Science of the Total Environment 915 (2024) 170174

Contents lists available at ScienceDirect

Science of the Total Environment

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

Review Article

The molecular determinants of pesticide sensitivity in bee pollinators

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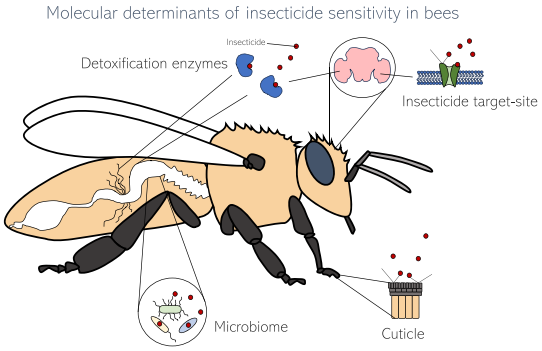
HIGHLIGHTS

- We review research on the molecular determinants of pesticide sensitivity in bees.
- A variety of molecular mechanisms influence bee sensitivity to insecticides.
- These include detoxification enzymes, insecticide targets, the cuticle and microbiome.
- Knowledge of insecticide sensitivity determinants can inform pesticide risk assessment.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Henner Hollert

Keywords:
Bee
Pesticide
Insecticide
Risk assessment
P450

ABSTRACT

Bees carry out vital ecosystem services by pollinating both wild and economically important crop plants. However, while performing this function, bee pollinators may encounter potentially harmful xenobiotics in the environment such as pesticides (fungicides, herbicides and insecticides). Understanding the key factors that influence the toxicological outcomes of bee exposure to these chemicals, in isolation or combination, is essential to safeguard their health and the ecosystem services they provide. In this regard, recent work using toxicogenomic and phylogenetic approaches has begun to identify, at the molecular level, key determinants of pesticide sensitivity in bee pollinators. These include detoxification systems that convert pesticides to less toxic forms and key residues in insecticide target-sites that underlie species-specific insecticide selectivity. Here we review this emerging body of research and summarise the state of knowledge of the molecular determinants of pesticide sensitivity in bee pollinators. We identify gaps in our knowledge for future research and examine how an understanding of the genetic basis of bee sensitivity to pesticides can be leveraged to, a) predict and avoid negative bee-pesticide interactions and facilitate the future development of pest-selective bee-safe insecticides, and b) inform traditional effect assessment approaches in bee pesticide risk assessment and address issues of ecotoxicological concern.

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https://doi.org/10.1016/j.scitotenv.2024.170174
Received 14 November 2023; Received in revised form 9 January 2024; Accepted 13 January 2024
Available online 19 January 2024

1. Introduction

Bees are a group of hymenopteran insects comprising approximately 20,000 described species (Michener, 2007). Bees are the world's most economically and environmentally important group of insect pollinators with 35 % of global food production dependent on their pollination services (Klein et al., 2007). Unfortunately, many bee species have been declining over recent decades threatening these essential ecosystem services (Potts et al., 2016; Sánchez-Bayo and Wyckhuys, 2019; Wagner et al., 2021). The possible reasons for these declines are complex and include habitat loss, climate change, parasites and pathogens, and pesticides (Wagner et al., 2021). In the case of pesticides, as insects, bee species may be particularly vulnerable to insecticides, and certain insecticides (*e.g.* certain neonicotinoids) have been banned in Europe after a review of their risk to bee health by the European Food Safety Authority (EFSA) (Bass and Field, 2018). However, as a group, insecticides currently play a key role in controlling insect disease vectors and securing quality and yield in plant production, and will remain an important component of integrated pest and vector management programs in the future. Given this, to safeguard the health of bee pollinators, it is essential that we understand the key factors that influence the toxicological outcome of insect exposure to these chemicals.

While bees have only been exposed to human-made pesticides over the recent past (last 80 years) they have co-evolved with plants and fungi which produce a range of xenobiotics, including plant allelochemicals and mycotoxins. This has led to the evolution of sophisticated systems that allow bees to detoxify or circumvent the natural xenobiotics they encounter in their environment (Berenbaum and Johnson, 2015; Gong and Diao, 2017; Johnson, 2015). These systems may have the potential to be co-opted to protect bees from certain synthetic pesticides if there is sufficient structural similarity between natural and synthetic xenobiotics. This is made more likely by that fact that, many pesticides, such as the neonicotinoids and pyrethroids, are derivatives of natural plant allelochemicals (Casida and Durkin, 2013).

Interestingly, recent work has shown that bees can exhibit profound variation in their sensitivity to different insecticides – including to compounds belonging to the same class (Beadle et al., 2019; Iwasa et al., 2004; Manjon et al., 2018; Reid et al., 2020) (Fig. 1). For example, honeybees (*Apis mellifera*) are >1000-fold less sensitive to the neonicotinoid thiacloprid than the neonicotinoid imidacloprid in acute contact bioassays (Iwasa et al., 2004) with the former classified as 'highly toxic' but the latter categorised as 'practically non-toxic' according to the official categories of the U.S. Environmental Protection Agency (USEPA et al., 2014). In another example, while many pyrethroid insecticides are highly toxic to insect pollinators, others, such as *tau*-fluvalinate, display such low acute toxicity to bees that they are used as in-hive treatments by beekeepers against parasitic *Varroa* mites (Mao et al., 2011). In contrast, the leaf-cutting bee *Megachile rotundata* is highly sensitive to many insecticides tested so far, including compounds categorised as moderately and practically non-toxic to honeybees, such as the cyano-substituted neonicotinoid insecticides acetamiprid and thiacloprid and the synthetic pyrethroid insecticide *tau*-fluvalinate. Why are some bees highly tolerant to some insecticides but not others, and what are the specific mechanisms that underpin this differential sensitivity? Recent toxicogenomic work has begun to address this question and identify the key factors that determine pesticide sensitivity in bee pollinators. In this review we summarise the current state of knowledge on this topic. We frame our review by addressing two primary questions: What are the molecular determinants of pesticide sensitivity in bee pollinators? How can an understanding of these factors be leveraged to predict and avoid negative bee-pesticide interactions, and inform bee pollinator pesticide risk assessment?

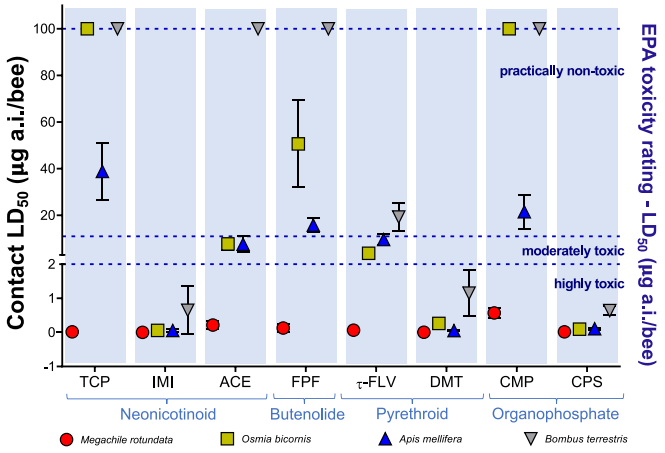

Fig. 1. Sensitivity of four managed bee species to different insecticides belonging to four different classes. Contact LD_{50} values for topical application of thiacloprid (TCP), imidacloprid (IMI), acetamiprid (ACE), flupyradifurone (FPF), tau-fluvalinate ($_t$-FLV), deltamethrin (DMT), coumaphos (CMP) and chlorpyrifos (CPS) are shown (error bars indicate 95 % CIs). Toxicity thresholds are depicted according to the Environmental Protection Agency toxicity ratings.

2. Mechanisms

A variety of different mechanisms have been implicated in the tolerance of bees to specific pesticides, and thus as determinants of insecticide sensitivity, these include metabolic detoxification, insecticide target proteins, the insect cuticle and bee gut microbiota. These mechanisms are illustrated in Fig. 2 and described in detail below.

2.1. Metabolic detoxification

The role of metabolic detoxification in bee sensitivity to pesticides was first implicated by the observation that pyrethroid insecticides such as *lambda*-cyhalothrin are more toxic to honeybees in the presence of cytochrome P450 monooxygenase (P450)-inhibiting fungicides, such as prochloraz (Colin and Belzunces, 1992; Pilling and Jepson, 1993). The mechanism by which prochloraz enhances pyrethroid toxicity was subsequently investigated by following the metabolism of radiolabelled *lambda*-cyhalothrin in whole bees and dissected midguts (Pilling et al., 1995). The metabolites identified were consistent with both esterase- and P450-mediated detoxification, and (especially oxidative) metabolism was found to be strongly inhibited in the presence of prochloraz. This provided further evidence that this fungicide enhances pyrethroid toxicity to honeybees as a result of inhibition of P450 and/or esterase metabolism (Pilling et al., 1995; Pilling and Jepson, 1993). Subsequent studies investigated the involvement of major detoxification enzyme systems in the differential tolerance of honeybees to different pyrethroid, organophosphate and neonicotinoid chemotypes using inhibitors of P450s (piperonyl butoxide, PBO), GSTs (diethyl maleate, DEM) and esterases (S,S,S-tributylphosphorotrithioate, DEF) (Iwasa et al., 2004; Johnson et al., 2009; Johnson et al., 2006). In the case of pyrethroids, toxicity was most profoundly synergized by PBO, implicating P450s in detoxification (Johnson et al., 2006). Furthermore, much greater synergism of the pyrethroid *tau*-fluvalinate (980-fold), which exhibits low acute toxicity to honeybees, was observed than for cyfluthrin (30-fold) and *lambda*-cyhalothrin (80-fold), which are extremely toxic to honeybees (Johnson et al., 2006). Similar work on the organophosphate coumaphos, used as an in-hive treatment against Varroa mites, showed that DEF and PBO synergised the toxicity of coumaphos to honeybees, enhancing toxicity 2.8-fold and 4.0-fold, respectively. Finally, in the case of neonicotinoids, PBO was found to increase the toxicity of acetamiprid and thiacloprid, which exhibit low acute toxicity to honeybees, 244- and 1141-fold, respectively, but had minimal effect on imidacloprid (1.85-fold) which is highly toxic to bees (Iwasa et al., 2004). Similar work on other bee species implicated P450 metabolism in the chemotype-specific neonicotinoid tolerance of both bumble bees,

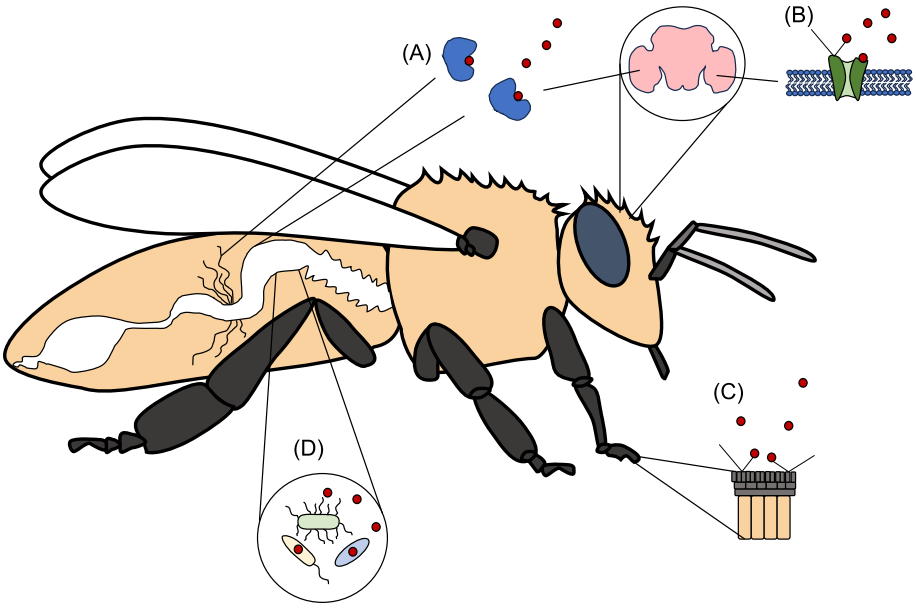

Fig. 2. Schematic of determinants of insecticide sensitivity in bees. Four different mechanisms have been associated with the tolerance of bees to specific pesticides including: (A) metabolic detoxification (most notably by P450s expressed in the Malpighian tubules, the gut or the brain), (B) insecticide target proteins (in this example in the bee nervous system), (C) the insect cuticle, and, (D) the gut microbiota of bees.

Bombus terrestris, and red mason bees, *Osmia bicornis* (Beadle et al., 2019; Manjon et al., 2018; Reid et al., 2020).

In addition to synergism studies, the role of P450s in the differential toxicity of different neonicotinoid insecticides was also evidenced by studies examining the *in vivo* distribution and metabolisation of [^{14}C]-imidacloprid and [^{14}C]-acetamiprid in honeybees (Brunet et al., 2005; Suchail et al., 2004). For both compounds, the profile of metabolites produced was consistent with the biotransformation of these compounds by P450s. However, the kinetics of metabolism suggested the low toxicity of acetamiprid may result, in part, from its much faster metabolism (Brunet et al., 2005). This has been confirmed by a recent pharmacokinetic study investigating the metabolic fate in honeybees of [^{14}C]-labelled neonicotinoids, including acetamiprid and thiacloprid, after topical application (Zaworra et al., 2019). Together, these results provided a first line of evidence that P450s play an important role in bee sensitivity to pyrethroid and neonicotinoid insecticides and may be especially important in the detoxification of chemotypes that exhibit low toxicity to bees.

Following the implication of P450s as determinants of insecticide sensitivity in bee pollinators, research turned to identification of the specific P450 gene(s) involved in insecticide metabolism. In a landmark study Mao et al. successfully functionally expressed eight honeybee P450s, and showed that three of these, CYP9Q1, CYP9Q2, and CYP9Q3, metabolise *tau*-fluvalinate and coumaphos (Mao et al., 2011). While not specifically identifying these P450s as determinants of the low toxicity of these insecticides to honeybees, this study was important as it identified specific insecticide metabolising P450s of honeybees for the first time. Mao et al. also showed that *tau*-fluvalinate exposure can induce *CYP9Q3* expression (by ~1.5-fold) (Mao et al., 2011). While the biological relevance of this modest change in expression is unclear, this finding suggested that it may be possible to identify bee P450s involved in the metabolism of other insecticides by using them as inducers of P450 gene expression, which could then be identified by transcriptome profiling. Indeed, this strategy has been employed to investigate the molecular basis of the low toxicity of thiacloprid to honeybees (Alptekin et al., 2016). Alptekin et al. showed that a measurable reduction in thiacloprid sensitivity could be induced in honeybees after exposure to a sublethal dose of this neonicotinoid for 24 h and correlated this with the induction of five P450 genes, *CYP6BE1*, *CYP305D1*, *CYP6AS5*, *CYP315A1* and *CYP301A1*, and a carboxyl/cholinesterase (CCE), *CCE8*, using microarray analysis. However, functional expression of four of these P450s and assessment of their ability to metabolise thiacloprid by liquid chromatography-mass spectrometry (LC-MS) analysis failed to provide any evidence of their ability to detoxify this insecticide. Manjon et al. therefore took a different approach, conducting an ambitious functional analysis of 27 of the 46 honeybee P450s, comprising the entire CYP3 clan (Manjon et al., 2018). This revealed that P450s belonging to the CYP9Q subfamily, most notably CYP9Q3, metabolise thiacloprid (and acetamiprid) with high efficiency but have limited activity against imidacloprid, providing a molecular explanation for the profound difference in honeybee sensitivity to *N*-nitroguanidine and *N*-cyanoamidine neonicotinoids. Work on bumble bees (*B. terrestris*) identified CYP9Q4 and CYP9Q6 as functional orthologs of honeybee CYP9Q3 and key metabolic determinants of neonicotinoid sensitivity in this species (Manjon et al., 2018; Troczka et al., 2019). Follow on work on the solitary red mason bee, *O. bicornis*, revealed that a P450 within the CYP9BU subfamily (CYP9BU1), with recent shared ancestry to the Apidae CYP9Q subfamily, metabolises thiacloprid *in vitro* and confers tolerance *in vivo* (Beadle et al., 2019).

Flupyradifurone and chlorantraniliprole are butenolide and diamide insecticides, respectively, which are considered moderately to practically non-toxic to honeybees based on acute contact and oral toxicity assays (EFSA, 2015; EFSA, 2013). Screening of the panel of 27 CYP3 clan P450s of honeybee against flupyradifurone identified three P450s involved in the detoxification of this compound: CYP6AQ1, CYP9Q2 and CYP9Q3 (Haas et al., 2021). Furthermore, transgenic *Drosophila melanogaster* lines ectopically expressing *CYP9Q2* and *CYP9Q3* were significantly less sensitive to flupyradifurone than control flies of the same genetic background without a transgene, demonstrating that these P450s confer resistance to this compound *in vivo* (Haas et al., 2021). In the case of chlorantraniliprole, similar work revealed that transgenic *Drosophila* lines expressing *CYP9Q2* and *CYP9Q3* exhibit enhanced resistance to this compound in insecticide bioassays and demonstrated that recombinant CYP9Q2 and CYP9Q3 have the capacity to metabolise chlorantraniliprole (Haas et al., 2022a).

In combination the studies detailed above reveal that members of the CYP9Q subfamily of P450s have the capacity to metabolise compounds belonging to five different insecticide classes (pyrethroids,

organophosphates, neonicotinoids, diamides and butenolides). This suggests that a handful of key P450s are critically important in defining the sensitivity of managed bees to pesticides. This finding has parallels with P450-mediated xenobiotic detoxification in humans, where two of the 57 functional P450s (CYP3A4 and CYP2D6) are responsible for the metabolism of approximately 40 % of clinically used drugs (Rendic and Guengerich, 2015).

The demonstration of the key role of CYP9Q/CYP9BU1 P450s (termed 'CYP9Q-type' hereafter) in defining the sensitivity of managed bee pollinators to insecticides led to several important questions on the potential importance of these enzymes across the wide diversity of >20,000 bee species. These include: 1) What is the level of evolutionary conservation of CYP9Q-type P450s in bees? 2) Do CYP9Q-type P450s from a broad range of bee species have the conserved capacity to detoxify certain insecticides? 3) If the presence of insecticide-degrading P450 enzymes is not universal to all bee species, what are the implications of this for insecticide sensitivity in species that lack these enzymes? Recent work has exploited the dramatic increase in bee genomic resources to address these questions. Haas et al. used a phylogenomic approach to identify >100 putative CYP9Q functional orthologs across 75 bee species encompassing all major bee families, revealing that the presence of CYP9Q-type P450s is generally well conserved across the diversity of bees (Haas et al., 2022b). Furthermore, functional analysis of 26 P450s from 20 representative bee species in this study revealed an evolutionarily conserved capacity of CYP9Q-type P450s to metabolise certain insecticides (including thiacloprid and flupyradifurone) across all major bee families (Haas et al., 2022b). However, *CYP9Q*-type genes were not found to be ubiquitous in all bee species in this study, with some Megachilidae species lacking such genes (Haas et al., 2022b). A follow-on study investigating the evolution and function of P450s in the Megachilidae in more detail revealed that several Megachilidae species, belonging to the Lithurgini, Megachilini and Anthidini tribes, including all species of the *Megachile* genus investigated, lack *CYP9Q*-type genes (Hayward et al., 2023). In contrast, species from the Osmiini and Dioxyini tribes of Megachilidae have CYP9Q-type P450s belonging to the CYP9BU subfamily. Furthermore, these P450s are able to detoxify thiacloprid (Beadle et al., 2019; Haas et al., 2022b; Hayward et al., 2023). Phylogenetic and syntenic analyses revealed that *Megachile* species have evolved phylogenetically distinct CYP9 genes, the CYP9DM lineage, in place of *CYP9Q*-type genes, and functional expression of these P450s from *Megachile rotundata* revealed they lack the capacity to metabolise thiacloprid and imidacloprid (Hayward et al., 2023). Consistent with this finding, native microsomes (a source of total cytochrome P450 enzymes localized to the endoplasmic reticulum) of this species were shown to have the capacity to metabolise the natural insecticide nicotine but not the neonicotinoid insecticides imidacloprid and thiacloprid, or the butenolide insecticide flupyradifurone or the pyrethroid insecticide *tau*-fluvalinate (Hayward et al., 2019). The implications of the loss of *CYP9Q*-type genes in *M. rotundata* was investigated using acute contact insecticide bioassays and revealed that this species is up to >2500-fold more sensitive to insecticides which are detoxified by CYP9Q-type enzymes in honeybees, bumblebees and red mason bees (Hayward et al., 2023; Hayward et al., 2019). Furthermore, while the three latter bee species exhibit marked differences (500–2000-fold) in their sensitivity to thiacloprid and imidacloprid only a 15-fold difference was observed in the sensitivity of *M. rotundata* to these two neonicotinoids (Hayward et al., 2019). These results clearly demonstrate that the intrinsic tolerance of other managed bee pollinators to certain insecticide chemotypes is not observed in *M. rotundata* and provides additional evidence of the role of CYP9Q-type P450s as key determinants of insecticide sensitivity.

Given the demonstrated importance of CYP9Q-type P450s in bee tolerance to pesticides, it is important to understand when and where these P450s are expressed in bees and how this might impact bee sensitivity to pesticides. Investigation of the spatial expression profile of *CYP9Q/CYP9BU* P450 genes in honeybees, bumblebees and red mason bees revealed high levels of expression in the Malpighian tubules and brain (Beadle et al., 2019; Manjon et al., 2018; Troczka et al., 2019). The expression of these P450s in Malpighian tubules, which are key osmoregulatory and detoxifying organs in many insect species, is consistent with a primary role in xenobiotic metabolism, while expression in the brain suggests a secondary or additional site of detoxification against xenobiotics that cross the blood-brain barrier. In another study, expression of *CYP9Q1–3* in antennae of adult honeybee workers performing different tasks revealed that *CYP9Q3* expression differed significantly according to age-related task performance, with low levels of expression in the antennae of newly eclosed workers, increasing to significantly higher levels in nurses and even higher levels in foragers (Mao et al., 2015). *CYP9Q1* and *CYP9Q2* expression was also higher in nurses and foragers than newly eclosed workers but no significant difference in expression was observed between nurses and foragers (Mao et al., 2015). The pattern of expression of *CYP9Q1–3* observed in honeybee legs was similar, increasing significantly with each behavioural stage (Mao et al., 2015). The finding that expression of *CYP9Q2* and *CYP9Q3* is highest in foragers is consistent with a role in xenobiotic protection as this behavioural stage is most like to routinely encounter phytochemicals while collecting nectar, pollen and resin. Recent work has characterized the gene expression profile of the inventory of detoxification genes in honeybees by analysing 47 transcriptomes across the honeybee life cycle, including different larval instars, pupae, and adults (Maiwald et al., 2023). This confirmed the results of earlier work on the tissue- and behavioural stage-specific expression pattern of *CYP9Q* P450 genes while also revealing a trend of incremental upregulation in developing larval stages until pupation and a marked increase in expression levels in adult stages. This finding supports the idea that these P450s are involved in the elimination of xenobiotics encountered by active life stages of honeybees. Related to this, *CYP9Q3* expression was shown to be strongly induced by constituents of honey and pollen, such as *p*-coumaric acid, that the active life stages of honeybees are exposed to (Mao et al., 2013). Furthermore, when *p*-coumaric acid was added to a diet of sucrose and fed to honeybees it was found to increase midgut metabolism of coumaphos by ~60 % demonstrating the functional significance of its effect as an inducer of P450 expression (Mao et al., 2013). Recent studies suggest that the influence of phytochemicals on the metabolic capacity towards pesticides is a broader phenomenon, probably based on the induction of detoxification genes such as P450s (Ardalani et al., 2021b; Ardalani et al., 2021a).

In addition to quantitative changes in the expression of CYP9Q-type P450s, recent work has provided evidence that qualitative variation in the sequence of these P450s in bee populations may also impact insecticide detoxification and bee tolerance (Tsvetkov et al., 2023). Previous work has shown that honeybee genotype can influence tolerance to neonicotinoids. For example, Rinkevich et al. examined the sensitivity of honeybees of different genetic backgrounds (Carniolan, Italian, and Russian stocks) to three insecticides and identified a < 1-fold difference in sensitivity to organophosphates, moderate differences in bioassays with pyrethroids (1.5- to 3-fold) and marked differences in neonicotinoid sensitivity (3.4- to 33.3-fold) (Rinkevich et al., 2015). More recently, Tsvetkov et al. investigated the genetic basis of neonicotinoid sensitivity in honeybees and showed that the survival of honeybee workers to an acute oral dose of clothianidin is heritable, with $H2 = 0.38$ (Tsvetkov et al., 2023). Tolerance to clothianidin was not associated with differences in the expression of CYP9Q-type P450s, however, mutations in *CYP9Q1* and *CYP9Q3* were strongly associated with worker survival following clothianidin exposure. More specifically nine mutations across seven haplotypes were identified for *CYP9Q3* and five mutations in six haplotypes for *CYP9Q1*. To date, the ability of CYP9Q-type P450s to metabolise clothianidin has not been demonstrated, and this, and the impact of the identified mutations on metabolism, requires functional validation. Nevertheless, the demonstration of genetic variation in CYP9Q-type P450s of bee populations in this study is important, as previous work on pest insects has demonstrated that standing genetic

variation in P450s of insect pest populations can be rapidly selected by insecticide exposure leading to resistance (Zimmer et al., 2018).

Only a handful of P450s beyond CYP9Q-type P450s have been implicated in insecticide metabolism in bees. The most well characterized example comes from recent work on the CYP336 family of cytochrome P450s and their role in protecting bees (and other hymenopteran species) from nicotine – a potent natural insecticide produced by plants (especially Solanaceae) (Haas et al., 2023). Honeybees are much more tolerant to this alkaloid than many insect pest species (Human et al., 2014; Steppuhn et al., 2004), with recent studies linking this tolerance to rapid and efficient metabolism in adults and larvae (du Rand et al., 2017b; du Rand et al., 2017a; du Rand et al., 2015). Investigation of the metabolic fate of nicotine in honeybees using radiolabelled nicotine and LC-MS/MS analysis provided evidence that nicotine is metabolised by P450–mediated C′2-oxidation of the pyrrolidine ring of this compound (du Rand et al., 2017b). Screening of the 27 honeybee P450s of the CYP3 clan for their capacity to metabolise nicotine identified just four P450s that exhibited a degree of activity against this compound (CYP9P2, CYP9Q1, CYP6AQ1 and CYP336A1) (Haas et al., 2023). However, marked differences in activity were observed for the different P450s, CYP9P2 and CYP9Q1, exhibiting basal activity (2.8 and 6.4 % depletion of 10 μM nicotine in 2 h), CYP6AQ1 exhibiting greater activity (22 % depletion), while CYP336A1 was the only P450 to deplete 10 μM nicotine entirely (*i.e.*, 100 % depletion). Further investigation of the substrate profile of CYP336A1 demonstrated that it can metabolise other alkaloids containing a basic nitrogen such as pyridine and tropane alkaloids that are closely related to nicotine from a biosynthetic perspective and appear in the same plant families, revealing this P450 is adapted to cope with concurrent alkaloid chemotypes (Haas et al., 2023). Remarkably, phylogenetic and syntenic analyses revealed that the CYP336 P450 family appears to be specific to *Hymenoptera* and highly conserved in this order, with representative genes found in all major hymenopteran lineages encompassing sawflies, wasps, ants, and bees. Furthermore, functional analysis of seven genes from four representative hymenopteran species that diverged over 281 million years revealed a conserved capacity to metabolise nicotine and related alkaloids. Using three-dimensional modelling and site-directed mutagenesis, CYP336A1 activity against basic alkaloids was linked to an aspartic acid residue (position 298) within the main access channel of CYP336 enzymes that is highly conserved within this P450 family (Haas et al., 2023). Remarkably, modification of this single residue completely abolished the metabolic activity of CYP336A1 against nicotine and atropine, revealing that D298 is a key determinant of the specific activity of the CYP336 family for protonated alkaloids. These findings demonstrate that insects from the same order, but with contrasting lifestyles, can recruit similar, conserved mechanisms to deal with potentially toxic plant allelochemicals. They also provide an example of the identification of a key structure-function determinant of P450-mediated insecticide metabolism at the amino acid level.

Beyond studies on CYP336 P450s and plant nectar alkaloids, it has been shown that members of the CYP6AS subfamily in honeybees metabolise flavonoids such as quercetin (Mao et al., 2009). This P450 subfamily has undergone substantial lineage specific expansion in a number of bee species, including *A. mellifera* (16 CYP6AS genes), *Melipona quadrifasciata* (a neotropical stingless bee; 17 CYP6AS genes) and *M. rotundata* (10 CYP6AS genes), which has been hypothesized to be a result from increased evolutionary exposure to a diverse range of flavonoids in nectar and pollen (Johnson et al., 2018). Computational homology modelling and docking studies with *A. mellifera* CYP6AS paralogs supported a role in flavonoid metabolism, which has been confirmed in substrate depletion studies with recombinantly expressed honeybee CYP6AS enzymes (Mao et al., 2009).

Work on *Apis cerana cerana* identified five P450 genes (named *Acc301A1*, *Acc303A1*, *Acc306A1*, *Acc315A1,* and *AccCYP6k1* by the authors) that were induced by several insecticides (Tan et al., 2023; Zhang et al., 2019). RNA interference-mediated knockdown of *Acc301A1*, *Acc303A1*, *Acc306A1* and *AccCYP6k1* expression significantly increased the mortality rate of *A. c. cerana* to pesticide treatment. However, P450 genes may have important endogenous functions. Thus, silencing such genes can reduce the overall fitness of dsRNA treated bees, which may, in turn, result in increased sensitivity to subsequent exposure to insecticides even if the gene of interest does not directly confer resistance. Furthermore, the CYPome of *A. cerana* was recently shown to include P450s of the CYP9Q subfamily (Haas et al., 2022b). Thus, further functional validation of the role of these non-CYP9Q-type P450s as determinants of insecticide sensitivity is required.

Finally, as detailed above, CYP6AQ1 was shown to be an important player in flupyradifurone metabolism in honeybees (Haas et al., 2021). In contrast to honeybee CYP9Q2 and CYP9Q3, this enzyme selectively hydroxylated flupyradifurone, and no metabolites other than flupyradifurone-hydroxy were formed by the activity of recombinantly expressed CYP6AQ1. Very recently Xiao et al. followed a toxicogenomics approach and investigated the potential role of functional CYP6AQ1 orthologs in flupyradifurone metabolism from eight different bee species, including stingless bees (Tribe: Meliponini) such as the neotropical species *Frieseomelitta varia* and *M. quadrifasciata* (Brazil) (Xiao et al., 2023). Recombinant expression of functional orthologs of honeybee CYP6AQ1, such as CYP6AQ56 (*M. quadrifasciata*) and CYP6AQ84 (*Tetragonula carbonaria*, a stingless bee endemic in Australia) revealed a common coumarin substrate profile and a conserved capacity of all CYP6AQ1-like orthologs to metabolise flupyradifurone by hydroxylation *in vitro*. This study highlights the significance of investigating the detoxification mechanisms of insecticides in non-*Apis* bee species by molecular means to potentially inform risk assessment and conservation efforts.

2.2. Insecticide target-sites

The sensitivity of bees to different insecticides within the same mode of action class can also reside in differences in the affinity of these insecticides for their target sites. An excellent example of this is for pyrethroid insecticides, which act on insect voltage-gated sodium channels. Wu et al. examined the sensitivity of the sodium channel (BiNav1–1) of common Eastern bumblebee (*Bombus impatiens*) to different pyrethroid insecticides by expression in *Xenopus* oocytes and electrophysiological assays (Wu et al., 2017). While BiNav1–1 was found to be highly sensitive to six commonly used pyrethroids, it was found to be significantly less sensitive to *tau*-fluvalinate. Specifically, based on calculated EC_{25} values, BiNav1–1 was 10- to 12-fold more tolerant to *tau*-fluvalinate than to type I pyrethroids tested, and 24- to 31-fold more tolerant compared to other type II pyrethroids. Additional assays revealed that BiNav1–1 is also tolerant to etofenprox, which, like *tau*-fluvalinate, has a chemical structure of extended length relative to other pyrethroids. Interestingly voltage-gated sodium channels from German cockroach (*Blattella germanica*), *D. melanogaster,* and the yellow fever mosquito (*Aedes aegypti*) were more sensitive to *tau*-fluvalinate and etofenprox than the BiNav1–1 channel, suggesting that sequences specific to BiNav1–1 may be responsible for its lower affinity for these insecticides. Alignments of the amino acid sequences of sodium channels from 11 bee species and 47 non-bee insect species identified three residues (T841 in domain IIS2, V926 in IIS5, and F1525 in IIIS6), which were conserved in sodium channel sequences of the analysed bee species, but not in the other species. Computational modelling of sodium channel three-dimensional structure and insecticide docking analyses identified four additional amino acid residues (P1007 and N1017 in IIS6, and V1528 and S1535 in IIIS6) as potential candidates for a role in *tau*-fluvalinate tolerance. Site-directed mutagenesis and functional analyses confirmed that these seven sites contribute to the tolerance of the bumblebee sodium channel to *tau*-fluvalinate *versus* other pyrethroids and thus are important determinants of pyrethroid target-site sensitivity in this species. The universality of this finding across bee species requires further work, however, as research examining the affinity of the

voltage-gated sodium channels of the honeybee (AmNav1) and *Varroa destructor* mite (VdNaV1) for pyrethroid insecticides did not reveal a similar pattern of *tau*-fluvalinate tolerance (Gosselin-Badaroudine et al., 2015; Gosselin-Badaroudine and Chahine, 2017). The lack of concordance in these two studies suggests that the role of insecticide target-sites as molecular determinants of insecticide sensitivity may differ between bee species, in contrast to the generally well conserved role of P450-mediated detoxification across bee diversity.

Amitraz is another acaracide and insecticide that is used as an in-hive treatment against Varroa mites due to its low toxicity to bees. Interestingly, the inhibitors DEM, DEF and PBO were found to have no impact on the toxicity of amitraz against honeybees, suggesting that the tolerance of bees to this compound is not due to rapid metabolism by P450s, GSTs or esterases (Johnson et al., 2013). Amitraz acts as an agonist of insect and mite octopamine receptors. Guo et al. recently identified one receptor subtype, Octβ2R, as the primary target of amitraz *in vivo* (Guo et al., 2021). Furthermore, in the same study, functional expression and pharmacological assays revealed that the Octβ2R of honeybee, AmOctβ2R, was 16-fold less sensitive to amitraz than that of Varroa mites, VdOctβ2R, and 6-fold less sensitive to its primary metabolite DPMF, based on calculated EC_{50} values. Investigation of the genetic basis of this difference using three-dimensional modelling of honeybee and mite Octβ2R, and amitraz docking studies, identified three amino acids (E208, I335, I350) within the potential ligand-binding domain that were unique to bees (Guo et al., 2021). Creation of a version of AmOctβ2R, in which these three amino acids were replaced with the corresponding amino acids in VdOctβ2R, followed by functional characterisation *in vitro* (cell-based assays) and *in vivo* (transgenic *Drosophila*) demonstrated the causal role of these amino acids in the tolerance of honeybees to amitraz. Further work is required to establish the relative importance of the three amino acids in determining sensitivity to amitraz and understand how they interact.

Diamide insecticides comprise two structurally different subtypes: phthalic acid derivatives such as flubendiamide and anthranilic acid derivatives such as chlorantraniliprole - both are potent activators of insect ryanodine receptors (RyRs) (Sattelle et al., 2008). As detailed above, the low toxicity of chlorantraniliprole to honeybees has been linked to metabolism by CYP9Q P450s (Haas et al., 2022a). However, in the same study the authors found no evidence for synergism between flubendiamide and the P450 inhibiting fungicide propiconazole suggesting that a different mechanism is responsible for its low bee toxicity. These differences might reside in phthalic acid diamide target site specificity as described for house flies (*M. domestica*), which were shown to have a high-affinity RyR binding site for chlorantraniliprole but not flubendiamide (Qi et al., 2014). Recent research has indeed provided evidence of determinants of bee sensitivity to diamides residing in the diamide target-site. This was initially evidenced by radioligand binding studies on thoracic muscle membrane preparations (an enriched source of RyRs) of honeybees and three other insect species (*Musca domestica*, *Heliothis virescens* and *Agrotis ipsilon*) (Qi and Casida, 2013). This revealed low levels of specific ^{3}H-chlorantraniliprole binding to honeybee preparations compared to the other three species, and no specific binding of ^{3}H-flubendiamide to honeybee RyRs. This is consistent with the tolerance of these compounds to honeybees, while also revealing a lower affinity of honeybee RyRs to phthalic diamides than anthranilic compounds. Intriguingly, work on the mechanisms of resistance to diamides in insect pests has shed light on the genetic basis of honeybee tolerance to diamides, and the differential selectivity of the two compounds for honeybee RyRs. Specifically, research on lepidopteran pests such as *Plutella xylostella* and *Tuta absoluta* has shown that resistance to diamides frequently results from target-site mutations in the RyR diamide binding site such as G4946E and I4790M (Richardson et al., 2020; Roditakis et al., 2017; Troczka et al., 2012). Functional characterisation of these mutations using CRISPR/Cas9 genome editing in *D. melanogaster* demonstrated their causal role in resistance (Douris et al., 2017; Richardson et al., 2020). In the case of I4790M, *D. melanogaster*, naturally wildtype for the I4790M mutation, were moderately resistant to flubendiamide (15.3-fold) but significantly less resistant to chlorantraniliprole (7.5-fold), and cyantraniliprole (2.3-fold) compared to a fly line engineered to carry the reverse mutation (M4790I). This finding is significant for bees, as honeybees also have a methionine at this position (M4764, *A. mellifera* numbering) suggesting that this amino acid is, at least in part, the molecular explanation of the tolerance of honeybees to diamides and lower affinity of phthalic diamides for honeybee RyRs (Zhou et al., 2020). Recently, both the crystal structure of the N-terminal domain A (NTD-A) of the RyR of honeybee and the cryo-EM structures of rabbit RyR1 in complex with chlorantraniliprole, were resolved (Ma et al., 2020; Zhou et al., 2020). These studies confirmed the importance of the amino acid at position 4790 (*P. xylostella* numbering) as a determinant of diamide sensitivity and allowed the identification of regions that are conserved within bees, but different in lepidopteran species, including a potential insecticide-binding pocket formed by loop9 and loop13 (Ma et al., 2020; Zhou et al., 2020). Further functional analysis is required to examine the role of these regions in determining bee sensitivity to pesticides.

For other insecticide classes, such as the neonicotinoids, evidence for the role of the target site in mediating chemotype-specific differences in insecticide sensitivity is limited. Neonicotinoids act on the nicotinic acetylcholine receptor (nAChR), and Manjon et al. examined the affinity of nAChRs for imidacloprid and thiacloprid in honeybee and bumble bee head membrane preparations, using radioligand binding assays. Both insecticides were found to bind to honeybee and bumblebee nAChRs with nM affinity, and no significant difference was seen in the specific binding of either compound at the receptor, demonstrating that the differences in bee sensitivity to these two neonicotinoids is not a consequence of variation in their affinity for the nAChR (Manjon et al., 2018). Radioligand binding assays on head membrane preparations of the solitary bees *O. bicornis* and *M. rotundata* also revealed equivalent affinity of nAChRs for these two insecticides (Beadle et al., 2019; Hayward et al., 2019). In the case of *M. rotundata* the equivalent specific binding at the receptor extended to flupyradifurone (Hayward et al., 2019). Recently, a new insect nAChR expression system employing auxiliary proteins has been established and has been used to functionally express neonicotinoid sensitive insect nAChRs in *Xenopus* oocytes, including heteromeric receptors from honeybees and bumble bees (Ihara et al., 2020). The authors demonstrated that expressed bee nAChRs were more neonicotinoid-sensitive than those of heteromeric Drosophila nAChRs. The agonist action of thiacloprid and imidacloprid on functionally expressed honeybee nAChRs was rather low compared to electrophysiologically generated patch-clamp data using native neurons. Because of this, and the fact that the subunit composition of insect nAChRs addressed by neonicotinoids *in situ* is still largely unknown, further work is required to establish how these findings translate to neonicotinoid action on insect nAChRs *in vivo*.

2.3. Insect cuticle

Compared to metabolic and target-site mechanisms, the role of the cuticle in differences in bee sensitivity to insecticides has been less well studied (Balabanidou et al., 2018). Zaworra et al. used radiolabelled neonicotinoids to show that imidacloprid penetrates the honeybee cuticle much more readily than thiacloprid and acetamiprid (Zaworra et al., 2019). For example, four hours after application 37 % of [^{14}C]-imidacloprid equivalents were detected in honeybee body extracts in contrast to 9.0 % and 18 % of internally cumulated [^{14}C]-thiacloprid and [^{14}C]-acetamiprid equivalents respectively. This variation in penetration speed and internal body concentrations of different neonicotinoids suggest that a pharmacokinetic component contributes to the different acute contact toxicity of these insecticides. However, in contrast to these findings when Beadle et al. compared the rate of penetration of [^{14}C]-imidacloprid and [^{14}C]-thiacloprid through the cuticle of *O. bicornis,* no significant differences in the amount of radiolabelled thiacloprid and

imidacloprid recovered from the cuticle or acetone combusted whole bees was observed at any time point post-application (Beadle et al., 2019). This suggests that the differential sensitivity of *O. bicornis* to imidacloprid and thiacloprid is not a result of variation in their speed of penetration through the cuticle. Thus, the pharmacokinetics of neonicotinoids may differ for different bee species, and further work on the role of the insect cuticle in influencing bee sensitivity to members of this insecticide class and others is required. In this regard, it has been shown that the butenolide flupyradifurone is taken up rather slowly by the honeybee cuticle upon contact exposure, possibly contributing to a > 50-fold difference between LD_{50}-values for acute contact and oral toxicity (Haas et al., 2021). Related to this, work on the influence of spray adjuvants on insecticide toxicity has suggested that increased toxicity of certain adjuvants in combination with insecticides may be due to increased uptake *via* the cuticle, however, a detailed evaluation of the mechanisms behind these observations is lacking (Shannon et al., 2023; Wernecke et al., 2022).

2.4. Bee microbiota

In addition to factors encoded by the genome of bees, emerging research is providing evidence that the sensitivity of bees to insecticides can also be influenced by their microbiome. Two possible mechanisms by which bee gut microbiota influence insecticide sensitivity of their host have been described, i) direct microbe-mediated detoxification of an insecticide, and, ii) indirect modification of host xenobiotic detoxification pathways. In relation to the first mechanism, work by El Khoury et al. isolated bacteria from the honeybee gut and demonstrated that seven bacterial species (*Edwardsiella sp.*, two *Serratia sp.*, *Rahnella sp.*, *Pantoea sp.*, *Hafnia sp.* and *Enterobacter sp.*) were able to grow *in vitro* in the presence of the neonicotinoid clothianidin, with clothianidin exposure actually promoting the growth of *Hafnia sp.* (El Khoury et al., 2022). Furthermore, the seven species were able to completely degrade this insecticide within 72 h under *in vitro* conditions, with some variation observed in the speed of degradation for different species. Related work on lactic acid bacteria (LAB), which are natural inhabitants of the digestive tract of honeybees, also demonstrated the capacity of bacteria to tolerate chlorpyrifos, coumaphos, and imidacloprid *in vitro* (Leska et al., 2022). Furthermore, tests of the cytotoxicity of these insecticides on an insect cell line (Sf9 cells) in the presence of LAB suggested these bacteria provide most effective protection against coumaphos. These findings are important as they demonstrate that microbiota derived from honeybee guts have the capacity to detoxify insecticides *in vitro*. However, further work is required to demonstrate microbe-mediated metabolism *in vivo* and understand the impact of this on the bee host's sensitivity to these insecticides. Given the variation in the ability of these bacterial species to tolerate and degrade different insecticides, it will also be important to understand the extent to which the microbiome of bee populations vary in terms of the relative abundance of these bacterial species and the impact of this on insecticide sensitivity.

In terms of indirect insecticide tolerance mediated by the microbiota of bees, Wu et al. compared the expression of P450 genes in honeybee workers that were prevented from establishing a normal gut microbiota (gut microbiota deficient (GD) workers) and workers with a normal gut microbial community (conventional gut community (CV) workers) (Wu et al., 2020). They found that several P450 genes, *CYP6AS1*, *CYP6AS3*, *CYP6AS4*, *CYP6AS10*, *CYP9Q2* and *CYP9Q3* were significantly upregulated in the midgut of CV bees compared to GD bees. This was correlated with increased tolerance to thiacloprid and *tau*-fluvalinate in CV bees compared to GD bees. Additional *in vitro* experiments, in which gut homogenates were isolated from CV or GD bees and cultured in the presence of thiacloprid and *tau*-fluvalinate prior to analysis using HPLC, revealed no significant changes in the level of these insecticides in CV gut homogenates when compared to GD gut homogenates after 2 days of culture (Wu et al., 2020). This suggested that under the tested conditions the honeybee gut microbiota cannot metabolise thiacloprid or fluvalinate directly. While these findings reveal a correlation between variation in honeybee gut microbiota and host resistance to pesticides, further work is required to provide additional evidence of the causal role of the microbiome in modulating P450 expression and the mechanisms involved.

3. Future perspectives

3.1. How can knowledge of the determinants of bee sensitivity to pesticides be leveraged to predict and avoid negative bee-pesticide interactions?

The knowledge generated by the studies reviewed here has strong potential to both inform pesticide risk assessment and underpin the development of tools and resources that can be used to identify and mitigate negative pesticide-insect interactions. This has been recently acknowledged by EFSA in its review of its guidance document on the risk assessment of plant protection products on bees, stating that *"Among the lines of research focusing on mechanistic explanation of the toxicity, developments of methods investigating the genetic and molecular basis of inter-species sensitivity […] are particularly promising"* (Adriaanse et al., 2023). Assessment of the risk of pesticides to bees is a regulatory requirement for pesticide registration. Risk is defined as a function of hazard (intrinsic toxicity of a chemical) and exposure (expected concentration an organism is exposed to). The hazard assessment is currently largely based on experimental data collected from a handful of 'model' bee species such as honeybees and bumble bees. However, bees (Anthophila) are an exceptionally diverse clade of insects with broad differences in ecology and life history traits, and, as demonstrated by the studies reviewed here, can exhibit marked differences in sensitivity to pesticides (Beadle et al., 2019; Haas et al., 2022b; Manjon et al., 2018). Thus, there is a fundamental challenge to meaningfully extrapolate from surrogate test species to the diversity of species meant to be covered by the scientific risk assessment. However, over the last decade there has been an explosion in the number of reference genome and/or transcriptome sequence assemblies available for bees. Furthermore, several ambitious sequencing projects such as the i5K initiative (which aims to sequence the genomes of 5000 arthropod species), the Earth Biogenome Project (which aims to sequence, catalogue and characterize the genomes of all of Earth's eukaryotic biodiversity over a period of ten years) and the Darwin Tree of Life project (which aims to sequence all eukaryotic organisms in Britain and Ireland) are releasing new assemblies all the time (Blaxter et al., 2022b; Blaxter et al., 2022a; i5K Consortium, 2013). These extensive 'omics' datasets offer an exceptional opportunity to understand the extent of variation in the molecular determinants of bee sensitivity to insecticides across the diversity of bees in order to predict the sensitivity of non-model species to pesticides. The recent work on bee sensitivity to neonicotinoids detailed above provides proof-of-principle data that genomic information for beneficial insects *can* be leveraged to predict their sensitivity to insecticides and thus has the potential to inform environmental risk assessment by implementing a molecular trait-based approach as previously proposed (Spurgeon et al., 2020). We envisage that this approach could be used as a component of a future Tier 0 molecularly informed risk assessment (Fig. 3). To employ this approach the molecular determinants of pesticide selectivity in the surrogate species, such as the metabolic profile of CYP9Q P450s in *A. mellifera*, must first be established. Following this, available genomic data can be screened to identify potential functionally orthologous genes/variants in related species and investigate their potential influence on toxicokinetic or toxicodynamic properties *in silico*. These *in silico* approaches include phylogenetic and syntenic analysis, and three-dimensional modelling of protein structure (Haas et al., 2023; Haas et al., 2022b). In regards, to the latter, recent advances in predicting protein structure directly from an amino acid sequence, without the requirement for a crystal structure template, such as employed by AlphaFold (Jumper et al., 2021) an artificial intelligence system

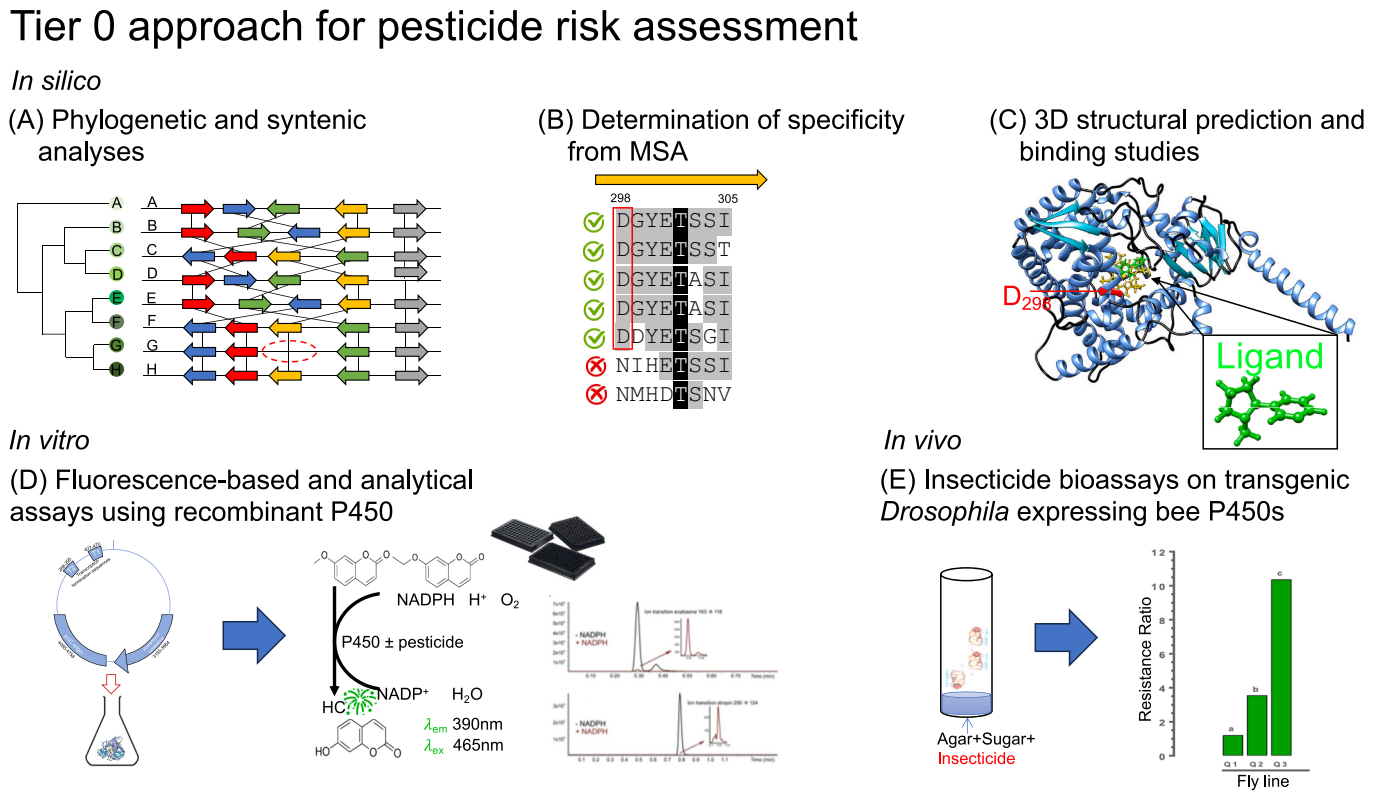

Fig. 3. A proposed Tier 0 toxicogenomic approach for pesticide risk assessment. In this approach knowledge of the molecular determinants of pesticide selectivity in model bee species is leveraged, in combination with genomic data for non-model species) to predict the sensitivity of non-model species to a pesticide of interest. A range of *in silico* approaches can be used to establish if non-model bee species have key sensitivity determinants present in the model species (such as CYP9Q-type P450s). These include: (A) phylogenetic and syntenic analysis, (B) determination of insecticide specificity from multiple sequence alignments (MSA), and, (C) three-dimensional modelling of protein structure. In concert with this genomic framework, there is a requirement for tools which can be used to provide experimental support and validation to *in silico* predictions. These include: (D) *in vitro* tools in the form of purified recombinant bee P450 enzymes that can be used to measure P450 activity (and pesticide-mediated inhibition of this activity) in simple fluorescent assays (using fluorescent model substrates) or analytical assays based on by liquid chromatography tandem mass spectrometry (LC-MS/MS), and, (E) transgenic fruit fly (*D. melanogaster*) lines each engineered to express CYP9Q-type P450s of different bee species.

developed by DeepMind, can enhance the accuracy of predictions of insecticide binding and/or metabolism where an insect crystal structure template is unavailable (such as for insect P450s).

In concert with this genomic framework, there is a requirement for tools which can be used to provide experimental support and validation to *in silico* predictions and enable the reliable identification of essential molecular determinants of pesticide selectivity. In the case of P450 enzymes, two complementary systems that facilitate functional validation of pesticide interactions with key protective P450s of bees have been developed. The first of these comprise *in vitro* tools in the form of purified recombinant bee P450 enzymes and associated fluorescent model substrates that can be used to measure P450 activity in simple fluorescent assays (Haas and Nauen, 2021). Together the enzymes and model substrates can be used in high-throughput screens of pesticides to examine if the P450 of interest is interacting with the test compound (providing an indication of its potential to be metabolised by P450s or inhibit P450 function). Such an approach would have parallels with molecular medicine approaches used to characterize P450–drug interactions in the pharmaceutical industry in order to predict impacts on human physiology and health (López-Osorio and Wurm, 2020). The utility of this approach was recently demonstrated in a study revealing certain fungicides as strong inhibitors of honeybee CYP9Q P450 enzymes - providing a molecular explanation as to why such fungicides can dramatically increase bee sensitivity to selected insecticides (Haas and Nauen, 2021). This serves as a demonstration of how such tools can be used to identify pesticide combinations that are harmful to bees. *In vivo* tools have also been developed that can be used to test *in silico* predictions of bee sensitivity to pesticides. An example of these are transgenic fruit fly (*D. melanogaster*) lines each engineered to express CYP9Q-type P450s of different managed bee species (Beadle et al., 2019; Manjon et al., 2018; McLeman et al., 2020). These genetically modified *Drosophila* lines are now resistant to the same pesticides as the native bee species, and their sensitivity to novel pesticides, or pesticide combinations, can be examined using simple pesticide bioassays. The ectopic expression of P450s in *Drosophila* provides information on the phenotype a P450 gene of interest confers (*e.g.* level of tolerance, cross-tolerance profile *etc.*). This system thus provides a useful complement to *in vitro* assays which provide information on enzyme catalytic activity but not the phenotypic level of tolerance a P450 confers (McLeman et al., 2020). Similarly, advances enabling the genomic manipulation of honeybees, *e.g. via* the CRISPR/Cas9 system provide a promising tool to study the impact of individual genes or genetic variation on pesticide sensitivity in the future (Chen et al., 2021; Grozinger and Zayed, 2020).

While *in silico*-based methods for 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 taxa that are not readily accessible for acute toxicity testing. Furthermore, integration of these approaches within the Aggregate Exposure Pathway (AEP) and Adverse Outcome Pathway (AOP) conceptual frameworks could be used to anchor the findings within a broader context (Teeguarden et al., 2016; Vinken et al., 2017). This would provide understanding of how insecticide detoxification by bee P450s relates to other factors leading from insecticide exposure to an eventual adverse

outcome endpoint. Longer term, as predictions on the sensitivity of bees to pesticides made using genomic data are refined through ongoing functional validation, it may be possible to move towards more automated approaches. An example of such an approach is the U.S. 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 (LaLone et al., 2016). Similarly, the SPDlight algorithm uses the specificity-determining positions (SDP) prediction method to determine the functional specificity of proteins with a common general function (Kalinina, 2004). This approach does not rely on any information about the protein family except its multiple sequence alignment (MSA) and specificity of some members of the family.

Finally, the identification of the molecular determinants of insecticide metabolism in bees also has potential to facilitate the rational design of future insecticides by informing structure-based design. In addition, the functional tools detailed above provide a filtering tool to examine the metabolic liability of future lead compounds to understand it they are likely to be rapidly metabolised by P450s of beneficial insects. This is of high value as live bioassays on beneficial insects such as bees are expensive and time-consuming to perform, with many species only available for a few months of the year (McLeman et al., 2020).

3.2. Knowledge gaps for future research

The literature highlighted in this review reveal the dramatic advances made in our understanding of the molecular determinants of bee sensitivity to pesticides over the last 5-10 years. However, the research conducted on this topic has inevitably led to a range of questions for further research. While it is not possible to list all of these, here we describe some of the knowledge gaps, which, in our view, are priorities for future research.

Firstly, our understanding and resolution of the full spectrum of genetic factors influencing bee sensitivity to pesticides, and their relative contribution to phenotype, remains limited. For example, while the role of P450 genes as insecticide sensitivity determinants is now well established, the role, if any, of other detoxification genes, such as esterases, glutathione S-transferases, UDP-glucosyltransferases and ATP-binding cassette (ABC) transporters remains largely unknown. In this regard, recent comprehensive RNAseq analyses of the expression profile of the entire detoxification gene inventory of honeybees may assist with the selection of candidate genes for further analyses (Maiwald et al., 2023). Related to this, several studies have revealed marked variation in the expression of molecular determinants of insecticide sensitivity in different bee life stages and after exposure to different environmental xenobiotics (Maiwald et al., 2023; Mao et al., 2013; Mao et al., 2011) and the impact of this on bee sensitivity requires further exploration. In this regard the finding that *p*-coumaric acid exposure can enhance metabolism of insecticides in honeybees by inducing CYP9Q3 expression (Mao et al., 2013) could be investigated in future as a potential means to enhance intrinsic pesticide tolerance of bees. In terms of factors that are not encoded by bee genomes, the role of the bee microbiome on pesticide sensitivity *in vivo* remains uncertain and warrants further investigation, including research into variation in the microbiome between bee populations/species. Furthermore, while some progress has been made in identifying structure-function determinants of insecticide binding/metabolism at the amino acid level (Haas et al., 2023; Tsvetkov et al., 2023; Wang et al., 2022; Wu et al., 2017), further work is required to enhance our resolution of insecticide-bee protein interactions.

Secondly, while genomic data have been effectively leveraged to understand inter-specific variation in key insecticide sensitivity determining genes of bees, knowledge of the extent of intra-specific variation in these genes and its impact on phenotype remains poor. However, efforts to sequence insect populations and new approaches that consider the genetic variation of a species as represented by its pangenome will facilitate investigation of this topic (Dogantzis and Zayed, 2019; Sherman and Salzberg, 2020).

Thirdly, future research needs to address how different insecticide sensitivity determinants contribute to phenotype in combination (*e.g.* are interactions additive, synergistic or epistatic). Such knowledge is important as it facilitates the development of increasingly sophisticated, and accurate, risk assessment frameworks in the future.

Finally, bees are exceptionally diverse in their ecology and life history. The expanding data available on the ability of these species to detoxify or circumvent natural and synthetic insecticides offers an excellent opportunity to understand the ecological factors influencing the evolution of xenobiotic detoxification genes in one of the most diverse and ecologically important group of insects on the planet.

CRediT authorship contribution statement

Chris Bass: Conceptualization, Writing – original draft, Writing – review & editing. **Angela Hayward:** Writing – original draft, Writing – review & editing. **Bartlomiej J. Troczka:** Writing – original draft, Writing – review & editing. **Julian Haas:** Writing – original draft, Writing – review & editing. **Ralf Nauen:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ralf Nauen reports a relationship with Bayer AG that includes: employment. Julian Haas reports a relationship with Bayer AG that includes: employment. J.H. and R.N. are employees of Bayer AG, a manufacturer of insecticides. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

C.B and A.H. are supported by funding (BB/V004093/1) from the Biotechnology and Biological Sciences Research Council of the UK.

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