
Eufriesea mexicana	CYP9Q8	35.63
Eufriesea mexicana	CYP9Q7	25.15
Melipona quadrifasciata	CYP9Q10	74.46
Tetragonula carbonaria	CYP9Q17	36.32
Tetragonula carbonaria	CYP9Q16	77.71
Habropoda laboriosa	CYP9Q9	56.04
Xylocopa violacea	CYP9Q18	109.76
Melitta haemorrhoidalis	CYP9FU3	28.34
Colletes cunicularius	CYP9FZ2	19.98
Andrena haemorrhoa	CYP9FT1	n.d.
Lasioglossum xanthopus	CYP9DL6	31.38
Bombus impatiens	CYP9Q6	n.d.
Bombus impatiens	CYP9Q5	59.64
Bombus impatiens	CYP9Q4	28.67
Macropis fulvipes	CYP9FU2	88.31
Dufourea novaeangliae	CYP9DL4	27.21
Eucera nigrescens	CYP9Q15	37.27
Nomada lathburiana	CYP9Q14	79.08
Nomia melanderi	CYP9DL5	123.40
Andrena vaga	CYP9FT2	8.68

Table S7. Steady-state kinetics data for BOMFC metabolism resulting in 7-hydroxy-4-(trifluoromethyl)coumarin (HC) by the selected recombinantly expressed bee P450s co-incubated with increasing concentrations of flupyradifurone (FPF). The inhibition type is based on kinetic characteristics of reversible inhibition models according to Fowler and Zhang (*The AAPS Journal* **10** (2008) 410-424).

P450 ± [FPF] (Inhibition type)	*K*m [µM]	CI[a]	*V*max pmol HC / min mg protein	95% CI[a]	Adjusted R2
CYP9Q18 (non-competitive)					
w/o	15.2	11.6 to 19.8	171	158 to 186	0.94
10 µM	14.2	10.9 to 18.6	155	142 to 168	0.93
30 µM	15.2	11.7 to 19.7	134	123 to 145	0.94
100 µM	16.9	12.9 to 22.3	92	84.5 to 100	0.93
CYP9FU3 (non-competitive)					
w/o	8.16	6.36 to 10.4	189	176 to 202	0.94
10 µM	8.71	6.78 to 11.2	190	177 to 203	0.94
30 µM	9.93	7.71 to 12.8	183	170 to 196	0.94
100 µM	12.2	9.27 to 16.1	155	143 to 168	0.93
CYP9FZ2 (non-competitive)					
w/o	14.7	11.1 to 19.4	69.0	63.3 to 75.1	0.93
10 µM	15.8	11.9 to 21.0	63.0	57.6 to 68.8	0.93
30 µM	17.3	13.2 to 22.6	56.0	51.4 to 61.1	0.93
100 µM	20.5	15.6 to 27.1	38.5	35.1 to 42.2	0.93
CYP9DL4 (non-competitive)					
w/o	11.0	9.27 to 13.0	78.0	74.4 to 81.8	0.97
10 µM	12.9	10.7 to 15.5	82.5	77.6 to 87.8	0.97
30 µM	12.7	10.4 to 15.4	74.9	70.7 to 79.3	0.96
100 µM	18.3	15.1 to 22.3	65.0	61.5 to 68.8	0.97
CYP9FT2 (non-competitive)					
w/o	7.43	5.65 to 9.74	50.6	46.9 to 54.4	0.92
10 µM	10.1	8.02 to 12.8	49.9	46.7 to 53.3	0.95
30 µM	10.4	8.18 to 13.2	46.5	43.4 to 49.8	0.95
100 µM	10.8	8.26 to 14.1	42.2	39.0 to 45.6	0.93

Table S8. Enzyme assays (2h) and subsequent UPLC-MS/MS analysis resulting in a significant gap between TCP-depletion and TCP-OH formation, suggesting additional metabolites not covered (values in pmol per mg protein) (unpaired t-test $p < 0.05$).

Enzyme	P value	Mean of TCP Depletion	Mean of TCP-OH
CYP9FU2 M. fulvipes	0,000039	5191	450,1
CYP9Q3 A. mellifera	0,000088	9092	6728
CYP9FU3 M. haemorrhoidalis	0,000242	5060	1302
CYP9Q15 E. nigrescens	0,001659	3177	441,5
CYP9Q14 N. lathburiana	0,003516	2121	255,2
AdCYP9Q3 A. dorsata	0,004755	3908	1658
CYP9Q18 X. violacea	0,005428	8323	2285
CYP9FT1 A. vaga	0,007835	1617	99,42
CYP9Q16 T. carbonaria	0,008379	1309	346,0
CYP9FZ2 C. cunicularius	0,010265	7683	7110
CYP9DL4 D. novaeangliae	0,012953	2432	1734
AfCYP9Q2 A. florea	0,015532	1819	318,2
CYP9Q8 E. mexicana	0,020614	6012	4615
CYP9Q17 T. carbonaria	0,024269	1168	51,48
CYP9DL5 N. melanderi	0,043334	3204	2498
CYP9Q10 M. quadrifasciata	0,044054	2745	1549

Table S9. Ion transitions and linear range of insecticide and their metabolites quantified via UPLC-MS/MS.

Compound	Ion Transition	Linearity [ng / mL]
TCP	253 > 187	0.2 - 100
TCP-OH	269 > 202	0.1 - 100
IMD	256 > 175	0.1 - 200
IMD-OH	272 > 191	0.5 - 200
FPF	289 > 126	0.1 - 100
FPF-AF	164 > 146	0.3 - 50
FPF-DFEA	207 > 126	0.5 - 100
FPF-AA	265 > 126	0.2 - 200
FPF-OH	305 > 126	0.1 - 100

Figure S1. Protein sequence identity matrix (%) of the selected CYP9Q-related enzymes.

	CYP9Q3 Apis mellifera	CYP9Q2 Apis mellifera	A. dorsata CYP9Q3	A. cerana CYP9Q3	A. florea CYP9Q19	A. florea CYP9Q2	B. impatiens CYP9Q6	B. impatiens CYP9Q5	B. impatiens CYP9Q4	E. mexicana CYP9Q8	E. mexicana CYP9Q7	T. carbonaria CYP9Q17	T. carbonaria CYP9Q16	M. quadrifasciata CYP9Q10	N. lathburiana CYP9Q14	H. laboriosa CYP9Q9	X. violacea CYP9Q18	E. nigrescens CYP9Q15	L. xanthopus CYP9DL6	N. melanderi CYP9DL5	D. novaeangliae CYP9DL4	C. cunicularius CYP9FZ2	M. haemorrhoidalis CYP9FU2	M. fulvipes CYP9FU3	A. haemorrhoa CYP9FT1	A. vaga CYP9FT2
A. Mellifera CYP9Q3	X	58	88	88	56	55	56	57	56	56	58	56	60	57	52	52	55	51	44	46	48	50	47	48	46	45
A. Mellifera CYP9Q2	58	X	60	59	87	84	59	62	60	57	57	60	62	63	56	55	55	58	45	48	49	52	50	49	47	46
A. dorsata CYP9Q3	88	60	X	91	57	57	56	57	56	58	58	57	59	57	52	51	55	52	44	48	48	49	52	48	45	45
A. cerana CYP9Q3	88	59	91	X	56	56	56	58	57	56	57	58	61	57	51	51	54	51	45	46	47	49	49	48	48	46
A. florea CYP9Q19	56	87	57	56	X	90	58	60	59	56	55	57	57	58	54	53	55	57	46	47	50	50	50	49	46	45
A. florea CYP9Q2	55	84	57	56	90	X	57	60	59	54	53	56	57	58	54	53	54	56	47	48	51	49	49	48	44	43
B. impatiens CYP9Q6	56	59	56	56	58	57	X	62	61	60	60	59	62	61	56	55	55	54	49	51	50	50	48	50	47	47
B. impatiens CYP9Q5	57	62	57	58	60	60	62	X	92	60	59	66	69	71	59	55	58	58	50	50	52	52	50	48	48	49
B. impatiens CYP9Q4	56	60	56	57	59	59	61	92	X	60	58	65	68	68	59	54	58	57	50	50	52	52	51	49	48	48
E. mexicana CYP9Q8	56	57	58	56	56	54	60	60	60	X	78	58	62	62	56	54	56	54	45	51	49	52	49	48	47	47
E. mexicana CYP9Q7	58	57	58	57	55	53	60	59	58	78	X	56	58	57	55	54	56	54	46	49	49	51	51	50	48	47
T. carbonaria CYP9Q17	56	60	57	58	57	56	59	66	65	58	56	X	78	76	55	52	54	53	47	46	49	49	46	46	45	46
T. carbonaria CYP9Q16	60	62	59	61	57	57	62	69	68	62	58	78	X	87	58	55	57	56	48	50	51	52	49	49	48	49
M. quadrifasciata CYP9Q10	57	63	57	57	58	58	61	71	68	62	57	76	87	X	57	55	57	56	46	48	50	51	49	47	48	48
N. lathburiana CYP9Q14	52	56	52	51	54	54	56	59	59	56	55	55	58	57	X	61	59	58	48	47	51	53	53	50	48	48
H. laboriosa CYP9Q9	52	55	51	51	53	53	55	55	54	54	54	52	55	55	61	X	56	56	48	49	50	53	52	49	47	48
X. violacea CYP9Q18	55	55	55	54	55	54	55	58	58	56	56	54	57	57	59	56	X	59	48	49	51	51	52	50	49	48
E. nigrescens CYP9Q15	51	58	58	57	56	57	59	66	62	58	54	56	56	58	56	56	59	X	48	47	50	50	53	50	49	49
L. xanthopus CYP9DL6	44	45	44	44	45	46	47	48	48	47	47	48	48	48	48	48	48	48	X	54	54	52	53	53	53	48
N. melanderi CYP9DL5	46	48	48	46	47	48	51	50	50	49	49	50	48	50	51	50	49	47	54	X	57	57	53	51	50	47
D. novaeangliae CYP9DL4	48	49	48	49	47	50	48	50	52	49	49	49	51	48	47	50	51	50	54	57	X	55	54	54	50	49
C. cunicularius CYP9FZ2	50	52	49	49	50	49	50	51	52	52	49	51	49	51	53	53	51	53	53	51	54	X	58	55	54	55
M. haemorrhoidalis CYP9FU2	47	50	48	48	50	49	48	50	51	49	51	46	49	49	53	52	52	53	53	50	54	58	X	61	54	54
M. fulvipes CYP9FU3	48	49	48	48	49	48	50	48	49	48	50	46	49	47	50	49	50	50	53	50	50	55	61	X	51	52
A. Haemorrhoa CYP9FT1	46	47	45	45	46	44	47	48	48	47	48	45	48	48	47	49	49	49	49	47	49	54	54	51	X	88
A. vaga CYP9FT2	45	46	45	46	45	43	47	49	49	47	47	46	49	48	48	48	48	49	48	47	48	55	54	52	88	X

Figure S2. Alignment of recomabinantly expressed CYP9Q related enzymes with focus on the five signature P450 motifs (red) in insects as described by Feyereisen (2019) with deviations in single amino acid residues highlighted in green. The transmembrane region (TMR) is depicted in yellow, and the I-helix region is marked in blue.

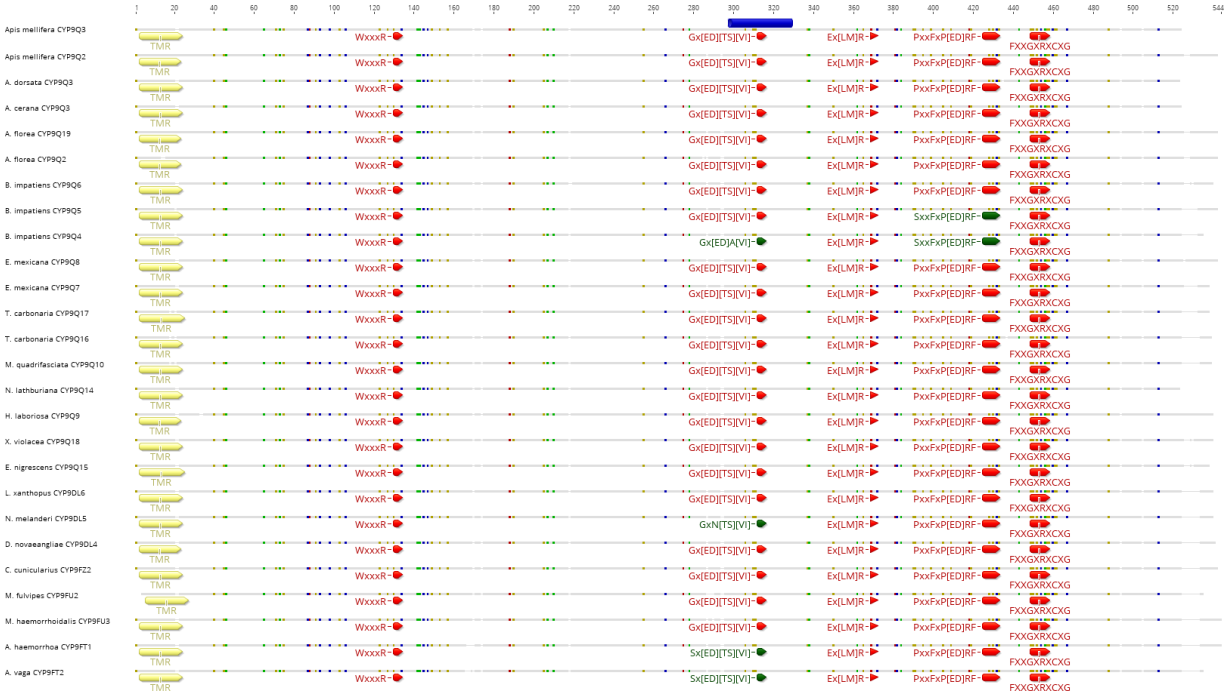

Figure S3. Correlation matrix (pearson r) of the coumarin model substrate profile of the investigated CYP9Q-related enzymes.

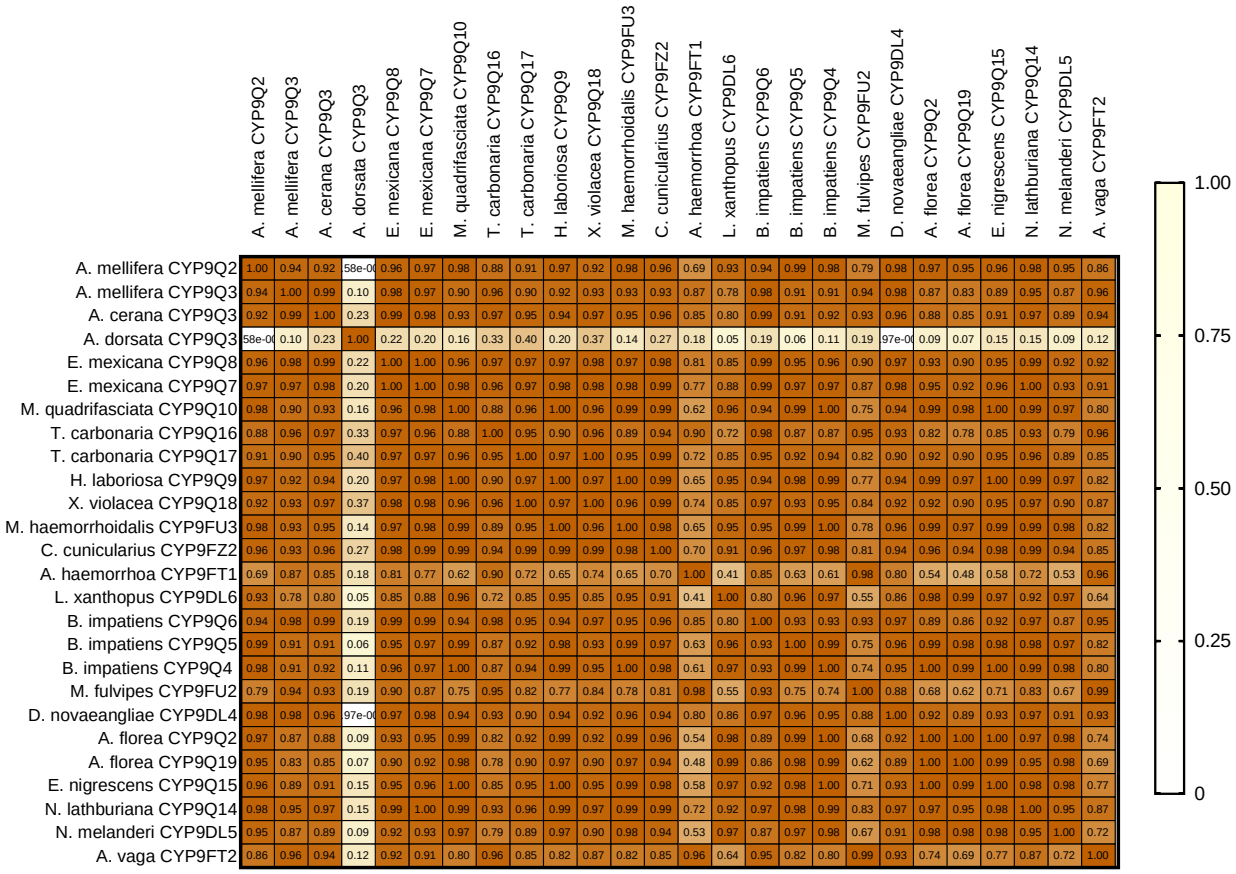

Figure S4. Fluorescence probe assay with BOMFC and increasing concentrations of flupyradifurone (FPF). Hanes-Woolf plot (large inset) shows allosteric behavior and heterotropic interaction between FPF and BOMFC. [S] = BOMFC concentration in µM, V =velocity in pmol product / min x mg protein.