Functionally characterized arthropod pest and pollinator cytochrome P450s associated with xenobiotic metabolism

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Abstract

The cytochrome P450 family (P450s) of arthropods includes diverse enzymes involved in endogenous essential physiological functions and in the oxidative metabolism of xenobiotics, insecticides and plant allelochemicals. P450s can also establish insecticide selectivity in bees and pollinators. Several arthropod P450s, distributed in different phylogenetic groups, have been associated with xenobiotic metabolism, and some of them have been functionally characterized, using different *in vitro* and *in vivo* systems. The purpose of this review is to summarize scientific publications on arthropod P450s from major insect and mite agricultural pests, pollinators and *Papilio sp*, which have been functionally characterized and shown to metabolize xenobiotics and/or their role (direct or indirect) in pesticide toxicity or resistance been functionally validated. The phylogenetic relationships among these P450s, the functional systems employed for their characterization and their xenobiotic catalytic properties are presented, in a systematic approach, including critical aspects and limitations. The potential of the primary P450-based metabolic pathway of target and non-target organisms for the development of highly selective insecticides and resistance-breaking formulations may help to improve the efficiency and sustainability of pest control.

Key words: cytochrome P450, xenobiotic detoxification, plant allelochemicals, insecticide selectivity, bees, agricultural pests.

1. Introduction

The P450 family of insects and mites includes diverse enzymes involved in endogenous physiological functions and the metabolism of xenobiotics (Nauen et al., 2022). The cytochrome P450 sequences (CYPome) are distributed into four major clans: CYP2, CYP3, CYP4 and mitochondrial. Insect and mite genomes and transcriptomes have facilitated researchers to "extract" their CYPome and study their function. The distribution of P450 genes is not homogenous and they are comprised of few families with many genes and many P450 families with few genes (Dermauw et al., 2020). P450 – based xenobiotic (insecticides and plant toxins) detoxification is typically mediated by hydroxylation, dealkylation and other oxidative reactions, rendering the xenobiotic molecule more excretable and less toxic (Feyereisen, 2012). Several arthropod P450 proteins and genes have been implicated in xenobiotic metabolism in arthropods, identified by a variety of techniques, including *in vitro* heterologous expression systems (Nauen et al., 2021) and *in vivo* (RNAi or transgenic techniques) functional characterization approaches. P450-xenobiotic metabolizers may also play an important role in the specific activation of pro-insecticides, to allow the development and use of low toxicity chemicals for pest control (Vlogiannitis et al., 2021). They also catalyze the detoxification of several active ingredients by pollinators and beneficial insects and determine insecticide selectivity (Jeschke, 2016; Manjon et al., 2018).

The goal of this review is to summarize the current literature on the insect and mite (pests and pollinators) cytochrome P450s, which have been functionally associated with pesticide toxicity, by *in vitro* or *in vivo* means. The phylogenetic relationships, the functional systems employed for the analysis of their role are systematically presented and limitations are discussed. The potential of cytochrome P450s to support the development of highly selective safe insecticides and resistance breaking compounds is discussed.

2. Methods

2.1 Identification / Search of validated P450s from agricultural pests, pollinators and Papilio sp.

Studies including P450s from agricultural pests and bees/pollinators, which have been functionally validated by either *in vitro* or *in vivo* techniques and shown to play a role in xenobiotic metabolism (natural or synthetic compounds) and/or pesticide toxicity/resistance have been included in this analysis. Veterinary pests such as *Musca domestica*, *Lucilia cuprina* and others, or insects regarded as occasional pests, such as Drosophilidae, were not included.

The database used for the literature search was Web of Science® and the latest search date was July 2021. The insect species that are involved in this review, the P450s, the types of methodology used to validate them (*in vitro*: *Escherichiacoli (E. coli)*, yeast, insect cell lines; *in vivo*: RNAi, genome modification: transgenic *Drosophila melanogaster*, CRISPR) were traced and the keywords used were (["pest" or "insect" or "mite" "P450s" or "CYPs"] and [validated* functional*]and [in vitro* E. coli* yeast* insect cell lines* RNAi* transgenic* Drosophila* CRISPR*]). Then the research articles that included functionally validated P450s either *in vitro* and/or *in vivo*/ genome modification were further categorized based on the species and the order. The data concerning substrate types for the P450s (chemical or natural compounds and the subgroup or exemplifying active ingredient in which they belong), types of methodology for the validation of the role of P450s (*in vitro*: *E.coli*, yeast, insect cell lines; *in vivo*: RNAi, genome modification: transgenic *D. melanogaster*, CRISPR) and the percentage of studies referring to each type of methodology were extracted from the studies with validated P450s, and tables, Venn graphs and pie charts were generated. Another literature search was carried using the Web of Science® database in order to trace the amount of all scientific papers that referred to each P450 either associating it with resistance or validating its contribution in resistance. Hence, a set of additional keywords was used: (["pest" or "insect" or "mite" "P450s" or "CYPs"] and [resistance*]). The results from this search were used for the generation of a heatmap depicting the number of studies in which each P450 has been studied in the literature.

2.2 Phylogenetic analysis

P450s were manually curated in selected Lepidoptera and Hymenoptera species, either publicly available or private (Table 1). The early-diverged CYP51A1 (Nelson, 1999) from *Homo sapiens* was used as an outgroup. Multiple sequence alignment was performed using the amino acid sequence of these P450s with MAFFT v7.271 (Katoh and Standley, 2013) with the default parameters and then trimming was done with Trimal v1.2rev59 (Capella-Gutierrez et al., 2009), with the parameter "-automated1". A Maximum Likelihood phylogeny with 100 bootstrap replicates was inferred with RAxML v8.2.11 (Stamatakis, 2014), with parameters "-m PROTGAMMAAUTO". Branches with <50% bootstrap support were collapsed with TreeGraph2 (Stöver and Müller, 2010) and the resulting Newick tree was loaded to EvolView v2 (He et al., 2016) for post-processing. The vector graphics editor Inkscape v0.92 was used for the final polishing. Two phylogenetic trees were reconstructed using the above approach; one for only the Lepidoptera P450s and another one for only the hymenopteran P450s.

The nomenclature for genes encoding P450s includes the root symbol CYP for the superfamily, a number indicating the gene family, a capital letter indicating the subfamily and a numeral for the individual gene name.

3. Identity and phylogeny of arthropod P450s implicated in xenobiotic metabolism and resistance

3.1 Identity

Several P450s have been functionally implicated in xenobiotic metabolism and insecticide resistance across different arthropod taxa, in agricultural pests, pollinators and mite species (Table 2A-D). More specifically, 56 Lepidoptera P450s; 73 non-Lepidoptera P450s from several orders (Homoptera, Diptera, Coleoptera and Orthoptera), 13 from mite species (Trombidiformes); and 23 from pollinator and *Papilio* species (Hymenoptera and Lepidoptera) have been functionally validated in the literature (Table 2A-D). These P450s (Table 2A-D) have been reported in individual, few or multiple studies (Figure 1A-D).

The Lepidoptera *Helicoverpa armigera* is one of the most well studied species in terms of the number of P450s (21 P450s) (Figure 1A). *H. armigera* CYP6B6 has been reported in more than 25 publications (Figure 1A), and CYP6B7 in 20. Several publications (6 to 19) focused on *H. armigera* CYP337B3, CYP9A12, CYP9A14 and CYP6AE14 and fewer (less than five) involve the CYP6AE cluster, while CYP6B2 has been mentioned in 15 publications and CYP337B1 in 8 publications (Figure 1A). Some *Spodoptera* species demonstrate an abundance of studied P450s: *S. exigua* has ten P450s (CYP6AE10, CYP6AB14, CYP9A98, CYP9A10, CYP9A21v3, CYP9A105, CYP321A16, CYP332A1, CYP321A8, CYP9A168) while *S. litura* has six functionally verified P450s (CYP321B1, CYP6B50, CYP6AB14, CYP9A40, CYP6AB60, CYP6AB12), all of them studied in less than five publications. *Helicoverpa zea* and *Chilo suppressalis* have three and four identified P450s respectively: CYP6B8 from *H. zea* has been studied in ten publications and CYP321A1 in nine scientific publications, while all *C. suppressalis* P450s have been mentioned in less than five (Figure 1A). *Plutella xylostella* and *S. frugiperda* have three functionally validated P450s each (CYP6BG1, CYP321E1, CYP340W1 and CYP321A8, CYP321A9, CYP321B1, respectively) (Figure 1A). They are all mentioned less than five times in the literature with one exception: *P. xylostella* CYP6BG1 appeared in the literature six times (Figure 1A). The rest of the species depicted in Figure 1A, *Amyelois transitella*, *Cydia pomonella* and *Depressaria pastinacella* include either one or two characterized P450s, appearing in less than five publications.

Among non-Lepidoptera pest species, 16 P450s belong to the Orthoptera species *Locusta migratoria*, nine P450s of *Laodelphax striatellus* (Hemiptera), with only CYP353D1 having been studied more than five times, eight P450s of *Leptinotarsa decemlineata* (Coleoptera) and nine P450s of *Aphis gossypii* (Hemiptera) have been implicated in xenobiotic detoxification (all of them mentioned in less than five publications each) (Figure 1B). Following up, five P450s from *Diaphorina citri* (Hemiptera) and *Bradysia odoriphaga* (Diptera) have been implicated to xenobiotic detoxification, while nine P450s from *Nilaparvata lugens* (Hemiptera) have been studied for their role in detoxification. All the above have been identified in the literature less than five times, with the exceptions of *N. lugens* CYP6ER1, CYP6AY1, CYP6CW1 which are mentioned in 19, 16 and 6 publications, respectively. *Sogatella furcifera* (Homoptera) and *Tribolium castaneum* (Coleoptera) have respectively three and four P450s contributing to xenobiotic metabolism, while *Myzus persicae*, the Diptera *Ceratitis capitata* and the Coleoptera species *Brassicogethes (Meligethes) aeneus* and *Sitophilus zeamais* have one P450 each (studied less than five times. The *M. persicae* CYP6CY3 appeared in approximately 13 publications (Figure 1B). Two P450s of the Hemiptera *Bemisia tabaci* have a validated role in metabolism/resistance respectively: CYP6CM1 and CYP6CX4. The *B. tabaci* CYP6CX4 is referred to less than five publications, while it is notable that CYP6CM1 from the same species is one of the most extensively studied P450s, with 40 publications (Figure 1B), referring also to its different biotype origins (CYP6CM1vB, CYP6CM1vQ).

From the agriculturally important mite species, seven P450s of *Tetranychus cinnabarinus* and six of *T. urticae* have been functionally validated for their contribution in xenobiotic detoxification and resistance (identified in less than five publications each) (Figure 1C). The impact of insecticides on the health of bee and bumblebee pollinators is a topic of intensive research and considerable current debate, as they are exposed to a wide variety of natural and synthetic xenobiotics (including pesticides) (Johnson, 2015). In the category of pollinators and *Papilio* sp., *Apis mellifera* (Hymenoptera) presents the largest number of studied P450s for their role in xenobiotic detoxification (n =8), which are studied less than five times each, except for CYP9Q1 (mentioned in seven publications) (Figure 1D). The hymenopteran species *A. cerana cerana*, *Bombus terrestris* and the Lepidoptera *Papilio glaucus* have each three P450s studied for their detoxifying role, reported less than five times (except for *P. glaucus* CYP6B4 which is reported in nine publications), while the hymenopteran *Osmia bicornis* and the Lepidoptera *Papilio polyxenes* have two xenobiotic metabolizing P450s each (*P. polyxenes* CYP6B1 has been mentioned in the literature 30 times) (Figure 1D).

3.2 Phylogeny

An overlay of literature reports on a phylogeny of complete Lepidoptera CYPomes of *H. armigera, S. littoralis* and *P. xylostella* (Figure 2A) shows that virtually all P450s that are known to be involved in detoxification of xenobiotics belong to Clan 3 (Figure 2A, genes with black circles), except for CYP340W1, a P450 belonging to Clan 4 which is involved in the detoxification of macrocyclic lactones. Within Clan 3, however, there is no particular clade that is associated with resistance to insecticides and many clades have genes involved in detoxification. The most studied P450 families with regards to pesticide resistance are CYP6B (pyrethroids, OPs (organophosphorus pesticides)), CYP6BG (pyrethroids, ryanoids), CYP6AE (pyrethroids and many classes of compounds), CYP9A (ryanoids, capsaicin), CYP332A (OPs), and CYP337B (pyrethroids; the CYP337B3 gene, specifically). In contrast, P450s that are involved either in known physiological functions (Figure 2A, genes decorated with black triangles), or are suspected to be involved in some physiological function (Figure 2A, black/red stars) are found in all four clans. It should be noted, however, that there are P450s in other clans that are involved in detoxification in non-Lepidoptera species as in case of the mitochondrial CYP12A1 of *Musca domestica* (Guzov et al., 1998).

Similarly, in the hymenopterans, the detoxification-related P450s are found in Clan 3 (Figure 2B, genes with black circles) and more specifically in one of two families; CYP9Q/CYP9BU (neonicotinoids, pyrethroids, quercetin) (Beadle et al., 2019; Mao et al., 2011; Troczka et al., 2019), or CYP6AS (quercetin) (Mao et al., 2009). Genes in the CYP9Q/9BU branch are present in the Apidae and some Megachilidae species, conferring selective protection from thiaclorprid, but not imidacloprid, even though both insecticides belong to the neonicotinoid class (Manjon et al., 2018; Troczka et al., 2019). Of particular importance is the expansion in the CYP6AS family, that appears to be Hymenoptera specific (Oakeshott et al., 2010). Nevertheless, not all CYP6AS genes are necessarily involved in detoxification, since in addition to the detoxifiers (CYP6AS1, 3, 4, and 10), others are involved in physiology (CYP6AS8, and 11) (Wu et al., 2017). More specifically, CYP6AS8 and CYP6AS11 are candidates for omega and omega-1 hydroxylations of short chain fatty acids to pheromones (Plettner et al., 1996), while recent RNAi experiments indicated that knock-down of CYP6AS8 affects the short chain fatty acid biosynthesis (Wu et al., 2020). Even though such omega hydroxylations are carried out by CYP4 enzymes in vertebrates, it has been proven for other CYP6 genes that this P450 family is also capable of this biochemical function (Helvig et al.,

2004). To date, however, neither CYP6AS8, nor CYP6AS11 have been biochemically characterized.

The key difference, however, when compared to Lepidoptera, is that there are far fewer cases of functionally characterized P450s in Hymenoptera. This is true even for Hymenoptera-specific P450s such as CYP6BC and CYP4AV that are present in all Hymenoptera and should therefore have an important and specific role in these insects. Similarly, Lepidoptera specific P450s are equally interesting, such as the CYP428A, which appears to be fast evolving according to our phylogenetic analysis (Figure 2A).

Concerted research efforts should be undertaken in order to develop selective insecticides, targeting major Lepidoptera pests on the one hand, which will be harmless to the ecologically and agriculturally important Hymenoptera, on the other hand. In this quest for selective insecticides, it is important to take into consideration two facts related to P450s. The first is that it is not possible to predict the catalytic competence of a P450, simply through similarity to another P450 that has been functionally characterized (Dermauw et al., 2020). The second fact is that there is no clear dichotomy between P450s involved in detoxification of xenobiotics, and P450s involved in physiological functions (Dermauw et al., 2020). Both facts stress that finding P450s that confer selective protection to an insecticide will not be a trivial task, and will require extensive expertise and most likely wet lab screening.

4. Systems used for functional validation.

Several approaches have been exploited to characterize the role of P450s from different species in xenobiotic metabolism, including *in vitro* expression in heterologous systems, RNAi-based reverse genetics, or transgenic approaches (*in vivo* overexpression in *D. melanogaster* or non-model organisms, or CRISPR/CAS9 knock out) which provided different levels of validation for the involvement of P450s in detoxification and implication in resistance (Nauen et al., 2022).

Eighty four P450s originating from all four categories of pests and pollinators/*Papilio* sp. reported in this review (Lepidoptera, non-Lepidoptera, mite pest species and pollinators/*Papilio sp.*) have been functionally validated to be associated to pesticide toxicity/resistance using only the RNAi approach (Figure 3A); thirty eight using only *in vitro* methodologies and one using only genome modification approaches; twelve P450s have been characterized utilizing both *in vitro* systems and RNAi, twenty three using both genome modification approaches (reverse genetics in non-model organisms or *D. melanogaster*

heterologous expression) and *in vitro*; two using genome modification and RNAi and five using all approaches (RNAi, genome modification and *in vitro*) (Figure 3A). The validation systems used for each P450 are mentioned in Table 2 (last two columns). It is notable that CRISPR in non-model organism has been so far utilized only for Lepidoptera P450 validation (*H. armigera* 6AE cluster, (Wang et al., 2018) and *S. exigua* 9A186 (Zuo et al., 2021)).

More specifically, looking at the different systems utilized for the functional validation of individual P450s in different arthropods ((a) Lepidoptera; (b) non-Lepidoptera insect species; (c) mites and; (d) pollinators/ *Papilio sp.*) the information is depicted in Figure 3 B-E:

(a) Twenty two P450s (Figure 3B) have been validated to resist/tolerate xenobiotics using only the system of RNAi in Lepidoptera, despite the debate in the literature concerning the effectiveness of RNAi in this order (Terenius et al., 2011). For example, all the P450s from *Spodoptera litura* (CYP321B1, CYP6B50, CYP6AB14, CYP9A40, CYP6AB60, CYP6AB12) have been validated for their contribution to resistance using RNAi (Lu et al., 2020, 2019b, 2019a; Sun et al., 2019; Wang et al., 2017; Wang et al., 2015b, 2015a). Eighteen Lepidoptera P450s have been validated using *in vitro* systems only, like *H. zea* CYP6B27 (Wen et al., 2009) and *H. armigera* CYP9A12 (Chen et al., 2019; Shi et al., 2021; Tian et al., 2019; Yang et al., 2008), while only one P450 (*H. armigera* CYP6AE20) has been validated using only genome modification approaches (Wang et al., 2018) (Figure 3B and Table 2A). Additionally, two P450s have been verified using both RNAi and *in vitro* systems: *H. armigera* CYP6B6 and CYP9A14 (Shi et al., 2021; Tao et al., 2012; Tian et al., 2017; Yang et al., 2008; Zhao et al., 2016) while 11 P450s have been verified using both *in vitro* and genome modification systems: the *H. armigera* 6AE cluster CYP6AE11, 6AE12, 6AE15, 6AE16, 6AE17, 6AE18, 6AE19 (both heterologous expression and CRISPR) (Wang et al., 2018) and *S. exigua* CYP321A16, CYP332A1, CYP321A8 (both heterologous expression and *D. melanogaster* transgenic expression) (Bo et al., 2020) and CYP9A168 both heterologous expression and *D. melanogaster* system (Zuo et al., 2021) (Figure 3Band Table 2A). *H. armigera* CYP6AE14 has been validated for xenobiotic tolerance and metabolism using all three categories: *in vitro* systems, genome modification and RNAi (Mao et al., 2007; Shi et al., 2018; Tao et al., 2012)(Figure 3B and Table 2A);

(b) Non-Lepidoptera pest P450s are distributed differently, with the great majority being characterized only with the use of RNAi (50/74 validated P450s- Figure 3C and Table 2B). For example, the *L. migratoria* P450s (CYP6FF1, CYP6FD2, CYP6FE1, CYP4G102, CYP4G62, CYP9AQ2, CYP409A1, CYP408B1, CYP9AQ1, CYP9A3, CYP6HC1, CYP6HL1,

CYP6HN1, CYP6HQ1 and CYP303A1 (except for CYP6FD1)) have been associated with insecticide toxicity/resistance with the use of RNAi. Exclusively *in vitro* systems have been exploited for only six P450s from this category- and one of them is *L. striatellus* CYP6FU1 (Elzaki et al., 2018) (Figure 3C and Table 2B). Notably, CRISPR is not detected in the literature so far for non-Lepidoptera pest P450 validation. It appears more common to use both RNAi and *in vitro* systems, as it is depicted in Figure 3C: nine P450s have been validated using both sets of techniques. For example, *A. gossypii* CYP6CY22 and CYP6CY13 assessed for its metabolic ability and involvement in resistance with *in vitro* expression system and RNAi (Chen et al., 2020; Hirata et al., 2017; Ma et al., 2019) (Figure 3C and Table 2B). Four P450s were studied using both *in vitro* and *D. melanogaster* transgenic expression (genome modification) approaches: *L. striatellus* CYP6AY3v2 (Wang et al., 2017), *M. persicae* CYP6CY3 (Bass et al., 2013; Nakao et al., 2019), *C. capitata* CYP6A51 (Tsakireli et al., 2019) and *B. (M.) aeneus* CYP6BQ23 (Samantsidis et al., 2020; Zimmer et al., 2014). One P450 has been validated for its role in resistance using both RNAi and genome modification systems: *N. lugens* CYP6CS1 using *D.melanogaster* heterologous expression system and the RNAi approach (Wang et al., 2021) (Figure 3C and Table 2B). Three P450s have been validated using *in vitro* and *in vivo* (RNAi and genome modification) methodologies: *B. tabaci* CYP6CM1 (Daborn et al., 2012; Nauen et al., 2013), *N. lugens* CYP6ER1 (Bao et al., 2016; Pang et al., 2016) and *T. castaneum* CYP6BQ9 (Zhu et al., 2010)(Figure 3C and Table 2B);

(c) The majority of mite pest P450s and their role in xenobiotic metabolism and/or toxicity/resistance has been investigated with the use of RNAi only, which is in fact nine out of the 13 validated P450s in the literature (Figure 3D and Table 2C): *T. cinnabarinus* CYP389B1, CYP392A26, CYP391A1, CYP384A1, CYP392D11, CYP392A28 were validated by Shi et al., 2016 using RNAi (Shi et al., 2016). Also, *T. urticae* CYP392D8, CYP392A12, CYP389C10 were evaluated for their contribution to resistance using RNA interference (Xu et al., 2021). *T. cinnabarinus* CYP389C16 has been validated for its role in metabolism and resistance using both *in vitro* and RNAi approaches (Feng et al., 2020). *T. urticae* CYP392E10 has been validated for xenobiotic detoxification using *in vitro* systems (Demaeght et al., 2013). Additionally, *T. urticae* CYP392A11 is involved in insecticide metabolism, demonstrated by the use of *in vitro* systems, *D. melanogaster* heterologous expression (genome modification), and also RNAi (Riga et al., 2015; Xu et al., 2021), while CYP392A16 was expressed *in vitro*, to prove its catalytic activity against insecticides (Riga et al., 2014) and genome modification techniques were also explored (Riga et al., 2020);

(d) Pollinator and *Papilio sp*. P450 functional validation seem to rely more on the *in vitro* systems, and also *in vivo*, mostly using genome modification set of techniques. Three P450s have been validated only using RNAi (*A. cerana cerana* CYP301A1, CYP303A1, CYP306A1 (Zhang et al., 2019))(Figure 3E and Table 2D). Thirteen P450s were validated using only *in vitro* systems. More specifically, *A. mellifera* CYP6AS1, CYP6AS3, CYP6AS4, CYP6AS10, *B. terrestris* CYP9Q5, *O. bicornis* CYP9BU2 and all the *Papillio* P450s (CYP6B1, 6B3, 6B4, 6B17, 6B21, 6B25, 6B33) were shown to contribute to xenobiotic metabolism using *in vitro* systems (Beadle et al., 2019; Hung, 1997; Li et al., 2003, 2004; Manjon et al., 2018; Mao et al., 2011, 2009; Wen et al., 2006, 2003) (Figure 3E and Table 2D). There have also been seven P450s identified in the literature for which the researchers exploited both *in vitro* and genome modification strategies: *A. melifera* CYP9Q1, CYP9Q2, CYP9Q3, CYP6AQ1, *B. terrestris* CYP9Q4, CYP9Q6 and *O. bicornis* CYP9BU1 (Beadle et al., 2019; Haas et al., 2021; Manjon et al., 2018; Mao et al., 2009; Troczka et al., 2019).

4.1 Heterologous expression systems used for in vitro validation

Different *in vitro* expression systems (bacterial, insect cell/baculovirus, yeast) and strategies (including modifications at the DNA or protein sequence or electron delivery, fusion enzyme systems) have been recruited in order to functionally characterize P450s associated with insecticide resistance and toxicity *in vitro* and confirmed their role of metabolizing several insecticides at different rates (Nauen et al., 2021).

The systems that have been exploited include different heterologous expression hosts: the prokaryote *Escherichia coli* and the eukaryotic systems *Saccharomyces cerevisiae, Pichia pastoris*, insect cells (baculovirus mediated) and stable insect cells (Figure 4). The *E. coli* system offers important benefits as an expression system, like the highly produced protein yields, the inexpensive culture media and most importantly, the lack of endogenously produced P450s (Nauen et al., 2021). The yeast systems that are recruited for P450 expression, especially *S. cerevisiae* and *P. pastoris*, offer the significant advantage of performing post-translational modifications combined with an environment of organelles similar to other eukaryotic organisms, enabling proper protein membrane anchoring (Hausjell et al., 2018). Insect cells also constitute a frequently utilized expression system, as they resemble the natural insect protein production system (Feyereisen, 2012), using either the baculovirus transient expression approach, or stable insect cell lines. The total distribution of the *in vitro* systems that are exploited in the literature for P450 expression leads to almost equal use of the baculovirus mediated system (47%) and the *E.coli* expression system (44%), while stable cell line comprise

only 6% and yeast systems 3% according to published research (Figure 4A). The set of methodologies used for the validation of each P450 are mentioned in the last two columns of Table 2.

There is a wide distribution of the *in vitro* systems used for the Lepidoptera P450 metabolism exploration (Figure 4B and Table 2A). The majority of the studies (72%) concerning Lepidoptera insect pest P450s took advantage of the baculovirus mediated transient expression system, as shown in Figure 4B. Fourteen percent of the studies exploited *E. coli* and 10% yeast systems, while only 4% of the studies used stable insect cell lines. For example, researchers using Sf9- baculovirus mediated expression, confirmed the detoxifying role of the *A. transitella* CYP6AB11 (Niu et al., 2011), *D. pastinacella* CYP6AB3v1 and CYP6AB3v2 (Mao et al., 2008, 2006), *H. zea* CYP6B27 (Wen et al., 2009)(*H. armigera* CYP6B2, CYP6B6, CYP6B7, CYP9A3, CYP9A12, CYP9A14, CYP9A16, CYP9A17 and CYP9A23 (Shi et al., 2021) and CYP321A16, CYP332A1, CYP321A8 from *S. exigua* (Bo et al., 2020; Hu et al., 2021). Additionally, P450s belonging to the *H. armigera* CYP6AE subfamily were proven to participate in the detoxification of several insecticides and phytochemicals using baculovirus mediated expression in Hi5 insect cells (Shi et al., 2018; Wang et al., 2018) The widely used *E.coli* system is also used for Lepidoptera P450 expression (14% of the studies) (Figure 4B). After heterologously expressing the proteins in *E.coli*, Calla et al., 2019 (Calla et al., 2020) described the ability of CYP6AE89 from *D. pastinacella* for metabolism. Similarly, Tian et al., 2019 expressed the *H. armigera* proteins CYP9A14, 9A12, 9A17 and CYP6B6 in the *E.coli* system, showing their contribution to detoxification. Close to the percentage of studies that used the *E.coli* expression system is the use of yeast (10%) (Figure 4B). For example, *H. armigera* CYP6B7 was proven to degrade chemical substances, using the *P. pastoris* expression system (Zhao et al., 2018). On the contrary, stable insect cells have been used only in 4% of the publications concerning Lepidoptera P450s (Figure 4B). Ha2302 stable cells were used to express CYP337B3 (Joußen et al., 2012; Rasool et al., 2014) and CYP337B1v1 (Joußen and Heckel, 2021) from *H. armigera*.

Non-Lepidoptera insect pest P450s are also mostly expressed in insect cells with the use of baculovirus (41% of the studies) (Figure 4C and Table 2B), while 34% of the studies used *E.coli* and 25% the stable insect cell line approach. Interestingly, expression in the yeast system was not identified in the literature (Figure 4C). *L. striatellus* CYP417A2v2, CYP439A1v3, CYP4C71 have all been successfully expressed and validated in Sf9 (baculovirus mediated) cell lines (Miah et al., 2019, 2017; Xiao et al., 2020). *N. lugens* CYP6AY1 on the contrary has

been expressed in *E.coli* cells by Ding et al., 2013, in an effort to assess xenobiotic metabolism. The smallest amount of publications (25%- Figure 4C) has exploited stable insect cell lines: Nakao et al., 2019 (Nakao et al., 2019) used S2 cells from *D. melanogaster* in order to express *M. persicae* CYP6CY3 and Kalsi et al., 2017 (Kalsi and Palli, 2017) handled SL1 stable cell lines to express *L. decemlineata* CYP6BJ variants, CYP9Z25 and CYP9Z29.

The great majority of mite P450s have been expressed in *E.coli* cells (83% of the studies), while 17% used baculovirus mediated expression in insect cells (Figure 4D and Table 2C). The yeast expression system as well as the stable cell lines are absent for the specific category. In fact, from all mite P450s that were evaluated for their metabolic capacity, only CYP392E10 from *T. urticae* was transiently expressed in Hi5 cell lines, showing its detoxifying ability (Demaeght et al., 2013). Concerning pollinator and *Papilio sp.* P450s, the distribution of the systems is completely different from the previous categories, as all the studies (100%) employed the baculovirus mediated expression system (Figure 4E).

Mosquito P450s (*Anopheles* and *Aedes* species), included as a separate category for comparison reasons only, have been elucidated for their metabolic activity utilizing mostly the *E.coli* system for their expression (90% of the studies), while the yeast and baculovirus mediated expression have been equally used (5% each) (Adolfi et al., 2019; Chandor-Proust et al., 2013; Edi et al., 2014; Ibrahim et al., 2018, 2016; Kasai et al., 2014; Mclaughlin et al., 2008; Mitchell et al., 2012; Müller et al., 2008; Riveron et al., 2017, 2014; Stevenson et al., 2012, 2011; Vontas et al., 2018; Yunta et al., 2019, 2016) (Figure 4F).

4.2 Reverse/functional genetic systems used for in vivo validation

In vivo functional validation of the role of P450s to xenobiotic tolerance and resistance has been facilitated by the RNA interference (RNAi) approach (Bai-Zhong et al., 2020; Ding et al., 2013; Gao et al., 2016; Kalsi and Palli, 2017; Mao et al., 2007; Pang et al., 2016; Wang et al., 2018; Wang et al., 2018) as well as *Drosophila* transformation tools (Daborn et al., 2012; Manjon et al., 2018; Riga et al., 2020; Samantsidis et al., 2020; Troczka et al., 2019; Tsakireli et al., 2019; Zhu et al., 2010; Zimmer et al., 2018)and the recent advances in genome editing technology in non-model organisms (Wang et al., 2018; Zuo et al., 2021).

RNAi has been used in various agricultural insect and mite species in order to validate the role of P450s in xenobiotic tolerance/resistance. For example, targeting of CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25, CYP9Z29 from *L. decemlineata* indicated their involvement in defense against natural and synthetic compounds (Kalsi and Palli, 2017), while RNAi mediated knockdown of

CYP6BQ9 from *T. castaneum* revealed increased susceptibility of the QTC279 resistant strain to deltamethrin (Zhu et al., 2010). This tool has been also used outside of the coleopteran species, such as the hemipteran species *N. lugens* where independent targeting of CYP6AY1 or CYP6ER1 indicated their involvement in imidacloprid tolerance (Ding et al., 2013; Pang et al., 2016). Moreover, P450s that are associated to xenobiotic resistance from major Lepidoptera pest species have been subjected to RNAi mediated knockdown, either through plant (Mao et al., 2007), diet or droplet feeding (Bai-Zhong et al., 2020; Wang et al., 2018) or injections (Gao et al., 2016; Hu et al., 2014) indicating their involvement in tolerance to natural and synthetic compounds. RNAi is the main P450 *in vivo* validation system in agricultural insect pests, while this methodology is equally used with *in vitro* systems in Lepidoptera species (Figure 3B) , despite the concerns about the effectiveness of this tool in Lepidoptera order (Terenius et al., 2011). Although, RNAi is a fast and easy approach linking P450 functional validation and resistance *in vivo*, dsRNA stability and/or cellular uptake may impair the methodology in certain insect species (Cooper et al., 2020).

The model organism *D. melanogaster* and the expansion of the genetic tools offer an alternative method for functional validation of P450s in resistance to xenobiotics. The employment of GAL4/UAS system has enabled the conditional expression of P450s in different tissues and investigation of their role in xenobiotic resistance and tolerance (McLeman et al., 2020). Examples from the Drosophila system include the heterologous expression of P450s from agricultural pests and the category of pollinators/ *Papilio sp.* such as, *C. capitata* CYP6A51 (Tsakireli et al., 2019), *P. xylostella* CYP6BG1 (Li et al., 2018), CYP6CM1vQ from *B. tabaci* (Daborn et al., 2012), *B. (M). aeneus* CYP6BQ23 (Samantsidis et al., 2020), CYP321A16 and CYP332A1 from *S. exigua* (Bo et al., 2020), *M. persicae* CYP6CY3 (Bass et al., 2013), *N. lugens* CYP6ER1 (Pang et al., 2016; Zimmer et al., 2018), *T. urticae* CYP392A11 and CYP392A16 (Riga et al., 2020, 2015), *A. mellifera* CYP9Q2 and CYP9Q3 (Manjon et al., 2018), *B. terrestris* CYP9Q4 and CYP9Q6 (Manjon et al., 2018; Troczka et al., 2019).

Although this system provides evidence for the functional link between P450s and their role in xenobiotic resistance, the levels achieved are usually less than 5-fold compared to the striking phenotypes observed in the original populations (often >100-fold). However, it is not clear if this is the actual contribution of the P450 or concerns limitations of the system. It has been suggested that additional factors may contribute to resistance. Recently, Samantsidis et al., 2020 combined transgenic expression and CRISPR/Cas9 modification in *D. melanogaster*. Transgenic flies expressing the *B.*(*M). aeneus* CYP6BQ23 while also bearing the L1014F

mutation in voltage gated sodium channel contributed greater resistance levels to the pyrethroid deltamethrin, than each mechanism separately, an indication of their synergistic role (Samantsidis et al., 2020).

CRISPR editing tool has been successfully employed not only in *D. melanogaster* and other model organisms, but also in the Lepidoptera species *H. armigera* and *S. exigua*. The reverse genetic approach followed by knocking out a cluster of CYP6AEs provided *in vivo* evidence for the involvement of this cluster in detoxification and tolerance to esfenvalerate, indoxacarb and phytochemicals (Wang et al., 2018), while knock out of *CYP9A186* from *S. exigua* restores susceptibility to emamectin benzoate (Zuo et al., 2021).

5. Xenobiotic specificity of functionally validated P450s

P450s from all arthropodal categories that are mentioned in this review (Lepidoptera pests, non- Lepidoptera pests, mite pests and pollinators/ *Papilio sp*.) have been associated with the metabolism of active ingredients and/or functionally linked *in vivo* with xenobiotic resistance/tolerance from two or more insecticide classes (pyrethroids, neonicotinoids, OPs, organochlorines, etc.), as well as diverse natural allelochemicals (furanocoumarins, phenylpropenes, ketones etc.). The substances are categorized and summarized in Table 2 for each P450.

5.1 Lepidoptera

Several Lepidoptera P450s have been identified to metabolize substances from only one chemical group. For example, *A. transitella* CYP6AB11 contributes to imperatorin (natural compound-furanocoumarin class) detoxification (Niu et al., 2011). The codling moth *C. pomonella* CYP9A61 contributes to the metabolism of cypermethrin, permethrin and λ-cyhalothrin, all belonging to the class of pyrethroid insecticides (Yang et al., 2017), while CYP6B2 was found to contribute to deltamethrin (pyrethroid) and azinphos methyl (organophosphate) resistance (Wan et al., 2019) (Table 2A). *C. suppressalis* CYP6CV5, CYP9A68, CYP321F3, CYP324A12 have been associated with chlorantraniliprole (diamide) resistance (Xu et al., 2019) (Table 2A). CYP6B8 originating from the generalist *H. zea*, has a broad insecticide- detoxifying role, being able to metabolize a great number of synthetic compounds from distant groups, such as cypermethrin (pyrethroids), aldrin (organochlorines), carbaryl (carbamates), diazinon (organophosphates), as well as natural substances belonging to the groups of furanocoumarins, flavonoids, indolyl alcohols, cinnamate esters (Li et al., 2004; Rupasinghe et al., 2007; Wen et al., 2009) (Table 2A). Similarly, *H. zea* CYP321A1 has

the ability to metabolize the above mentioned compounds (except for carbaryl (carbamates) and indole carbinol (indolyl alcohols) with various efficiencies (Rupasinghe et al., 2007; Sasabe et al., 2004). Additionally, P450s belonging to the *H. armigera* CYP6AE subfamily were proven to participate in the detoxification of a broad spectrum of insecticides and phytochemicals. For example, CYP6AE17 and CYP6AE18 contribute to the metabolism of pyrethroids, neonicotinoids, organochlorines, oxadiazines and carbamates, but also furanocoumarin compounds (Shi et al., 2021, 2018; Wang et al., 2018)(Table 2A). The *H. armigera* CYP6B6 is capable of detoxifying both natural (capsaicin) and chemical compounds (the pyrethroid esfenvalerate) while also contributing to resistance to chlorpyriphos (organophosphate) and bifenthrin, cyfluthrin (pyrethroids) (Shi et al., 2021; Tian et al., 2019, 2017; Zhao et al., 2016). The *H. armigera* CYP6B7 has been shown to metabolize two different insecticide classes: pyrethroids (esfenvalerate, fenvalerate and bifenthrin) and OPs (chloryrifos) (Shi et al., 2021; Zhao et al., 2018, 2017). CYP6BG1 from *P. xylostella* has been functionally implicated in chlorantraniliprole (diamide) as well as b-cypermethrin, permethrin (pyrethroid) resistance *in vivo* (Bautista et al., 2009; Li et al., 2018) (Table 2A). *S. exigua* CYP6AB14, CYP9A98, CYP9A10 contribute to pyrethroid resistance (deltamethrin for CYP6AB14, CYP9A98 and a- cypermethrin for CYP9A10) (Hafeez et al., 2020a, 2019), while CYP6AE10 is implicated in resistance to lamda- cyhalothrin (pyrethroid) and metabolic adaptation to the its plant host defense allelochemicals (quercetin (flavonoid)) (Hafeez et al., 2020b). *S. litura* CYP9A40 is implicated in resistance/tolerance of both insecticides (deltamethrin (pyrethroids), methoxyfenozide (diacylhydrazines)) and natural compounds (cinnamic acid, quercetin (flavonoid)) (Wang et al., 2015b).

5.2 Non- Lepidoptera pests (Homoptera, Hemiptera, Diptera, Coleoptera, Orthoptera)

L. striatellus CYP353D1v2 is able to degrade the chemically unrelated insecticides buprofezin and imidacloprid (neonicotinoid) (Elzaki et al., 2017), while CYP417A2v2, CYP439A1v3, CYP4C71 have been found to metabolize chemical substrates: imidacloprid (neonicotinoid), deltamethrin (pyrethroid) and imidacloprid (neonicotinoid), respectively (Miah et al., 2019, 2017; Xiao et al., 2020). CYP6CW1 has been associated with buprofezin and pymetrozine (pyridine azomethine derivative) resistance, CYP4DE1 and CYP6W3v2 with ethiprole (phenyl pyrazole) resistance (Elzaki et al., 2015; Zhang et al., 2015). Three *L. decemlineata* P450s have been associated with imidacloprid resistance (neonicotinoid): CYP4Q3, CYP9e2- like and CYP9Z26 (Clements et al., 2017; Kaplanoglu et al., 2017; Naqqash et al., 2020) while CYP350D1 has been associated with chlorantraniliprole (diamide) resistance (Dumas et al.,

2020). *A. gossypii* CYP6CY14, CYP6CY22 and CYP6UN1 have been found to be involved in resistance to dinotefuran (CYP6CY14 also in acetamiprid resistance- (Ullah et al., 2020)) and potentially involved in its detoxification (Chen et al., 2020), while CYP380C6 is involved in spirotetramat (tetronic and tetramic acid derivative) resistance (Pan et al., 2018) and CYP6A2 in a- cypermethrin (pyrethroid) and spirotetramat (tetronic and tetramic acid derivative) (Peng et al., 2016)(Table 2B). The *N. lugens* CYP4DE1, CYP353D1, CYP439A1, CYP6AY1v2 have been associated with chlorpyriphos and imidacloprid resistance (organophosphate and neonicotinoid, respectively)(Xu et al., 2020). Additionally, CYP4CE1 and CYP6CW1 metabolize and are also involved in resistance of imidacloprid (neonicotinoid) (Zhang et al., 2016). CYP6ER1 detoxifies several members of the neonicotinoid insecticide family (imidacloprid, thiamethoxam, dinotefuran and nitenpyram), as well as sulfoxaflor (group of sulfoximines) (Hamada et al., 2020; Liao et al., 2019; Mao et al., 2019; Pang et al., 2016; Sun et al., 2018). Several CYP6ER1 variants have been investigated for their capacity to detoxify imidacloprid, with positive results (Zimmer et al., 2018). A follow up study on the aforementioned CYP6ER1 variants that bear deletions was published by Hamada and colleagues (Hamada et al., 2020) and assayed for their ability to metabolize/detoxify several neonicotinoid insecticide (acetamiprid, thiacloprid, clothianidrin, thiamethoxam, nitenpyram) and one butenolide (flupyradifurone) (Table 2B). *S. zeamais* CYP6MS1 knockdown experiments revealed its role in terpinen-4- ol (terpineol) susceptibility (Huang et al., 2020). The *S. avenae* CYP6A14-1 and CYP307A1 contribute to imidacloprid (neonicotinoid) resistance (Zhang et al., 2020). *S. furcifera* CYP6FD1 and CYP4FD2 contribute to sulfoxaflor (sulfoximine) resistance (Wang et al., 2019) and CYP6ER4 to chlorpyriphos (organophosphate) resistance (Ruan et al., 2021). *T. castaneum* CYP346 has been experimentally associated with phosphine resistance (Wang et al., 2020). Five *D. citri* P450s (CYP4C67, CYP4DA1, CYP4C68, CYP4G70, CYP4DB1) are involved in imidacloprid (neonicotinoid) resistance (Killiny et al., 2014). Chen and colleagues depicted in several studies the relationship of *B. odoriphaga* CYP9b2, CYP49a1, CYP12b1 and CYP6FV12 and imidacloprid resistance (neonicotinoid), while CYP3356A1 has been related to imidacloprid, thiamethoxam (neonicotinoids) and b- cypermethrin (pyrethroid) resistance (Chen et al., 2018; Chen et al., 2019a, 2019b) (Table 2B).

The whitefly CYP6CM1 is capable of metabolizing a broad range of compounds that belong to neonicotinoids (imidacloprid, thiacloprid, nitenpyram, clothianidin), pyriproxyfen and pyridine azomethine- derivative (pymetrozine) groups (Daborn et al., 2012; Hamada et al.,

2019; Karunker et al., 2009; Nauen et al., 2015, 2013; Roditakis et al., 2011). Additionally, *B. tabaci* CYP6CX4 has been found to contribute to flupyradifurone (butenolide) and imidacloprid (neonicotinoid) resistance (Wang et al., 2020). The *L. migratoria* CYP303A1 is implicated in pyrethroid (deltamethrin), organophosphate (malathion, chlorpyriphos) and carbamate (carbaryl) resistance (Wu et al., 2020) while CYP9AQ2 from the same species has been functionally associated with detoxification of pyrethroids (Guo et al., 2015). Also, CYP6FD1 has been shown to metabolize deltamethrin (pyrethroid)(Liu et al., 2019) (Table 2B). *T. castaneum* CYP4BN6 and CYP6BQJ have been shown to contribute to essential oil (from *Artemisia vulgaris*) metabolic detoxification (Gao et al., 2020; Zhang et al., 2021).

5.3 Mite pests

Several more specialized P450s of the CYP392 family in *T. urticae* are capable of metabolizing specific acaricides showing narrower range metabolism in comparison to other arthropod P450s. For instance, CYP392A16 and CYP392E10 are metabolizing abamectin and ketoenols, respectively (Demaeght et al., 2013; Riga et al., 2014) (Table 2C). *T. urticae* CYP392A11 is able to detoxify certain acaricides b- ketonitrile (cyenopyrafen) and METI (fenpyroximate), while RNAi studies indicated the involvement of the specific P450 in abamectin resistance (Riga et al., 2015; Xu et al., 2021). Resistance to abamectin (avermectin) has also been attributed to some *T. urticae* P450s: CYP392D8, CYP392A11, CYP392A12, CYP389C10 (Xu et al., 2021). *T. cinnabarinus* P450s have been validated *in vivo* to be implicated in fenpropathrin (pyrethroid) resistance: CYP389B1, CYP392A26, CYP391A1, CYP384A1, CYP392A11 and CYP392A28 (Shi et al., 2016), while CYP389C16 is able to metabolize b-ketonitrile (cyflumetofen and its de-esterified metabolite) and METI (pyridaben) acaricides (Feng et al., 2020)(Table 2C). Recently, *T. urticae* CYP392A16 has been found to metabolize a metabolite of pyflubumide (carboxanilide metabolite) (Fotoukkiaii et al., 2021).

5.4 Pollinators and Papilio sp.

The *A. mellifera* P450s 9Q1, 9Q2, 9Q3 have all demonstrated metabolic activity against tau-fluvalinate (pyrethroid), thiacloprid, acetamiprid (neonicotinoids), coumaphos (organophosphate) and flupyradifurone (butenolide) as well as natural compounds of the flavonoid group (quercetin) (Haas et al., 2021; Manjon et al., 2018; Mao et al., 2011) (Table 2D). Also, *A. mellifera* P450s 6AS1, 6AS3, 6AS4 and 6AS10 have been implicated in natural compound metabolism, from the group of flavonoids (quercetin) (Mao et al., 2009). The eastern honey bee *A. cerana cerana* P450s 301A1, 303A1, 306A1 showed to be functionally associated

with a wider xenobiotic spectrum, involving thiamethoxam (neonicotinoid), dichlorvos (organophosphate), deltamethrin (pyrethroid) and a herbicide, classified as viologen (paraquat) (Liu et al., 2019). The *B. terrestris* P450s 9Q4, 9Q5, 9Q6 and the *O. bicornis* CYP9BU1 and CYP9BU2 have also been found to be involved in neonicotinoid detoxification: thiacloprid, acetamiprid (Manjon et al., 2018; Troczka et al., 2019) and imidacloprid, thiacloprid (Beadle et al., 2019) respectively (Table 2D).

Among the most well characterized examples of P450-mediated detoxification of plant allelochemicals is that of furanocoumarin metabolism within the genus *Papilio* (swallowtail butterflies). *Papilio polyxenes* CYP6B1, can metabolize plant allelochemicals: the fouranocoumarins xanthotoxin, psoralen, angelicin and flavone, the simplest member of the class of flavones, as well as the OP diazinon (Li et al., 2003; Wen et al., 2003) Interestingly, the comparison between the greater substrate range of the generalist *H. zea* CYP6B8 and the narrower substrate range of the specialist *P. polyxenes* CY6B1 indicated that generalist detoxification proteins have the ability to metabolize more structurally diverse compounds (Li et al., 2003). *P. polyxenes* CYP6B3 was observed to metabolize alpha-naphthoflavone and furanocoumarins (Li et al., 2003; Wen et al., 2003) (*P. glaucus*, CYP6B17, CYP6B21, *P. canadensis* CYP6B25 and *P. multicaudatus* CYP6B33 have all been involved in furanocoumarin metabolism (Hung, 1997; Li et al., 2003). Notably, P450s from *P. glaucus*, which feeds occasionally on furanocoumarin-containing host plants, showed higher activities against furanocoumarins than those from *P. canadensis*, which normally does not encounter furanocoumarins. These P450s in turn catalyze a larger range of furanocoumarins at lower efficiency than CYP6B1, a P450 from *Papilio polyxenes*, which feeds exclusively on furanocoumarin-containing host plants (Li et al., 2003).

Although the aforementioned studies provide an overview of the number of substrates catalyzed by each P450, drawing conclusions concerning their substrate specificity (narrow or broad metabolizer) should be determined on the basis of the range of substrates explored in each study. For instance, *T. urticae* CYP392A16 has been shown to metabolize abamectin only, although other insecticides belonging to different classes were also tested without detecting metabolism. On the other hand, other P450s may show metabolism to specific xenobiotics, while may or may not be tested for their catalytic activity against other compounds, i.e. CYP6A51 from *C. capitata*, CYP6BQ23 from *B. (M.) aeneus* and others.

6. Conclusions

Arthropods have expanded the limits of P450 sequence diversity, with many variations on the sequences diverse approaches have been used to characterize P450s in arthropods. Each approach comes with unique strengths and weaknesses and most of the time frequently a clear picture can only be drawn upon successful completion of parallel efforts. In principle the P450 characterization approaches can be divided into *in situ* approaches e.g. genome editing or RNAi in the species of interest and *ex situ* approaches e.g. *in vivo* ectopic expression in model organisms or heterologous expression *in vitro*. The toolbox in biology has never been richer than it is today. To decipher the contribution of P450s (and other players in pathways) multidisciplinary approaches should be combined. While the *in situ* approaches offer the advantages of the context the danger maybe to overlook compensatory processes (regulation of other genes as an unintended consequence of the primary desired changes). On the other hand, the *ex situ* approaches may be less convincing e.g. low resistance ratios of transgenic *D. melanogaster* because the relevant context is missing. Rarely are measured turnover rates *in vitro* put in a clear context to what it means for the toxicokinetics *in vivo*. The toolbox that allows us to study single genes/enzymes in detail may distract us with describing the principal component (in the absence of certainty about how many there are) in detail.

Studying the evolution and function of arthropod P450s could inspire genomics-based ecotoxicology and pest control research, while the catalytic activity of unusual P450s could serve as an inspiration for green chemistry pesticide leads (Figure 5). For example, bees and pollinators are equipped with P450-based defense that define their sensitivity to insecticides and this knowledge can be exploited to avoid negative off-target effects (Manjon et al., 2018). Indeed, previous studies demonstrated that honeybees exhibit differences in their sensitivity to pesticides, and that certain compounds display very low toxicity to bees, even when used as in-hive treatments against *Varroa* mites (Tomizawa and Casida, 2005). The negative cross-resistance between various insecticide/pro-insecticide classes, due to overproduction of P450s in resistant insects which detoxify certain active ingredients but activates others is another example that has also been explored (Adolfi et al., 2019). P450 -biotechnology based applications, including testing pipelines for screening the selectivity and liability of active compounds against insecticidal leads have been considered and/or developed at certain extend, for pest and vector control. These include robust pipelines for standardized microsomal preparations for the monooxygenase blend based metabolism, libraries of recombinant insect (pests and pollinators) P450s, as well as transient and stable reverse genetic approaches in insects and/or insect cell lines. However, the potential of those tools for

industrial applications needs to be further validated and can only be realized if sufficient and consistent yields of recombinant proteins are achieved or robust reverse genetic systems or cell based assays are established. With a toolbox as rich as today's we should aim at fusing the knowledge of principle components with the bigger picture. Knocking out a P450 and running a bioassay is a good step, preparing microsomes form the knock-out strain and measuring metabolism vs. wildtype microsomes (and/or *in vivo* metabolism) should become a logical second step to put a finger on the rate of metabolism in the relevant context (where no reliable knock-outs can be achieved a similar principle may be applied to model species). If more holistic studies are conducted in tandem to heterologous expression, it may allow translation of results and bring us closer to extrapolation of toxicity thus enabling *in vitro* screens for desired selectivity based on differential metabolism.

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Competing interests' statement

The authors have no competing interests to declare

Declarations of interest

None

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Tables

Table 1. Species used for the phylogenetic analysis.

Species	Insect order	Number of P450s	Source
Helicoverpa armigera	Lepidoptera	112	Publicly available; manually curated
Spodoptera littoralis	Lepidoptera	137	Private Syngenta genome; manually curated
Plutella xylostella	Lepidoptera	88	Private Syngenta genome; manually curated
Apis mellifera	Hymenoptera	49	Publicly available; manually curated
Megachile rotundata	Hymenoptera	50	Publicly available; manually curated
Osmia bicornis	Hymenoptera	60	Publicly available; manually curated
Bombus terrestris	Hymenoptera	6*	Publicly available; manually curated
Nasonia vitripennis	Hymenoptera	90	Publicly available; manually curated

* only partially curated

Table 2 A-D: P450s from agricultural insect pest species that are involved in xenobiotic metabolism (synthetic or natural compounds) and the validation systems (*in vitro*/ *in vivo*/ genome modification) identified in the literature. A. Lepidoptera pest species, B. Non-Lepidoptera insect pest species, C. Mite and D. Pollinator/*Papilio sp.* species.

A			Synthetic compounds									Natural compounds									Validation systems	
Order	**Species**	**P450**	**PYR**	**NN**	**OC**	**OX**	**AV**	**CAR**	**OP**	**DM**	**DCH**	**FC**	**KT**	**PA**	**FLV**	**IA**	**AFL**	**CR**	**CA**	**CN**	**GA**	***in vitro***	***in vivo/ Genome mod.***
Lepidoptera	*Amyelois transitella*	CYP6AB11										✓										X	
	Chilo suppressalis	CYP6CV5								✓													R
		CYP9A68								✓													R
		CYP321F3								✓													R
		CYP324A12								✓													R
	Cydia pomonella	CYP9A61	✓																			X	
		CYP6B2	✓						✓														R
	Depressaria pastinacella	CYP6AB3										✓										X	
		CYP6AB3v2										✓										X	
		CYP6AE89										✓										X	
	Helicoverpa armigera	CYP337B3v1	✓																			X	
		CYP6B6	✓						✓											✓		X	R
		CYP6B7	✓						✓													X	
		CYP6AE11	✓	✓	✓	✓		✓				✓	✓									X	C
		CYP6AE12	✓		✓			✓				✓										X	C
		CYP6AE14	✓	✓	✓	✓		✓				✓	✓	✓								X	R/C
		CYP6AE15	✓		✓			✓				✓										X	C
		CYP6AE16	✓	✓	✓			✓				✓										X	C
		CYP6AE17	✓	✓	✓	✓		✓				✓										X	C
		CYP6AE18	✓	✓	✓	✓		✓				✓										X	C
		CYP6AE19	✓	✓	✓	✓		✓				✓	✓									X	C
		CYP6AE20	✓			✓						✓	✓										C
		CYP6AE24	✓	✓	✓																	X	
		CYP9A12	✓											✓						✓		X	
		CYP9A14	✓																	✓		X	R
		CYP337B1	✓																			X	
		CYP9A16	✓																			X	
		CYP9A3	✓																			X	
		CYP9A23	✓																			X	
		CYP6B2	✓																			X	
		CYP9A17	✓																	✓		X	
	Helicoverpa zea	CYP6B8	✓		✓			✓	✓			✓			✓	✓			✓			X	
		CYP321A1	✓		✓				✓			✓			✓		✓					X	
		CYP6B27	✓		✓			✓	✓													X	
	Plutella xylostella	CYP6BG1	✓							✓													R/D
		CYP321E1								✓													R
		CYP340W1					✓																R
	Spodoptera frugi-perda	CYP321B1								✓													R
		CYP321A8								✓													R
		CYP321A9								✓													R
	Spodoptera exigua	CYP9A105	✓																				R
		CYP6AE10	✓												✓								R
		CYP6AB14	✓																				R
		CYP9A98	✓																				R
		CYP9A10	✓																				R
		CYP9A21v3								✓													R
		CYP332A1							✓													X	D
		CYP321A8	✓						✓													X	D
		CYP321A16							✓													X	D

		CYP9A186					✓														X	C
	Spodoptera litura	CYP321B1	✓						✓													R
		CYP6B50									✓											R
		CYP6AB14									✓			✓			✓					R
		CYP9A40	✓							✓				✓				✓				R
		CYP6AB60															✓			✓		R
		CYP6AB12	✓																			R

PYR:Pyrethroids; NN:Neonicotinoids;; OC: Organochlorines; OX: Oxadiazines; AV:Avermectins; CAR: Carbamates; OP:Organophosphorus pesticides; DM:Diamides DCH: Diacylhydrazine ; FC: Furanocoumarins; KT: Ketones; PA: Phenolic aldehydes ; FLV: Flavonoids; IA: Indolyl alcohols; AFL: Aflatoxins; CR:Coumarin; CA: Cinnamate ester; CN: Capsaicinoids; GA: Glycoalcanoids X: validated with in vitro systems; C: Validated with the use of CRISPR; D : Validated with the use of D. melanogaster heterologous expression system; R: Validated with the use of the RNAi system

B			**Synthetic compounds**													**Natural compounds**			**Validation systems**	
Order	**Species**	**P450**	**PYR**	**NN**	**SUL**	**CAR**	**OP**	**PPF**	**BF**	**TTA der**	**PA der**	**DM**	**PHP**	**BTL**	**PHO**	**T**	**AL**	**EO**	***in vitro***	***in vivo/ Genome mod.***
Homoptera	*Sogatella furcifera*	CYP6FD1			✓															R
		CYP6ER4			✓															R
		CYP4FD2			✓															R
Hemiptera	*Aphis gossypii*	CYP380C6								✓										R
		CYP6A2	✓							✓										R
		CYP6CY13		✓	✓														X	R
		CYP6CY14		✓																R
		CYP6CY19			✓															R
		CYP6CY22		✓															X	R
		CYP6UN1		✓																R
		CYP6DC1		✓																R
		CYP6CZ1		✓																R
	Bemisia tabaci	CYP6CM1*		✓				✓			✓			✓					X	R/D
		CYP6CX4		✓				✓			✓			✓						R
	Diaphorina citri	CYP4C67		✓																R
		CYP4DA1		✓																R
		CYP4C68		✓																R
		CYP4G70		✓																R
		CYP4DB1		✓																R
	Laodelphax striatellus	CYP6AY3v2		✓															X	D
		CYP6FU1	✓																X	
		CYP353D1v2		✓					✓										X	
		CYP417A2v2		✓															X	
		CYP439A1v3	✓																X	
		CYP6CW1							✓		✓									R
		CYP4DE1											✓							R
		CYP6CW3v2											✓							R
		CYP4C71		✓															X	
	Myzus persicae	CYP6CY3		✓													✓		X	D
	Nilaparvata lugens	CYP6AY1		✓			✓												X	R
		CYP6ER1**		✓	✓									✓					X	R/D
		CYP4CE1		✓															X	R
		CYP6CW1		✓															X	R
		CYP4DE1		✓			✓													R
		CYP353D1		✓			✓													R
		CYP439A1		✓			✓													R
		CYP6CS1		✓							✓									R/D
Diptera	*Bradysia odoriphaga*	CYP9b2		✓																R
		CYP49a1		✓																R
		CYP12b1		✓																R
		CYP6FV12		✓																R
		CYP3356A1	✓	✓																R
	Ceratitis capitata	CYP6A51	✓																X	D
Coleoptera	*Leptinotarsa*	CYP4Q3		✓																R

	decemlineata	CYP9e2-like		✓															R
		CYP6BJ[a/b]		✓														X	R
		CYP6BJ1v1		✓														X	R
		CYP9Z25		✓														X	R
		CYP9Z26		✓															R
		CYP9Z29		✓														X	R
		CYP350D1									✓								R
	Brassicogethes (M.) aeneus	CYP6BQ23	✓															X	D
	Sitophilus zeamais	CYP6MS1													✓				R
	Tribolium castaneum	CYP6BQ9	✓															X	R/D
		CYP346												✓					R
		CYP4BN6												✓			✓		R
		CYP6BQJ															✓		R
Orthoptera	*Locusta migratoria*	CYP6FD1	✓															X	
		CYP6FF1	✓																R
		CYP6FD2				✓													R
		CYP6FE1				✓													R
		CYP4G102	✓			✓	✓												R
		CYP4G62	✓			✓	✓												R
		CYP9AQ2	✓																R
		CYP409A1	✓																R
		CYP408B1	✓																R
		CYP9AQ1	✓																R
		CYP9A3	✓																R
		CYP6HC1	✓																R
		CYP6HL1	✓			✓													R
		CYP6HN1	✓			✓													R
		CYP6HQ1	✓			✓													R
		CYP303A1	✓			✓	✓												R

*CYP6CM1 includes CYP6CM1vB/ CYP6CM1-B/ CYP6CM1vQ **CYP6ER1 includes CYP6ER1-del3 and CYP6ER1-T318Sdel3.

PYR:Pyrethroids; NN:Neonicotinoids; SUL: Sulfoximines; CAR: Carbamates; OP:Organophosphorus pesticides; PPF: Pyriproxifen; F:Bu-profezin; TTA-der:Tetronoc and tetramic acid derivatives; PA-der: Pyridine azomethine derivatives; DM: Diamides; PHP: Phenylpyrazoles; BTL: Butenolides; T: Terpineol; AL:Alkaloids; Phosph: Phosphine; EO: Essential oils X: validated with in vitro and/or in vivo systems; D : Validated with the use of D. melanogaster heterologous expression system; R: Validated with the use of the RNAi system.

C			**Synthetic compounds**						**Validation systems**	
Order	**Species**	**P450**	**PYR**	**AV**	**TTA der**	**b-KT**	**CRN**	**METI**	***in vitro***	***in vivo/ Genome mod.***
Trombidiformes	*Tetranychus cinnabarinus*	CYP389C16				✓		✓	X	R
		CYP389B1	✓							R
		CYP392A26	✓							R
		CYP391A1	✓							R
		CYP384A1	✓							R
		CYP392D11	✓							R
		CYP392A28	✓							R
	Tetranychus urticae	CYP392A16		✓			✓		X	D
		CYP392A11		✓				✓	X	R/D
		CYP392E10			✓				X	
		CYP392A12		✓						R
		CYP392D8		✓						R
		CYP389C10		✓						R

PYR:Pyrethroids; AV: Avermectins; TTA der:Tetronoc and tetramic acid derivatives; b-KT: beta ketonitrile derivatives; CRN: Carboxanilides; METI: Mitochondrial complex I electron transport inhibitors

X: validated with *in vitro* systems;; D : Validated with the use of *D. melanogaster* heterologous expression system; R: Validated with the use of the RNAi

D			Synthetic compounds					Natural Compounds		Validation systems	
Order	**Species**	**P450**	**PYR**	**NN**	**OP**	**BTL**	**VL**	**FC**	**FLV**	***in vitro***	***in vivo/ Genome mod.***
Hymenoptera	*Apis cerana cerana*	CYP301A1	✓	✓	✓		✓				R
		CYP303A1	✓	✓	✓		✓				R
		CYP306A1	✓	✓	✓		✓				R
	Apis mellifera	CYP9Q1	✓	✓	✓				✓	X	
		CYP9Q2	✓	✓	✓	✓			✓	X	D
		CYP9Q3	✓	✓	✓	✓			✓	X	D
		CYP6AS1							✓	X	
		CYP6AQ1				✓				X	D
		CYP6AS3							✓	X	
		CYP6AS4							✓	X	
		CYP6AS10							✓	X	
	Bombus terrestris	CYP9Q4		✓						X	D
		CYP9Q5		✓						X	
		CYP9Q6		✓						X	D
	Osmia bicornis	CYP9BU1		✓						X	D
		CYP9BU2		✓						X	
Lepidoptera	*Papilio polyxenes*	CYP6B1			✓			✓	✓	X	
		CYP6B3						✓	✓	X	
	Papilio glaucus	CYP6B4						✓		X	
		CYP6B17						✓		X	
		CYP6B21						✓		X	
	Papilio canadensis	CYP6B25						✓		X	
	Papilio multicaudatus	CYP6B33						✓		X	

PYR:Pyrethroids; NN:Neonicotinoids;; OP: Organophosphates; BTL: Butenolides; VL:Viologen; FC: Furanocoumarins; FLV: Flavonoids
X: validated with *in vitro* systems;; D : Validated with the use of *D. melanogaster* heterologous expression system; R: Validated with the use of RNAi

Figure Legends

Figure 1: Heat map of the studied P450s per species and the number of scientific studies referring to them. **A.** Lepidoptera pest species **B.** Non-Lepidoptera pest species. **C.** Mite pest species and **D.** Pollinators/*Papilio* sp. The scale bar indicates the amount of studies referring to each P450 within each insect category.

Figure 2: Phylogenetic analysis of Lepidoptera and Hymenoptera P450s. **A.** Phylogenetic analysis of Lepidoptera P450s from three major pest species; *H. armigera* (Harmi), *S. littoralis* (Slitt), and *P. xylostella* (Pxylo). Virtually all known insecticide metabolizers are found in Clan3. This is in contrast to the P450s implicated in physiological functions that are almost equally distributed across all four clans. **B.** Phylogenetic analysis of hymenopteran P450s from five species; *A. mellifera* (Amell), *B. terrestris* (Bterr), *O. bicornis* (Obico), *M. rotundata* (Mrotu), and *N. vitripennis* (Nvitr). Similarly to Lepidoptera, most insecticide metabolizers are found in Clan 3, whereas CYPs involved in physiological functions are found in the remaining clans. The shapes that are next to the genes indicate the following: black circles – P450s involved in the metabolism of insecticides; black triangles – P450s involved in known physiological functions; black stars – P450s belonging to families with few members, and are thus suspected to be involved in physiological functions; red stars – same as in the previous category, but these are also Lepidoptera or Hymenoptera-specific, respectively. Branch color denotes CYP clans: cyan – Clan2; yellow – Mito; orange – Clan4; green – Clan3.

Figure 3: Venn graphs depicting the number of functionally characterized P450s with *in vivo* and *in vitro* systems. *In vivo* functional characterization has been categorized into RNAi and genome modification with the latter one including CRISPR and transgenic *Drosophila melanogaster*: **A.** Total species, **B.** Lepidoptera insect pest species, **C.** Non-Lepidoptera insect pest species, **D.** Mite pest species, **E.** Pollinator/ *Papilio sp.* species.

Figure 4: Percentage of studies* that characterize P450s of insects and mites of economic and public health interest (including Lepidoptera species, other insect species, mite species, pollinators/*Papilio sp.* and mosquito species (*Anopheles* and *Aedes*)) using different *in vitro* systems (*E.coli*, insect cells- baculovirus mediated, yeast and stable insect cells). **A.** Total insect species, **B.** Lepidoptera insect pest species, **C.** non -Lepidoptera insect pest species, **D.** mite pest species, **E.** pollinator/ *Papilio sp.* species and **F.** mosquito species.

*Studies that use two or more *in vitro* validation systems are considered as two or more different studies. Also a study for two or more P450s are considered as two or more different studies.

Figure 5: *Left*: novel highly selective insecticides and synergists; **A**. **Synergists** (green structure with bar) that are not intrinsically toxic, will inhibit the target species CYPs (blue), to increase / maintain the effectiveness of the insecticides (green chemical); **B**. **Pro-drug** (green chemicals) will only become toxic, when enzymatically activated inside target insects (red dots), by specialized and resistance conferring P450s; **C. Safe chemicals**: synergists & pro-insecticides will be non-toxic to bees and non- target organisms, since they will be readily degraded by their P450s. *Right*: Innovative protein and cell biotechnology based tools for insecticide development; **D. High-throughput plate with immobilised enzymes, cells, or micro-patterned cells** for screening metabolic liability/activation, toxicity and selectivity of low risk/safe (pro)insecticides.

Figures

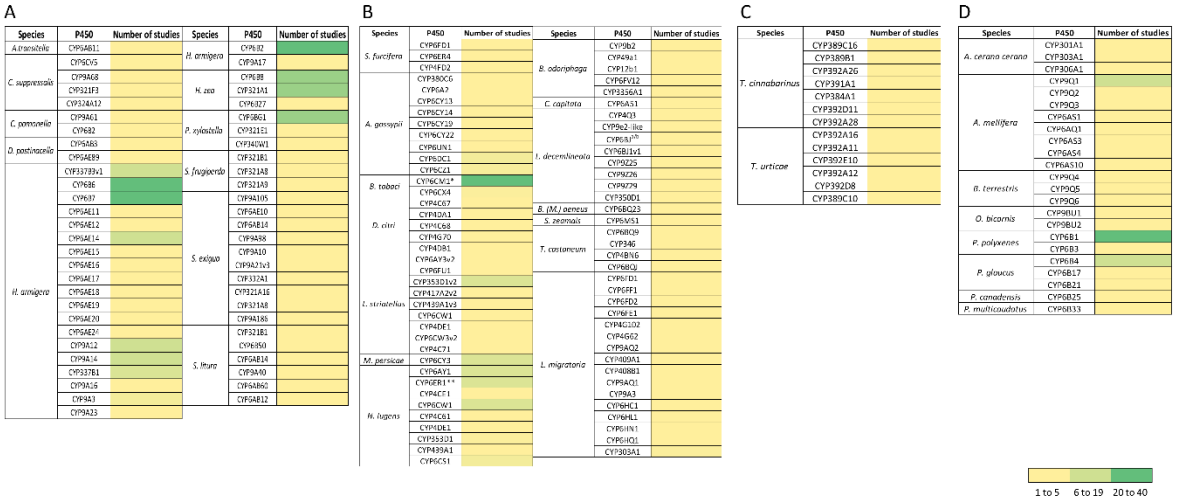


Figure 1

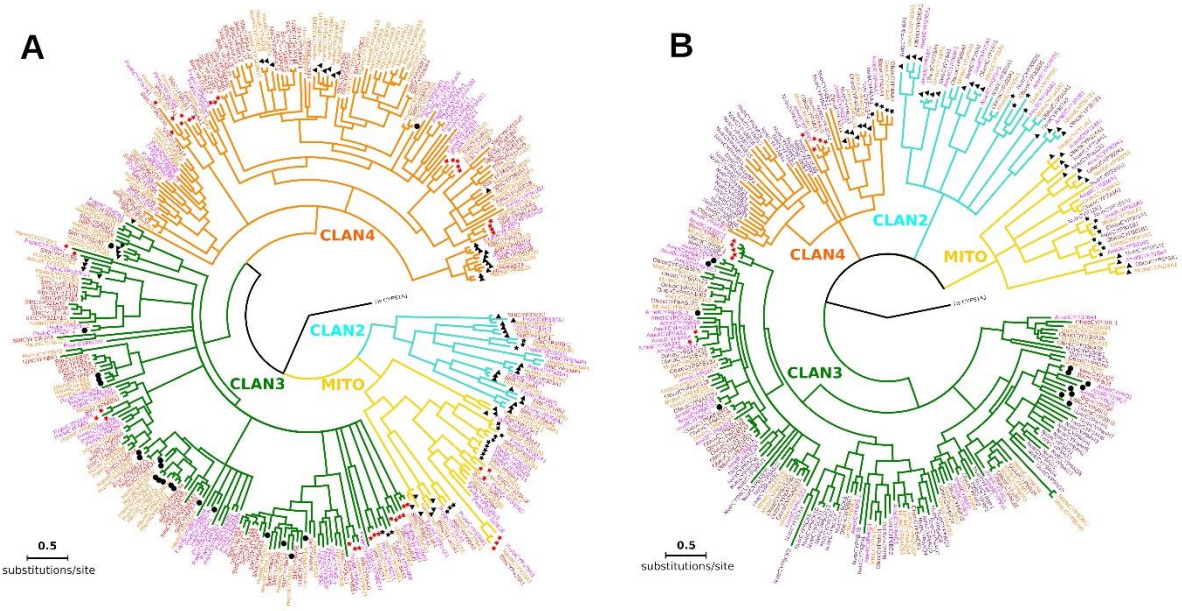
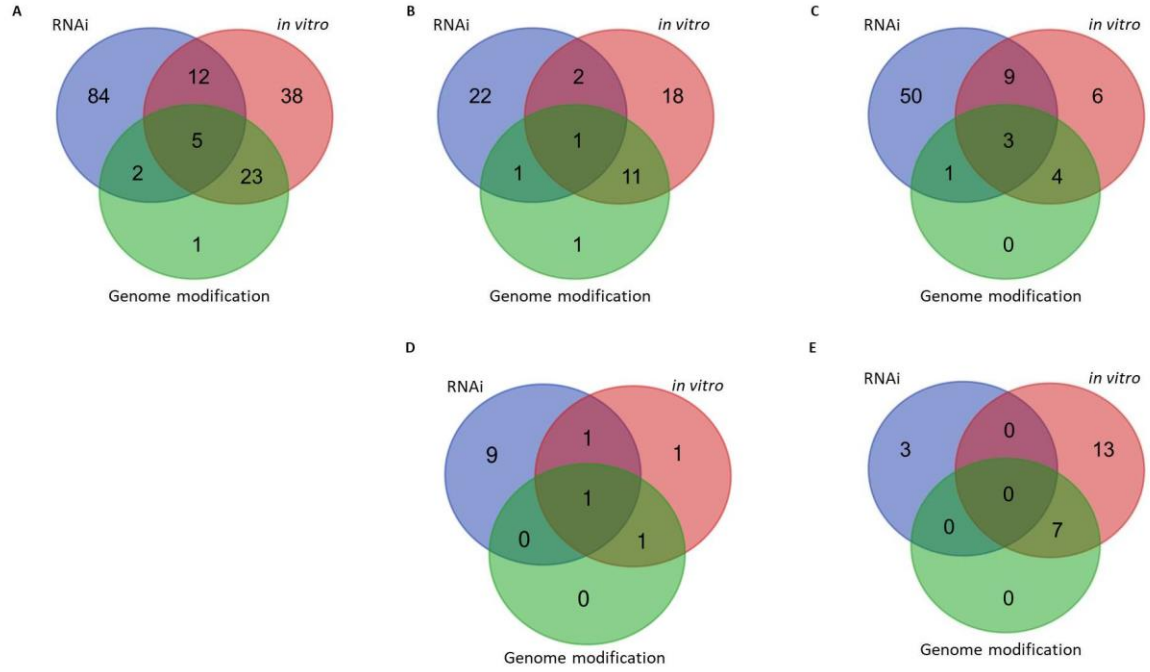
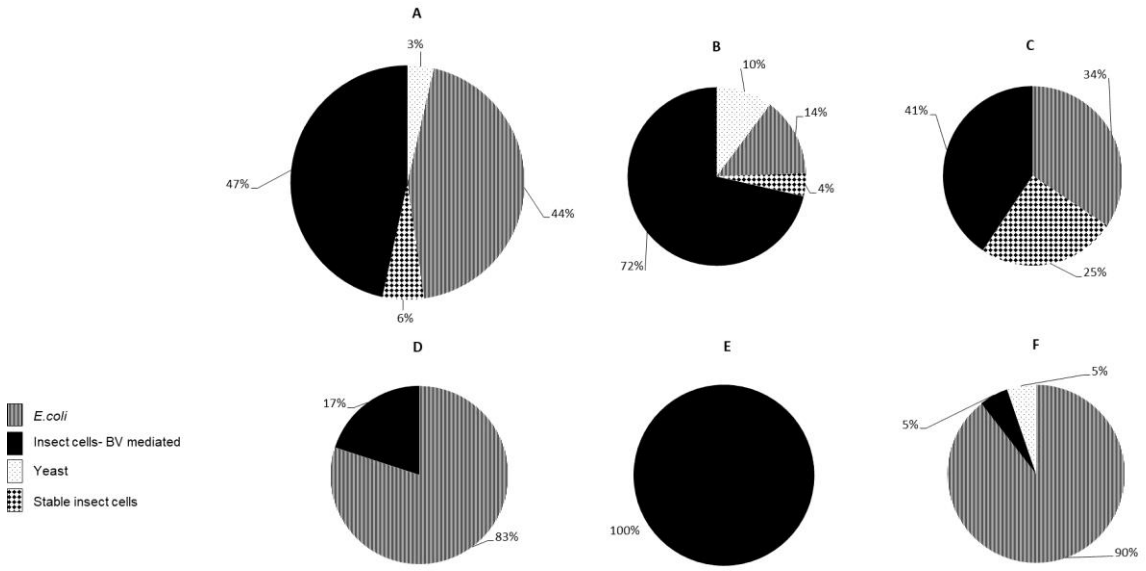


Figure 2



1448

1449 **Figure 3**



1450

1451 **Figure 4**

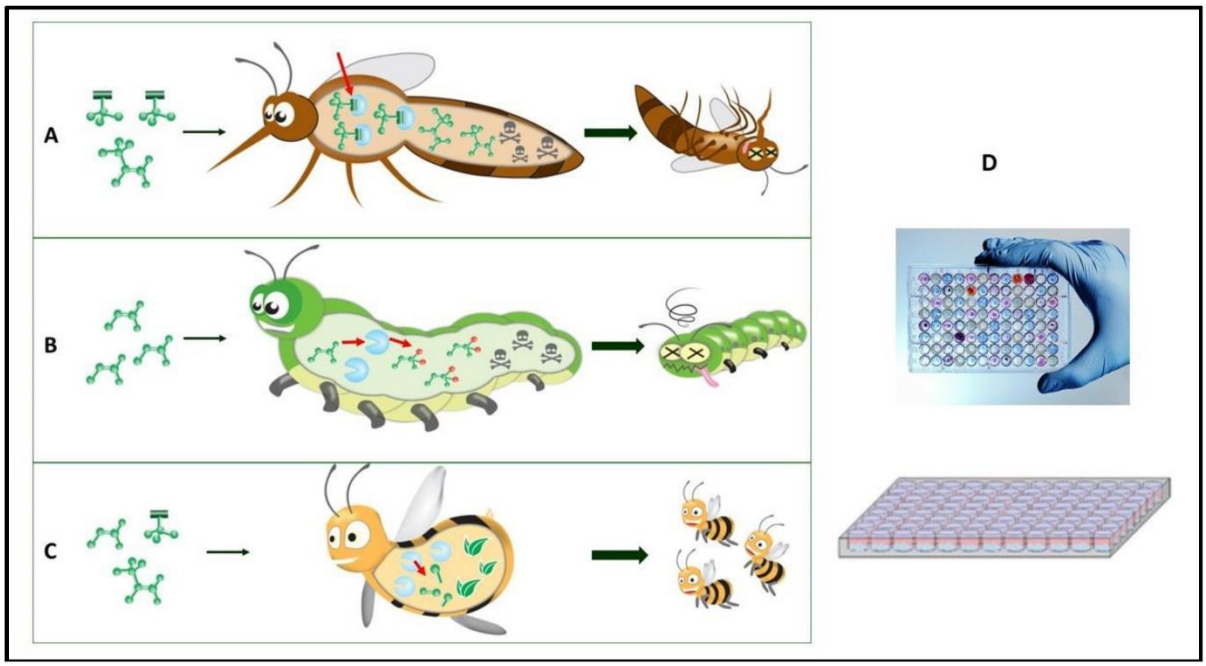


Figure 5