Developing screening tools to identify novel, resistance breaking pesticides.

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

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Abstract

Pesticide resistance is estimated to cost the USA $1.4 billion annually. Not only is there a huge economic cost, but the loss of crop yield and higher doses of pesticides needed to control pests damages the ecosystem [1,2]. The development of resistance to chemicals is a universal phenomenon and within insect pests more than 440 species are now resistant to one or more pesticidal compound [3,4]. As increasing levels of resistance arise and new molecular tools become available the understanding of resistance mechanisms grows and the limitations of pesticides are clarified [5,6]. Understanding resistance is vital to counter it [5,7,8].

Still facing high levels of pesticide resistance and the damaging effects of the remaining effective compounds, I here look to identify a novel pesticidal compound to overcome current resistance mechanisms [9]. Synthetic compounds made by industrial partner Darr House M.I. were tested for activity against *Drosophila melanogaster* and *Myzus persicae.* The first 18 compounds were expected to act on the nicotinic acetylcholine receptor using imidacloprid as a positive control. Four competitively active compounds were found but, following a ban on neonicotinoids in the EU in 2018 and a knock-on lack of interest on the part of major agrochemical companies in novel nAChR compounds, this part of the project was pursued no further [10,11]. The next 30 compounds were then tested for activity against the neurotransmitter gamma-aminobutyric acid (GABA) receptor. Here activity was only found against *Drosophila* not *Myzus.* Five compounds showed activity against *D. melanogaster* susceptible strain

Canton-S, four then showed activity against metabolic resistant strain Hikone-R with compound 47 being close to resistance breaking.

While synthetic compounds are popular, natural sources are not only a source of inspiration for synthetic products but natural products used for pest control have advantages of being environmentally friendly and constantly evolving with their pests. I tested 9 botanical sources for insecticidal and repellent activity against *D. melanogaster* and the Peach potato aphid *M. persicae.* Extracts from samples were taken using a methanol extraction technique. Rosemary extract results suggest potential lethal effects on *Drosophila* but development of this product would be required to concentrate the lethal effects above 40%. All extracts: basil, chilli, garlic, lemongrass, nasturtium leaves, flowers and seeds and rosemary showed repellent activity against *Myzus* except dill extract which had no effect. An increase in nymph droppings was seen for *Myzus* treated with basil suggesting possible problems for use of this compound as aphid control.

To address the problem of cost and identification of novel active pesticides a Fly-Tox panel was developed using *D. melanogaster* as a model screening tool containing metabolic P450 resistance genes from multiple economically important pests and pollinator species. In this thesis four lines were developed containing *Cyp6cm1, Cyp6bq23, Cyp6bq9* and *Cyp337b3* but conferral of resistance was unsuccessful. Alternative lines from the published Fly-Tox panel were used to test the use of the screening tool with novel insecticides from chapter 2 and 3; one nAChR and one GABA targeting compound. These novel compounds were compared against positive controls; imidacloprid and fipronil, and showed a successful test run of a section of the screening tool.

No resistance breaking bee-safe compounds were identified in this thesis but there was a successful trial of the Fly-Tox screening tool of transgenic *Drosophila* showing the value of this new resource in pesticidal discovery science.

There were also findings of broad metabolic capabilities of the gene *Cyp6er1*, known to metabolise neonicotinoids, but also found to be active against suspected GABA targeting novel compound 47.

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Authors Declaration

Amy Elizabeth McLeman was funded by BBSRC SWBio DTP in a case partnership with Darr House M.I.. All work presented in this thesis was completed under the supervision of Professor Richard ffrench Constant, Professor Chris Bass and Dr Alistair Miller. All writing and data collection was completed by Amy McLeman unless otherwise stated below.

Chapter 2 & 3

Dr Alistair Miller provided synthetic chemical compounds 1-47 details of which can be found in supplementary table 1.

Chapter 5

Emma Randall provided pre-extracted DNA from *Bemisia tabaci* which was extracted prior to my working on this thesis. Additional support was provided by Bartek Troczka and Adam Pym instructing on proving overexpression of transgenes in developed lines.

Chapter 1:

Introduction

1.1 The current importance of pest management

1.1.1 Food Security

Global population records show dramatic increases in population sizes from 2.5 billion in 1950 to a current day estimate of 7.5 billion and predictions of 9.6 billion people by 2050 [12,13].

Figure 1.1 Global population, 1950-2100, using various projections from source: population division of the department of economic and social affairs of the United Nations Secretariat (2013). World population prospects: The 2012 Revision. New York: UN.

Despite current improvements, 793 million people still do not have food security; "when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life." [14].

There are three considerations to improve global food security: food availability, concerning a need to increase crop yields, food accessibility, concerning a

household or individuals access to a balanced diet, and utilization of food [15–17]. To address food security as a whole it is important to consider all factors; efficient food distribution, minimal waste and increased availability.

Accessibility to food is a major argument for how to improve food security and is effected by socioeconomics and politics [15,16]. Of the people suffering from food insecurity in 2014-2016 98.6% lived in developing countries while only 1.4% lived in developed countries [17]. This can be partially explained by Engels law; rich countries have a financial buffer of non-food expenses available should food prices increase, poorer countries do not [15]. It would be possible to relieve food shortages and increase food security in low-income countries with political interventions [15,17].

Policies are also suggested as a way to reduce food waste [18]. Every year 65kg of food is wasted per person across the globe; that's 18 days of a healthy diet for one person wasted per capita [19]. Of that waste most comes from high income countries and scales down with wealth of the country; the daily waste of food from high income countries is 6 times that of low income countries [19]. This again highlights the socioeconomic impacts on food security. The terms food loss and food waste are often used interchangeably but are in fact two different problems to address; food waste looks at food that was fit for consumption but has not been utilised whereas food loss looks at quantity and quality of food that causes a reduction in food suitable for human consumption [18].

A solution from The World Bank (2013) and FAO (2012) to improve global food security for the growing human population is for agriculture to produce 50 to 70% more food [13,17]. One way to keep on top of global food requirements is to protect crop yield from pests [20,21]. This thesis focusses on protecting crop

losses from pests via pesticides and therefore increasing the availability of food for consumption.

1.1.2 Pest effects on crop yield

The effect of pests on crops can be varied and significant [22]. Major food crops like wheat can lose 50% while potatoes, maize and rice can lose 40% of their yield due to pests [22]. Pre-harvest pests decrease crop yield potential by an average of 35% globally [13]. Though crops are vulnerable to other factors limiting yield it is important to develop effective pest management first as higher available yields make crops more vulnerable to pest damage [22]. A review on food security from India notes a loss of 20-30% of food crops due to pests and disease while noting the ever increasing need for effective crop protection due to their growing population and increasingly limited space available for arable land [23].

1.1.3 Pesticide use

Pesticides are useful to increase crop yield, crop quality and maintain affordable food prices [13,23–25]. The effect of pesticides alone contributing to increased crop yield is demonstrated below, we can see an approximate increase of a third in crop yield when comparing actual yields between the current situation in the central bar, to a scenario without pesticide use, the right hand side bar [22,25].

Figure 1.2 Crop yield losses and effects of crop protection and pesticide use on overall yields. Source: [22]

Pesticide use has been continuously increasing and has a dual function to control insects to protect crops and control the spread of insect-borne disease [20,24,26].

1.1.4 Pesticide discovery:

The process of novel pesticide discovery is extremely expensive and comes with high risk [27]. Of the ten agribusiness companies that monopolised the research and development of novel agrochemicals in 2015; Monsanto, Syngenta, DuPont, Bayer, Dow, BASF, MAI, Nufarm, FMC and Sumitomo Chemicals there are now only 7: Monsanto is now owned by Bayer, DuPont is no longer involved but has a spin off company Corteva Agrisciences and MAI and Nufarm are no longer involved [28]. Mergers and high research and development (R&D) costs make it difficult for smaller businesses [27,28]. The average novel insecticide cost of discovery to commercialisation in 2014 was $256 million [28].

Identification of novel compounds with useful insecticidal activity comes from multiple different experimental strategies. The strategies are here listed. Screening large numbers of compounds to look for activity is a common method. As many compounds are made quickly sampling of a collection is often required, either randomly or by testing compounds expected to show activity due to structurally recognised active moieties within a compound. Working off background research from current patents can be used to speed up discovery of novel compounds. Virtual design and crystallography are also popular for 1D, 2D and 3D chemical design. These methods provide information on a compounds physiochemical properties such as lipophilicity and molecular weight, the number or aromatic bonds and connectivity and finally the shape of a compound and the potential interactions between it and a binding site [29]. The use of synthetic chemical control of pests started in 1940 with an aim of finding novel compounds with reduced negative impacts in the 1970s [30]. There are multiple challenges facing novel pesticide discovery [28]. One which is key is the need to discover novel insecticidal compounds that overcome known resistance mechanisms in important pesticide targets, which is much harder due to the sheer quantity of pesticides in the commercial market and resistance development, spread and maintenance in wild pest populations [27,30].

1.1.5 Problems with Pesticides

The side effects of pesticides have been a concern for a long time, notably Carson's publication of 'Silent Spring' in 1962 following DDT use brought to light some of the serious effects of non-discriminate pesticides (pesticides that affect non-target organisms) [31]. Despite increased use pesticides contain the risk of

not only effecting target pest insects but non-target organisms including endangered species and humans [20,24,32]. Another notable problem of pesticides is that of emerging resistance. High usage of pesticides causes strong selection pressure which can result in highly resistant pest populations in a short time. Resistant traits evolved during strong selection pressures are then sometimes capable of conferring cross resistance to other pesticides [22,33].

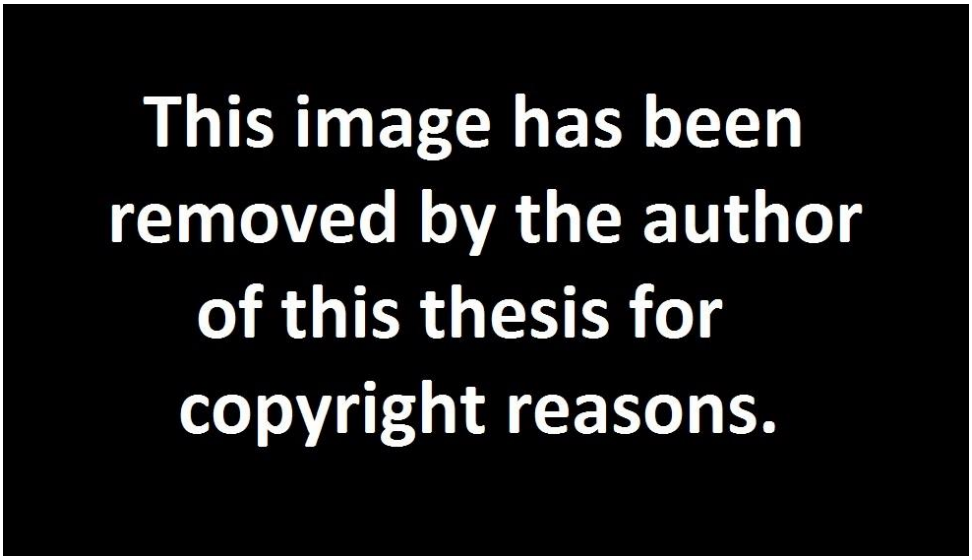


Figure 1.3 Increase in the number of species resistant to one or more insecticides alongside the number of insecticides that resistance has been found for in one or more species and finally the number of resistance traits found against GMO's in 2015. Source: [34].

Figure 1.3 shows how prevalent the rising resistance to pesticides is and that this concern is important to address when looking for novel compounds.

1.1.6 Control of Pesticide Use

The control of pesticide use needs to be evaluated by determining the risk of compounds often with data gaps [32]. It is, therefore, important when looking at potential pesticidal compounds to understand as much as possible about the compound and the potential harmful effects. The negative effects of compounds

such as neonicotinoids are an example of this lack of information on the compounds before widespread use. There is an ongoing need for new pesticide discovery due to the retirement of old pesticides, once they are no longer effective due to emerging resistance, or, discovery of environmentally damaging effects [35,36]. On rare occasions pesticides are found to have human toxicity effects following misuse of a chemical; for example organophosphate pesticides used by farmers for sheep dip was causally linked to neurological symptoms, or, the highly controversial current concern surrounding herbicide glyphosate (Roundup) having possible human health concerns and questions about proper handling of the substance [37–39].

The ‘pesticide treadmill’, a coined expression for our dependency on synthetic pesticides, states the continuous need for either higher doses of current pesticides as pests become resistant and higher doses are required to control pest populations, or new compounds all together needed to replace previous ineffective chemicals [13]. The ideal pesticide will be effective while using low quantities [23]. To understand where thinking on pesticide discovery and activity lies currently we will evaluate two important pesticide classes; neonicotinoids and phenylpyrazoles.

1.2 Methods of resistance evolution

1.2.1 Target site resistance

There are three factors that affect the rise of resistance to pesticides in insects; detoxification of compounds, the ability of a compound to pass through the insects cuticle and changes in the target site [4,40]. Binding sites of the pesticidal compound may either be modified or absent altogether in resistant pests [4]. An

example of target site resistance can be seen in the brown plant hopper to imidacloprid [41]. Imidacloprid is known as an agonist to the nicotinic acetylcholine receptor (nAChR) and research has shown two high affinity binding sites within the receptors of susceptible strains of pests affected by imidacloprid [41]. Liu *et al.,* 2005, [41] demonstrated supporting evidence that resistance to imidacloprid within the brown plant hopper could be due to target site insensitivity. The binding of imidacloprid was shown to be lower on membranes isolated from resistant strains when compared to susceptible strains and they noted cross resistance to other pesticides that had the same target binding site of the nAChR [41].

Further examples of point mutations conferring both resistance to imidacloprid, the selecting chemical initiating resistance responses, and cross resistance to similarly acting pesticides are noted in *Aphis gossypii* [42]. Three mutations occur within the nAChR: the R81T mutation, which also provides *M. persicae* with resistance to imidacloprid, and the V62I and K264E mutations [42]. Chen *et al.,* 2016, also reported down regulation of the β1 subunit within the nAChR of resistant strains, suggesting both point mutations and this down regulation of the β1 subunit aid in imidacloprid resistance within *A. gossypii* [42].

A single point mutation can also be seen in the GABA-gated chloride channel when looking at strains of *Nilaparvata lugens* that are resistant to ethiprole and fiprole [43]. Despite locating the point mutation, Alanine^{301}Serine (or A^{301}S), in fiprole resistant strains, this mutation was found to not confer resistance to fiprole and the resistance mechanism to this compound was not identified [43]. In experiments run by Garrood *et al.,* 2016, the A^{301}S mutation was fixed in *N. lugens* populations that were evolved with ethiprole. These strains conferred resistance to fiprole too, but when specifically looking at this mutation the fiprole

resistance was not retained, meaning there must be an alternative method of resistance to fiprole [43]. There are alternative means of resistance such as metabolising toxic compounds [4,40].

1.2.2 Metabolic resistance

There are three types of metabolic resistance mechanisms, involving three gene superfamilies capable of producing enzymes to metabolise xenobiotics, a chemical product that is foreign to the organism, such as a pesticidal compound [44,45]. Esterase-based resistance can be upregulated by gene amplification from susceptible strains to resistant strains, as more esterases are produced more of the toxic chemical can be sequestrated [44]. Esterase enzymes are specific to toxins that have an ester bond [44]. Glutathione S-transferases (GSTs) are capable of conferring resistance to a wider range of toxins [44]. GSTs are also upregulated in resistant strains and can interact with P450's, esterases or alone as a resistance mechanism [44,46]. GST variation suggests plenty more information has not yet been discovered [44].

P450's, the third genetic superfamily producing enzymes to metabolise xenobiotics, are part of a natural defence system used ubiquitously across the animal kingdom [40,47,48]. The P450 metabolic resistance mechanism is claimed to be one of the most important methods of resistance in insects [1]. These enzymes can vary from having broad to narrow specificity to the compounds they are able to metabolise [49]. Insect P450's naturally evolve to confer counter adaptation against plant toxins that evolve to protect the plant from its predators [40]. Pesticide use is often intensive causing a high pressure selection on insects, their natural resistance mechanisms of P450's adapt well to these conditions

resulting in fast rise in resistance [40]. Local adaptation via spontaneous mutations, or the spread of a mutation already present in the populations conferring resistance, are two theories of how the P450's adapt so quickly to pesticides [40].

P450's are named as such due to their optical absorption at 450nm which was recognised in 1962 [1,47]. One year later the function of these genes was identified and following this the literature boomed [1,47]. The P450 gene superfamily came from a single ancestral protein which has since diversified [47,49]. There are more than 70 gene families within the P450's, they are grouped into a family if there is a >40% match in sequence, or into the same subfamily if the is >55% match [1]. Within a species there are multiple P450 genes, for example in *Drosophila* there are 86 P450 genes from 25 different P450 families [1,49].

To understand the function of P450 enzymes it was first necessary to understand their structure [47]. Original research on P450's concentrated on mammals and so the following research into insect P450's were based on what was already known from the mammalian proteins [49]. Mammalian P450's have a similar 3D structure with a conserved core but contain a variable binding region (Figure 1.4)[47,50].

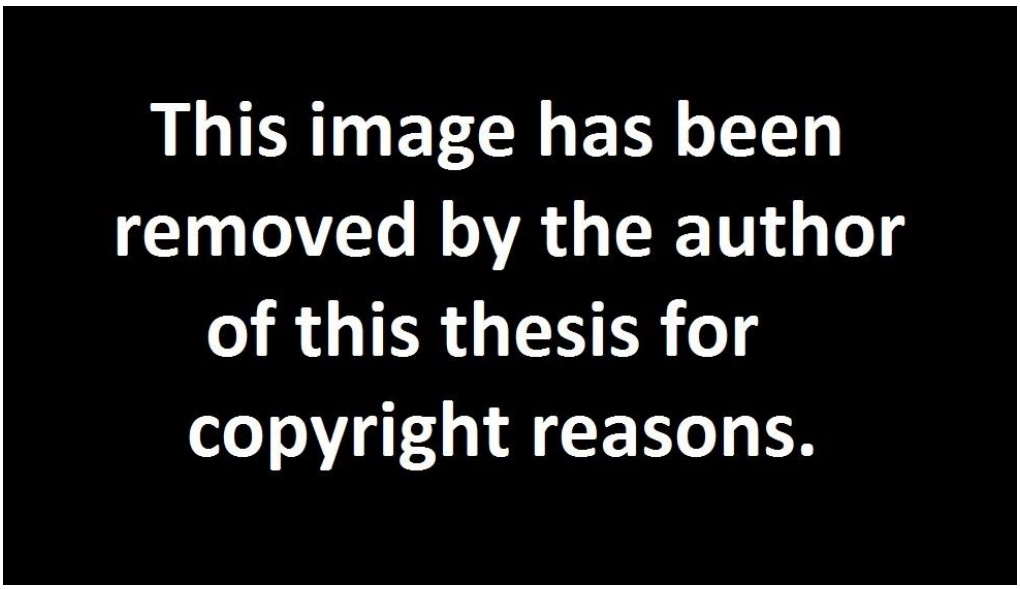


Figure 1.4 from Peterson and Graham, 1998 [50] showing an illustration of the P450 protein structure. Green rectangles represent helices, blue arrows represent the strands of β sheets, and orange lines represent the 'random' coils connecting the helices and strands. The α and β domains contains the grouped elements as originally described by Poulos *et al.*,1987 [51]. Substrate recognition sites, found in helix I, K, B', F, G and the B'-C interhelical loop and strands of the β-sheets 1 and 4, are highly conserved [52](See figure 1.5 for another view of these conserved regions).

Karunka *et al.,* 2009, analysed the structure of the *Cyp6cm1* gene from *Bemisia tabaci.* They showed high conservation in core and substrate binding sites with similar folding (See Figure 1.5), this concurs with the mammalian P450 structural similarities. The folding of the P450 proteins is conserved more than amino acid sequences and modelling tools have been effective to analyse structural-activity relations [53–55].

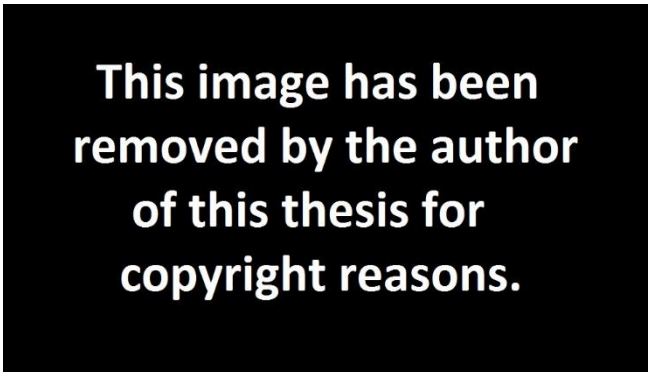


Figure 1.5 shows the conserved substrate recognition sites in yellow in this figure from Schuler and Berenbaum, 2013, are shown alongside the variable regions in brackets on this P450 protein.

A comparison of *Cyp6b1* and *Cyp6b8* from *Papilio polyxenes* also show similarities in secondary structure but note the interesting structural differences that change the P450 from a specific to generalist enzyme [56]. The *Cyp6b* subfamily of genes have the purpose of protecting insects from plant allelochemicals; the narrow, specialist feeding range of *P. polyxenes* means *Cyp6b1* is suitable for feeding on plants containing furanocoumarin whereas *Cyp6b8* enables *Helicoverpa zea* to feed on a wider range of plants by protecting against multiple plant allelochemicals [56]. Rigidity in the *Cyp6b1* catalytic pocket shows the reason for a narrow range of target substrates, whereas the flexible catalytic pocket in *Cyp6b8* defines the reverse, confirmation of this expected substrate range was confirmed by the experiments carried out by Li *et al.,* 2003. Three similarities in the *Cyp6b1* and *Cyp6b8* proteins are noted, all surrounding the heme ring: the two positively charged arginine pairs close to the two negatively charge carboxyl groups of the heme ring, a hydrophilic region on one side of the heme ring, and a hydrophobic region containing a group of highly conserved amino acids [56]. Differences and similarities between *Cyp6b* proteins can be visualised in figure 1.6.

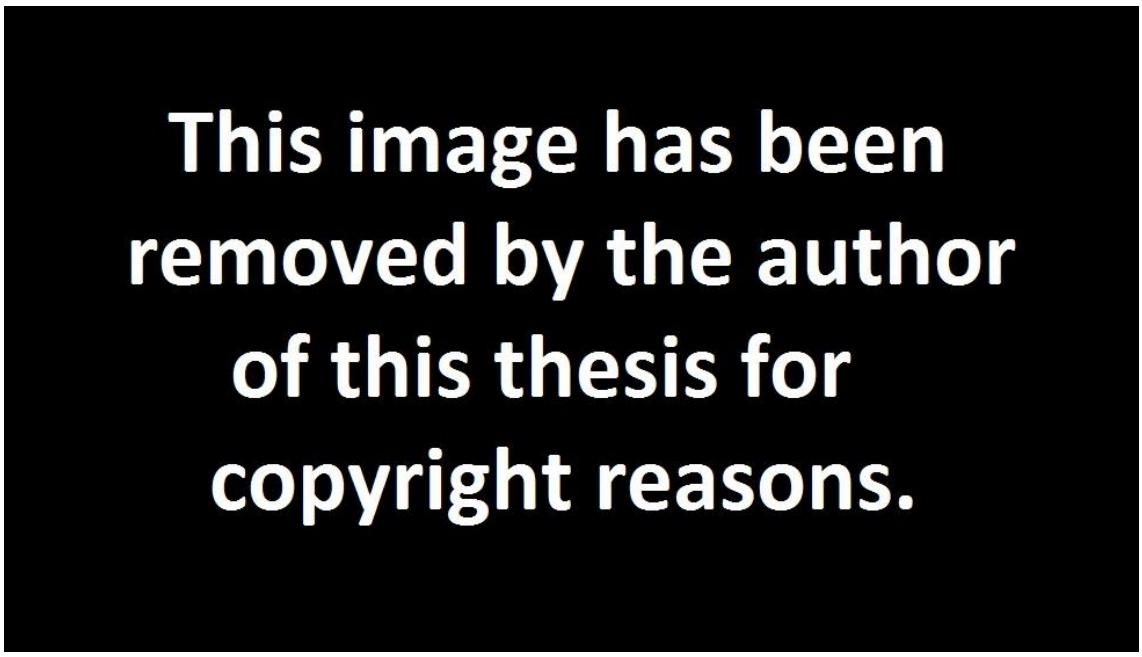


Figure 1.6 taken from Li *et al.,* 2003. Structural models for the *Cyp6b1* (A) and *Cyp6b8* (B) proteins. The hydrophobic (white) and hydrophilic (red) sites are displayed as spheres. Both proteins shown superimposed (C) with the *Cyp6b1* backbone in red, the *Cyp6b1* channel spheres in green, the *Cyp6b8* backbone in purple, and the *Cyp6b8* channel spheres in yellow.

There are seven methods by which any particular P450 gene can evolve to increase the rate at which its corresponding protein breaks down a particular substrate; (1) mutations in the promoter sequence causing increased production of transcripts, (2) mutations causing increase expression of transcripts, (3) environmental factors leading to increased responsivity to transcriptional inducers, (4) mutations in the catalytic site,(5) substrate access channel, (6) mutations affecting electron transfer by changing surface proteins and (7) mutations affecting coupling [53]. 1, 2 and 3 in this list refer to methods that increase the amount of a P450 protein whereas 4, 5, 6 and 7 change the activity of the protein to become more efficient. The insect species and the xenobiotics to which they are exposed to both affect the likely mechanism(s) of resistance in specific pest species [53].

1.3 Transgenics

Researching P450 mechanisms can be difficult. One method used to help understand these genes and their functions is by creating transgenic organisms [47]. P450's are expressed in complex organisms such as mice or fruit flies as their activity requires compatible P450 reductase and cytochromes to function; the more similar the host of the P450 gene to the original organism the more likely the gene will be functional [47]. Mouse lines were used to understand functionality of human P450's (*Cyp* genes), these lines consisted of mice with *Cyp* knockouts [47]. Transcription regulators of *Cyp* genes were also altered in some mice lines and, importantly, human *Cyp* genes were inserted into mice while the mouse P450's were removed, a great model to understand P450's and their function *in vivo* without having to use the original organism [47]. Transgenic lines of *Drosophila melanogaster* have been made to research the function of multiple insect P450's [57–59].

D. melanogaster is a well-known model system in genetics [60–62]. In order to create transgenic lines a tool is necessary to insert a new gene into the organism of choice. One of the developed tools enhancing the genetics field is that of the GAL4/UAS system, designed to control expression of specific genes within the organism [61,63]. This system enables analysis of the function of specific genes [64], originally with the intent of looking at developmental genes but [65], in this case, to look at the function of various P450 genes. The GAL4/UAS system relies on two transgenic strains, the effector and the activator [64]. The activator strain containing the GAL4, and the effector strain containing the gene of interest and the upstream activating sequence (UAS) [63,64]. GAL4 is a regulatory protein found in yeast, this binds to the UAS, which, in turn, causes transcription of linked genes [61,66]. When the two strains, activator and effector, are crossed

the offspring displays the transgenic phenotype [61] (Figure 1.7). There are multiple advantages to having separate activator and effector parental lines; specific genes that may reduce fitness are silent in the parental line and activator lines can be designed to target specific tissues, crosses can also be done multiple times with activation of the genes in various tissues allowing great diversity within this tool [61].

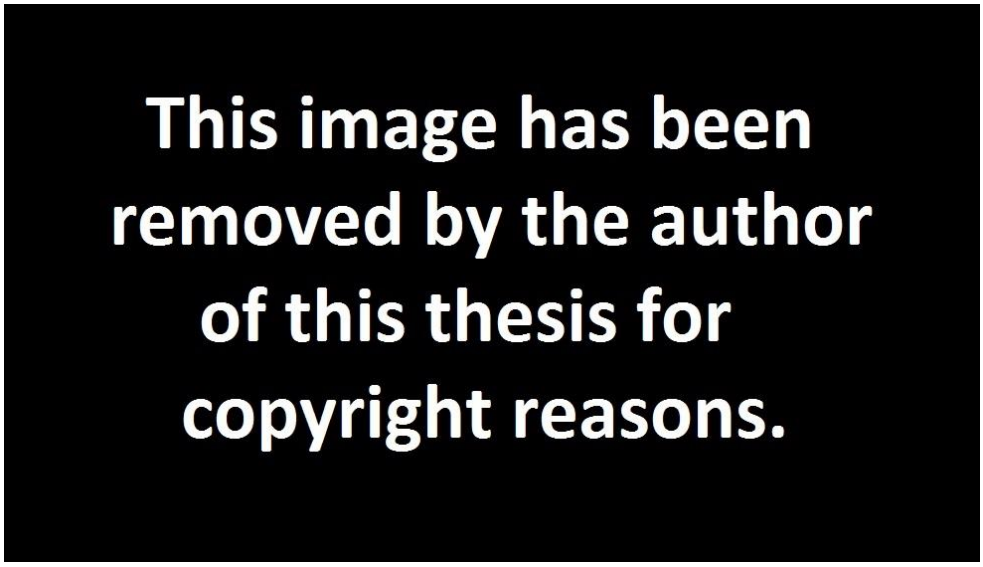


Figure 1.7 shows the GAL4/UAS system demonstrating the separate parental lines of activator (GAL4) and effector (UAS with target P450 gene) and the binding of GAL4/UAS to result in transcription of the target gene in the specific tissue defined by the GAL4. Figure adapted from Brand and Perrimon, 1993 [65].

Transgenic *Drosophila* containing *Cyp6d1* from houseflies demonstrated potential for limited efficacy of transgenic organisms due to interactions with P450 cofactors, but activity can be enhanced by increasing copy number or expression of the P450 gene [57]. Over-expression of certain P450's in *D. melanogaster* enabled transgenic lines to define individual P450 effects on pesticide resistance [67]. After eight P450's were individually over-expressed in

transgenic *D. melanogaster*, five were found to have no effect on survival when exposed to DDT, nitenpyram, dicyclanil and diazinon but *Cyp6g1* conferred increased survival against DDT, nitenpyram and dicyclanil, *Cyp6g2* conferred resistance to nitenpyram and diazinon and *Cyp12d1* demonstrated a higher survival rate for insects exposed to DDT and dicyclanil [67]. Transgenic organisms and their use to identify important P450 genes can also be useful to look at disease vectors, such as *Aedes albopictus*, a type of mosquito known to pass on dengue fever and chikungunya [58]. *Cyp6p12* in these mosquitos passed on pyrethroid resistance to transgenic *Drosophila,* the information gained from transgenic flies has given an insight into potential ways to overcome pyrethroid resistance in *Ae. albopictus* [58]. The value of an open resource of transgenic *Drosophila* can be seen by the information gained from these studies. It would be a great tool for continued research into important P450 genes, their activity and potential to overcome resistance. Future research could be carried out without further cost of time and resources to recreate transgenic lines, streamlining this area of research.

Chapter 2:

Testing novel synthetic compounds targeting the nicotinic acetyl choline receptor.

2.1 Abstract

Neonicotinoids are effective against sucking and chewing pests and bind at the postsynaptic nicotinic acetylcholine receptor and do not suffer from cross resistance affecting carbamates, organophosphates or synthetic pyrethroids which made them a commercially competitive pesticide [68]. They are used globally and account for twenty-five percent of the global pesticide market but there is growing concern around this class of pesticide [69–73]. Rise of resistance and cross resistance to neonicotinoids is high so there is a need to find novel resistance breaking compounds. Here we test 18 synthetic compounds for activity against *D. melanogaster* susceptible (Canton-S) and resistant (Hikone-R) strains and *M. persicae* susceptible (NS), metabolic resistant (*Cyp6cy3*) and target site resistant (FRC) strains. Compounds 1, 3 and 6 are competitive against imidacloprid and have limited resistance breaking activity against DDT resistant strain *D. melanogaster,* Hikone-R. These same four compounds show activity against susceptible *Myzus* strain but following restrictions in the EU for compounds targeting the nAChR the potential for these compounds to be used commercially is unlikely. There is accumulating evidence on the environmentally damaging and non-target organism effects of this type of compound [70–73]. Follow up assays to determine activity of these compounds against P450 and target site resistance *Myzus* clones were not continued with due to lack of commercial potential.

2.2 Introduction

It has been stated that understanding the mechanisms of how resistance occurs is vital to design effective ways to counter insecticide resistance [5,7,8]. In the

1970's research around how and why resistance occurs gained more interest for three reasons; high levels of resistance arose to once very effective compounds, showing, in turn, the limitations of pesticidal compounds and important new tools aiding the molecular understanding of pesticide resistance mechanisms were gaining pace [5,6]. Still facing high levels of pesticide resistance and the damaging effects of the remaining effective compounds we here look to identify novel potential compounds useful for pest control [9].

The need to control pest species is of increasing importance as the human population grows, approximately adding 2.1 billion people by 2050 [12,13] leading to a 70% increase in food productivity demand [13]. A constraining factor of current food productivity is attributed to losses from pest damage [21]. Crops are vulnerable to other factors limiting yield but it is important to develop effective pest management first as higher available yields make crops more vulnerable to pest damage [22]. Pesticides prevent crop losses, thereby increasing the crop yields actually achieved. The global boost to production potentially available if all damage were prevented varies according to crop and varies from year to year but has been calculated to be potentially as much as 56% although the level of loss actually prevented is only 26% (figures for global production of 5 major staple crops – see Oerke, 2006[22]) [13,22–25].

There are eight orders of insects that are of economic importance as crop pests; coleoptera (beetles, weevils), diptera (flies), hemiptera - suborders heteroptera (true bugs) and homoptera (aphids, whiteflies, leafhoppers, scales), lepidoptera (moths, butterflies), orthoptera (grasshoppers and crickets), thysanoptera (thrips) and acarina (mites) [74]. These are twenty six families of pests [74] showing some individual species resistant to extensive lists of active pesticide compounds shown on the Arthropod Resistance Pesticide Database[9].

M. persicae for example is shown to be resistant to eighty active compounds and *D. melanogaster* has been reported to have resistance to ten active compounds from global sites [9].

2.2.1 Drosophila melanogaster

Drosophila is both a genetic model and also of growing utility as a model insect itself [75]. *D. melanogaster* itself is not a pest species but is a close relative of commercially important species of pest fruit fly [76]. *Drosophila* can cause losses to fruit and vegetable produce and the movement of infested crops are under strict international regulations [76]. The value of research using this species is the vast extent of knowledge about its evolution, development, behaviour and genetics [75,76]. The species has been described as a good model system for insecticide resistance research [8]. A model system must have certain qualities such as easy rearing, simple analysis, ease of investigating your given question and enough background research on your model organism [8]. *D. melanogaster* has a wealth of information reported and, importantly for resistance research, has had the genes associated with resistance mapped [8,33,75,77]. Not only this, but the relative ease of genetic manipulation of *Drosophila* makes it advantageous over other pest species [8].

D. melanogaster has two important strains valuable for experiments on resistance assays; Canton-S and Hikone-R. Canton-S is a susceptible strain useful as a reference of a wild type field population yet to develop resistance mechanisms [77]. Hikone-R on the other hand is a strain collected in the 1960's containing DDT resistance and also demonstrates cross-resistance to

imidacloprid, this strain is useful to show whether novel compounds will be effective against current resistance mechanisms in *Drosophila* populations [33,77].

Hikone-R contains a resistance mechanism located at *Cyp6g1,* a P450 gene that in this strain is over transcribed [78]. P450's are ubiquitous enzymes across the animal kingdom, in insects they are responsible for a diverse range of functions including the metabolic breakdown of pesticides [49]. The P450 gene family is very old and used to regulate insect growth, reproduction, development and aids adaptation of insects to host plant toxins, for example, nicotine in tobacco plants [49]. The ability of this gene family to help insects break down natural plant toxins has been adaptable to synthetic pesticide toxins. There are 100's of P450 genes and their substrate specificity varies between narrow and broad; a change of even a single amino acid can change specificity. Thus, selective pressure from the environment can be easily adapted to by minor evolutionary change [49].

2.2.2 Myzus persicae

Unlike our fly strain, the Peach potato aphid or *M. persicae*, is an economically important pest species capable of infesting a wide variety of host plants spreading over 40 different plant families including some important crop plants, for example sugar beet, tobacco, potato, peach, even ornamental plants [79–81]. The aphid causes damage to its host plant in three ways; transmitting viruses, directly by leaf damage and reduced crop yield and indirectly by honeydew excretion on the leaves which leads to reduced photosynthetic capability of the host plant [79,80,82–84]. It is described as having great economic importance as a pest, primarily for its ability to spread viruses to host plants [81].

Biocontrol of *Myzus* has proven difficult and costly for multiple reasons; natural enemies can be limited by the crop the aphid infests, other more common aphids may be preferred as prey and the spread of the biocontrol through the crops limit where aphids are affected [79,80,85,86]. *Myzus* is ephemeral and naturally occurs at low densities making it a poor food source for biological control, predators have limited food supply and are unable to establish their own population in the absence of aphids meaning multiple release of biocontrol is required for this aphid [79,80,86]. Fungal pathogens were also used but the density at which they were required to effectively control *Myzus* caused more serious problems, natural enemies were starved allowing repeat infestations by other pests [79].

Resistance to insecticides is one of the biggest problems when it comes to trying to control pest insects [7]. *Myzus* is particularly interesting in the discovery of novel insecticides due to its natural resistance to a wide range of insecticides [87]. Clones within the *Myzus* family can be resistant to a variety of insecticides and insecticide combinations of 80 insecticides from multiple classes; neonicotinoids, pyrethroids, carbamates, organophosphates and organochlorines [84].Three mechanisms of resistance have been identified; the first discovered mechanism was duplication of a structural gene leading to higher production of carboxylesterases, later a second target site resistance mechanism at nicotinic acetylcholine receptors (nAChRs) and sodium channels and finally overexpression of a P450 gene [84,87–89]. The gene duplication resulted in a higher level of production of carboxylesterases, specifically E4 or FE4, this enzyme degrades the insecticide providing *Myzus* with a insecticidal resistance to organophosphates, carbamates and some resistance to pyrethroids [84,87,88]. An advantage of this mutation is that the costly production of these enzymes is

quickly lost in the absence of insecticidal pressure [84,90]. Mutations to the sodium channel provide the primary resistance to pyrethroids [84]. Neonicotinoids still remain as an effective control for *Myzus* but there are two specific resistance mechanisms that affect the activity of these insecticides [84,91]; a single point mutation in nAChR β subunit gene inhibits neonicotinoids binding to this receptor thus reducing effectivity of the insecticidal activity [91] and neonicotinoid catabolism due to the overexpression of a P450 gene, *Cyp6cy3* [84,90,92]. P450's are part of a metabolic system that can catabolise and anabolise pesticides and other xenobiotics, this is what confers resistance to pesticides [7].

2.2.3 Imidacloprid

Neonicotinoids are one of the most modern insecticidal class of compounds [93]. Of the five major classes, neonicotinoids currently account for a quarter of the global insecticidal market [93]. Nithiazine, was the first neonicotinoid lead structure occurring in the 1970's but was unstable in hydrolytic and photolytic conditions and had limited efficacy [94,95]. Shinzo Kagabu improved the photostability and insecticidal activity of nithiazine and his optimisation of the structure led to imidacloprid and thiacloprid [93,95].

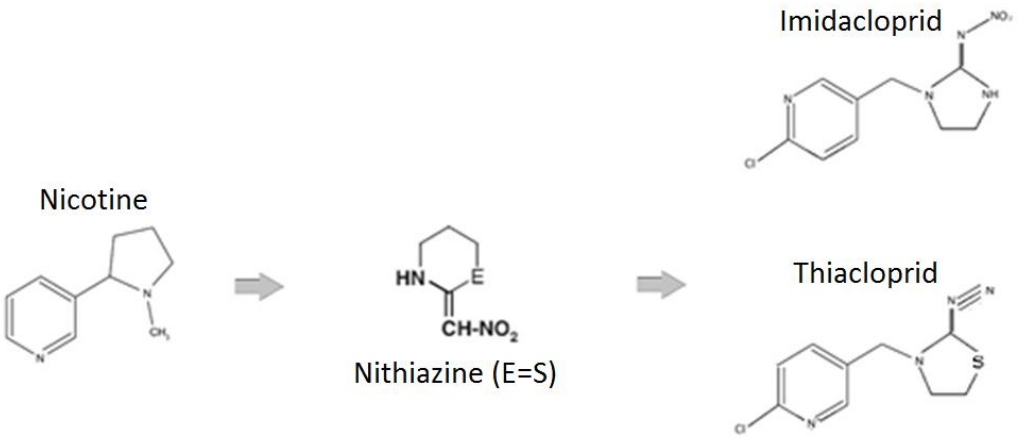


Figure 2.1 Structural changes between the initial toxic compound nicotine into its first neonicotinoid lead structure and then its commercialised photo-stable products imidacloprid and thiacloprid.

Like their original nicotine basic structure, neonicotinoids act as an agonist specifically on the post-synaptic nAChR's within an insect's central nervous system [94–96]. Imidacloprid has been shown to affect the post-synaptic membrane by depolarising and blocking transmission and a sub-conductance state in the nAChR's is activated [95]. Sensitivity to imidacloprid is dependent on the presence of an α-subunit and the strength of the charge from the negative subsite on non-α-subunits in the nAChR model [95,97,98].

2.2.4 Why replace imidacloprid?

Despite the efficacy of imidacloprid there is a need to look for alternatives due to environmentally damaging and non-target organism effects and rising resistance [71,72,99]. These are driving forces behind looking for a novel pesticidal compound, this study particularly focusses on overcoming resistance [99]. Two years after the first commercial use of imidacloprid resistance had already developed within the Colorado potato beetle and cross resistance to imidacloprid was noted from the resistance mechanism already developed

against DDT [33,99]. Resistance to neonicotinoids is linked to a change of target-site and/or metabolic resistance [100]. A resistance mechanism identified in the brown plant hopper, *Nilaparvata lugens,* showed that the resistant strain of insects had a single point mutation in nAChR subunits, Nlα1 and Nlα3 [41]. This mutation reduced imidacloprid toxicity by 250 times [41,100]. Knockdown of Nlα8 also shows a reduced insecticidal activity of neonicotinoids, this is because Nlα8 combined with Nlβ2 has high affinity for neonicotinoids and knocking out Nlα8 interferes with this [100]. A second single point mutation found to confer reduced activity of imidacloprid was identified in *M. persicae* in the D-loop of the nAChR subunit β1 (Figure 2.2)[91].

P450's are also an important mechanism of tolerance to neonicotinoids. Overexpression of the P450 gene *Cyp6cy3* directly corresponds to neonicotinoid resistance by metabolising the toxic pesticides [90]. Upregulation of cuticle proteins also reduced toxicity of neonicotinoids by limiting the penetration of the pesticide into the insect [90].

To understand the sensitivity of insects to neonicotinoids the understanding of the nAChR and how neonicotinoids bind to this site is vital. Interactions with non-α-subunits determine the sensitivity to neonicotinoids [95]. The positively charged ammonium nitrogen of acetylcholine (ACh) is proposed to interact with a negative subsite on non-α-subunits in the nAChR model [95,97,98]. The sensitivity to neonicotinoids is said to change depending on the strength of the negative charge on the nAChR's, this is down to repulsion between the nitro group within the insecticide and the electronegative charge on the non-α-subunit of the target receptor [95]. Figure 2.2 gives a visual aid in order to show how the nAChR is formed, where alpha and beta subunits are and the D-loop previously mentioned as well as how ACh is linked to these subunits.

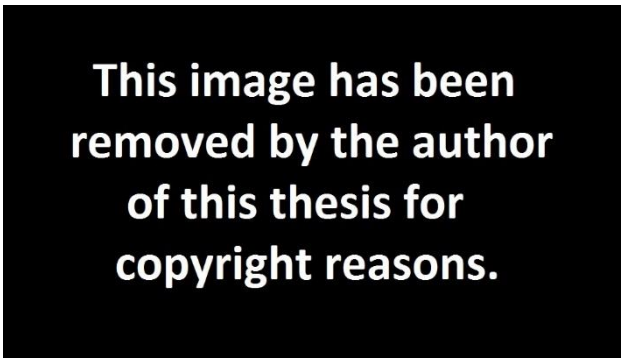


Figure 2.2 Visual aid of an nAChR. (a) Representations of a heteromer and homomer nAChR molecule, (b) above view of homomer and heteromer molecules of nAChR's with binding sites of ACh, (c) more detailed depiction of an α and non-α subunit and their binding to ACh. Figure edited from Matsuda *et al.,* 2001 [95].

Imidacloprid's high toxicity to bees is also of increasing concern contributing to the drive to find alternative compounds with neonicotinoids being associated as a driver of bee population decline [10,99,101]. The regulation leading to the restriction and ban of neonicotinoids came from actors usually not involved in this process; the public, politicians, beekeepers and NGO's who produced and used ecotoxicological data to intervene [11]. Public engagement on ecological matters and their opinions on new pesticides and associated bee safety of these compounds is now a particularly important consideration.

The global importance of bees to food security can be demonstrated by the number of crops they are responsible for pollinating, approximately 60% of the food supplying 146 countries are pollinated by bees [102]. Imidacloprid is spread through the plant via the roots and is spread to pollinators by the pollen and nectar [99,102]. It is also persistent and mobile in the soil long after treatment application and there is evidence to suggest neonicotinoid persistence in soil correlates with lower species richness of wild bees [99,102–104]. The LD_{50} for bees is 100 times higher than the estimated ingestion of a single day of foraging but

the bees are subject to repeat exposure [105]. In either case the trace neonicotinoid from ingestion is not high enough to be the sole cause of colony collapse disorder (CCD) but may be a contributing stressor [105]. Despite this, seed treatment using imidacloprid can still be systemically lethal to bees via non-floral plant substances [105]. Another concern of the effect of imidacloprid on bees is that of performance measured by the mass of the colony, number in subsequent broods and behaviour at the hive and foraging site, performance can be decreased by up to 20% after treatment [105]. Temperature effects of pesticide toxicity to bees is also of concern, with doses of imidacloprid and thiamethoxam having increased negative effects on bee colonies at 24 degrees or a day:night 24:13 degrees versus 35 degrees [106]. This again highlights the importance of more thorough understanding of pesticides on pollinators before use, especially in concurrence with global warming concerns.

2.3 Methods

2.3.1 Synthesis of novel compounds

Compounds were synthesised by Dr Alistair Miller at Darr House M.I.. The first ten compounds were derived from imidacloprid and contained examples that were previously patented such as imidaclothiz. These compounds allowed for thorough testing of the methods accuracy and safety when handling unknown, potentially hazardous compounds. A further eight compounds were sent designed with the intention of attacking this receptor. Eighteen compounds in total were tested for pesticidal activity but only three showed activity below 500ppm. These three compounds were tested further to determine their activity.

2.3.2 Drosophila bioassay

Prior to use in bioassays *Drosophila* strains were kept in standard conditions; 24ºC with 18:6 light:dark cycle in a *Drosophila* stock bottle containing 45ml Nutri-Fly BF. The bioassay for *Drosophila* strains Canton-S (susceptible) and Hikone-R (resistant) were carried out in plastic scintillation vials. Each vial contained 2 ml sugar agar at the base consisting of 2 % agar and 1.2 % granulated sugar, the agar was pipetted into each vial and allowed to set for a minimum of 4 hours. Pesticide dilutions were made with 50 % water and 50 % acetone, initial concentrations of 0, 0.1, 0.5, 1, 10 and 100 µg per vial was added in 200 µl volume and allowed to evaporate onto the sugar agar surface overnight. Twenty Canton-S flies were then added per vial and each vial was stopped with a cotton wool plug.

After 72 hours scores were taken for numbers of dead and alive individuals within each vial. The scores were used to narrow down the LD_{50} for each compound and the experiment was repeated with a narrower range. The narrower range of dilutions were carried out for Canton-S and Hikone-R strains of *D. melanogaster.*

2.3.3 Myzus bioassay

The bioassay for *Myzus* was carried out using a leaf dip method. 1.2 % W/v water agar was added to the base of small pots and were allowed to set. Leaf discs were cut using a cookie cutter of a diameter of 3.5 cm from *Brassica rapa pekinensis,* Chinese cabbage. Dilutions of the compounds were made in Triton

water (0.2 %) and leaf discs were dipped for ten seconds with slight agitation with care to avoid damage to the discs. Due to a lack of human toxicological data surrounding the novel compounds they were treated cautiously, leaf discs were dried in a fume hood on absorbent desk protectors and allowed to dry for 30 minutes. Dry discs were then placed on the agar with the underneath of the leaf facing upwards and ten adult aphids were added to each disc. This first experiment looked at the variation in susceptibility of three *Myzus* strains to imidacloprid, a susceptible strain NS (5557), a strain with P450 resistance mechanism with over transcription of *Cyp6cy3* (5410R) and a homozygote target site resistant strain (FRC). Before use the strains were maintained on whole Chinese cabbage plants; they were maintained and tested in a 16:8 light: dark regime, 55% relative humidity and at 20ºC.

The initial assay was completed using imidacloprid at concentrations of 100, 10, 1, 0.1, 0.01, 0 µg/ml to confirm methods and expected range of mortality for our positive control. Mortality of aphids were scored after 96 hours. Following the initial assay and due to unforeseen fungal infection of many cabbage plants the initial assay on our ten compounds was narrowed to the highest dilution, 100 µg/ml, for each compound. Any compounds not seen to reach their LD_{50} at this concentration was removed from further assays. The six remaining compounds were tested on the susceptible *Myzus* strain at concentrations of 100, 10, 1, 0.1, 0.01, 0 µg/ml. After the initial results concentrations were altered to surround the suspected LD_{50}'s for each active compound.

2.3.4 Statistical analysis

Probit analysis was run on each dataset to determine the LD_{50} value and associated 95% confidence interval for each compound using POLO Plus [107]. Before loading datasets into POLO Plus datasets were corrected using Abbott's formula [108]. Compounds showing a t-ratio of lower than 1.96 for the slope were removed from subsequent analysis as this showed the treatment as having no effect at the tested concentrations [109].

Resistance ratios are shown for the comparison of a susceptible versus resistance strain and this is calculated by dividing the LD_{50} of the resistant strain by the LD_{50} of the susceptible strain.

2.4 Results

2.4.1 Drosophila bioassays: Toxicity

The initial *Drosophila* bioassay was used to get an idea of each compounds activity in comparison to imidacloprid. Our results showed compound 2, 4, 7 – 10 as having a t-ratio for the slope as 0 so these compounds were immediately removed from further analysis. Compounds 1, 3, 5 and 6 were then compared showing significantly different intercepts demonstrating the significant difference in the insecticidal activity of the compounds (Chi-square: 6860, degrees of freedom: 6, tail probability: 0.000, $P<0.05$). Figure 2.3 shows the activity of the four active compounds and how their potency compares.

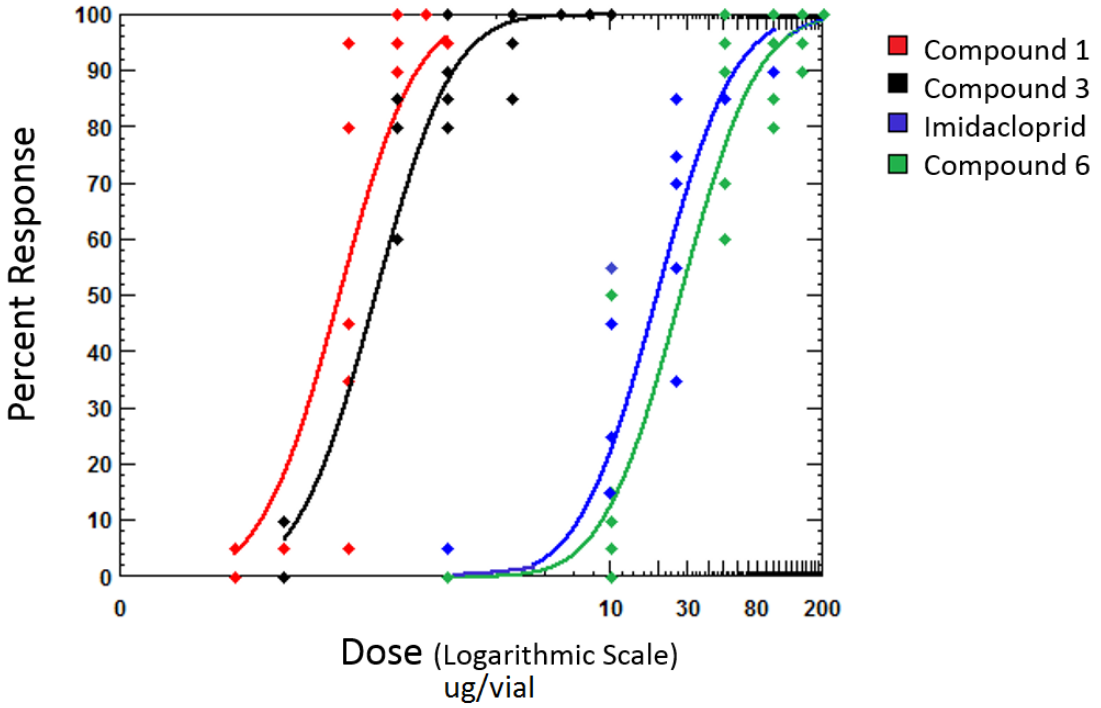


Figure 2.3 Shows the comparative activity of 3 compounds in comparison to imidacloprid on *D. melanogaster*. Compound 1 and 3 show a high activity compared with imidacloprid while compound 6 shows slightly lower activity than imidacloprid.

The relative activity of these compounds to imidacloprid is shown by the comparative LD_{50} doses. Compared to imidacloprid we see compound 1 as 75 times more toxic: compound 3 was 48 times more toxic, both showing significantly higher activity than imidacloprid and compound 6. Imidacloprid was only 1.5 times more toxic than compound 6 making it a competitive compound at this stage.

2.4.2 Drosophila bioassays: Resistance

Compound 1 showed an LD_{50} of 0.2µg per vial for the susceptible Canton-S strain and an LD_{50} of 6.4µg per vial for the resistant Hikone-R strain (Figure 2.4, Table 2.1) (Chi-square: 416, degrees of freedom: 2, tail probability: 0.000,

P<0.05) and a high resistance ratio of 32, this result shows the high increase in quantity needed to cause 50% mortality in a resistant population versus a susceptible population. This shows that this compound is relatively unsuccessful at breaking the resistance conferred by Hikone-R. Compound 3 shows similar responses as shown by the resistance ratio of 31.

Comparing these two compounds to imidacloprid we can see the higher LD_{50} of 17.1µg per vial for the susceptible Canton-S strain and no LD_{50} reached for the resistant Hikone-R strain at the doses we tested (Figure 2.4, Table 2.1). This is as expected as we know the *D. melanogaster* strain Hikone-R confers resistance to imidacloprid [33]. Compound 6 equally has problems of current resistance with an undetermined resistance ratio, despite the appearance of figure 2.4. A lower resistance ratio is best for novel compounds as it shows reduced cross resistance conferred by current resistance mechanisms in wildtype flies.

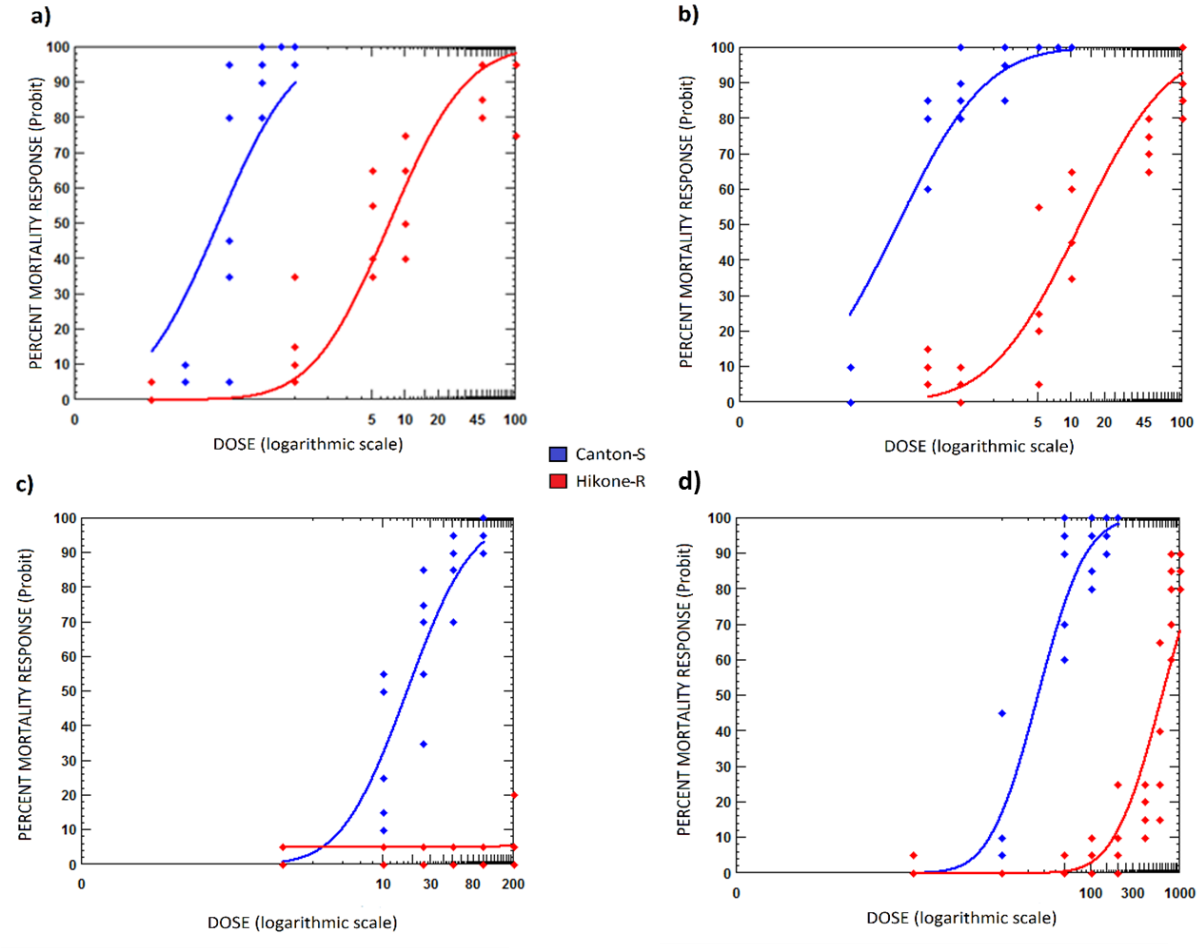


Figure 2.4 Shows the comparative toxicity of compound 1 (a), compound 3 (b), imidacloprid (c) and compound 6 (d) on *D. melanogaster* susceptible Canton-S strain and resistant Hikone-R strain.

2.4.3 Myzus bioassays: Toxicity

The first *Myzus* bioassay was used to be sure of methods and to get an estimate of lethal doses of imidacloprid on the three strains of *M. persicae*. Our results showed our susceptible and FRC strain as having a slope t-ratio of less than 1.96, meaning we were unable to get LD_{50} values from this dataset (in future experiments this can be corrected for with replicates which would minimise the effect of outlying data points). Figure 2.5 shows the effect of resistance mechanisms in *M. persicae* against imidacloprid.

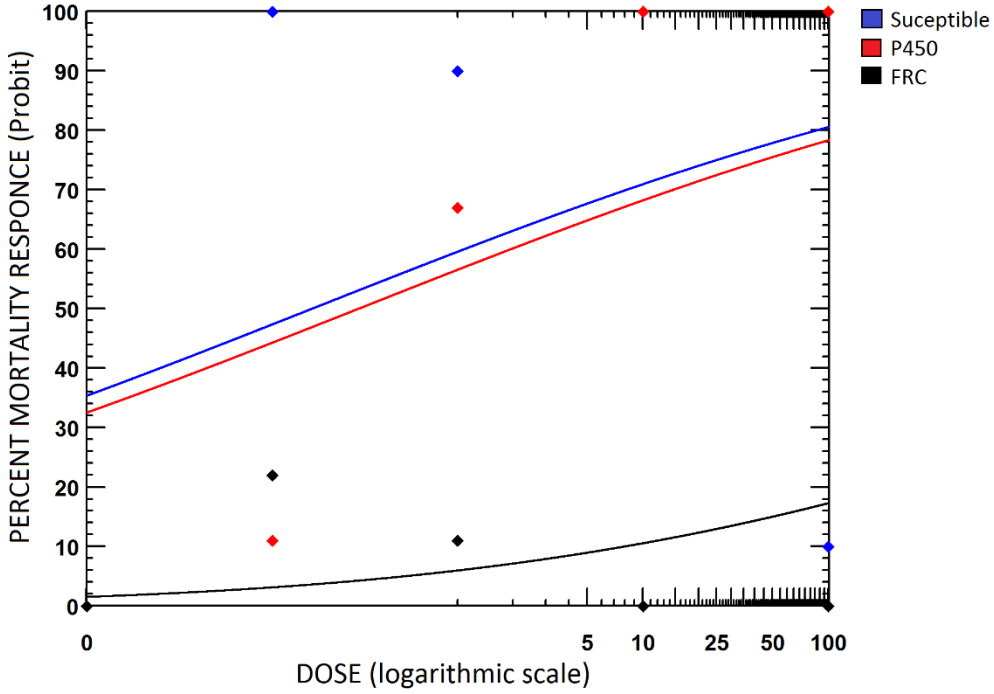


Figure 2.5 Shows the comparative mortality responses of three strains of *Myzus*; one susceptible strain, one strain with P450 enzymes and one FRC strain. Imidacloprid is used to test various responses at five concentrations ranging from 0.01 µg/ml to 100 µg/ml.

The relative mortality of the resistant strains is compared to the susceptible strain visually showing significant resistance conferred by the FRC target site resistance and minimal resistance from the P450 mechanism.

2.4.4 Myzus bioassays: Resistance

To test the compounds active against *Myzus* we first tested 100 µg/ml of each compound on the susceptible strain. Every compound that caused at least 50% mortality response at this concentration was tested further with the dose response range; 0.01, 0.1, 1, 10, 100 and 0 µg/ml. Compounds 2, 7, 8 and 9 were eliminated at this stage. After testing compounds 1, 3, 4, 6 and 10 against imidacloprid we found that the compounds did not have equal LD_{50} intercepts

(Chi-square: 0.226E+04, degrees of freedom: 10, tail probability: 0.000, P<0.05). Compounds 1, 3 and 6 look promising with similar activity to imidacloprid while compound 4 and 10 show lower activity and will, therefore, be eliminated from further experiments (Figure 2.6). From these results we do not have estimates of the LD_{50}'s for the compounds as dose response was covering too wide a range and went from very low response to complete mortality. The statistical software is, therefore, unable to predict an LD_{50} based on limited data points relevant to the mortality response.

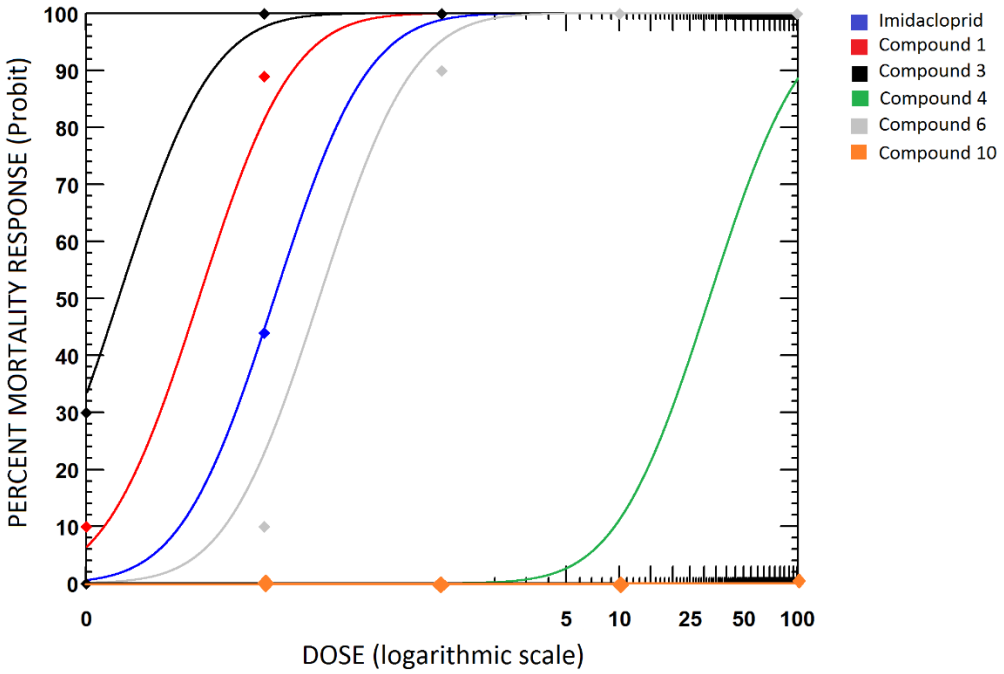


Figure 2.6 Dose responses of the susceptible strain of *Myzus* to imidacloprid and Compounds 1, 3, 4, 6 and 10 at five concentrations ranging from 0.01 µg/vial to 100 µg/vial.

2.5 Discussion

The search for a novel pesticidal compounds has been increasingly important as the rise of resistance occurs [99,110] and the importance of food security against pests increases [13,20,21]. Imidacloprid being an effective and widely used

pesticide currently is a good place to start comparing activity [93,110]. Using this as a starting point the compounds in this experiment were derived from imidacloprid. Following compounds will start to diverge from this structure but an initial understanding of what small differences make to the toxicity of a current pesticide is important to understand.

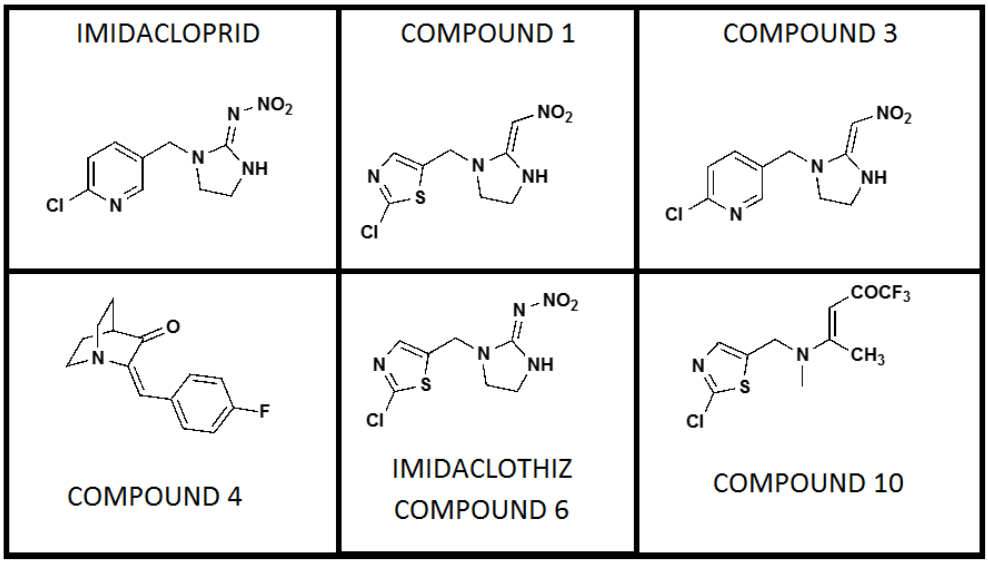


Figure 2.7 Structural representations of test compounds showing activity against *Drosophila* and *Myzus*.

Note two surprising differences considering the results of *D. melanogaster* bioassay comparing the first 10 compounds on the susceptible Canton-S strain. Imidacloprid and compound 3 are similar structures with the exception of a nitrogen to carbon atom exchange in compound 3 (Figure 2.7), this manifests itself in a comparative LD_{50} dose of 42 times more toxic when compared to imidacloprid, a significantly increased toxicity. The exchange of nitrogen to carbon changes imidacloprid, a N-Nitroguanidine class of neonicotinoid, to compound 3, a nitromethylene class which can be considered an closed-ring variant of nitenpyram. Similarly compound 1 and compound 6 are similarly structured but with the same nitrogen to carbon atom exchange, present in

compound 6 and absent in compound 1 (Figure 2.7). The absence of this nitrogen between compound 1 and 6 shows an increase the LD_{50} toxicity of 260-fold. The change in cyclic ring in these 4 compounds shows minimal difference when the nitrogen is present but a significantly higher toxicity of (2-chloro-1,3-thiazol-5-yl)methyl compared to (6-chloropyridin-3-yl)methyl in the absence of the nitrogen. The higher toxicity of (2-chloro-1,3-thiazol-5-yl)methyl group in the bioassays supports the early research into neonicotinoids showing that the smaller the ring size the more toxic the compound [94].

The structural properties of compounds 1, 3 and 6 possessed shared characteristics of known neonicotinoids in the binding group and tail group of the compounds, whereas compound 10 was less similar by means of the binding group. Although compound 4, whilst being notably dissimilar, was assayed on the basis that the quinuclidine ring is present in many compounds that have demonstrated activity in the central nervous system of vertebrates and it was, therefore, considered worthwhile to test activity among invertebrate pests [111]. The primarily shared structures between our compounds and neonicotinoids means we assume the receptor our compounds are affecting within the insects are the post-synaptic nicotinic acetylcholine receptors (nAChRs).

The exchange of the nitrogen to carbon atom in compound 1 and 3 that increases the effectivity of these compounds compared to their counterparts containing the nitrogen may be affecting the manner in which the compounds bind to the nAChRs. Sensitivity of these receptors is affected by the coulombic interaction between the negative charge on the compounds binding site and the positively charged site on the receptor [95,112]. By replacing the nitrogen with carbon, a less electronegative element, this perhaps, aids binding by

concentrating the electronegative charge to one place on the binding group of the compound. Alternatively, perhaps the coulombic interaction between the receptor and compound has been optimised.

Compound	**Canton-S LD_{50} (µg/vial)**	**Hikone R-LD_{50} (µg/vial)**	**Resistance ratio**
Imidacloprid	17.1	Not determined	Not determined
Compound 1	0.2	6.4	32-fold
Compound 3	0.4	12.5	31-fold
Compound 6	25.5	Not determined	Not determined

Table 2.1 Displaying comparative LD_{50} and resistance ratios in comparison to imidacloprid on *D. melanogaster* for all compounds active below 100µg/vial.

The first aim of this project was to find compounds that are more or equally toxic to pests when compared with imidacloprid, we can see from table 2.1 that only compound 1 and 3 are more toxic than imidacloprid with lower doses causing 50% mortality. The second aim is that these compounds also show lower resistance ratios than imidacloprid, as this would mean that the dose needed to cause 50% mortality in resistant fly strains would be lower than imidacloprid and that current resistance mechanisms are not working on these compounds. Despite no statistically valid resistance ratio for imidacloprid we know the resistance ratio for this compound is higher than that of compound 1 and 3 as the doses measured of imidacloprid did not kill more than 0.5% of the population at any measured dose.

Compound 10 showed potential promise working against *Myzus*, possibly due to the electronegative $COCF_3$ binding group of compound to replace NO_2 of other active compounds, this structural difference is also found in flupyrimin, one of the newest nAChR targeting insecticides, but the activity from compound 10 was much lower than other tested compounds here [113,114]. Compound 4 also showed some promise in activity against *Myzus* but the significantly different structure of this compound likely explains why our results indicate the lower mortality response.

In these tests it is not only important to discuss promising compounds but those that were not active in order to narrow down potential compound structures that show no activity. The non-active compound structures are shown below in figure 2.8.

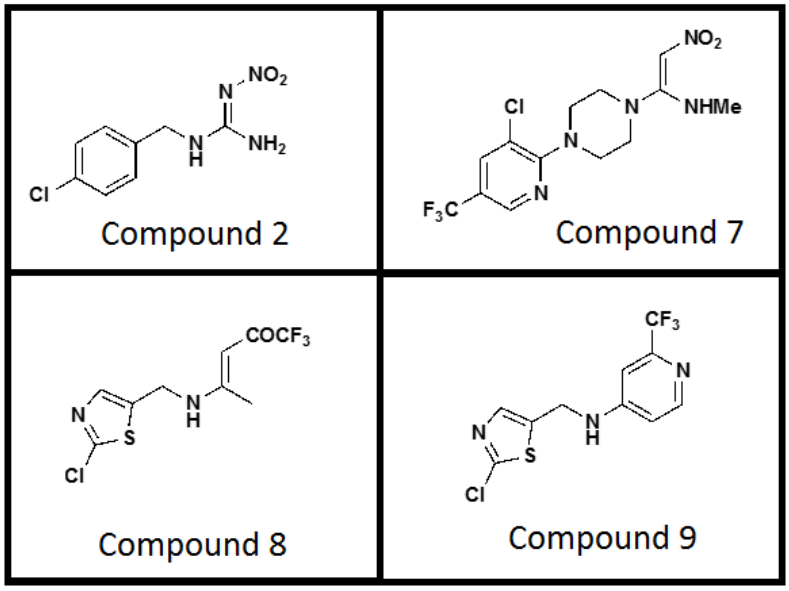


Figure 2.8 Structural representations of non-active compounds.

Compound 2 emphasizes the importance of the tail group in the binding of these compounds, the switch from the pyridine ring of imidacloprid to the benzene ring in compound 2 severely affects the activity of the compound. Compound 7 also looks at the importance of the tail group which is a particularly

different structure to the neonicotinoids. Compound 7 is also a larger molecule than our other compounds with the addition of a piperazine ring between the tail and binding group.

Compound 8 has a comparative tail group to the neonicotinoids and electronegative warhead similar to compound 10. Compound 9 was derived from compound 8, sharing the pyridine ring in the tail group with neonicotinoids we here looked at the effect of a cyclic binding group compared with the acyclic binding group of compound 8 [115]. The nitrogen in the cyclic binding group has been kept in the same position as the neonicotinoid binding groups to compare the two but still shows no activity against *Myzus* or *Drosophila*.

Following these experiments we are now confident in expected result ranges, typical resistance ratios and the overall methodology. Further novel compounds will be analysed with the aim of identifying a novel pesticidal compound with higher potency than imidacloprid and a lower resistance ratio. A decreased or very low resistance ratio will demonstrate current resistance mechanisms are not preadapted to our novel compound.

Chapter 3:

Testing novel synthetic compounds targeting the GABA receptor.

3.1 Abstract

There is an ongoing need to find and develop novel active pesticides to protect food security for an ever-growing population [12,13]. Neurotoxic compounds are popular in pest control products with the GABA target identified as an underutilised target for pest control [27,116,117]. Here we screen the activity of thirty synthetic compounds expected to act on this target site; finding five compounds with potential activity. Compounds 30, 36, 39, 40 and 47 were screened in comparison to fipronil, a highly active GABA antagonist. No activity was found for these compounds on *M. persicae* longevity or fecundity. These compounds were also tested against three strains of *D. melanogaster*; susceptible (Canton-S), metabolic resistant (Hikone-R, over-expressing *Cyp6g1*) and target site resistant (GABA receptor subunit RDL). None of the compounds overcame *Rdl* resistance but compounds 30, 39, 40 and 47 showed activity against Hikone-R with compound 47 being closest to a 'resistance breaking' compound.

3.2 Introduction

Synthetic pesticides have benefitted crop production since the 1940's and were very effective [27,118]. The use of pesticides, although not always effectively used, can reduce loss of crop production by 26% [22]. Early pesticides were developed from botanicals. For example, picrotoxin was developed to produce the synthetic compounds lindane (1942) to the competitive fipronil (1992), these products act on the gamma-aminobutyric acid (GABA) neurotransmitter [27,119]. Despite the rise and fall of popular insecticides, like DDT or the neonicotinoids, neurotoxic compounds have been most popular for the control of pest populations and are expected to remain an important method of pest control

[27,116]. In 1993 John Casida identified the GABA target as an underutilised system for novel pesticides as the continued study of these compounds significantly reduced following restrictions of use in the 1970's [117]. Since Casidas' paper there has been renewed interest in this system [116,120].

Despite problems using pesticides such as environmental concerns, resistance among pest species, and damaging non-target organisms, the benefits of pesticides to living standards across the world suggests that they will carry on being used [13,27]. An important obstacle to overcome is that of resistance. The GABA receptor has four targets (see figure 3.1), although they overlap and are paired, they are known to have little cross resistance [116]. The first three sites, the NCA's (non-competitive antagonist), are targeted by blockers or non-competitive agonists [116]. Targets NCA-IA and NCA-IB are attacked by non-competitive agonists, the first by long or large compounds such as the cyclodienes or fiproles and the second by smaller compounds such as convulsants [116]. The third target, NCA-II, has selective toxicity to insects, does not affect mammals, and is currently targeted by isoxazolines and meta-diamides [116]. The last target site is for allosteric modulators of this channel called the avermectin site which is also known to be selective for insects over mammals due to compounds being unable to cross the blood-brain barrier [116,121].

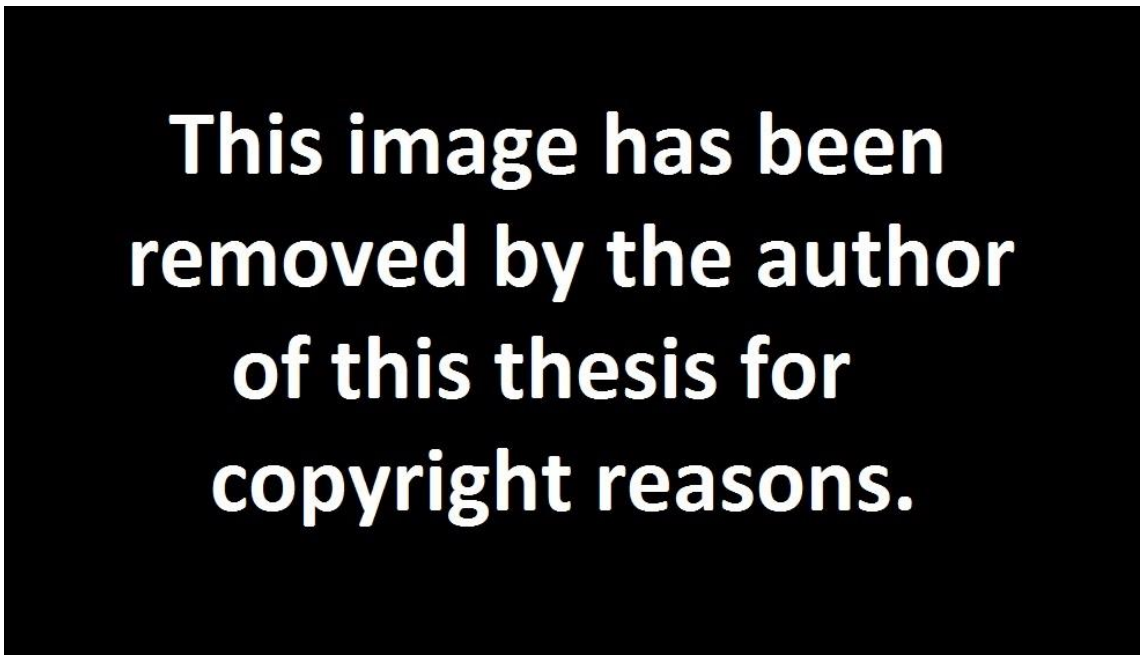


Figure 3.1 The four *D. melanogaster* RDL GABA-R binding sites (based on 3RHW). Starting left is one subunit (green) with helices M3,M1, M2 and a second subunit (red) is left to right M3, M2, M1. The three NCA pesticides are shown in the channel pore together (interactions with M2) and separately on the right maintaining the same relative positions. Also shown in the channel pore is avermectin at the interface of two transmembrane subunits. Reference [116].

Cross resistance for the RDL subunit containing GABA gated chloride channel can occur by as little as a single amino acid change at the target site but can be reduced by using GABA agonists, for example avermectins, instead of GABA channel blockers [27,121]. Resistance to the first target of the GABA receptor is via a single base pair mutation causing an amino acid substitution from Alanine to Serine or Glycine resulting in the loss of the Hae II site [122]. The second site relates to toxic compounds to mammals and does not have overlapping resistance with pesticides such as dieldrin [116,121,123], it is therefore, of less interest when looking at overcoming pesticide resistance. The third site shows no cross resistance with compounds acting at the first site but there are three mutations that reduce sensitivity here, all are located in the pore and are expected to change the pores shape thus limiting binding of compounds [116]. Finally the avermectin site can be blocked by high levels of chloride flux [116].

Fipronil is an example of one of the GABA targeting compounds that, initially, had no resistance in target pests, it is highly toxic, and often used for pest control across the globe [124–126]. Fipronil was developed by Bayer Crop Science and made it to market by 1993 as a very effective insecticide [124]. Fipronil was well liked for being selective to invertebrates with high persistence and versatile methods of treatment [124,127]. Despite previous beliefs that fipronil had minimal effect in the aquatic system it has now been identified as highly toxic to fish and aquatic invertebrates [124,126]. There are also concerns that the dust created from seed treatments have affected losses of honeybee populations and due to fipronil being bio accumulative even low doses can cause mortality [127,128].

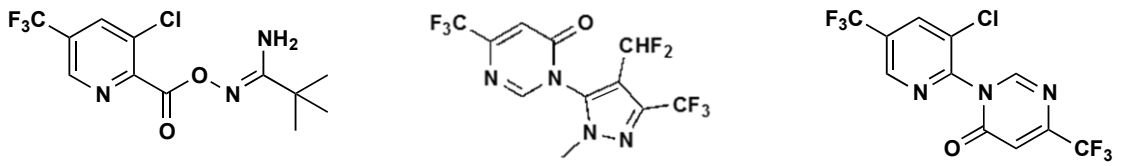
Resistance to fipronil began to arise due to increased use over time and frequency of application [125,129]. Two mutations have been identified within the RDL GABA receptor in *Drosophila simulans;* the $A^{301}G$ *Rdl* mutation and the T350M in the third transmembrane domain [130]. Low to mid-level resistance arose approximately 15 years ago in the rice stem borer and mechanisms included low permeability of the cuticle, increased metabolism, and detoxification activity [129]. It is also noted that there are signs of cross resistance, an important problem when looking at effectivity of pesticides [129]. Resistance to fipronil found in houseflies, aphids and other insects has also been attributed to increased activity of detoxification enzymes in these insects [129], suggesting that when looking for new pesticides it will be important to test them against current P450 resistance mechanisms.

3.3 Methods

3.3.1 Synthesis of novel compounds

Compounds were designed by Alistair Miller at Darr House M.I.. Of thirty compounds tested, five compounds showed potential activity, the other twenty-five compounds did not show activity below 500ppm (Figure 3.2) (other tested compounds can be found in supplementary information, Table 1).

Compound 30 Compound 36 Compound 39



Compound 40 Compound 47 Fipronil

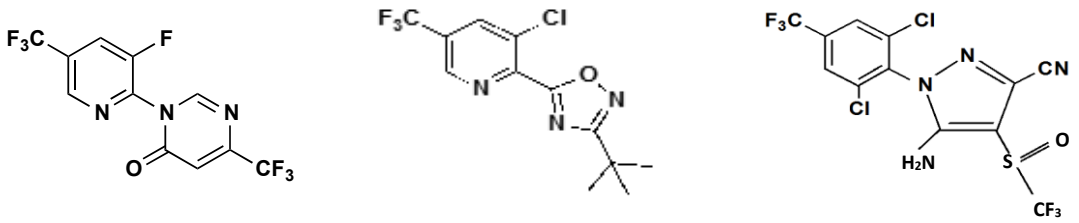


Figure 3.2 showing the chemical structure of the five active novel synthetic compounds in comparison to fipronil.

3.3.2 Drosophila strains

Three strains of *D. melanogaster* were tested for potential susceptibility to each new compound. A susceptible strain, Canton-S (Bloomington Stock number 1) is used as a reference of a wild type field population yet to develop resistance mechanisms [77]. A resistant strain, Hikone-R (Bloomington Stock number 4267)

containing DDT resistance, cross-resistance to current and novel pesticides, via over transcribed P450 gene, *Cyp6g1* [33,77,78]. A second resistant strain *Rdl* [MD-RR] (Bloomington Stock number 1675) contains a single point mutation in the RDL GABA receptor subunit and confers resistance to cyclodienes and other channel blockers [131–133]. This range of strains allows a comparison of a control strain, a metabolic resistant strain with high levels of cross-resistance and target site resistance.

3.3.3 Drosophila bioassay

The bioassay for *Drosophila* strains were carried out in polystyrene plastic scintillation vials. Each vial contained 2 ml sugar agar at the base consisting of 2 % agar and 1.2 % granulated sugar. The agar was pipetted into each vial and allowed to set until dry. Compound dilutions were made with acetone, spanning concentrations between 0 and 500 ppm per vial added in 200 µl volume and allowed to evaporate onto the sugar agar surface overnight. Five replicates of each concentration were made and fifteen flies were then added per vial and stopped with a cotton wool plug. After 72 h scores were taken for numbers of dead and alive individuals within each vial. The scores were used to narrow down the LD_{50} for each compound and the experiment was repeated with a narrower range until the LD_{50} could be calculated.

3.3.4 Myzus persicae bioassay

Aphid cups were made with a single cabbage leaf from *Brassica rapa pekinensis,* and 10 adults were placed on the leaf. After 24 hours the adults were removed, and the nymphs were allowed to grow for 12 days from initially putting in adults before using in the assay. An aphid 'pot' was used for this assay; using a soldering iron to create a hole in the lid and secure metal mesh over the gap this created a breathable test container. Leaf discs of 3.5cm in diameter were soaked in a 0.2% Triton water suspension of a novel compound for 10 seconds before air drying for 20-30 minutes. The pot base was filled with 15ml of 1.2% water agar and leaf discs were added underside of the leaf up before the agar set, adding before the agar set stops aphids settling underneath the leaf for ease of scoring results.

Once set up 10 adult aphids were added onto the leaf discs. After 24 hours the number of nymphs dropped on each disc were recorded as a response to account for effect on fecundity. Mortality scores were taken at hours 24, 48, 72 and 96.

3.3.5 Statistical Analysis

Probit analysis was run on each dataset from the *Drosophila* setup to determine the LD_{50} value and associated 95% confidence interval for each compound using POLO Plus [107]. Compounds showing a t-ratio of lower than 1.96 for the slope were removed from subsequent analysis as this showed the treatment as having no effect at the tested concentrations [109]. The LD_{50}'s were calculated from scores taken at 96 hrs including a 95 % confidence interval with resistance ratios compared to fipronil. *Myzus* assays were analysed using statistical

software R and running a GLM with Chi square post hoc test. This was done for comparing effect on fecundity and mortality at a single concentration. POLO Plus was not needed to calculate LD_{50}'s due to initial results.

3.4 Results

Drosophila bioassays showed compounds 30, 36, 39, 40 and 47 as being active against the susceptible strain of *D.* melanogaster, Canton-S. LD_{50} concentrations can be seen in table 3.1. All of these active compounds, except compound 36, also showed activity against the resistant strain Hikone-R but none were able to break the resistance of the *Rdl* strain. Only fipronil was active against *Rdl* resistant strain with an LD_{50} of 1.24 ppm but a resistance ratio of over 11-fold showing this resistance mechanism is effective against fipronil too. In addition, none of the strains were as effective as fipronil which was used as our current competitor in the market.

Compound	**Canton-S** **LD_{50} ppm** **(±95% CI)**	**Hikone-R** **LD_{50} ppm** **(±95% CI)**	**Resistance ratio**
Fipronil	0.11 (0.09 - 0.13)	0.19 (0.17 - 0.21)	1.73-fold
30	6.94 (6.33 - 7.50)	14.04 (11.70 - 15.45)	2.02-fold
36	169.97 (131.16 - 208.39)	>500	Not determined but value is >2.94
39	4.62 (4.28 - 4.93)	10.67 (9.81 - 11.77)	2.31-fold
40	17.63 (16.35 -18.74)	26.66 (24.22 - 28.92)	1.51-fold
47	3.86 (3.55 - 4.20)	5.63 (4.75 - 6.23)	1.5-fold

Table 3.1 shows Hikone-R mediated resistance for 5 novel GABA targeting compounds compared to fipronil. LD_{50} values alongside 95% confidence intervals are shown with resistance ratio. This shows the extent of Hikone-R mediated resistance against each novel compound compared to fipronil.

Initial tests on these novel compounds also looked at mortality effects on *M. persicae* as an example of the activity of these compounds on sucking pests but in these assays we found no activity at the highest concentration dose of 100µg/µl. At this concentration 100% of an aphid population was dead at 96hrs for fipronil and so no follow up experiments were carried out due to a lack of toxicity of the novel compounds to aphids under the conditions tested. It was also noted that there was no significant effect on fecundity after 24 hours of aphid adults at this high dose, including on fipronil.

3.5 Discussion

Here we show the activity and limitations of five novel compounds provided by Darr House M.I. in comparison to fipronil as a positive control. Fipronil is highly effective at low concentrations and provides long term protection across many crops to major crop pests like the *Lepidoptera* and *Orthoptera* [134]. Fipronil is used as a positive control against these compounds as it is highly competitive as a current commercial product and has the same mode of action as that expected of the novel compounds: targeting the GABA receptor [124].

To understand the toxicity results of our novel compounds it is important to understand the structural differences that may be behind the differences we see in resistance levels. I will refer to the compounds as having a 'head' and 'tail' group; as an example here is fipronil separated into its two groups in figure 3.3.

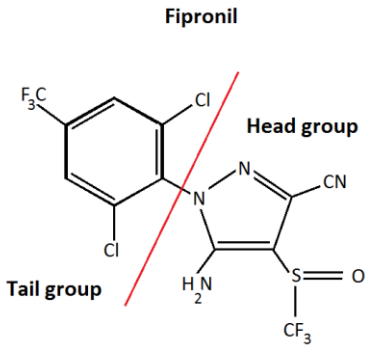


Figure 3.3 Fipronil structure[134,141].

The only compound to already display evidence of being affected by pre-existing resistance in *Drosophila* strain Hikone-R is compound 36. Hikone-R is a *D. melanogaster* strain with DDT resistance via overexpression of the P450 *Cyp6g1* gene[135]. *Cyp6g1* has spread ubiquitously worldwide and has been shown to confer cross resistance to the neonicotinoid, imidacloprid and here it

shows potential for metabolising other compounds[135]. The resistance to compound 36 may be due to in vivo oxidation of the methyl group on the head group of this compound enabling metabolic breakdown and excretion of this xenobiotic. Oxidation of methyl groups can be protected against by changing the methyl into a fluorine atom which can be seen in the development of atorvastatin, an inhibitor of the HMG-CoA reductase enzyme [136]. Due to the difference in toxicity of compound 36 between the susceptible Canton-S and Hikone-R strain we attribute this to the overexpression of *Cyp6g1*. The high level of resistance of Hikone-R to compound 36 shows that this compound is particularly vulnerable to P450 mediated attack.

Though the vulnerable methyl groups can also be found on compound 30 and 47 it is not an individual methyl group like compound 36, it is a tri-methyl group which results in a sterically encumbered environment. Heme, an oxidative catalyst in blood[137], cannot get close enough to the methyl groups to oxidise them meaning we don't see the resistance conference from Hikone-R to these compounds.

Compound 30 in fact turns into compound 47 in vivo via cyclisation by losing water see figure 3.4 and yet interestingly compound 47 is 2.5 times more active against Hikone-R than compound 30, and 1.8 times more active when looking at the susceptible Canton-S strain. This difference in activity is perhaps because compound 47 does not have to go through any chemical process in vivo before being active at the binding site. The speed at which compound 30

turns into compound 47 in vivo may be making this compound vulnerable to metabolisation or it may simply be slowed by the rate of reaction time.

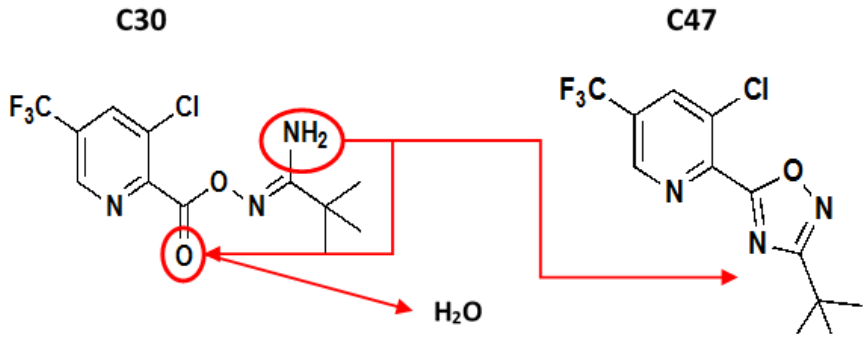


Figure 3.4 showing the loss of water when compound 30 changes into compound 47 in vivo.

The conversion of compound 30 to 47 is similar to that of malathion to malaoxon; whereby malathion is the precursor and malaoxon the active metabolite. Malathion is a widely known and heavily used organophosphorus pesticide which is activated in vivo by oxidative desulfuration changing it into malaoxon [138–140]. Malathion is not particularly toxic before this bio-activation as can be seen by the LD_{50} scores when tested against *Daphnia magna* by Zhang *et al.*, 2011[139,140]. They show that malathion is 4-5 times less toxic than malaoxon and state that previous studies have found up to 10-fold difference in toxicity in insects [139].The activation into the toxic malaoxon is exclusive to insect metabolism as mammal metabolism hydrolyses the compound into non-toxic metabolites with esterase's which are in low concentration in insects [138,139].

Lastly compound 39 to 40 simply changes a chlorine atom to a fluorine in the tail group but this difference causes a notable change in activity. Compound 39 is 2.5 times more active against Hikone-R than compound 40, and 4.2 times more active against the Canton-S.

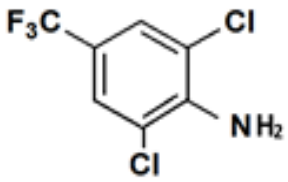


Figure 3.5 'Parnellophore'/'Magic Aryl'
2,6-dichloro-4-(trifluoromethyl)aniline

The tail group in these compounds is important. It is a 2, 6-dichloro-4-(trifluoromethyl)phenyl group, otherwise known as the "Parnellophore" or "Magic Aryl" (figure 3.5), this group is recognised to be part of compounds with good insecticidal activity [141].This group is lipophilic which allows it to cross the waxy cuticle of plants, cross cell membranes and be taken up by the roots and translocated to other areas of the plant [142,143]. These abilities stop the compound from being washed away or lost in leaf fall but they are less well adapted to partition into xylem sap so lipophilicity is a trade-off [142]. The 'Parnellophore' is a static structure in GABA targeting compounds due to its reliable activity and the structural development space is often found in the head group for novel product development. The strength of this group, 2,6-dichloro-4-(trifluoromethyl)aniline, shown in figure 3.4, can be seen by its use as an insecticidal intermediate for multiple insecticides targeting the GABA receptor[144,145].

All of the novel compounds tested here change this magic aryl group and it is, therefore, possible that the significantly higher toxicity of fipronil compared to our novel compounds is, in part, down to this. Trading out a single chlorine atom reduces activity by 35-63 times for Canton-S and 29.6-73.9 times for Hikone-R seen in compounds 30, 39 and 47, while the extra trade of the last chlorine to fluorine further reduces activity by a total of 160.3/140.3 times for Canton-S and Hikone-R respectively for compound 40.

Compound 36 is a highly divergent compound so activity here was exciting but showed 1550 times less activity for Canton-S than fipronil and no activity against Hikone-R. Ultimately none of these novel compounds tested showed the ability to overcome *Rdl* resistance which is a highly conserved gene in insects and so these compounds would not be competitive against the currently used compound fipronil [146].

Rdl resistance is named as such as it confers resistance to dieldrin and through genetic mapping it was found that this resistance is achieved by a single point mutation causing an amino acid change at position 302/301 from alanine to either serine of glycine [147–150]. The loss of alanine at this position is vital to insecticide resistance as it is necessary for cyclodiene insecticide binding [130,147,151]. There is varying levels of cross resistance reported from the Ala^{301} mutation to fipronil [149–152]. $Ala^{301}Gly$ mutation for example confers stronger cross resistance to fipronil than the $Ala^{301}Ser$ [149]. The *Rdl* resistance does not confer complete resistance to fipronil suggesting the binding of fipronil to the GABA receptor is slightly different than the binding site of the cyclodienes (e.g. dieldrin) themselves [130].

Our results show a resistance ratio of 11.8-fold between *Rdl D. melanogaster* compared to Canton-S for fipronil, but high mortality at comparatively low dose is still noted supporting the literature than low levels of resistance to fipronil is conferred by *Rdl* [148]. Our novel compounds showed no valuable levels of resistance breaking capabilities to the *Rdl* $A^{301}S$ replacement suggesting that they, like cyclodienes, require the presence of Ala^{301} to successfully bind to the GABA target site. As this mutation is widespread in nature this means these novel compounds did not pass this level of screening and will no longer be studied for commercialisation.

Chapter 4:

Novel compounds extracted from plants.

4.1 Abstract

Botanicals have long been sources of inspiration for pest control with 50% of the insecticidal modes of action identified from nature inspired synthetic compounds [153,154]. A methanol extraction technique was used to identify insecticidal and repellent effects of basil, chilli, dill, garlic, lemongrass, nasturtium and rosemary samples. Rosemary showed significant insecticidal properties against *D. melanogaster* but only up to 40% mortality of the population was recorded at the highest extraction concentration so active components will need to be identified and concentrated for potential insecticidal value. No samples were insecticidal against *M. persicae*. All extracts except dill showed significant repellent properties to adult *M. persicae* only, not nymphs, suggesting short-term repellent activity or a lack of activity against nymphs. Basil, lemongrass, nasturtium seed and rosemary extracts showed significant increases in the number of nymphs dropped by aphids suggesting possible problems in using these extracts as aphid controls.

4.2 Introduction

Controlling pest outbreaks is vital both economically and for global food security [13,23–25]. *D. melanogaster* are a useful insect to test control methods due to the extensive research done on the organism [75,76]. It is described as a good model system for insecticide resistance research but it is not a pest species and so a comparison to crop pest organism is a useful addition [8,76]. Aphids are considered one of the most damaging crop pests across the globe [155]. They multiply fast, transmit viruses, damage the leaf by feeding and by reducing

photosynthesis by leaving honeydew excretions [79,80,82–84,155] . Controlling *Myzus* outbreaks has proven difficult for multiple reasons; resistance among pesticides and limits to biological control due to natural enemies being restricted by population size of the aphids or other aphid species being preferred as their diet [79,80,85–87]. Due to the primary negative effect of *Myzus* being the transmission of viruses repellent effects of insecticides are of equal importance for crop protection [81].

Nature has inspired pest control since 1200B.C. where insecticides from plants were used in China[153]. Although discoveries of natural products are rare it is notable that nature inspired synthetic products account for 50% of the mode of action products currently in use [154]. Research on natural compounds continues to be of importance for insect control partially due to the value of their environmentally friendly nature and biodegradability when compared to synthetic compounds [156]. Many well-known and widely used pesticide products used today arose from botanical sources such as pyrethroids from chrysanthemums [157]. Discovering novel natural products can be difficult and so it is important to use a wide variety of sources to identify potential leads. Delving into both scientific and public domains potential sources can be identified using the broad knowledge of whole communities.

Oils extracted from plants have contained amides, imides, esters and other compounds known to be successful pest repellents; as such they are considered a good potential source for other repellents [156,158,159]. One of the sources for novel pesticidal compounds could be derived from companion plants; this is a method of pest control without using synthetic chemicals [155]. This can work in multiple ways; by attracting pests away from the crop, by

attracting natural predators or by interacting with the crop plant so that is no longer a suitable host for the pest species [155]. Companion plants can mask the smell of another plant making it more difficult for pests to identify their host plant or the companion plant can produce a repellent [155]. Aphid behaviours can be impacted by olfactory cues and using repellent as a field control for these pests has been effective [160].

Ocimum species, basil, have been tested and used as pest repellents against aphids, grain weevils, mosquitoes and more using extracts from the essential oil and their activity as repellents has been highly cited [155,159,161]. *Ocimum* plants are used in the home to repel flies and insect pests and have been experimentally shown to repel *D. melanogaster* strains [156,162]. Hori, 1998, notes no notable repellent effect of basil oil on *M. persicae* but one of his earlier papers show a toxic effect of Holy Basil on the same aphids [160,163]. Stein *et al.*, 1988 show *Ocimum sanctum,* Holy Basil*,* ethanol and methanol extracts as having strong insecticidal activity against multiple species of aphids, with increased activity when mixed with 0.1% Tween 20 [164]. They attribute the mortality seen to compounds other than eugenol, the main component of the essential oil [164].

Garlic has also been shown to display insecticidal activity from the plant itself and its extracts [158,163,165]. *M. persicae* is a crop pest that has been shown to be controlled by garlic companion planting to protect tobacco crops [166]. An active part of garlic oil, allitin, has been shown to effect mortality and fecundity in *M. persicae* [163]. Garlic has been tested against *Drosophila suzukii* and showed an adverse effect on emergence from both eggs and larvae but a repellent effect was also tested on *D. melanogaster* with no significant effect [167,168].

Rosemary oil has been used against the Peach potato aphid *M. persicae* but its effectivity required the presence of multiple components from its oil [160]. Nia *et al.*, 2015, found that using an petroleum ether extraction on *Rosmarinus officinalis* resulted in 60% mortality in *M. persicae* after 24 hours at the highest concentration [169]. It has also been noted that reproduction of *M. persicae* is significantly reduced if present on the rosemary plant, and *M. persicae* avoid settling on these plants [163]. Mortality and repellent effects do not appear to have been studied for *D. melanogaster* but rosemary extract has an interesting use to reduce mortality effects of high fat diets in *D. melanogaster* suggesting it could actually benefit *Drosophila* rather than the adverse [170].

The capsicum (pepper) family of plants contains capsaicinoids, the substance making the 'heat' in chilli peppers, this substance has been used for its insecticidal properties [171]. *Capsicum frutescens*, otherwise known as bird chilli, has been shown to be an effective pesticide against the larval stages of *Aedes aegypti* with the fruiting body shown to be most potent [172]. No known studies have been carried out on *Drosophila* or *Myzus* for insecticidal activity of the chilli plant and it is important to note that *M. persicae* is referred to as the chilli aphid making this an unlikely repellent for this pest [173].

Dill, *Anethum graveolens*, has also been found to possess insecticidal qualities against beetles, cockroaches and the housefly, but has shown limited repellent effect against *M. persicae*, at least in the context of companion planting [159,174,175].

Cymbopogon citratus, lemongrass, is often used for mosquito repellent but can also repel cockroaches, ants, flies, wasps and beetles [159,176,177]. Avoidance of citronellal, a key component of *Cymbopogon* essential oils, is noted in *D.*

melanogaster [178,179] but toxic capabilities of the lemongrass essential oil have been demonstrated towards *M. persicae* [180]. But, Costa *et al.*, 2013, brings up a point of concern; essential oils are acting on the central nervous system on insects by inhibiting acetylcholinesterase, meaning they could affect non target insects such as pollinators [180].

Nasturtium, *Tropaeolum majus*, has been found to contain insecticidal properties via 2-phenylethylisothiocyanate, mustard oil [181]. Research by Lichtenstien *et al.*, 1962, shows promise for high mortality of this compound against *D. melanogaster* when it was extracted from turnip [182]. Research into pesticidal qualities of Nasturtium primarily focus on its use as a companion plant; it is used to protect multiple crops such as tomato and squash [183]. As part of the Brassica family it is unsuitable for use against pests of these crops but it has shown effectivity against other pests such as the coffee leaf miner [184]. Nasturtium showed both larvicidal effects and repellent effect for feeding behaviour but the work done by Alves *et al.*, 2013 [184], highlighted the dramatic effect of preparation of extracts and time of collection on the activity of extracts from the plants they tested.

Methanol extraction has been used as a technique to identify potential pesticidal compounds from the capsicum plant, bird chilli and the technique was used and evaluated in the research by Alves *et al.*, 2013 [172,184]. Using an adapted methanol extraction technique published by Colclough *et al.*, 2019 [185] we will here asses the pesticidal activities of the above plants on *M. persicae* and *D. melanogaster*.

4.3 Methods:

4.3.1 Novel compound sources

Sample	Source
Basil	Schwartz Basil ASDA product code: 13054
Chilli	ASDA Bird's Eye Chilli Flakes ASDA product code: 3877916
Dill	ASDA fresh
Garlic	ASDA dried chopped garlic ASDA product code: 5384020
Lemongrass	Schwartz Lemongrass ASDA product code: 6450718
Nasturtium (Flower, leaf, seed)	Campus grown
Rosemary	ASDA Rosemary ASDA product code: 544330

Table 4.1 Showing the sources for the compounds used in this chapter.

4.3.2 Methanol extraction technique

Samples were sealed in individual bags and stored at -20°C until extraction. Samples were lyophilized using a freeze drier (Scanvac, Labogene, Lynge, Denmark). Basil, lemongrass, chilli, rosemary and garlic were already dried samples so this step was omitted. Dried samples were ground to powder using a household spice grinder (James Martin ZX809X). The resulting powder was then mixed with 60% methanol in water in the ratio 1:10 (m/v) and incubated for 2 hours at 40°C, 100 rpm. After extraction, samples were centrifuged at 2500 rpm for 15 minutes, after which the supernatant was removed and evaporated

to a final volume of 5 mL in a laminar flow hood. Concentrated extract was aliquoted and stored at -80°C until further analysis. This method was adapted from Colclough *et al.* 2019 [185].

4.3.3 Mortality bioassays: *Myzus persicae*

The bioassay for *Myzus* Clone NS (5557), which is a fully susceptible strain was carried out using a leaf dip method. 1.2 % W/v water agar was added to the base of small pots. Aphids were reared and tested in a 16:8 light: dark regime, 55% relative humidity and at 20ºC.

Leaf discs were cut using a cookie cutter of a diameter of 3.5 cm from *Brassica rapa pekinensis,* Chinese cabbage. Dilutions of the compounds were made in 60% methanol and leaf discs were dipped for ten seconds with slight agitation with care to avoid damage to the discs. Leaf discs were dried in a fume hood on absorbent desk protectors and allowed to dry for 15-20 minutes. Discs were then placed on the agar just before it set with the underneath of the leaf facing upwards, this meant aphids could not get underneath the disc for ease of scoring results. Ten adult aphids were added to each disc and mortality was scored after 96 hours.

4.3.4 Mortality bioassays*: Drosophila melanogaster*

The bioassay for *Drosophila* strains Canton-S (susceptible) was carried out in plastic scintillation vials. Each vial contained 2 ml sugar agar at the base consisting of 2 % agar and 1.2 % granulated sugar, the agar was pipetted into each vial and allowed to set for a minimum of 4 hours. Novel compound

extractions were then added to the set agar surface and allowed to evaporate onto the agar surface overnight. Fifteen Canton-S flies were then added per vial and each vial was stopped with a cotton wool plug. After 72 hours scores were taken for numbers of dead and alive individuals within each vial.

4.3.5 Choice test: *Myzus persicae*

The choice test needed to cover protocols designed for behavioural testing and, therefore, aphids for the experiment were synchronised in age. To do this aphid cups were made with a single cabbage leaf from *Brassica rapa pekinensis,* and 10 adults were placed on the leaf. After 48 hours the adults were removed and the nymphs were allowed to grow for 10 days from initially putting in adults before using in the choice assay.

To test repellent effect of novel compounds a choice assay was designed. A petri dish was altered; using a soldering iron to create a hole in the lid and secure mesh over the gap this created a breathable test container. Leaf discs of 2.5 cm were soaked in the methanol suspension of a novel compound for 10 seconds before air drying for 15-20 minutes. Each petri dish had one leaf disc treated with 60% methanol only (M), and the other with a compound (C). Or control plates using M and M treated leaves. The petri dish base was filled with 25ml of 1.2% water agar and leaf discs were added underside of the leaf up before the agar set, adding before the agar set stops aphids settling underneath the leaf for ease of results.

Once set up 10 adult aphids were added in between the two leaf discs and the leaf they settled on after 20 hours was recorded as their response. The repellent response was measured by comparing numbers of aphids settled on

agar or control leaf discs compared to those settled on treated leaf discs after 20 hours. The number of nymphs dropped on each disc were also taken as a response to account for effect on fecundity.

4.3.6 Statistical analysis

Mortality and choice test assays were analysed using statistical software RStudio, version 0.98.994 © 2009-2013. An anova test was run for both data sets with a post hoc tukey test to compare activity of each extraction to each other and to the negative control. The analysis of nymph data was carried out using a GLM model with data fit into a Poisson distribution.

4.4 Results

4.4.1 Mortality

Figure 4.2 shows the comparative mortality scores for the highest concentration of each extraction against *D. melanogaster.* Rosemary shows the only extraction of interest for mortality effects but the levels are too low to be of any value for pest control.

Analysis of variance of all the data showed that there were significant differences in mortality among treatments (Anova, df=9, f value = 9.511, $p<0.001$) but that this was due solely to the effect of the rosemary extract (figure 4.2). Using a post hoc Tukey test we confirmed that rosemary showed a significant increase in mortality compared to that of the control (Tukey, difference = 0.28, 95%CI = 0.1-0.46, $p<0.001$) but no other compounds did (figure 4.1). Despite the significant mortality effect of rosemary on average less

than 40% of the population died at the highest concentration so repellent effects were also of interest here to explore alternative values of these extracts.

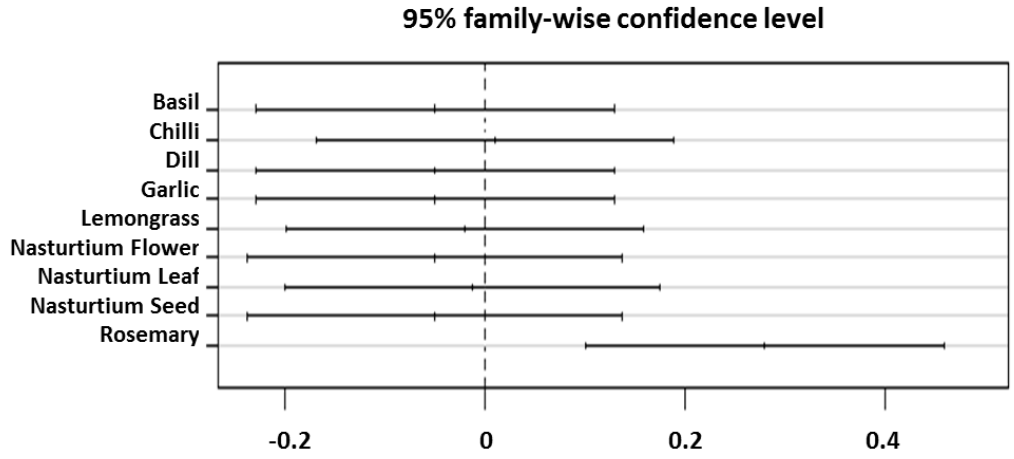


Figure 4.1 showing the directional difference of proportional mortality response between the control and treatment extracts including ±95% confidence intervals.

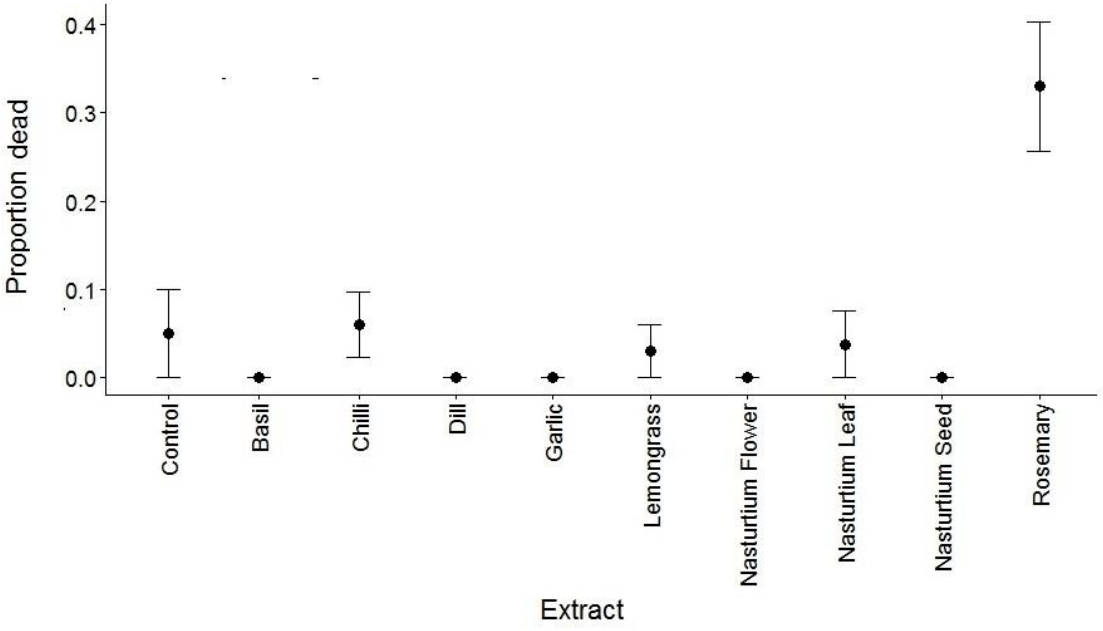


Figure 4.2 showing the proportional mortality with ± 95% confidence intervals for *D. melanogaster* for each treatment and the control.

There was no mortality observed for any of the extracts against *M. persicae.*

4.4.2 Choice

The effects of extracted samples show a significant difference between the effects of treatments (Anova, df=9, f value = 23.29p<0.001). All extracts except dill showed significant repellent effects as shown in table 4.2 and figures 4.3 and 4.4.

Treatment	Difference to control	Lower 95% CI	Upper 95% CI	P value
Basil	-0.87	-1.19	-0.55	>0.001
Chilli	-0.73	-1.05	-0.41	>0.001
Dill	0.08	-0.24	0.4	1
Garlic	-0.9	0.58	1.22	>0.001
Lemongrass	-0.62	-0.94	-0.3	>0.001
Nasturtium Flower	-0.77	-1.09	-0.45	>0.001
Nasturtium Leaf	-0.87	-1.19	-0.55	>0.001
Nasturtium Seed	-0.75	-1.07	-0.43	>0.001
Rosemary	-0.85	-1.17	-0.53	>0.001

Table 4.2 showing the directional differences in repellence between the treatment extracts when compared to the control, including ±95% confidence intervals and p values.

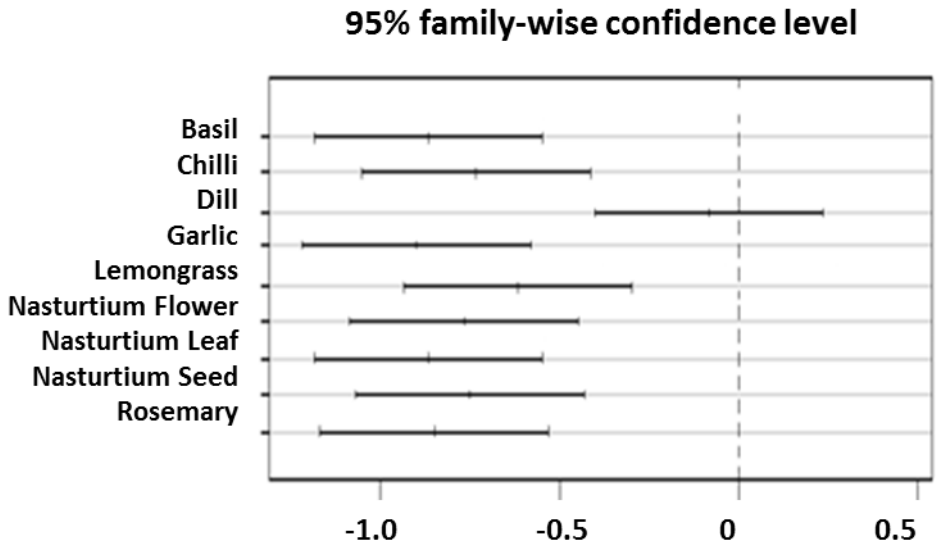


Figure 4.3 showing the directional difference of repellent response between the control and treatment extracts including ±95% confidence intervals.

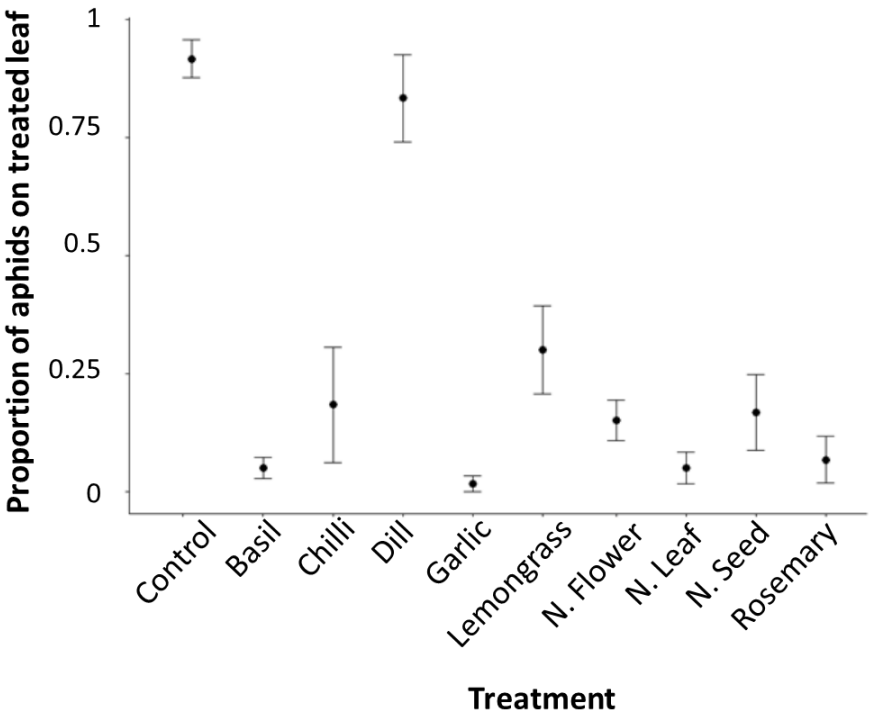


Figure 4.4 showing the proportion of aphids on the treated leaf within the choice experimental setup including the ±95% confidence intervals

Another impact of natural compounds is the effect on progeny. The choice test also gathered offspring data after 72 hours. The raw data on adult aphid and nymph settling response and numbers of nymphs dropped shows possible effects on nymph repellence and fecundity of adult aphids see table 4.3. Following statistical analysis it was clear that there were confounding factors from control treatments which meant repellent and fecundity effects could not be categorically stated from this experimental design. The control treatment with methanol only leaf discs showed a significant negative impact on the number of nymphs dropped (GLM, SE = 0.378, t value = -2.97, $p < 0.01$) so all other significant negative effects are masked by this. One result that is not masked by the control treatment is a positive fecundity effect from aphids treated with basil (GLM, SE = 0.187, t value = 17.82, $p<0.01$) suggesting that this would be a particularly poor crop protection treatment.

As for repellent effects of the natural product extracts these are also interfered with by a significant random effect as seen by the number of nymphs settling on odd versus even leaf discs in the methanol only control (GLM, SE = 0.503, t value = -3.13, $p<0.01$). This means, although the raw data looks like there may be effects on attraction or repellence there is significant interference seen in our control treatment.

Treatment	Adults on Treated Leaf Disc	Adults on Agar or Control Leaf Disc	Offspring on Treated Leaf Disc	Offspring on Control Leaf Disc	Total Offspring
Basil	1	9	1	14	15
	1	9	0	23	23
	0	10	0	25	25
	0	10	0	32	32
	0	9	1	32	33
	1	9	3	38	41
Chilli	0	10	0	7	7
	0	10	0	13	13
	0	10	0	19	19
	0	10	0	9	9
	7	1	14	1	15
	4	5	5	0	5
Dill	9	1	11	0	11
	10	0	6	3	9
	5	5	1	1	2
	10	0	10	0	10
	10	0	4	0	4
	6	4	3	5	8
Garlic	0	10	3	8	11
	0	10	0	8	8
	0	10	0	6	6
	0	10	1	6	7
	0	10	0	5	5
	1	9	0	29	29
Lemongrass	5	5	16	14	30
	3	7	3	14	17
	6	4	20	18	38
	0	10	1	35	36
	1	9	4	28	32
	3	7	20	29	49
Nasturtium Flower	1	9	2	34	36
	2	8	1	11	12
	1	9	0	15	15
	3	7	8	20	28
	2	8	1	11	12
	0	10	6	36	42
Nasturtium Leaf	0	10	0	26	26
	0	10	0	31	31
	1	9	3	19	22
	0	10	0	20	20
	2	8	3	18	21
	0	10	0	19	19
Nasturtium Seed	3	7	0	27	27
	0	10	0	29	29
	1	9	4	24	28

	5	5	13	14	27
	0	10	0	35	35
	1	9	3	21	24
Rosemary	0	10	0	32	32
	1	9	2	28	30
	3	7	8	35	43
	0	10	1	39	40
	0	10	1	8	9
	0	10	0	46	46
Methanol Control	10	0	0	0	0
	8	0	0	3	3
	9	0	0	0	0
	8	0	0	1	1
	10	0	2	30	32
	10	0	19	0	19

Table 4.3 showing the raw data for the *M. persicae* choice test. The information shown here includes the 10 adult aphids and where they settled, either on the plant extract treated disc or the methanol control disc. In the case of the methanol only control odd discs were arbitrarily given the identity of treated or control. The number of nymphs dropped and where they settled was also recorded and can be seen in this table.

4.5 Discussion:

Given that one of the central findings of this thesis is that it is difficult (in our case impossible) to find novel resistance breaking compounds looking for 'alternative' methods of pest control, such as repellence, may be the best option for the control of multiple resistant pests such as *M. persicae*. Here I screen a range of primary extracts from plants to look at their ability to repel aphids from treated plant surfaces (leaf discs).

4.5.1 Mortality effects

There was no aphid mortality response to any of the extractions. In context of the results found by Nia *et al.*, 2015, it is possible this was due to the extraction technique used here [169]. They showed rosemary at its highest concentration using an ether extraction to cause 60% mortality in *M. persicae* but when using an ethanol extraction no mortality effects were seen [169].

A significant mortality response was seen in *D. melanogaster*, though only up to approximately 40% of the population, not high enough for significant pest control. Mortality could possibly be improved by concentration of a sample and fractionation to identify the effective compound(s). Oddly rosemary extract was shown to improve the longevity of *D. melanogaster* by Wang *et al.*, 2017, for those compensating for a high fat diet [170]. The control individuals had slightly better survival than those encumbered by a high fat diet, but this diet was aided by the presence of rosemary extract [170]. This would be something to be aware of in future investigation into rosemary extract as a control method. The insecticidal properties of rosemary oil has been previously noted against a variety of pests with gas chromatography – mass spectrometry to identify the components of the oil [186,187]. Of the 9 components of rosemary oil identified by Isman *et al.*, 2008, all except Camphene showed insecticidal activity against *Pseudaletia unipuncta* and *Trichoplusia ni*. The mix and components of rosemary oil had varying effects against the two different pest species. Important to note is the method of extraction for compounds, Isman *et al*., 2008, used essential oil while Wang *et al.*, 2017, used an unidentified solvent extraction by Jianfeng Natural Product Co. Ltd [170,188] and I used methanol extraction. These differences likely account for the different toxicity found by each experiment.

Extraction technique is a key aspect of finding novel insecticidal properties from natural products. It can affect the quantity of phytochemicals extracted from material such as phenols [183]. The three Nasturtium samples, for example, were expected to work via three potential compounds; 2-phenylethylisothiocyanate, glucosinolate and glucotropaeolin [181,184]. High mortality effects were seen for *D. melanogaster* via a component of nasturtium, 2-phenylethylisothiocyanate, but

the differences between these two studies maybe attributed to differing concentrations of 2-phenylethylisothiocyanate in turnip vs. Nasturtium [182]. Extraction technique used by Lichtenstein included anhydrous sodium sulphate, pentane, ether and acetone but no data was shown for control treatments so whether mortality is due to treatment or remnants from the extraction technique is not clear [182]. The methodology used in this experiment also describes putting *Drosophila* directly onto turnip puree exposing them to a stronger pressure than would be applicable in pest control on other crops [182].

Another important consideration when using natural product extracts is the environmental conditions surrounding the growth of the plant which can affect the activity of extracts significantly and must be considered as a possible factor here in our results [184]. It was also noted that freeze drying samples, part of my methanol extraction technique, can be less active due to a loss of volatiles during this process [184].

While the results here showed no mortality effect of chilli, a 2020 study has looked at the effect of capsaicin from chilli on health and longevity on *D. melanogaster* [189]. Li *et al.*, 2020, found that capsaicin reduced fitness of progeny by reducing pupae survival by 30% and survival into adulthood by 39% and also showed significant toxicity to adult flies with males being more susceptible than females [189]. Another important finding by this study indicated other fitness costs to adult flies by affecting their locomotion ability making them more vulnerable to predation [189]. Unlike our study Li *et al.*, 2020, used capsaicin (MACKLIN) rather than whole chilli extract, therefore, quantities of capsaicin and any other chilli extract components may not have been at high enough concentrations to see the effects found by Li *et al.*, 2020, yet again highlighting the important effects of extraction techniques and purity of compounds.

The remaining extracts showed no activity and previous research either does not exist or does not suggest this would be expected. Previous work on lemongrass has shown little mortality against *D. suzukii* in line with our findings [190]. Research on dill shows genotoxic effects on *D. melanogaster* but the research focused on genetics insights and did not show mortality effects [191]. Basil and garlic have no literature suggesting previous mortality effects on pests, and, though our results do not show mortality, this cannot be ruled out due to the strong differences in effects of extracts due to techniques used.

4.5.2 Repellent effects

Our study showed significant effective repellent qualities of all extracts except dill which had no effect. Research into the effects of dill have looked primarily from a genetic insight showing genotoxic effects against *D. melanogaster* via chromosome aberrations and somatic mutations but no apparent research was found on its insecticidal or repellent potentials [191]. Dill was looked into due to extensive recommendations on gardening websites for use as insect repellent, specifically listing aphids as one of the insects it repels [192,193]. Our findings do not support this claim.

Basil repellence has been shown previously against *D. melanogaster* by using the essential oil containing linalool, methyl cinnamate, eugenol and estragole [156]. The results here add to this finding by Onar *et al.*, 2010, by showing the repellent effects of basil extract on *M. persicae* as well as *Drosophila*. An odd addition to these results do show that offspring numbers of *M. persicae* increase suggesting basil has an increased effect on nymph dropping. This would be something to be considered should basil extract be studied further for *Myzus*

control. Longer term and multiple dose effects of basil treatment would need to be evaluated in case the short term showed repellence but long term may increase the numbers of pests.

The repellent effect against *Drosophila* has been previously recognised using garlic though the effects are more complicated [167,168]. Garlic extract shows an increase in adult and larval numbers of *D. suzukii* after 3 days, a reduction of approximately 50% after 6 days and then a return to normal after 10 days [168]. Our results concerning *Myzus* show repellence to adults but fecundity and nymph repellence was not possible to confirm from our results due to methanol control interference. An important note from comparing this study to Perez-Guerrero and Mateus, 2019, is the differences in possible results when looking at a laboratory experimental design and a field trial. The aphids in this study could not leave the environment where the repellent was which may affect the natural behaviours of movement and nymph dropping.

Chilli compound capsaicin has been described as having repellent properties against *D. melanogaster* via feeding repellence and oviposition repellence but it was noted that flies still laid comparable numbers of eggs but not on the capsaicin covered surface [189]. The data gathered in this study could not give sufficient information on fecundity or repellent effects on nymphs but it would be interesting to know if the repellent effect on adults is as successful on nymphs, how long the effects last and if competition for food was a stronger driving force than the repellent effect of the extract.

The repellent effects of lemongrass is commonly referred to, specifically for mosquitoes [177]. This is attributed to citronellol and citronellal components of lemongrass oil from the species *Cymbopogon winterianus* [177,178,194]. It is

important to note that cooking lemongrass is often *C. citratus* or *C. flexuosus,* likely to have been the species tested here due to samples being bought in supermarket groceries, though we also found repellent effect from this sample[177,178,194]. *Drosophila* has been shown previously to avoid feeding when citronellal and citronellol are used but they do not avoid lemongrass oil, just these components [178,190]. Eben *et al.*, 2020, also noted that though there was an effect on feeding there was no effect on offspring from citronellol or citronellal but there was significantly reduced oviposition in the presence of lemongrass oil [190]. This highlights the importance of further rigorous testing of the effects on *M. persicae* offspring, a potential problem that would need to be understood before using lemongrass extract as *Myzus* control.

Surprisingly, despite the potential of nasturtium as a source for pest control substances little research is available. The 2013 study by Alves *et al.* showed activity of nasturtium extract against the coffee leaf miner in the form of a feeding deterrent and larvicide for extracts from the leaf and flower but, of key note, was the complete loss of activity from samples collected at different times [184]. Our results also show repellent effects of nasturtium samples, which were separated out into leaves, flowers and seeds due to the difference in concentrations of known insecticidal components of nasturtium like glucosinolate and glucotropaeolin found in high quantities in the leaves [184] but no significant differences in avoidance was seen between the three sample types for adult avoidance.

The repellent effects of rosemary have been long known and previous repellent effects against *M. persicae* was found by Hori, 1998, which the results found here support [195]. Methodology used by Hori, 1998, bring to attention the different requirements for pesticidal treatment, as they focus on greenhouse like

settings; keeping rosemary oil containers open in an enclosed space to cause aphids to move to different areas in the space [195]. Our methodology uses particularly controlled settings where numbers of adult aphids cannot increase or leave a relatively small area. Though this was useful for initial testing of extracts, larger scale testing would be valuable to test valid application methods of these compounds and longer term effects on pest population with particular focus on the effects of extracts on offspring numbers and behaviour.

Chapter 5:

Construction of transgenic *Drosophila melanogaster* lines as a screening tool for metabolic resistance.

5.1 Abstract

Identification of novel insecticidal compounds is a multimillion dollar industry costing $256 million to get an individual product from discovery to market. To reduce R&D costs screening methods to quickly and cheaply identify resistance breaking compounds would be a valuable resource. Developed here are four transgenic *D. melanogaster* lines which were be added to a larger collaboration called the Fly-Tox panel. The lines developed contain P450 genes that have been previously implicated in metabolic resistance to insecticides. First is *Cyp6cm1* from *Bemisia tabaci* which confers resistance to neonicotinoids, second *Cyp6bq23* from *Meligethes aeneus* conferring pyrethroid resistance. *Cyp6bq9* was taken from *Tribolium castaneum*, also conferring pyrethroid resistance specifically to deltamethrin. The last line contains *Cyp337b3* from *Helicoverpa armigera*. These lines were expressed using the GAL4/UAS system and functional expression was proven for *Cyp6cm1, Cyp6bq9* and *Cyp337b3* but resistance was not conferred by these genes to *Drosophila* transgenic lines.

5.2 Introduction

Getting compounds to the market comes with high costs and an ever increasing chance of cross resistance already present from pre-existing resistance mechanisms [27]. This research will provide a screening tool to identify compounds with the potential to overcome known resistance mechanisms in target insects, in a cheaper and quicker way.

5.2.1 Chapter aims

Developing resistance to chemicals is a universal phenomenon from bacteria to mammals[4]. The first recorded instance of resistance to a pesticide was documented in 1914 and has since become a widespread problem, with more than 440 pest species now resistant to one or more pesticidal compounds [3,4]. To counter insecticide resistance it is important to understand the mechanisms of resistance and how they occur [5,7,8]. Research on resistance gained more interest in the 1970's; high levels of resistance arose to once very effective compounds, and new molecular tools were available to understand resistance mechanisms, and the limitations of pesticidal compounds became clear [5,6]. Pesticide resistance was estimated in 2001 to cost the USA $1.4 billion annually [1]. Not only is there a large economic cost but the knock on effects of resistance reduce our ability to control pest species, in turn reducing crop yield and requiring higher doses of pesticide to be used which damages the surrounding ecosystem and wildlife [1,2].

Still facing high levels of pesticide resistance and the damaging effects of the remaining effective compounds, I here look to create a test panel of *D. melanogaster,* containing multiple resistance genes from the P450 gene superfamily [9]. The focus here is on metabolic resistance conferred by P450s, as it is one of the most important mechanisms of resistance in insects [1]. P450 enzymes can vary from having broad to narrow specificity to compounds they are able to metabolise, and so a variety of these enzymes will be important to show their range of capabilities [49]. Genes will be chosen from economically important pests and inserted into the genome of *Drosophila*. By screening novel compounds against this panel it will be easier to identify current resistance mechanisms that may reduce the effectiveness of a new pesticide, or pest

species that will be more or less susceptible to the new compound. *D. melanogaster* was chosen as the host for the resistance genes as it is a model organism, cheap to maintain, and allows for high throughput experiments.

5.2.2 Insecticide resistance and key P450 genes

The screening tool produced here will take four P450 genes from economically important crop pests; *Helicoverpa armigera, Tribolium castaneum, Meligethes aeneus* and *Bemisia tabaci.*

Whitefly, *B. tabaci* infestations cause yellowing of leaves, twisting of stems, curling of leaves and stunted growth [196]. Plants may also wilt and turn black from honeydew production, this allows mould to grow on the plant and enhances virus transmission [196]. *B. tabaci* and *T. vaporariorum* are considered the two most common and destructive species of whitefly [197]. Whitefly infests 900 crop plants spreading approximately 100 viruses and are considered one of the most severe global pest species [196,198]. The host range of this already polyphagous species is increasing and adapting to control methods easily, they are under strong selection pressure causing rapid spread of resistance [198].

Whitefly, such as *B. tabaci*, have been responsible for extensive crop losses every year, for the past 30 years, causing great economic losses, but it is difficult to estimate the precise monetary losses due to the global spread and large range of host plants affected [198]. The viruses transmitted by whitefly also result in economic loss, geminiviruses affecting tomatoes alone has cost Florida $140 million in one year, Puerto Rico $40 million and Brazil 11,000 jobs [198]. This is only from one of the viruses the pest transmits and its effect on one crop. The cost of control also adds to the economic losses this pest causes, insecticide

treatment often fails, due to the quick development of resistance [198]. Control methods often include an insecticide, and resistance to this is expected so insecticide resistance management (IRM) programs are applied [198]. Limiting applications of insecticides helps to maintain their effectiveness [198].

Imidacloprid was an effective control for *B. tabaci* but this species has evolved mechanisms which confer resistance to this compound [199]. Imidacloprid resistance, which arises due to high exposure from repeat doses and residual persistence causes constant selectivity, and confers cross resistance to neonicotinoids such as thiamethoxam and acetamiprid [200,201]. Synergised pyrethroids were also heavily depended on for controlling *B. tabaci* outbreaks, but high doses of a fenpropathrin and acephate combination caused reduced susceptibility of field populations [200].

Crop damage reached a point during these control measures where the pyrethroids were no longer controlling whitefly to an acceptable level [200]. Though alternatives of buprofezin and pyriproxyfen were subsequently used to control the infestation, returning to intensive synergised pyrethroid control of fenpropathrin and acephate combination is expected to result in a rapid selection for resistant *B. tabaci* [200,202]. Buprofezin, another effective control, is also selecting for resistance in intensive use programs, lowering susceptibility, but populations using single treatment rather than intensive treatments are showing much slower adaptation of *B. tabaci* to this chemical [200,203,204]. Within one year, pyriproxyfen use in greenhouses resulted in an over 500-fold increase in resistance, but controlled use of the insecticide, limited to one dose per season, showed a much lower selection for resistance [200,203,205]. Unfortunately, despite this, five years after use, resistance levels reached a high level where pyriproxyfen was no longer an effective control [200,205]. It is hypothesised that this

resistance level occurred due to geographic isolation within the study site, Ayalon Valley [200]. Pyriproxyfen is still effective but IRM programmes are essential to control resistance [200].

One of the resistance mechanisms against pyriproxyfen, in glasshouse whitefly *T. vaporariorum,* is the overexpression of a P450 gene, *Cyp4g61* [206]. Mild resistance to pyriproxyfen was found in a strain of *T. vaporariorum* in Germany where the insecticide is not registered for use [206,207]. Resistance could have occurred via cross resistance mechanisms or through trade of contaminated plants, after no cross resistance was identified, the latter explanation seems more likely [206,207]. Twelve P450's were identified as overexpressed in the resistant strain compared to a susceptible strain, of these genes only one was found to be highly overexpressed in the resistant strain by 81.7-fold, *Cyp4g61* [206,207]. The *Cyp4g* subfamily of P450's is known to confer resistance in other pest species and the level of resistance between wildtypes, partially resistant (1.41-fold more than wildtype susceptible), to fully resistant (81.7-fold increase compared to susceptible) in *Cyp4g61* suggests this is the likely candidate conferring resistance to pyriproxyfen [197,206,207].

Neonicotinoid resistance in whitefly has been linked to overexpression of *Cyp6cm1* in *B. tabaci* and *Cyp6cm2* and *Cyp6cm3* in *T. vaporariorum* [197]. The P450, *Cyp6cm1,* will be added to the reference panel as not only does it confer resistance to the neonicotinoid, imidacloprid, over expression of this P450 gene also confers cross resistance to pymetrozine [208,209].

The second pest species from which a P450 gene was selected, was the pollen beetle, *Meligethes aeneus*. The pollen beetle is harmful to turnip, cabbage, mustard, and particularly oilseed rape [210]. Economic harm occurs as low as two

insects per plant, while twenty insects per plant causes 50% yield loss [210]. Pollen beetles are noted as one of the most important and destructive pests to oilseed rape and are able to reduce crop yield by up to 80% [211–213]. Pollen beetles damage flowering pods on the plant; adults affects buds from green to yellow stages which prevents them from developing while larvae cause abscission of buds [211,213].

Countries growing both winter and spring rape suffer more from pollen beetle infestations due to the extended period of time in which the pests have a food source and breeding site [212]. Due to adaptability of the plant, the effect of infestations can vary, low density infestations can either result in no loss or even increased yield due to spare pods on the plant that are available to compensate for disease, frost damage or pest damage, but once the infestation crosses a certain threshold the yield loss becomes severe [213].

Pollen beetles are controlled by insecticide treatments, primarily pyrethroids but, due to intensive use of pyrethroids as a control measure, resistance is becoming more and more widespread, initiating in 1997 in France and now found across Europe at high frequencies [211,212]. By 2000 a population study in Denmark found 90% of a pollen beetle population survived standard pyrethroid treatment [212]. Resistance to pyrethroids can occur by both target site resistance at the voltage-gated sodium channel and an increase in P450 genes able to metabolise the pesticide [211]. I will be looking at the specific *Cyp6bq23* P450 gene which is highly overexpressed in resistant pollen beetle strains by several hundred fold [211].

The red flour beetle, *T. castaneum,* is the third crop pest. It affects grains such as flour, cereals, seeds, beans and more and is deemed one of the most

prominent pests to storable crops [214,215]. The red flour beetle, adults and larvae, only target grain dust or broken kernels, not undamaged grain [216]. Infested crop yields tend to go mouldy, and are contaminated reducing product quality and value [216,217]. The red flour beetle is noted as a model organism with much information on its importance, development, and genome [218]. It has evolved to interact with a diverse set of chemicals, one of its methods for coping with this being P450 genes [218].

Pesticide resistance in *T. castaneum* has occurred through intensive selection pressures of previously effective chemical treatments. Resistance to malathion, an organophosphate, was found at higher frequencies in populations from Oklahoma where the use of malathion was more intensive than comparative sites and laboratory strains [219]. The red flour beetle is also shown to be resistant to pyrethroid, deltamethrin [211,215]. Zhu *et al.,* 2010, found that the cause of resistance to deltamethrin was the overexpression of *Cyp6bq9* which metabolises the chemical and is found at a 200-fold increase in resistant *T. castaneum* compared to the non-resistant strain [211,215]. The *Cyp6bq9* gene will be another addition to the P450's used for this screening panel.

The final addition to the screening panel originates in the cotton bollworm, *H. armigera*. It is polyphagous, severely affecting cotton and tomatoes and is estimated to cost over $2 billion every year [220–222]. The cotton bollworm bores into plants damaging or consuming flower buds, seed, grain and leaves and can lead to secondary infections [220,221]. It has been argued that the cotton bollworm is one of the most agriculturally important pest species in Africa, Australia, the People's Republic of China and subcontinental India [222–225].

H. armigera is known to be resistant to a diverse range of insecticides from pyrethroids, organophosphates, carbamates, cyclodines, endosulphan and chlorofenapyr [222–225]. Intensive use of these pesticides, sometimes over 20 applications per season, increased selection pressure and resulted in resistant pest populations [222]. Transgenic cotton plants are now relied upon to manage the cotton bollworm [222].

Specific P450's in *H. armigera* conferring resistance to pyrethroids had not been identified until the *Cyp337b3* gene identified by Jouβen *et al.,* 2012 [226]. An Australian strain of cotton bollworm was resistant to fenvalerate but the resistance was supressed using piperonyl butoxide, suggesting that the resistance mechanism was a P450 [226]. Further investigation led to the identification of the P450 *Cyp337b3* as the causal enzyme in resistance [226]. The P450 gene encoding this gene resulted from unequal crossing over of the *Cyp337b1* and *Cyp337b2* genes [225,226]. Resistance to cypermethrin is also determined by the presence and upregulation of this P450 gene [225].

5.3 Methods

5.3.1 P450 gene plasmids

Previously extracted insect RNA was provided by Emma Randall who used the TRIzol® reagent (Molecular Research Inc.) following the manufacturer's protocol. Live insects were snap-frozen in liquid nitrogen and ground using a homogeniser in a 1.5 ml sterile microcentrifuge tube. cDNA was reverse transcribed from this sample using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) following manufacturers protocol. Forward and reverse primers were designed using Geneious version 8.1.9 and sequences

for the pUAST plasmid and the *Cyp* gene of interest. I identified restriction sites within the multiple cloning site of the pUAST plasmid that were not replicated in the *Cyp* gene of interest. Primers were then designed to add the restriction site at either end of the gene to create 'sticky ends'. This was used later in the process to insert the gene into the plasmid. The gene was PCR amplified using Phusion buffer and quality and presence of the gene was confirmed using an electrophoresis gel. Quantity was measured using the NanoDrop One.

In cases where the insect was not available to directly extract the gene, the sequence of the gene was obtained and a synthetic copy of the gene commercially synthesised. When doing this codon optimisation was applied to enhance expression in *Drosophila*. Each species has a preferred codon sequence for the same amino acid, by designing a synthetic P450 with the optimal codons for the transgenic organism instead of the original host, the P450 functionality should be maintained in the transgenic host [227,228]. Any difficulties found with genes extracted directly from the host were returned to this step and a synthetic, codon optimised version was used in its place.

The *Cyp* gene was then amplified by PCR and reactions were made using DreamTaq Green PCR Master Mix (Thermo Scientific) (final volume, 20µl) contained 10µl DreamTaq, 1µl of each primer, 1µl cDNA and 7µl water. PCR conditions were 95ºC for 3min for enzyme activation, followed by 40 cycles of 95ºC for 15s, 59ºC for 20s and 72ºC for 20s and then a final 95ºC for 15s and 65ºC for 5s. Once the gene was amplified both the P450 gene and pUAST plasmid were digested using BglII and Xba1, these enzymes adhere to the two restriction sites that were added into the *Cyp* gene using the primers.

This digestion was carried out for 4 hours at 37ºC and at 65ºC for 20 minutes to denature the enzymes. The product was purified using the GeneJET PCR purification kit (Thermo Scientific) and an electrophoresis gel confirmed the quality of the digested product. Quantity of product was measured by the NanoDrop One.

The digested plasmid and *Cyp* gene were then ligated using a 4:1 ratio and a ligation buffer. Sticky ends of the plasmid and *Cyp* gene enabled ligation to occur at room temperature for 4 hours and the ligase was then inactivated for 10 minutes at 65 ºC. Ligated plasmids with the *Cyp* gene were then transformed into *E. coli* for replication. Competent *E. coli* cells were thawed and the plasmid was added. The mixture was incubated for 30 minutes on ice and heat shocked at 45 ºC for 45 seconds before being returned to the ice for 2 minutes. Heat shock causes cells to uptake free DNA. S.O.C. medium was added and the mixture was incubated at 37 ºC for 1 hour shaking at 225rpm. The reaction was diluted before plating on 100µg/ml ampicillin LB plates overnight at 37 ºC. Colonies that grew on the plates contained the plasmid insert which included ampicillin resistance. 12 colonies were stabbed onto a new ampicillin plate for 6 hour incubation at 37 ºC. A second attempt used 39 colonies to consider a low efficacy of ligation success. A PCR was run using DreamTaq Green PCR Master Mix (Thermo Scientific) (final volume, 20 µl) contained 10ul DreamTaq, 1µl of each primer, 1µl cDNA and 7µl water. PCR conditions were 95ºC for 3min for enzyme activation, followed by 35 cycles of 95ºC for 30s, 64ºC for 30s and 72ºC for 1min and then a final 72ºC for 10min and held at 4 ºC. The PCR product was electrophoresed on a 1% agarose gel as a visual check for the presence of the insert in the colonies.

The colonies that were stabbed onto a new plate were used to make micro-cultures, consisting of LB broth with ampicillin (100µg/ml) and the samples were incubated overnight before using the Miniprep protocol. In this protocol cells were lysed and the plasmid was extracted. The DNA concentration of the plasmid preparation was determined using a Nanodrop spectrophotometer. Next a double digest was repeated to confirm that the plasmid taken up by the *E. coli* contained the chosen *Cyp* insert and this was run again on an electrophoresis gel.

5.3.2 Injecting embryos

D. melanogaster embryos were then injected with the plasmids at Cambridge University by Kadri Oras. The fly stock chosen for the injections was stock 13-20: vas-int; attp40. This stock uses the PhiC31 integrase-mediated transgenesis system, this means the transgene is directed to a specific site on a specific fly locus. The system is highly efficient, irreversible and creates stable transformants [229].

Fly embryos were collected within 30 minutes to ensure similar age of embryos and the correct phase for injecting. Embryos were collected by making a 3% agar plate, scoring slightly and adding a small spread of yeast. This mimics rotten fruit surfaces to encourage laying. The surface of the plate was covered in a layer of water and lightly brushed using a small paintbrush to dislodge embryos from the plate surface. Ideally the yeast sample is not resuspended in the water as crystals get in the way during injection. The water was then poured into a small sieve which caught the embryos and was then towel dried. The

sieve containing embryos was then placed in a 50:50 household bleach and water solution to remove the outer layer of the embryos for 3 minutes and then rinsed with deionised water. This is called dechorionation which removes the tough outer layer of the embryo known as the chorion (eggshell). Within 30 minutes embryos must be lined up on a pre-prepared slide; this is a slide with a cover slip that has 3 rows of glue from sellotape, this keeps the eggs in position for injection. Embryos should be aligned on the slide with their posterior on the left. After 30 minutes the aligned eggs need to be coated in a small amount of Voltalef 10S oil to stop desiccation of the embryo. Embryos were injected in the posterior end where the pole cells are. These cells pick up the plasmid construct and replicate it around the body.

The cover slip containing the injected embryos was transferred to another agar plate with yeast spread and incubated at 18ºC for three days. This temperature is slightly lower than the optimum for *Drosophila*, this means the embryos have a little extra time developing which helps the plasmid construct spread around the embryo. The larvae tend to go into the yeast but any that are around the plate were moved into the yeast paste. A cornmeal tube with fresh yeast paste was then made up and larvae were transferred to this tube. Survival was measured by deducting dead embryos from the coverslip from the total number of embryos injected. 50% survival was expected for this strain.

5.3.3 Activating P450s within the transgenic flies

Adult *Drosophila* containing the P450 transgenes were returned from Cambridge and a homozygote population was reared. The adults show phenotypic markers of the presence of the P450 transgene, in this case red

eyes, and heterozygote flies show orange eyes. Females and males were chosen to multiply the population with red eyes only for a homozygote stock of inactive transgenic flies. Female virgin flies were collected over multiple days every 2-3 hours from the activator stock, strain 20 + pUAS-attP containing *Cyp12/SM6a*. Fifteen virgin females were then crossed with 10 transgenic males in a large fly bottle containing 25ml Nutrifly food. After 1 week the adults were transferred into new large bottles with 25ml Nutrifly, this produced two batches of 'activated' P450 offspring on which to run follow up bioassays. The offspring can be collected two weeks after putting in the adults. Active offspring were identified by straight wings and red eyes.

Control activated lines were also made by crossing virgin females with males from strain 20 + pUAS-attP containing *Cyp35/SM6a*. Once these offspring emerged the active individuals containing the same genetic cross just without the P450 gene were identified with straight wings but white eyes. These controls provided a baseline resistance for *Drosophila* having undergone this genetic manipulation, resistance then caused specifically by the P450 gene was easily identified.

5.3.4 Confirming P450 overexpression

To confirm overexpression of the target gene in crossed *D. melanogaster* lines a qPCR was conducted using a real-time detection system (BioRad, CFX connect). Transgenic flies were kept at 24ºC with 18:6 light:dark cycle in a *Drosophila* stock bottle containing 45ml Nutri-Fly BF. Adults were snap frozen in liquid nitrogen at 18 days old ground using a pestle in a 1.5ml Eppendorf tube and RNA was extracted using Isolate II RNA mini kit from Bioline following the manufacturer's protocol. cDNA was then made using Maxima H minus First

Strand cDNA synthesis kit from ThermoFisher Scientific also following manufacturer's protocol. Prior to PCR, each cDNA sample was made up to a concentration of 50ng/µl using a qubit to measure concentrations and reactions were made using SYBER Green JumpStart Taq Readymix (Sigma) (final volume, 15µl) contained 7.5µl SYBER Green, 1µl of each primer, 4µl cDNA and 1.5µl ultra-pure water.

PCR conditions were 95ºC for 3min for enzyme activation, followed by 40 cycles of 95ºC for 15s, 60ºC for 20s and 72ºCfor 15s and then a final 95ºC for 15s. Fluorescence was measured after each cycle.

The expression of each gene was measured in comparison to two housekeeping genes, *SdhA* and *RpL32* and the parental and offspring lines were compared to confirm overexpression of the target transgene once the *Drosophila* cross had taken place. Each gene had four biological replicates with three technical replicates of each

The real-time PCR primers used were designed using the Primer3 program [230] to amplify a 150-250bp fragment for each gene. These primers are listed in the supplementary table 2.

5.3.5 Bioassays

The bioassay for *Drosophila* strains was carried out in polystyrene plastic scintillation vials. Each vial contained 2 ml sugar agar at the base consisting of 2 % agar and 1.2 % granulated sugar, the agar was pipetted into each vial and allowed to set until dry. Compound dilutions were made with acetone and added in 200 µl volume and allowed to evaporate onto the sugar agar surface overnight. Five replicates of each concentration was made and fifteen flies were then added per vial and stopped with a cotton wool plug. After 72 h scores were

taken for numbers of dead and alive individuals within each vial. The scores were used to narrow down the LD_{50} for each compound and the experiment was repeated with a narrower range until the LD_{50} could be calculated. The transgenic *Drosophila* lines were tested against the compounds the genes conferred resistance to in the original pest species. *Cyp6cm1* from *B. tabaci* was tested against imidacloprid [231]. *Cyp6bq9* (*T. castaneum*) and *Cyp6bq23* (*M. aeneus)* were tested against deltamethrin and *Cyp337b3* (*H. armigera)* was tested against fenvalerate which it is known to confer resistance to [215,232,233].

5.4 Step by step example

5.4.1 *Cyp6cm1* from *Bemisia tabaci*

Below is the sequence of the *Cyp6cm1* gene from *B. tabaci*. The coding region (in yellow) is as sequenced by Diao,Y.Z., Wu,G. and Zhang,Y.J. and was obtained from NCBI Genbank. The primers designed are annotated, the light green regions show the restriction enzymes added to create sticky ends with an overhang (figure 5.1).

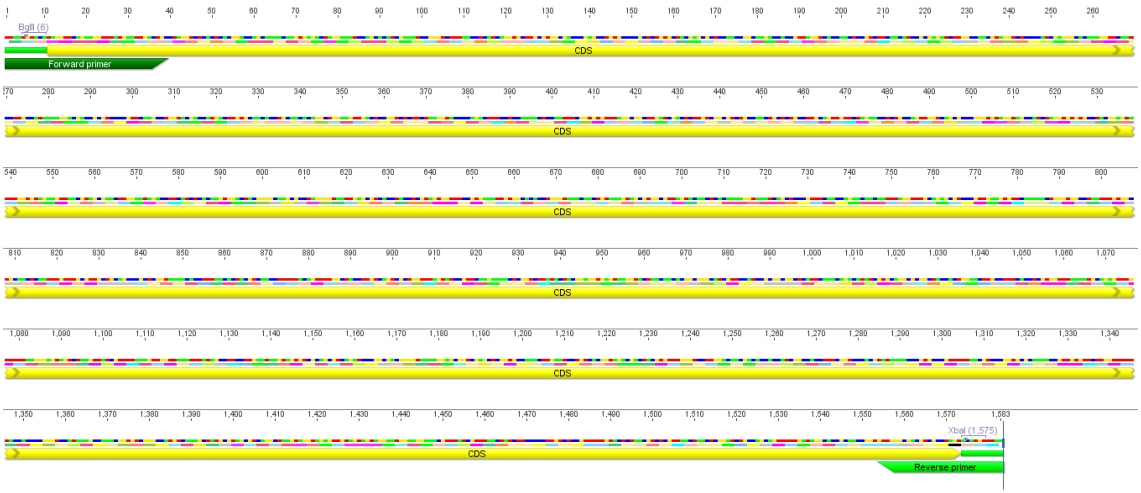


Figure 5.1 *Cyp6cm1* gene sequence with primer annotations.

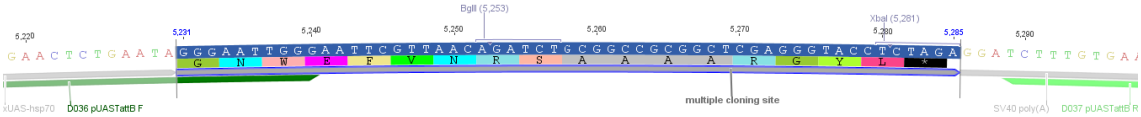


Figure 5.2 The multiple cloning site (highlighted in blue) of the pUAST plasmid, can be seen here with the BglII restriction enzyme also found on the forward primer, and the Xba1 found on the reverse primer for the *Cyp* gene.

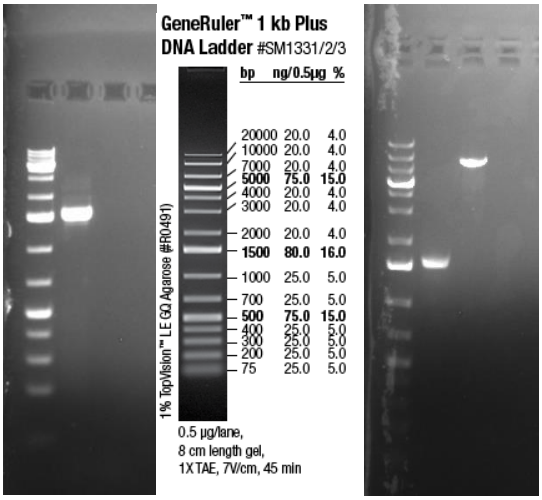


Figure 5.3 Electrophoresis gel showing; *Cyp6cm1* gene (left), both the plasmid and *Cyp* gene with the two restriction enzymes to create the sticky ends for ligation (right).

The gel on the left of figure 5.3 shows the gene of interest at the expected 1500bp while the gel on the right shows both the *Cyp6cm1* gene at 1500 bp and the plasmid running between 7000 and 10,000 (as expected for the digested plasmid measuring 8467bp). Following this the sequences were ligated.

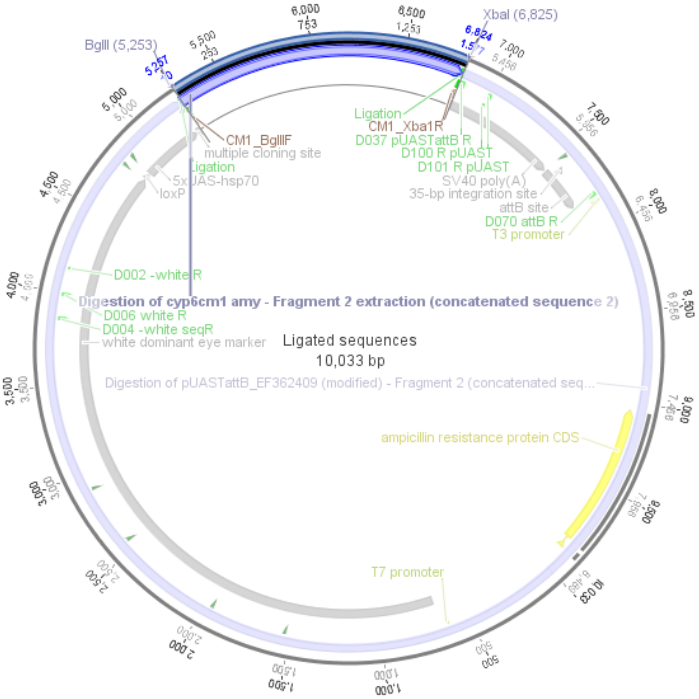


Figure 5.4 A representation of the ligated plasmid and gene created in Geneious, the *Cyp* insert is highlighted in blue.

Ligated samples were then transformed into *E. coli* and transformants were selected using ampicillin plates. See figure 5.4 for the ampicillin resistance code within the pUAST plasmid but note that this is not connected to the gene insert, it is important to check transformants for not only the plasmid but the *Cyp* gene too.

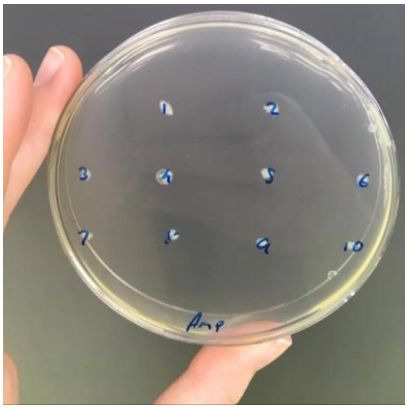


Figure 5.5 LB/ampicillin plate showing transformant samples 1 – 10.

5.4.2 The first attempt

A colony PCR was performed on the transformants using the *Cyp* primers and a gel was run. There was no plasmid or gene but what was predicted to be hairpin replication of the primers themselves (Figure 5.6). The primers designed were long, with two potential hairpin formations in the forward primer, this was unavoidable while creating the primers. The minimum number of bases required to attach to the correct *Cyp* gene combined with the restriction enzyme and equalizing the two primers to have similar melting temperatures all contributed to the long primer size. Because of this more checks were carried out.

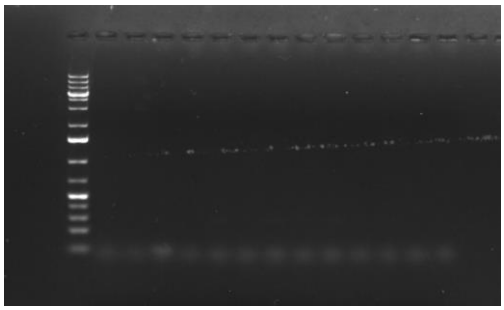


Figure 5.6 Electrophoresis gel of colony PCR on 10 *Cyp6cm1* transformants.

Continuing with the miniprep and isolating the plasmids from the transformant *E. coli*. A NanoDrop confirmed high concentrations of plasmid, so another gel was run. Using a small sample of the miniprep plasmid and another double digest was carried out, this way the gel should have shown both a plasmid and *Cyp* gene bands if they were present in the sample.

Though there was a band to confirm the expected size of the digested pUAST plasmid there was no band to show the *Cyp6cm1* gene (Figure 5.7). From this it can be deduced that ligation step did not work and this step was repeated.

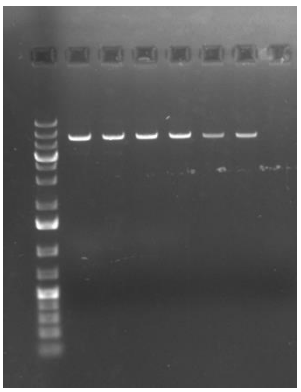


Figure 5.7 Electrophoresis gel of digested pUAST plasmid with no visible *Cyp6cm1* insert.

5.4.3 Second attempt

Once ligation and transformation was repeated, checks on the efficacy of ligation could be carried out again. This time 39 transformants were sampled for colony PCR in case of low efficacy, an electrophoresis gel showed promise for the plasmid at the right fragment size on the gene ladder (Figure 5.8).

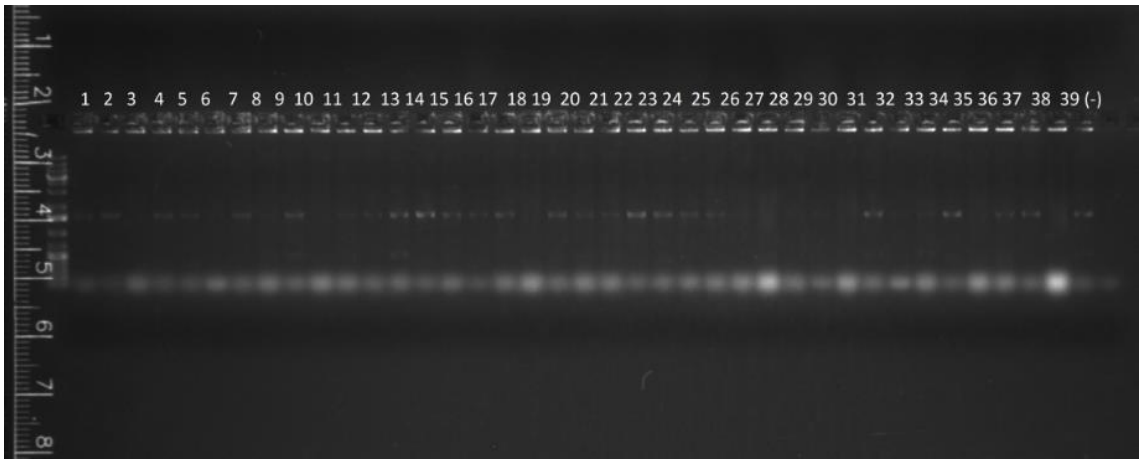


Figure 5.8 Second attempt electrophoresis gel of colony PCR on 39 *Cyp6cm1* transformants.

Six samples with the brightest bands at 1.5kb were selected for mini cultures (13, 14, 22, 23, 31, 34 (figure 5.8)). These samples were run through the mini prep protocol and double digested. This next gel should have shown a band for the insert and plasmid (figure 5.9).

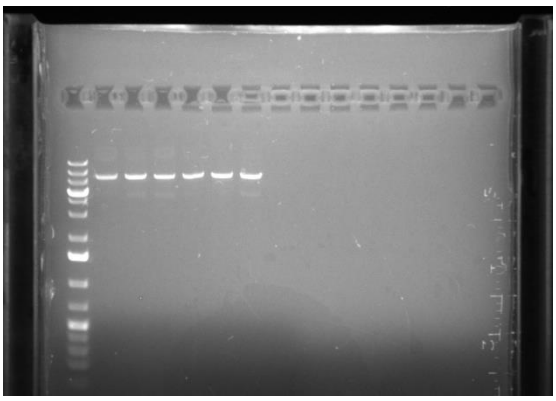


Figure 5.9 Second attempt electrophoresis gel of digested pUAST plasmid with no visible *Cyp6cm1* insert.

The plasmid was visible but again no insert was found for these samples. Following this result a synthetic copy of all four P450 genes was ordered with codon optimisation [234].

These four transgenic lines, *Cyp6cm1, Cyp6bq23, Cyp6bq9* and *Cyp337b3,* I contributed to the paper McLeman, A., Troczka, B. J., Homem, R. A., *et al*. (2020). Fly-Tox: A panel of transgenic flies expressing pest and pollinator cytochrome P450s. *Pesticide Biochemistry and Physiology*, *169*.

5.5 Results

5.5.1 Expression of transgenes

Following activation of expression by crossing the UAS:P450 line with the GAL4 driver line a qPCR test was used to confirm overexpression of target genes in the transgenic fly lines made here. *Cyp6cm1* was expressed 112 times more than in the parental line (Table 5.1). *Cyp6bq9* was expressed 191 times more and *Cyp337b3* was expressed 140 times more than the parental lines showing the successful overexpression on the target genes once activated by the GAL4/UAS system. See figure 5.10.

Fly Line	Gene	Biological set	Fold change
Cyp6cm1	*Cyp6cm1*	Offspring	112
		Parent	1
	Rpl32	Offspring	0.48
		Parent	1
	SdhA	Offspring	0.80
		Parent	1
Cyp337b3	*Cyp337b3*	Offspring	139
		Parent	1
	Rpl32	Offspring	0.63
		Parent	1
	SdhA	Offspring	0.72
		Parent	1
Cypbq9	*Cypbq9*	Offspring	190
		Parent	1
	Rpl32	Offspring	0.47
		Parent	1
	SdhA	Offspring	0.39
		Parent	1

Table 5.1 showing the relative quantity of P450 mRNA compared with housekeeping mRNA from *Rpl32* and *SdhA*. Here is also shown the offspring lines compared to parental lines.

Unfortunately, despite testing 5 different sets of primers for *Cyp6bq23*, no usable primers were found, and so it was not possible to test this genes expression. Due to the lack of a positive control to confirm primer activity it cannot be ruled out either that this gene was overexpressed or that it was not

present in this crossed line. Plasmid inserts were confirmed upon arrival of the transgenic lines post embryo injections (supplementary figure S.1) but there is no confirmation that *Cyp6bq23* was still present during bioassays.

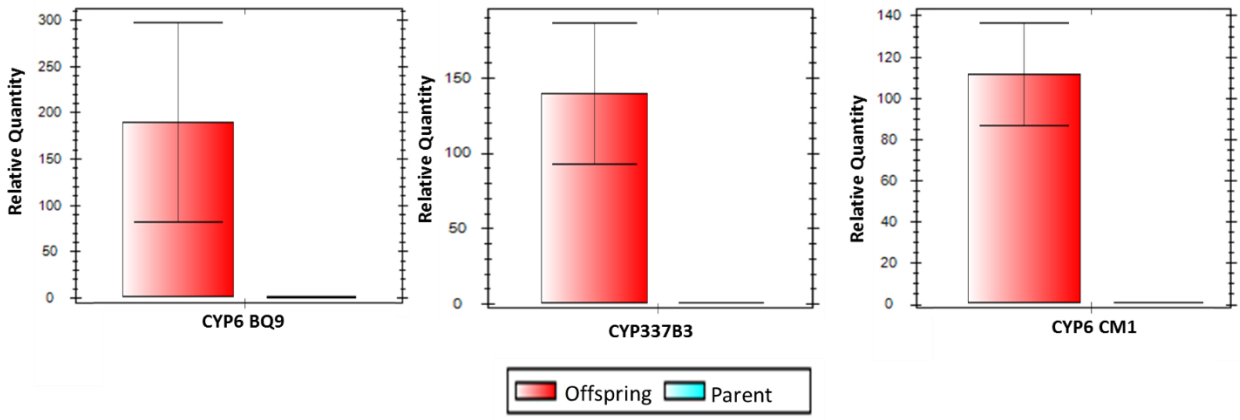


Figure 5.10 showing the relative quantity of P450 genes in offspring lines compared to parental lines.

5.5.2 Bioassay results

Bioassays were run on transgenic fly lines using the pesticides that selected for resistance in the original pest species. The hypothesis was that the transformed fly lines would now be resistant. Resistance ratios in comparison to the control *Drosophila* line (Table 5.2) show that lines expressing *Cyp6cm1* and *Cyp6bq9* showed no significant increase or decrease in resistance while *Cyp6bq23* and *Cyp337b3* showed reduced resistance to the intended compound.

Transgenic line	Resistance ratio	Lower 95% CI	Upper 95% CI
Cyp6cm1	0.92	0.86	0.99
Cyp6bq9	1.29	1.21	1.38
Cyp6bq23	0.69	0.61	0.77
Cyp337b3	0.64	0.58	0.71

Table 5.2 Shows the resistance ratios to control line and ±95% confidence intervals for each transgenic line versus the control line for the respective pesticide the gene showed resistance to in the original pest species.

5.6 Discussion

5.6.1 Bioassays and expression

Surprisingly, adult flies expressing *Cyp6cm1* of *B. tabaci* showed no significant resistance to imidacloprid compared to control fly lines in insecticide bioassays (Table 6.1) [231,234,235]. Work done by Karunker, *et al.*, 2008, confirmed that *Cyp6cm1* confers resistance to imidacloprid in *B. tabaci* strains when overexpressed 5 to 17-fold more than the susceptible strains so the transgenic *Drosophila* line expressing this P450, 112-fold more, was expected to show resistance [231]. In previous work by Daborn, *et al.*, 2012, transgenic *D. melanogaster* containing this gene did confer 2.3 to 2.6-fold resistance to imidacloprid but they carried out larval bioassays instead of adult bioassays [236]. There are multiple possibilities why our line may not have conferred the expected resistance. We used codon optimised genes unlike Daborn*, et al*., 2012, and while it is expected that this would improve upon the translation efficiency of the gene the conferral of resistance was not seen [236]. There is also a possibility that the lack of resistance may be due to the adult bioassay used in this research versus the larval bioassay used by Daborn, *et al*., 2012, we can confirm this by the extended results on this line published in Mcleman *et al*., 2020, showing conferred resistance to imidacloprid in the larval bioassay. Another possibility is an effect of epistasis from the different genetic background of lines 51C and 86Fb used by Daborn, *et al.*, 2012, as their *Drosophila* host line for their transgene [236].

The over-expression of the *Cyp6bq9* gene was linked to deltamethrin resistance in *T. castaneum* by Zhu, *et al.* 2010 [215]. The assay showed very little increase in resistance to deltamethrin when compared to the control fly line. In the

deltamethrin resistant *T. castaneum* strain, QCT279, *Cyp6bq9* is expressed 200-fold more than in the susceptible strain and despite variation our transgenic *Drosophila* show similar levels of overexpression of this gene, therefore, this is unlikely to be the cause of the lack of resistance in our transgenic line [215]. Expression in the central nervous system is sufficient to confer deltamethrin resistance to transgenic *Drosophila* in a study by Zhu, *et al.*, 2010, meaning that the whole body expression used in the GAL4/UAS system used for these transgenic lines should be sufficient to see resistance [215]. Zhu, *et al.*, 2010, created transgenic *Drosophila*, without codon optimisation and carried out adult bioassays and confirmed resistance of their *Drosophila* transgenic line to deltamethrin [215]. Similarly to *Cyp6cm1*, it is possible the strain made in this chapter had epistatic interference from the background genetics of the transgenic host line.

Cyp6bq23 also confers resistance to deltamethrin in *Meligethes* but importantly for this transgenic line it was not possible to confirm overexpression of this gene in the transgenic line due to lack of working primers. Not having a positive control to test primers on meant it was not possible to confirm whether these results were due to the gene not being overexpressed in the offspring lines or simply the primers not working. It is, therefore, a possibility that the gene is not present or activated in this line. *Cyp6bq23* is correlated with resistance to pyrethroids, particularly deltamethrin, in *M. aeneus*, where the level of overexpression is up to 900-fold higher than in susceptible populations [233]. It is possible, given the levels of overexpression of the other P450 genes in this study that, if this transgene is present in our *Drosophila* line, it is not expressed highly enough to confer resistance. Samantsidis, *et al.*, 2020, made a transgenic *D. melanogaster* containing *Cyp6bq23* using the GAL4/UAS and

codon optimisation and showed that this conferred strong resistance to deltamethrin [237]. They used the same transgene host line as in this study but instead of using sugar agar with evaporated insecticide of the surface of the agar they coated the whole of the scintillation vial and kept cotton wool plugs moist with 5% sucrose solution but this is unlikely to account for the difference between the results [237]. It is then another consideration that the resistance gene could have been dropped by mutation from this line as supported by the finding from Samantsidis, *et al.*, 2020, that their transgenic line came with a fitness cost.

Lastly *Cyp337b3* confers resistance to fenvalerate in *H. armigera* [232,233]*.* The resistance conferred by this gene does not seem consistent across all *H. armigera* strains. In Brazil *Cyp337b3* is very common in *H. armigera* and though levels of resistance to pyrethroids vary the species are described as pyrethroid tolerant and through phylogenetic analysis this gene is shown to share 99% identity with the Chinese and Pakistan allele [238]. The Chinese strain of *H. armigera* shows a correlation of fenvalerate resistance and high frequencies of *Cyp337b3v2* but that frequency of the gene does not correlate with resistance levels suggesting other P450 genes may be more important in conferring resistance to pyrethroids here[239]. In Australia *Cyp337b3v1* has been shown to confer strong resistance to fenvalerate and produce metabolites of the pesticide [225,226]. Literature does not currently show other transgenic *Drosophila* lines being made with this gene. As with *Cyp6bq23* we see reduced resistance to the fenvalerate suggesting a general cost to the fitness of the host when carrying this gene. Though a fitness cost is not yet noted for *Cyp337b3v1* there was a suggested cost to carrying *Cyp337b3v2* as once pesticide pressure is no longer present the gene frequency dropped significantly [239].

The Fly-Tox panel is an important and valuable tool with multiple applications. It can be used to assess cross-resistance profiles which is particularly useful when widespread resistance to an insecticide requires a new compound to control pest populations. This is of particular relevance to this thesis as it can determine whether or not a new compound should be perused in research and development. Early warning of pre-existing cross-resistance saves wasted time and resources on a compound that ultimately may not be able to control resistant pest populations.

This tool can also help to identify synergistic insecticide activity that may restore susceptibility of a resistant insect population to particular pesticides [240]. An example of this is piperonyl butoxide (PBO) which binds to the active site of some P450 enzymes but inhibition by PBO is not universal and the inhibitor does not act equally for different P450's neither is it the only insecticide synergist [240]. Transgenic *Drosophila* can help to understand metabolism of P450's both temporally, by expression of P450's in different tissues, structurally and in combination with other P450's by expressing multiple genes in a single transgenic line which was done in the extension of this work in the McLeman *et al.,* 2020, paper. A limitation of the Fly-Tox panel is the effect on P450 activity and efficacy in *Drosophila* due to differential need for endogenous co-factors such as P450 reductase and cytcochrome b5 [49,234].

Chapter 6:

Testing Transgenic *Drosophila* against the Novel Compounds.

6.1 Abstract

Finding novel pesticides can be an expensive and slow process. Here we show a demonstration of a sample from the Fly-Tox panel being used to screen novel pesticides compound 1 and compound 47 in comparison to imidacloprid and fipronil. Transgenic *D. melanogaster* containing either pest P450 *Cyp6er1* or bee P450 *Cyp9q3, Cyp9q4* or *Cyp9bu1* were selected to compare pesticidal activity against pest metabolic resistance versus bee metabolic resistance. A successful compound would be resistance-breaking against the pest P450 and unsuccessful against the bee P450's. Though no successful compounds were found here the outputs of the bioassays and cross resistance conferred by *Cyp6er1* to expected GABA antagonist compound 47 are discussed alongside the limitations of the screening tool.

6.2 Introduction

Pesticides are still considered a vital tool for pest control but rapid evolution of resistance to pesticides remains a global hurdle [241,242]. Rapid evolution of resistance in insect populations can happen due to short life histories and the heavy selection pressure caused by intensive pesticide use [242,243]. However, resistance arises far quicker if resistance genes are already present in a population [242,243]. For this reason when developing novel pesticides it is important to test them against current modes of resistance. Regulation on the development of novel pesticides now require testing on both pest and pollinator species which can be both costly and time consuming [234]. To determine likely activity of new compounds testing them on the Fly-Tox panel of *D. melanogaster* created by McLeman *et al.* 2020, is a quicker, more cost-

effective method. This panel contains *Drosophila* with P450 resistance genes from economically important pest species and pollinators alike.

The Brown plant hopper, *Nilaparvata lugens*, is an important crop pest of rice with neonicotinoid resistance conferred by the *Cyp6er1* gene [234,244,245]. Maintaining and testing *N. lugens* populations is costly, so prior to testing novel pesticides against the pest organism, bioassays completed against the *Cyp6er1* gene will indicate whether a novel compound shows promise. *Cyp6er1* was shown by McLeman *et al.*, 2020, to confer significant levels of resistance to imidacloprid and so the ability of a candidate pesticide to overcome resistance due to this gene can be used as an indicator of resistance breaking potential.

Bees provide essential pollinator services to wild plants and crops and their economic value is predicted to increase with human population growth [246]. Pesticide poisoning is a concern for conservation efforts for bee species and so it is important to test activity of new compounds against current bee resistance mechanisms to aid in population conservation of these important pollinators [246]. Social bee sensitivity to pesticides is moderated by the *Cyp9q* family of P450 genes which confer resistance to xenotoxins [247–249]. *Cyp9q3* is a P450 resistance gene in honeybees, *Apis mellifera*, that confers resistance to the neonicotinoid thiacloprid, but it does not confer resistance to imidacloprid [248–250]. The equivalent gene in the bumblebee, *Bombus terrestris, Cyp9q4* shows similar sequence to the honeybee resistance gene *Cyp9q3* and also confers resistance to thiacloprid but not imidacloprid [248]. The *Cyp9q* P450 family shows metabolic resistance across three groups of pesticides; the neonicotinoids, pyrethroids and organophosphates, making them an important group to consult when looking for novel pesticides that are bee safe [248]. Previous work has suggested that honeybees in particular are more sensitive to pesticides than

other insects but an analysis of toxicology data shows that genes like the *Cyp9q* family can provide protection for bees against potential pesticidal products [248]. It is, therefore, important to address this family of P450 genes when assessing novel pesticides. Although the *Cyp9q* family of P450 genes is important to social bee species this group is not present in most solitary bee species and so separate resistance genes need to be considered to cover this group [247,251].

The solitary (in contrast to the more 'colonial' *Apis* sp.) bees are also a very important group to assess the safety of new pesticides as more than 85% of bee species are solitary, not social [252]. The solitary bee *Osmia bicornis* is a common and economically important pollinator species of Central Europe [247]. *Cyp9bu1* was identified as the most effective neonicotinoid metabolising gene in this species and was shown to share an ancestor with the *Cyp9q* family of resistance genes in social bees [247,251]. Osmia bees bearing this gene also show not only resistance to thiacloprid but also some ability to metabolise imidacloprid but the transgenic *Drosophila* line containing the resistance gene *Cyp9bu1* only conferred resistance to thiacloprid [251].

Novel pesticides need to be competitive against current products. Together fipronil and neonicotinoids account for a third of the pesticide market with imidacloprid as the most popular insecticide in 2008 and so they are important competitors when developing new products [124]. Imidacloprid is a commonly used pesticide which is high effectivity against sucking and some biting pests [99,253]. When it was first produced in 1991 it showed a resistance breaking, novel mode of action but now resistance has arisen in many pest species [7,99,124,253,254]. The novel compound 1, identified as having potential pesticidal properties in chapter 2 in this thesis, is believed to act in a similar way to imidacloprid by targeting the nicotinic acetylcholine receptor (NAChR) [124]. Fipronil will be the

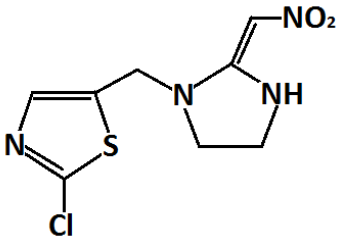
second control as it is expected to have a similar mode of action to the second novel compound 47, identified as having potential pesticidal properties in chapter 3 of this thesis. Fipronil reached the market in 1993 and works at low doses on the GABA gated chloride channel but pests have also developed resistance to fipronil [124,255,256]. The novel compounds will be directly compared to their market competitors to assess the potential of the novel compounds.

6.3 Methods

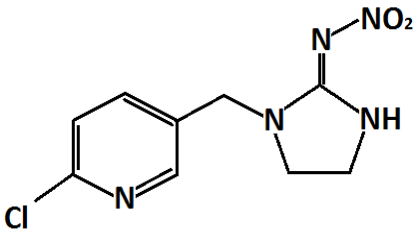
6.3.1 Compounds

Synthetic compounds were created by Alistair Miller at Darr House M.I.. Compound 1 was derived from imidacloprid and is expected to target the nAChR. Compound 47 was designed with similarities to fipronil and is expected to act on the GABA receptor. These compounds will be tested against their market competitor's imidacloprid and fipronil.

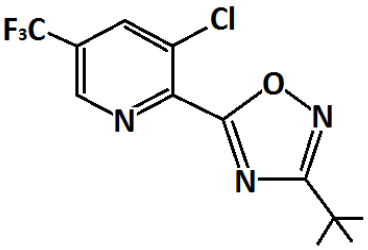
Compound 1



Imidacloprid



Compound 47



Fipronil

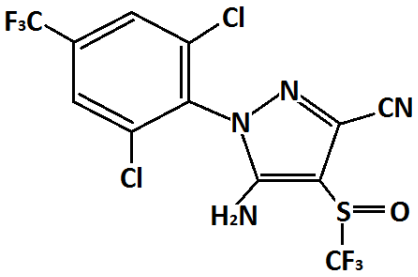


Figure 6.1 The structures of novel compound 1 and compound 47 compared to their commercial competitors.

6.3.2 Fly Strains

A selection of fly strains from the Mcleman *et al.* Fly-Tox paper [234] were chosen to cover a range of pest and pollinator resistance genes. *Cyp6er1* was chosen due to its economic importance in the brown plant hopper pest, *N. lugens*, and the high resistance shown to imidacloprid. This makes it a good example to test the use of the Fly-Tox panel as a screening tool against novel compounds and its resistance to imidacloprid makes it ideal to test for possible cross resistance to other compounds targeting the nAChR.

To cover important pollinator resistance genes from social and solitary bees a gene was chosen from *A. mellifera*, *B. terristrus* and *O. bicornus. Cyp9bq3* and *Cyp9q4* are from the important *Cyp9q* subfamily in social bees which confer

resistance to xenotoxins but this family is not found in most solitary bee species and so *Cyp9bu1* was added to this subset of Fly-Tox. By testing these four genes we can show the use of the Fly-Tox panel and the value of these two novel compounds against economically important pests and social and solitary bee species.

6.3.3 D. melanogaster crosses

Female virgin flies were collected over multiple days every 2-3 hours from the activator stock, strain 20 + pUAS-attP containing *Cyp12/SM6a*. Fifteen virgin females were then crossed with 10 transgenic males in a large fly bottle containing 25ml Nutrifly food. After 1 week the adults were transferred into new large bottles with 25ml Nutrifly, producing two batches of activated P450 offspring on which to run follow up bioassays. Offspring were collected 2 weeks after putting in the adults. Active offspring were identified by straight wings and red eyes.

Control activated lines were also made by crossing virgin females with males from strain 20 + pUAS-attP containing *Cyp35/SM6a*. Once these offspring emerged the active individuals containing the same genetic cross without the P450 gene were identified with straight wings and white eyes. These controls provide a baseline resistance for *Drosophila* having undergone this genetic manipulation, resistance then caused specifically by the P450 gene can be easily identified.

6.3.4 *D. melanogaster* bioassays

The bioassays for *Drosophila* were carried out in plastic scintillation vials. Each vial contained 2 ml sugar agar at the base consisting of 2 % agar and 1.2 %

granulated sugar. The agar was pipetted into each vial and allowed to set for a minimum of 4 hours. Pesticide dilutions were made with acetone and were added in 200 µl volume to each vial and allowed to evaporate onto the sugar agar surface overnight. Fifteen flies were then added per vial and each vial was stopped with a cotton wool plug.

After 24, 48 and 72 h scores were taken for numbers of dead and alive individuals within each vial. The scores were used to narrow down the LD_{50} for each compound and the experiment was repeated with a narrower range until a statistically significant LD_{50} value was obtained. Statistical analysis was run on data from 72 hrs as this time point showed most consistency.

6.3.5 Statistical analysis

Probit analysis was run on each dataset to determine the LD_{50} value ±95% confidence interval for each compound using POLO Plus [107]. Compounds showing a t-ratio of lower than 1.96 for the slope were removed from subsequent analysis as this showed the treatment as having no effect at the tested concentrations [109].

6.4 Results

6.4.1 Activity of novel compounds vs. known compounds

Novel compound 1 and compound 47 showed increased activity when compared to imidacloprid across all tested transgenic fly lines tested here (Table 1). This data shows that these novel compounds are indeed competitive against this commercial product.

Compound 1 in particular shows extremely high toxicity working at anywhere from 55 to 144 times lower concentration than the commercial product imidacloprid (Table 1). It is important to note that this high toxicity is also seen for the bee P450 genes suggesting this product will not be bee safe, an important quality of any novel pesticidal compound.

Compound 47, with a structure and presumed mode of action related to that of fipronil, also shows increased toxicity to imidacloprid. It is 8 times more toxic to the *N. lugens* P450 *Cyp6er1* than imidacloprid but this increase in toxicity is also seen in *Cyp9q3* and *Cyp9bu1* suggesting this compound is also bee toxic. The *B. terrestris* gene *Cyp9q4* suggests high toxic effect of this compound but to get a clear idea of the potential use of these novel compounds it is important to look at the comparative resistance conferred by each P450 gene.

Strain	Compound	LD50 ug/vial	95%CI lower	95%CI upper	Potency compared to Imidacloprid	Potency compared to Fipronil
Control	Imidacloprid	8.28	7.38	9.11	-	251 x less
	Compound 1	0.15	0.14	0.17	55 x more	5 x less
	Fipronil	0.033	0.028	0.036	251 x more	-
	Compound 47	2.15	2.00	2.31	4 x more	65 x less
Cyp6er1	Imidacloprid	30.37	25.07	35.94	-	1085 x less
	Compound 1	0.29	0.23	0.36	105 x more	10 x less
	Fipronil	0.028	0.023	0.032	1085 x more	-
	Compound 47	3.67	3.31	4.56	8 x more	131 x less
Cyp9q3	Imidacloprid	21.78	19.21	25.37	-	1089 x less
	Compound 1	0.19	0.16	0.22	115 x more	10 x less
	Fipronil	0.02	0.016	0.023	1089 x more	-
	Compound 47	3.31	2.97	3.64	7 x more	166 x less
Cyp9q4	Imidacloprid	20.22	16.67	25.29	-	722 x less
	Compound 1	0.14	0.13	0.17	144 x more	5 x less
	Fipronil	0.028	0.024	0.031	722 x more	-
	Compound 47	1.42	1.30	1.52	14 x more	51 x less
Cyp9bu1	Imidacloprid	11.55	10.20	12.91	-	550 x less
	Compound 1	0.14	0.13	0.15	83 x more	7 x less
	Fipronil	0.021	0.019	0.023	550 x more	-
	Compound 47	2.50	2.30	2.73	5 x more	119 x less

Table 6.1 LD_{50} values including ±95% confidence intervals showing the competitive activity of each compound when compared to imidacloprid and fipronil for each transgenic line.

6.4.2 Resistance shown by the Fly-Tox panel

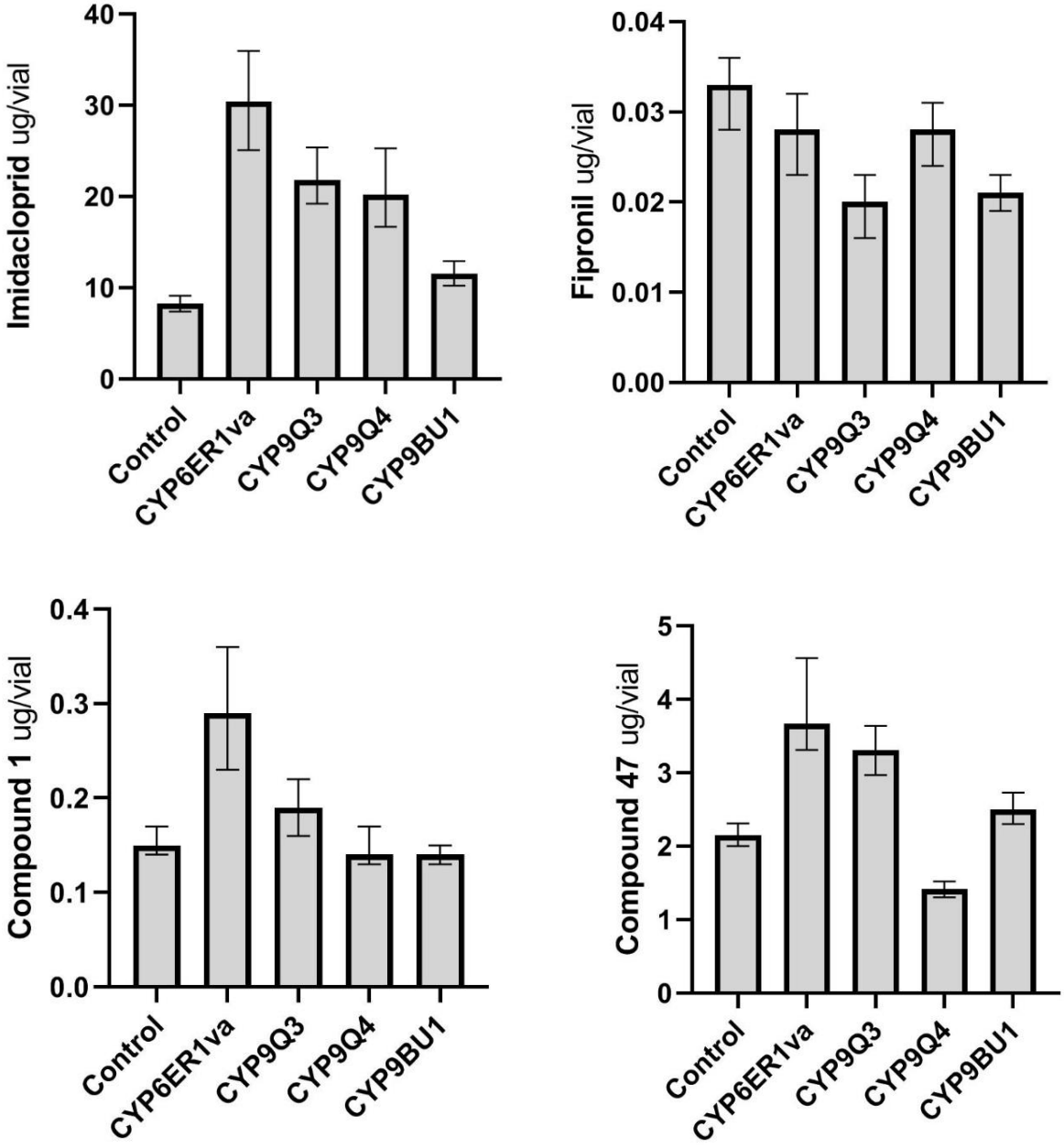


Figure 6.2 shows LD_{50} values for individual P450 genes to imidacloprid (top left), fipronil (top right), compound 1 (bottom left) and novel compound 47 (bottom right). Error bars show the ±95% confidence intervals.

Testing the range of transgenic lines against each compound found that all transgenic strains showed resistance to the commercial product imidacloprid. The pest resistance P450 gene *Cyp6er1* conferred 3.67-fold increased resistance and the social bee P450 genes *Cyp9q3* and *Cyp9q4* conferred

Compound	**Strain**	**LD50 ug/vial**	**95%CI lower**	**95%CI upper**	**Resistance ratio to Control fly strain**
Imidacloprid	Control	8.28	7.38	9.11	-
	Cyp6er1	30.37	25.07	35.94	3.67 fold
	Cyp9q3	21.78	19.21	25.37	2.63 fold
	Cyp9q4	20.22	16.67	25.29	2.44 fold
	Cyp9bu1	11.55	10.20	12.91	1.39 fold
Compound 1	Control	0.15	0.14	0.17	-
	Cyp6er1	0.29	0.23	0.36	1.94
	Cyp9q3	0.19	0.16	0.22	1.27
	Cyp9q4	0.14	0.13	0.17	0.94
	Cyp9bu1	0.14	0.13	0.15	0.94
Fipronil	Control	0.033	0.028	0.036	-
	Cyp6er1	0.028	0.023	0.032	0.85
	Cyp9q3	0.02	0.016	0.023	0.61
	Cyp9q4	0.028	0.024	0.031	0.85
	Cyp9bu1	0.021	0.019	0.023	0.64
Compound 47	Control	2.15	2.00	2.31	-
	Cyp6er1	3.67	3.31	4.56	1.71
	Cyp9q3	3.31	2.97	3.64	1.54
	Cyp9q4	1.42	1.30	1.52	0.66
	Cyp9bu1	2.50	2.30	2.73	1.16

Table 6.2 LD_{50} values including ±95% confidence intervals showing the relative resistance of each transgenic fly line to each pesticidal compound when compared to a control fly line.

2.63 and 2.44-fold increase respectively in imidacloprid resistance when compared to the control strain. The solitary bee gene *Cyp9bu1* on the other hand only showed a 1.39 resistance ratio showing minimal resistance to imidacloprid (Table 2, Figure 2). This data shows that imidacloprid is more detrimental to bees than the pest species.

This trend is followed by the results of bioassays against novel compound 1. Flies bearing the bee P450 genes show no significant resistance to this compound but flies expressing *Cyp6er1* do show close to 2-fold resistance (Table 2, figure 2). Finally, pre-existing resistance to novel compound 47 is conferred by *Cyp6er1* (1.71-fold), *Cyp9q3* (1.54-fold) and *Cyp9bu1* (1.16-fold) (Table 2). A particularly negative result for the trial of this compound shows that the overexpression of *Cyp9q4* reduced tolerance to this compound by almost half suggesting it would be particularly toxic to *B. terrestris.*

Table 2 and figure 2 shows *Cyp6er1* conferring consistently higher resistance to all compounds when compared to the three bee P450 genes. This shows that the novel compounds tested here would have a detrimental effects on pollinator species at lower does than those that would affect the target pest species *N. lugens*.

6.5 Discussion

Resistance to pesticides is a rising global problem as is the need to protect crop products, therefore, there is a current need for the discovery of novel resistance breaking pesticides [234,257]. It is also important that these novel compounds are competitive against the current commercial products. This work shows that the novel compounds tested here were competitive against imidacloprid, compound

1 being active at over 100-fold lower concentrations. Despite this the resistance profiles of these novel compounds show they would be unlikely to be commercial products.

Compound 1 results show just under 2-fold resistance conferred by the *N. lugens* resistance gene *Cyp6er1*. This suggests resistance to this compound could arise quickly in the pest as the resistance mechanism is already present. This result is not unexpected due to the fact that this gene confers neonicotinoid resistance as was specifically confirmed in this transgenic fly line to imidacloprid by McLeman *et al.*, 2020 [234,244,245]. Our expectations of compound 1 are that it acts on this receptor; its structure having been derived from imidacloprid. The structural similarities between these two compounds can be seen in figure 6.1. Compound 1 possesses shared characteristics of known neonicotinoids in the binding group and tail group of the compounds. A nitrogen atom changes to carbon in compound 1 changing imidacloprid, a N-Nitroguanidine class of neonicotinoid, to compound 1, a member of the nitromethylene class which can be considered an open-ring variant of nitenpyram. The change in cyclic ring in the compound 1 significantly increases toxicity of (2-chloro-1,3-thiazol-5-yl)methyl compared to (6-chloropyridin-3-yl)methyl in the absence of the nitrogen. The higher toxicity of (2-chloro-1,3-thiazol-5-yl)methyl group in the bioassays supports the early research into neonicotinoids showing that the smaller the ring size the more toxic the compound [94].

Including the already present resistance shown by the *N. lugens* P450 gene, compound 1 shows additional problematic results in that it is resistance breaking for all three bee metabolic resistance genes. Commercial products must go through screening to show they do not cause damage to bee species

otherwise a product is not marketable. Because it overcomes the resistance conferred by the three bee P450s, this product seems unlikely to pass this test.

Compound 47 was also assessed against this small sample of the Fly-Tox panel. Though it was 4 times more active than imidacloprid it was shown to be 65 times less effective than its target specific competitor fipronil.

Phenylpyrazoles are potent insecticides acting at the GABA gated chloride channel at particularly low concentrations as seen here in the 251-fold difference in LD_{50} toxicity between imidacloprid and fipronil [258]. It would be expected that compound 47 would act at more similar concentrations to those of fipronil and yet between the two novel compounds tested here its activity is much lower than that of compound 1. Despite this initial observation the importance of this screening tool is to inform the user on potential resistance breaking activity against pests and bees. This is most easily observed via figure 6.2

The pest resistance gene *Cyp6er1* confers significant resistance to compound 47 compared to our control fly line suggesting there is some cross resistance conferred by this gene to this novel pesticide. This was unexpected due to the activity of *Cyp6er1* against neonicotinoids not this class of insecticide [234,244]. The *Cyp6er1* P450 gene was taken from *N. lugens* which has been shown to display resistance to phenylpyrazoles [244,259,260]. In particular, resistance to fipronil has been identified from *N. lugens* but the resistance mechanism was as yet unidentified by Zhao *et al.*, 2011 [244,259,260].

The target site mutation $A^{301}S$ RDL was found in 2017 to confer strong resistance to ethiprole (resistance ratio to control fly strain >4300) but only limited resistance was seen against fipronil (resistance ratio 6.9) [43]. The addition

of a secondary mutation R300Q, only present alongside the *Rdl* mutation Ala^{302}, increases this resistance by 100 times [148]. Underlying mechanisms were still deemed unclear for the synergistic effects of multiple mutation effects by Li *et al.*, 2020, so their study demonstrates the increasing resistance to fipronil from wild type to Ala^{302} to R300Q to strongest resistance found for double mutations but all of this research focusses on target site resistance [261].

Metabolic resistance of *Cyp6er1* only focusses on the effects on neonicotinoid compounds but a study by Pang *et al.*, 2016, looks at the functional analysis of the gene and how it works [259]. They looked at the catalytic function of this gene using molecular modelling. Due to the size of this protein, like many P450's, it was too large to successfully use X-ray crystallography for this analysis [259]. By comparing functional shared amino acids with other P450's they made 20 models of *Cyp6er1* noting shared structures to elucidate possible binding sites. Though this paper focusses on molecular binding of imidacloprid to *Cyp6er1* it would be interesting to analyse the structures they modelled for this P450 with insight into potential binding sites for other molecules. Resistance to compound 47 found in the transgenic drosophila containing *Cyp6er1* suggests that this P450 is able to metabolise this compound. The fact that compound 47 and imidacloprid differ markedly in their solubility properties (the former is lipophilic, the latter is hydrophilic), suggests that binding sites would certainly be different between the two molecules given that imidacloprid binds at a hydrophobic interface stabilised by hydrogen bonding between imidacloprid and *Cyp6er1* [259].

P450's *Cyp9q3* and *Cyp9bu1*, both derived from hymenopteran pollinators, also show some metabolic activity against compound 47. A number of members of the *Cyp9* family of P450's are known to provide protection against neonicotinoids. *Cyp9q3* from *A. mellifera* metabolises compounds from multiple

insecticidal classes, the neonicotinoids (thiacloprid), the pyrethroids (tau-fluvalinate) and the organophosphates (coumaphos) [248,262]. *Cyp9bu1* from solitary bee species *O. bicornis* is also well known for its strong resistance to neonicotinoids but also shows activity on flupyradifurone, a butenolide also active on the NaChR [263]. *Cyp9bu1* is labelled a generalist detoxification enzyme by Beadle, 2018, [251] and so the resistance conferred by these two P450 genes is less unexpected and shows promise for compound 47 as being bee safe were it to break pest resistance mechanisms. It is also important to note the limitations of this screening tool which specifically applies here. Although *Cyp9q4* was not seen to confer resistance to compound 47 on the corresponding Fly-Tox line, within the whole organism *B. terrestris* may display resistance to this compound via other mechanisms.

The purpose of this screening tool is a guide. Resistance of bee P450's signals a compound that may potentially be bee safe, for lethal effects, but it does not rule out other mechanisms of resistance in the whole organism or an interaction of resistance mechanisms. It is possible to address this issue in the Fly-Tox screening panel by engineering *Drosophila* lines to express multiple P450s. An example of this is where the three honeybee P450s *Cyp9q1, Cyp9q2* and *Cyp9q3* are co-expressed in a transgenic fly line described by McLeman *et al*. (2020) [234]. Although this screening tool may indicate resistance breaking compounds for certain genes, results are subject to differ in the whole organism which is why this tool cannot replace the later testing required on original pest and pollinator species. Overall the results here do not find resistance breaking compounds but do show a successful demonstration of the use of the Fly-Tox panel.

Chapter 7:

Discussion: the way forward for pesticide development?

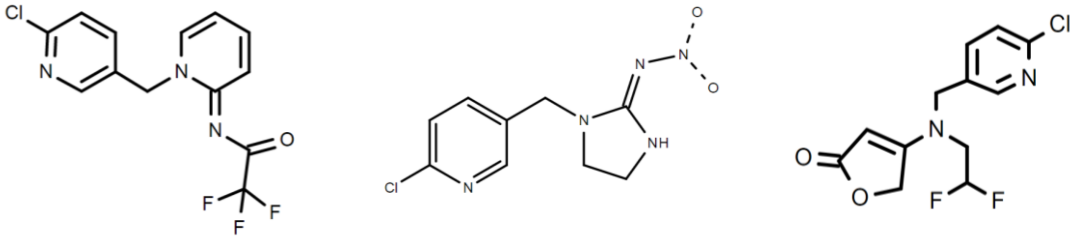
7.1 Novel insecticides

The global business of crop protection including insecticides, herbicides, fungicides and biotechnology was valued at $56.7 billion in 2014 by Maienfisch and Stevenson, 2015 [28]. 2013 saw an annual research and development cost of just under $7 billion between the top 10 agribusiness companies who control 90% of global annual crop protection product sales[28]. Bringing a new product to market costs an average of $85 million to research, $146 million to develop and $25 million to register; a total of $256 million per product [28]. A time scale for discovery to commercialisation is also slowly increasing to approximately 10 years as of 2005 likely due to increased regulations for new products [264]. This is a huge cost with high risks since in the past decade approximately only 34 new insecticides were brought to market [27,30]. An additional risk when developing novel pesticides is the likelihood that they will work on already identified target sites increasing the chances of cross resistance to a novel product [27]. Insecticide resistance is a widespread problem with just under 600 insecticide resistant species in 2014 causing dramatic impacts on global economics, food security and ecosystem effects [1–4]. The aims of this thesis were to make a contribution to the reduction of time and cost needed to identify and develop novel resistance breaking pesticides starting with the screening of potential pesticidal compounds.

In chapter 2 I began to look at novel synthetic compounds targeting the nicotinic acetyl choline receptor (nAChR). The compounds chosen for screening in this chapter had shown promise of activity previously from collaborators with Darr House M.I. but had been released back to Darr House M.I. enabling further work on the samples. A randomised screening of these compounds was used to look for activity against metabolic resistance in *Drosophila* and metabolic and

target site resistance mechanisms in *Myzus*. According to a study by Umetsu and Shirai, 2020, insecticide development trends are moving towards nicotinic and diamide compounds [30]. For example a novel insecticide flupyrimin (figure 7.1) registered in 2019, Japan, is claimed to target the nAChR with strong resistance breaking activity but pollinator safe [30,114,265].

Flupyradifurone is another recent compound working on the nAChR and breaks the resistance conferred by *Cyp6cm1* [266,267]. These compounds have been shown to bind in a different way than other neonicotinoids making them of particular interest, they have been classed as butenolides [30,114,266,267].



Flupyrimin Imidacloprid Flupyradifurone

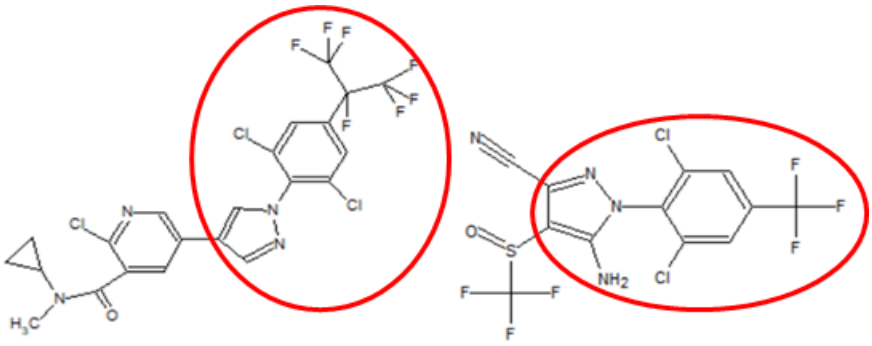
Figure 7.1 Comparative structures of novel resistance breaking compounds flupyrimin, flupyradifurone and imidacloprid.

Flupyrimin (FLP) and flupyradifurone (FPF) conserves the chloropyridine tail group, unlike our results and previous work which suggests the theory that the smaller this cyclic ring the more toxic the compound [94]. This is not what was found for FLP which had activity of 5 to 13-fold higher than imidacloprid against *Nilaparvata* and in fact showed more similar activity to that of fipronil [114]. The presence of nitrogen on the offshoot from the head pyridine group in FLP, N-trifluoroacetyl, also does not track with our findings of higher toxicity of our novel compounds when this nitrogen is absent. The differences in activity found for our novel compounds versus FLP may be due to the binding site of the

compounds. FLP was shown to bind to the nAChR at an overlapping but separate site to imidacloprid [114].

Despite the success of FLP, FPF and a handful of other new neonicotinoids there are often similar appendages to the structures suggesting there is little room for development of structurally diverse compounds in the classes of pesticides targeting the nAChR. It is important to note that the toxicity of neonicotinoids to non-target species like pollinators has led to bans in the EU in 2018 for key compounds such as imidacloprid [30,70]. Recent research on FPF, perhaps unsurprisingly due to its structural similarities to the neonicotinoids, despite being named a bee safe compound has been shown to have lethal effects against bee species at field doses with particular vulnerability seen by *A. mellifera* [268,269].It was as a result of this new evidence that market opinion on nAChR-targeting candidate compounds began to change during my studentship, and the decision was taken to discontinue further discovery and development work on the Darr House compounds in this area of chemistry. Work continued, however, on screening of synthetic compounds designed to target the GABA receptor as described in chapter 3.

Despite the opinion of Umetsu and Shirai, 2020, that combinations of neonicotinoids and diamides are the focus of novel insecticides, it has emerged that there is currently emerging interest in combining the chemistries of diamides and fipronil-like compounds to make novel GABA targeting compounds [30,270]. Nicofluprole developed by Bayer in 2020 has the structural combination of fipronil and traditional diamides as can be seen in figure 7.2. It is a highly lipophilic compound and larger than most insecticides [271]. Though the patent claims insecticidal activity no papers currently discuss the resistance breaking capabilities of this compound.



Nicofluprole, 2020. Fipronil

Figure 7.2 showing structurally similar groups of new compound nicofluprole to fipronil.

The GABA targeting compounds were again chosen at random by Darr House M.I. for screening. An advantage for these compounds is that structures working on the GABA receptor are much more structurally diverse than agonists of the nAChR, increasing the chances of finding a novel compound that binds to this target. Ironically despite this very few active compounds at this site break current resistance mechanisms unlike novel compounds found for the nAChR. The RDL target site resistance is key to overcome for novel compounds targeting the GABA receptor.

Target site resistance frequently comes with associated costs as these mutations often affect protein function which is usually highly conserved and this assumption of cost is relied upon for the loss of target site resistance over time [148,272]. The study by Zhang *et al.*, 2016, noted a co-mutation of Ala^{301} increasing the resistance to fipronil, R300Q, but this gene has never been found alone in populations due to the high fitness cost. It is suggested the Ala^{301} compensates for the deleterious effects of R300Q [148]. The point mutation at Alanine [301] causing *Rdl* resistance has arisen in multiple pest species from various orders of insects and is highly conserved [122,273,274].

Zhang *et al.*, 2016, found *Rdl* resistance conferred by replacements of Ala^{301} with either Serine or Glycine is associated with fipronil resistance to approximately 230-fold when subjecting insects to fipronil selection [148]. In the present work *Rdl Drosophila* were not subjected to selection and an 11-fold resistance to fipronil was already seen upon initial exposure. By contrast, working with *N. lugens*, Zhang *et al.*, 2016, found a resistance ratio of <5 after 72 hours which would be the comparable time scale for our results [148]. Their research also indicated the important rise of frequency of the Ala^{301} mutation over generational time when subject to fipronil selection. Our results indicate a faster rise of resistance to fipronil in *D. melanogaster* than *N. lugens* due to the already higher resistance recorded. Our novel compounds, however, showed no activity against *Rdl* resistance. An inability to overcome this mechanism of resistance means these compounds will not be effective against most wild pest populations.

Regardless of this it is still important to analyse our compounds in context of metabolic resistance mechanisms. Our metabolic resistant fly strain, Hikone-R, in which *Cyp6g1* is overexpressed, originally conferred resistance to DDT [135]. DDT was considered a breakthrough in pest control, used widely after World War II the chemical showed promise in tackling problems like malaria but DDT also caused long lasting negative environmental effects highlighted by Rachel Carson's book Silent Spring [31,275,276]. One of the remaining problems left by the extensive use of DDT is that of metabolic pesticide resistance particularly P450 gene *Cyp6g1* which confers cross resistance to a wide range of insecticides [147].

Our results show that compound 36, the most novel structure, cannot break the resistance conferred by overexpression of *Cyp6g1* in Hikone-R. Compounds 30, 39 and 40 show 1.5 to 2.3-fold increased resistance conferred by Hikone-R

versus Canton-S which although relatively low would, like *Rdl* resistance, need to be monitored in the case of selection pressure over time. Of particular interest is compound 47, Hikone-R mechanisms confer <1.5-fold resistance making this compound closest to breaking metabolic resistance. Again it would need to be monitored in the context of selection pressure over time but due to the lack of activity against *Rdl Drosophila* this line of investigation was not pursued.

When looking at novel insecticidal products and their target sites there is a trade-off in compound qualities. For example the GABA compounds are lipophilic whereas the nAChR compounds are hydrophilic, each come with pros and cons. Hydrophilic compounds must be available inside the plant to be eaten by the insect, whereas lipophilic compounds can display contact toxicity [277]. This means lipophilic compounds are faster acting than hydrophilic compounds. But hydrophilic compounds that prove soluble in water are useful due to the preferred method of spraying pesticides for treating crops [271].Neonicotinoids are highly water soluble and this type of compound has good uptake through plant roots and translocation via the xylem but they do not cross the membrane barriers easily like the lipophilic compounds [142,266]. A notable disadvantage to lipophilic compounds is the potential for bioaccumulation in food chains; a property that was responsible for the environmentally damaging effects of DDT [278,279]. These qualities deal with important trade-offs contributing to the bioavailability of the pesticide; the uptake and translocation of compounds into and through the plant, and translaminar activity through the leaf [142,143,267,277]. Key to uptake of pesticides by plants is being able to pass through plant cell cuticles which is enabled by being lipophilic [143]. This quality also increases translaminar activity, meaning that sucking pests living on the underside of

leaves, such as aphids, still get affected by chemical sprays on the plant leaf upper surface by moving through the leaf [267]. This is demonstrated by the novel FPF compound [267].

When considering bioavailability of compounds to pests it is vital to take into account their feeding sites. Aphids and whitefly behaviour demonstrates vascular feeding in the phloem whereas mites and thrips feed in the mesophyll, within cells [277,280]. This means these pests will only be subject to the concentration of pesticides that are present in these positions in the plant. The $\log K_{ow}$ value can be used to determine lipophilicity and polarity of compounds which helps to determine the areas of a plant a compound may travel through or get 'trapped' in [281]. A positive value means it is lipophilic, the higher the value the more lipophilic inversely the lower and negative $\log K_{ow}$ often relates to more polar compounds [281].

Polar, hydrophilic compounds as previously discussed tend to be less well suited to be taken in by plant cells and persist mostly in the apoplast making them more suitable for control of aphids and whitefly but there is then the trade-off for translaminar activity which is more successful by lipophilic compounds [277,280,282]. Lipophilic compounds pass easily through cell walls into the mesophyll layer where they can successfully control mites [277,280]. These lipophilic basic compounds are also subject to vacuole, or ion, trapping causing accumulation of compounds in the vacuole of a cell [283].

Another important note for novel insecticide design is that of particular structural similarities for active, or non-active compounds. For example alcohols and amines are usually avoided in all agrochemicals due to easy metabolisation via oxidisation which was a problem we hypothesised for compound 36 due to its

methyl group [271]. Acidic groups are also avoided as they rarely show insecticidal activity [271]. Aromatic rings on the other hand are considered valuable in insecticides due to their stability and the need for insecticides to remain active in field sites for days to weeks [271].

7.2 Methods of novel compound discovery

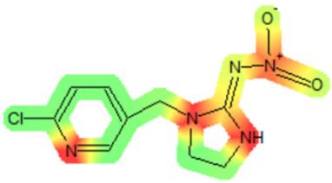
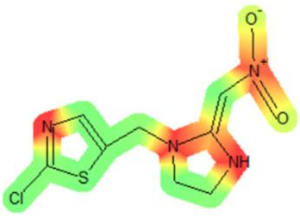
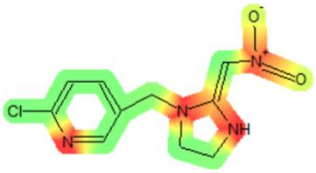
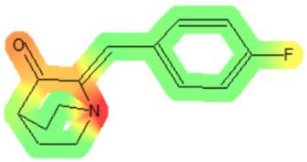
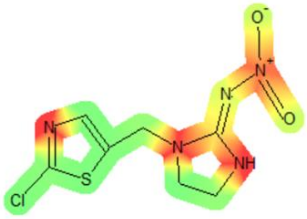
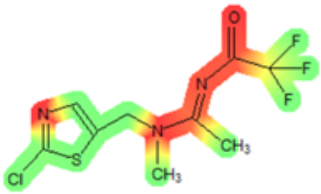
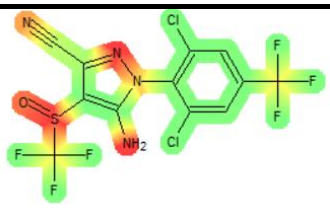
Discovery of novel compounds consists of a few methods; large scale screening, virtual design, chemistry based discovery, which relies on chemists to design new compounds around known molecular starting points, target based discovery, and breaking patents to use background work previously done [264,284,285]. Target based discovery has never been successful in the development of marketable agrochemicals despite remaining a core part in the developmental process of discovery [284,285]. Our compound testing worked off random screening of compounds expected to be effective due to shared chemical properties of known pesticides. This is a chemistry based design starting from a known active molecular starting point. There is a downside to this approach; mode of action (MoA) is assumed though multiple or different MoA's may be affected, and bioavailability in a natural setting is assumed [29]. Natural bioavailability would need to be tested further into the development process.

An important method for compound design is that of virtual screening. Virtual screening, whereby compounds are tested against a range of models and predictions, allows for compounds to be assessed virtually before having to even be synthesised and can inform on chemical composition, physiochemical properties such as lipophilicity, polarity, bonds and shapes of compounds [29].

This can also eliminate compounds already in the corporate repository [29]. There are filters that can be applied to large scale virtual screening to reduce the number of compounds with potential; Briggs rule of three that indicates loss of bioavailability should three or more of his limits be broken, Clarke-Delany's guide of 2 gives filters for physiochemical properties and Tice provides a guide for insecticidal activity [29]. After these filters are applied it is often necessary to choose a subset of the remaining compounds to remain within research and development (R&D) budget [29].

Tice discusses the important screening filters and the differing requirements between herbicides and insecticides by filling in parameters for known commercially active compounds[271]. Focussing on insecticidal qualities Tice looks into molecular mass, commonly in the range of 300-400, when >500 it was noted previously, by Lipinski's rule of 5 for pharmaceutical drug screening, that this larger molecule significantly reduces membrane permeability [271,286]. $LogK_{ow}$ values for pesticides are often more than 3 which is above the expected balance for hydrophilic/lipophilic balance of -0.5 to 3 for membrane crossing but these advantages have already been discussed explaining this trend for lipophilic compounds that get trapped in membranes [271]. Hydrogen bond donors and acceptors are also important with 73% of insecticides with no hydrogen bond donors at all and low values for remaining compound donors and acceptors [271]. This affects solubility, with high values for either of these characteristics compounds tend to be less soluble, a clear disadvantage for insecticidal spray application requirements [271]. Finally the number of rotational bonds are considered important in biological activity with highly rotatable compounds often showing poor activity [271].

Comparative scores for fipronil and imidacloprid and the novel compounds showing any activity are shown in table 7.1 using the parameters from Tice's paper and Lipinski's work on pharmaceutical compounds. Tice notes the comparative novelty of fipronil and imidacloprid at the time of his study and the higher than normal H acceptor bonds and molecular weight compared to the majority of pesticides [271].

Compound	Molecular weight	LogP	H donor	H acceptor	Rotational bonds	Structure and lipophilicity
Imidacloprid	255.7	0.38	1	7	3	
Compound 1	260.7	-0.19	1	6	3	
Compound 3	254.7	-0.41	1	6	3	
Compound 4	231.3	2.37	0	2	1	
Compound 6	261.7	0.57	1	7	3	
Compound 10	299.7	1.49	0	4	5	
Fipronil	437.2	3.90	2	5	5	

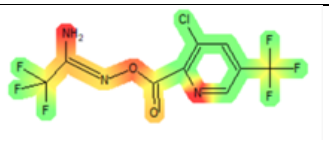
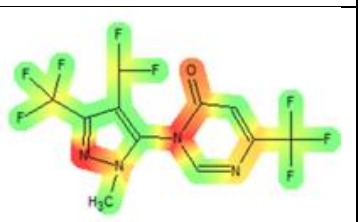
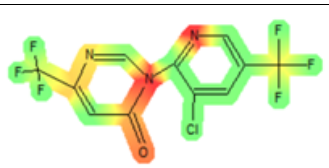
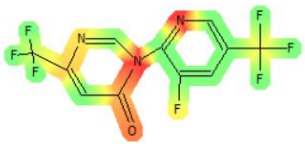
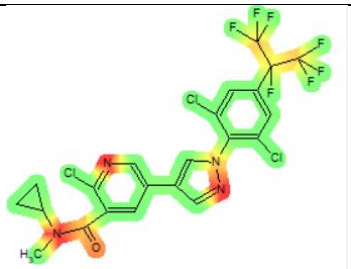
Compound 30	335.6	2.44	2	5	5	
Compound 36	362.2	2.09	0	5	4	
Compound 39	343.6	3.08	0	4	3	
Compound 40	327.2	2.24	0	4	3	
Compound 47	317.6	3.62	0	4	3	
Nicofluprole, 2020 (Bayer)	589.7	7.07	0	5	7	

Table 7.1 showing virtual screening scores for imidacloprid, fipronil and novel compounds, including new commercial product nicofluprole using Tice and Lipinski's parameters. Lipophilic groups are shown in green while hydrophilic groups are in red with colour intensity relating to strength of the characteristic. Values obtained using the EPSRC funded Physical Sciences Data-science Service hosted by the University of Southampton and STFC under grant number EP/S020357/1.

The virtual screening parameters show the comparatively high molecular weights (MW) of the GABA targeting compounds, which are significantly higher than those of the nAChR targeting compounds. Though from the lowest MW of

231.3 to the highest 362.2 of our novel compounds sit closely around the average of known pesticides leading to this parameter of active insecticides being met according to the information amalgamated by Tice, 2001 [271]. The H bond donor is at the 95th percentile (2) for compound 30 but otherwise is at the expected 0 to 1 and the H bond acceptors all sit between the 5th and 95th percentiles (1-7) though it is of note than compound 4 has no H bond donors and only 2 H bond acceptors, this may have caused reduced binding capabilities in the target site. Though a high number of these bonds is considered a hindrance, too few may also be a disadvantage [271]. Rotational bonds here also sit on the lower end of the average (6.1) according to Tice's research. The logP otherwise known as log K_{ow} is harder to put into context of Tice's research as he uses two different methods to calculate this value, neither of which are the methods used by the EPSRC funded Physical Sciences Data-science Service. Using the method most closely related to Lipinski's calculations alogP used by Tice, fipronil is at 5.23 and imidacloprid is at 2.29 both sitting between the 5th and 95th percentiles (0-6.4) around the average (3.5) [271]. Compound 4 and 10 are significantly more lipophilic than the rest of the nAChR targeting compounds possibly explaining their reduced activity. Other than compound 47, the closest to breaking resistance in the GABA targeting compounds, the rest show reduced lipophilicity potentially being the cause of their reduced activity compared to fipronil.

As a comparison nicofluprole currently an ISO published insecticide from Bayer, discussed earlier, shows exceptionally high comparative molecular weight compared to most insecticides, very high lipophilicity and higher than average H bond acceptors and rotational bonds [271,287]. The activity and resistance breaking

results from this compound will be of interest due to the differences seen compared to previous compound characteristics.

Virtual screening consists of 1D, 2D and 3D. 1D and 2D screening comprising of the type of information discussed above is fast and cheap to calculate but for structural information such as shape and how this then might fit into target binding sites 3D modelling is required [29]. A 3D agrophore model can more accurately indicate active compounds by visualising key molecular functions in specific places on the compound [29]. It is then possible to see which compound (key) best fits into the target site (lock) by applying docking algorithms which incorporate information like the lipophilic contacts and hydrogen bonds between target site and compound [29,288,289].

7.3 Modes of action

Of the big-selling insecticides in 2009 there was a primary focus on only four of the well-known modes of action for insecticidal activity, acetyl-cholinesterase, the voltage gated sodium channel, the acetylcholine receptor and the GABA receptor [290]. Thus making the identification of novel target sites important for agrochemical companies [291]. Identification of novel target sites and development of insecticides for novel compounds would minimise the concern of cross-resistance particularly that of target site resistance mechanisms. An example of a new mode of action insecticide is flubendiamide, the first in the novel insecticide class phthalic acid diamides developed by Bayer Crop Science and Nihon Nohyaku [291,292]. Flubendiamide showed multiple counts of resistance breaking activity particularly useful for the control of lepidopterans and acts on the ryanodine receptor in the endo(sarco)plasmic reticulum [291]. After the release

of flubendiamide in 2007 there have already been problems with rapid resistance evolution in wild pest populations via a target site mutation in the ryanodine receptor which has caused cross resistance to other diamides such as chlorantraniliprole [291,293]. This resistance arose as quickly as 18 months after the first use, it is important to note here though that ryanodine itself was used as a pesticide extensively previously and so perhaps the evolution of resistance may have begun much earlier than the rollout of flubendiamide [293,294].

Modification of flubendiamide has led to discovery of broflanilide (Tenebenal) by Mitsui Chemicals Agro, Inc. called a meta-diamide with yet again another separate mode of action [295,296]. This compound again looks to control lepidoptera, notoriously difficult to control due to high genetic plasticity, heavy use of novel effective pesticides and fast generation times [293,295]. Broflanilide acts as a GABA receptor antagonist with non-competitive RDL and distinct from the binding method found by fipronil allowing it to break fipronil resistance mechanisms in pest populations [297]. This compound has also been shown to be useful for public health via control of susceptible and resistant strains of malarial vectors *Anopheles gambiae* and *Aedes aegypti* mosquitos [296].

Despite these promising discoveries finding novel resistance breaking compounds is uncommon. As previously mentioned, the aftermath of extensive DDT use left multiple resistance mechanisms still causing problems today. One of which is knockdown resistance (kdr) a target site resistance mechanism [40,147,298,299]. Kdr resistance is found in domain II of the voltage gated sodium channel, evolving due to overuse of DDT and conferring cross resistance to pyrethroids that also use this target site [299]. Target site resistance can incur heavy fitness costs due to the altering of target sites and, therefore, potentially compromising their function [148,272]. When a target site resistance gene comes

with heavy costs this can trigger selection pressure for a second target site resistance gene that can compensate for the costs of the first gene [135]. This makes the loss of such resistance genes less likely. Target site resistance mutations can be difficult to overcome and different MoA compounds would be helpful.

An important consideration when screening for novel pesticides is to be market focussed [284], which we incorporated into our research with communications with market agrochemical giant Bayer. They informed us about a lack of current interest in neonicotinoids due to negative social opinions. Despite strong interest in compounds targeting the nAChR in Japan it is not a popular class in Europe or the UK due to negative effects on our pollinators [30,251,268]. Bee safe insecticides are not always as labelled as found by Siviter and Muth, 2020, when they reported lethal and, equally important, sub-lethal effects of two newer pesticides FPF and sulfoxaflor [268]. Unfortunately, a lack of new effective pesticides that are safe for bees has led to a temporary, and controversial, lift on the ban of thiamethoxam in the UK this year for “emergency use” highlighting the importance and urgency of finding novel bee safe pesticides [300,301].

7.4 Natural compounds

Across multiple disciplines a constant and ongoing source of resistance breaking materials is found in nature and in the environment. They are considered one of the most effective pest control options and being more environmentally friendly gives them a significant advantage over synthetic products [154,156]. The combination of nature with intelligent product design have already produced important products for pest control and is expected to

continue doing so [154,302,303]. A more comprehensive study of the extent to which natural products have inspired and contributed to current insecticides is the review by Gerwick and Sparks, 2014, showing a table of the IRAC class, market percentage, MoA, class and natural product compound and source [302]. As consistently shown here that resistance breaking compounds are hard to design I also investigated a range of natural plant derived extracts for their repellent effects on aphids.

Natural product interest is highly driven by their constant co-evolution with their pests and their eco-friendly advantages [304]. Perseanol taken from natural product diterpene, which is similar to ryanodine, has been synthesised recently for research into its insecticidal properties and mode of action, though is too complex for use directly as an agrochemical [304]. This is a potential inspiration for novel semi-synthetic insecticides.

Despite the known insecticidal properties of nicotine from tobacco plants, the development of neonicotinoids arose separately [302]. One of the most notable natural inspiration for an important class of insecticides is that of the *Chrysanthemum* flowers containing pyrethrum which evolved into the insecticidal class pyrethroids [305]. The natural product pyrethrum is still extracted directly from dried flowers with 10,000 tons used in 2010 for global productions and while synthetic pyrethroids have high levels of resistance and cross resistance there is low resistance to the natural pyrethrins [305]. This is an advantage of natural evolution of plant protection mechanisms against their pests.

The difficulty around natural products is that of slow and complex extraction methods needed as well as relatively complex structures compared to synthetic

products [154]. Long chain compounds are more difficult to synthesise and these structures tend to also be difficult when using virtual screening parameters to detect possible activity. For example Tice's parameters often have active outliers when looking at natural compounds making their activity difficult to predict using virtual screening [271]. This reduces a potential filter for testing large ranges of compounds and, therefore, increasing the cost of research in this area [271]. Among natural products, otherwise known as biologics, there are multiple categories of which natural products are considered most useful due to high effectivity and compatibility with current application methods of other pesticide treatments [154]. These natural products are defined as secondary metabolites of organisms such as spinosads whereas the products studied here would be classed as botanicals such as essential oils and pyrethrum which are recognised as having significant disadvantages by way of the complex nature of supply [154].

Another significant downside to natural product development is the rarity with which a new product may be identified making this venture particularly expensive for wide scale R&D [154]. Instead semisynthetic products are of much more interest as natural products can be extremely potent but chemical synthesis of natural product variants is much more promising [154]. Semi synthetic product expansion aims to address difficulties identifying novel modes of action and has the potential to create new classes of nature inspired insecticides but the slow, expensive and risky attributes of this line of research tends to make wholly synthetic insecticide design more attractive and attainable for developmental agrochemical companies [154].

The findings made here mostly find repellent activity of the extracts made making them inefficient for the testing of screening fly lines as mortality is the

key parameter for this screening tool. Though there is potential interest in the rosemary extract for a potential insecticide which would be interesting to delve into as a future project.

7.5 Screening tool

The difficulties discussed for the identification of a novel insecticide above, and the hurdles new compounds must overcome to be deemed successful, highlighted the need for a screening tool to aid R&D of new pesticides. A particular hurdle to overcome was the high cost of R&D. While rational design and computer screening techniques may identify potentially active compounds the resulting chemicals must be tested against current mechanisms of resistance. Rather than testing on each species being able to screen compounds against one species with genetic modifications to include economically important resistance genes from pest species would be helpful. Model organisms have a long history of advantageous use in multidisciplinary research of which *D. melanogaster* has been used for over 100 years [8,306]. *D. melanogaster* was used as the model system for this concept for multiple reasons; they are cheap and easy to rear, and, due to so much being known about their genetics, it is possible to genetically modify them and their short lifecycle helps with the speed at which results can be gained [8,307].

Genetic manipulation of *Drosophila* was of particular importance due to the need to insert various resistance genes into new lines. The field of genome editing has been one of rapid evolution in the past few decades [61,308]. We used the GAL4/UAS system which is the most commonly used system for targeted transgene expression in *Drosophila* model systems [309]. The advantages of this

system is the inactivity of transgenes before activation by crossing the UAS line containing the transgene with the GAL4 driver line, so any potential side effects of carrying the transgene are not present until activation [61,309]. This means should there be a significant fitness cost of the resistance genes it will not affect the stock parental lines [309]. The system also allows for the stock UAS line to be crossed with any GAL4 driver line which can change the spatial expression of the transgene, for example whole body or the brain or the Malpighian tubules [61,66].

There are multiple enhancements of gene editing. Multiple systems allow for spatial and temporal control of transgenes for example heat shock promotor combined with FLP recombinase can be used in conjunction with GAL4/UAS to enable the user to activate the transgene at a specific point in time which can be useful if the transgene has any toxicity to the organism [309]. As this was not the case with the P450 genes in our transgenic lines this extra control was not needed. Another advantage of the lines made here is that, though we used Actin5C which expresses the genes in the whole body, other GAL4 driver lines are available from the Drosophila Stock Centre at Bloomington enabling different spatial and temporal control [234]. The GAL4/UAS system has been used to functionally express P450 genes in *Drosophila* before for the *Cyp6g1* gene using multiple driver lines; one expressing the transgene in the midgut, Malpighian tubules and fat body another using a heat shock driver [77,310,311]. The extent to which driver lines differ can be seen in the paper by Jenett *et al.*, 2012, where they generated 7000 GAL4 driver lines which were made available by Bloomington [312].

7.6 Gene expression

An important part of the construction of a transgenic line is proving the overexpression of the target gene which was done for *Cyp6cm1*, *Cyp337b3* and *Cyp6bq9* showing overexpression between 100 to 200-fold compared to the control and parental lines. Comparison with housekeeping genes means we can be sure the only gene overexpressed was the P450 target, therefore, making this gene responsible for any resistance seen in these offspring lines.

The lack of functional qPCR primers for *Cyp6bq23* meant we were unable to prove its overexpression after testing with 5 sets of primers designed. Despite this, initial tests to prove the presence of *Cyp6bq23* were carried out on the parental line and trial offspring lines, when it arrived from Cambridge Fly Lab and the insert was seen via PCR and gel electrophoresis (figure S.1, supplementary information) suggesting it is likely the gene remained present despite the lack of proof of overexpression. Bioassay results showing no conferral of deltamethrin resistance, as with the other three transgenic lines showing no resistance to the pesticide the genes confer resistance to in their host species, suggests a larger problem.

Cyp6cm1 has been functionally expressed in *D. melanogaster* by Daborn *et al.*, 2012, using 6g1HR-GAL4-6c driver which expresses the gene in the gastric cecum, midgut, Malpighian tubules and fat body or the tubP-GAL4 expressing transgenes in all cells [236,313,314]. Though there was some variation for resulting resistance levels between the two drivers at different levels of DDT, there was no significant difference, so our driver choice is unlikely to have affected resistance as it was expressed ubiquitously across all tissue [236]. Zhu *et al.*, 2010, confirmed functional expression of *Cyp6bq9* in *D. melanogaster* using

heat shock expression activation and drivers expressing the gene in the central nervous system only and using the Actin 5c for whole body. Zhu *et al.*, 2010, like Daborn *et al.*, 2012, showed some variation but no significant difference in resistance levels between the drivers used.

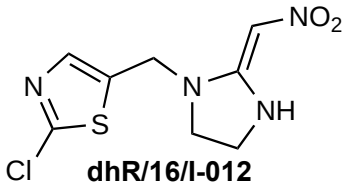
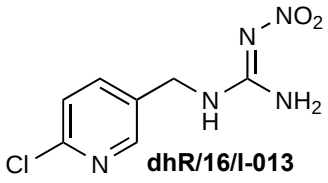
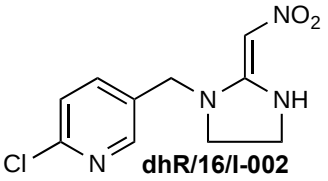
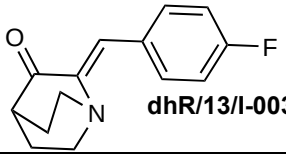
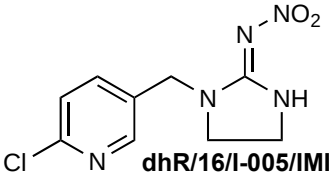
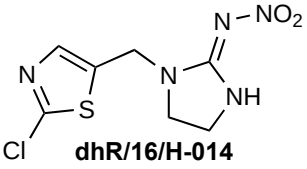
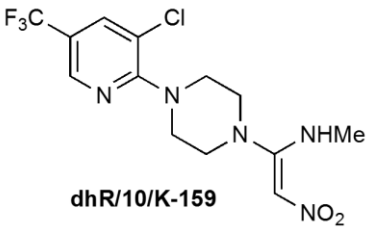
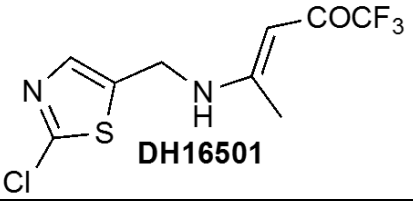
Functional expression of *Cyp337b3* was achieved in microsomes using *E. coli* cells and the P450 gene cloned from *H. armigera* [225]*.* The functional expression of metabolic resistance genes in microsomes is extremely useful showing the ability of a gene to metabolise given concentrations of pesticide quickly and cheaply. Had there been conferred resistance of the four genes worked on in chapter 5 of this thesis I would have expanded the screening tool to include a microsomal screening tool for initial testing of novel compounds against resistance genes.

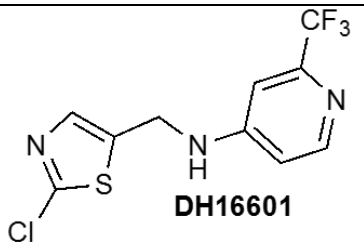
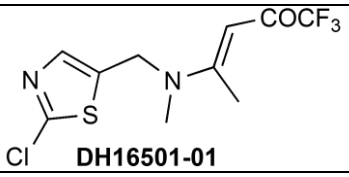
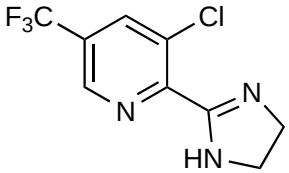
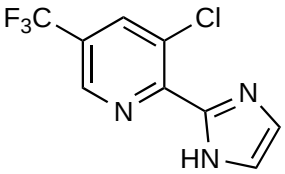
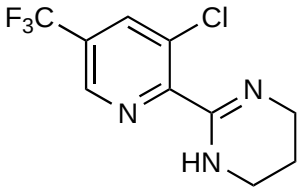
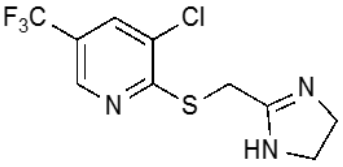
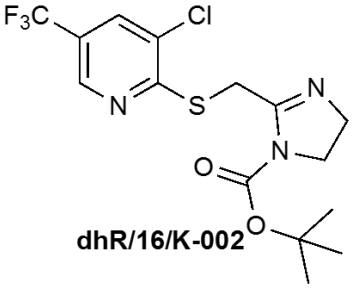
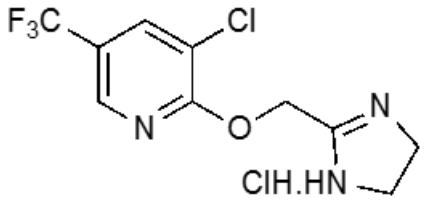
7.7 Testing the screening tool

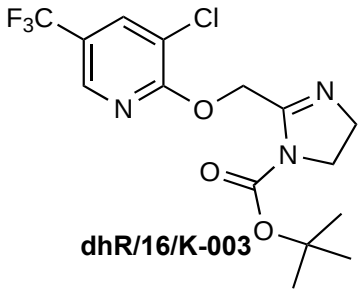
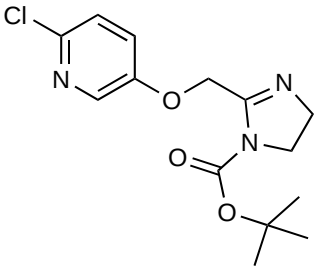
Chapter 6 was a test run of the screening tool developed in chapter 5 using novel compounds identified in chapter 2 and 3. Only synthetic compounds were used to test the screening tool as, although rosemary extract showed some lethal effects they were not strong enough to do dose dependent bioassays without identifying and concentrating the active component. Transgenic lines *Cyp6er1*, *Cyp9q3*, *Cyp9q4* and *Cyp9bu1* were taken as examples from the screening tool developed in a collaboration; see McLeman *et al.*, 2020, supplementary information. The reason these lines were selected is they cover pests and beneficial insects and this information is required by law for registration of compounds.

Results already known about the selected, most successful, synthetic compounds means their lack of commercial viability was expected from the run through of the screening tool but was carried out to show the information possible to gain from using this screening tool directly. Following successful screening of a novel compound using this tool bioassays would need to be carried out with the original pest species and with bees to check lethal and sub-lethal effects. Tests would also be needed to check effects of mammalian cells to confirm any toxic effects to wildlife or humans and chemical tests would be required to confirm stability in the environment.

Supplementary information

C1	dhr/16/I 012	 **dhR/16/I-012**
C2	dhr/16/I 013	 **dhR/16/I-013**
C3	dhr/16/I 002	 **dhR/16/I-002**
C4	dhr/13/I 003	 **dhR/13/I-003**
C5	dhr/16/I 005	 **dhR/16/I-005/IMI**
C6	dhr/16/H 014	 **dhR/16/H-014**
C7	dhr/10/K 159	 **dhR/10/K-159**
C8	DH 16501	 **DH16501**

C9	DH 16601	 **DH16601**
C10	DH 16501 01	 **DH16501-01**
C11	dhr/11/F 014	 **dhR/11/F-014**
C12	dhr/17/C 004	 **dhR/17/C-004**
C13	dhr/14/C 014	 **dhR/14/C-014**
C14	dhr/17/C 005	 **dhR/17/C-005**
C15	dhr/16/K 002	 **dhR/16/K-002**
C16	dhr/17/C 010	 **dhR/17/C-010**

C17	dhr/16/K 003	 **dhR/16/K-003**
C18	dhr/17/D 001	 **dhR/17/D-001**
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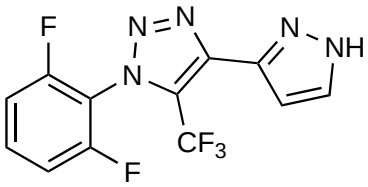
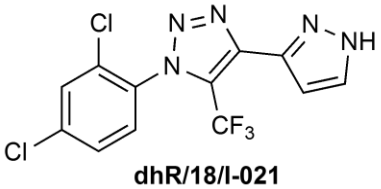
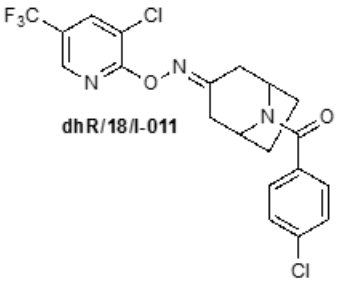
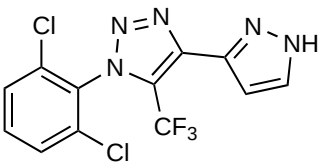
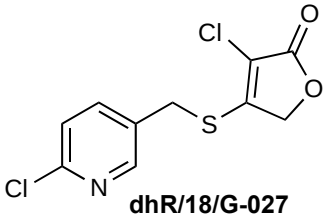
C20	dhr-18-I-020	 **dhR/18/I-020**
C21	dhr-18-I-021	 **dhR/18/I-021**
C22	dhr-18-I-011	 **dhR/18/I-011**
C23	dhr-18-H019	 **dhR/18/H-019**
C24	dhr-18-G-027	 **dhR/18/G-027**
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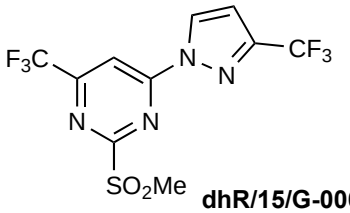
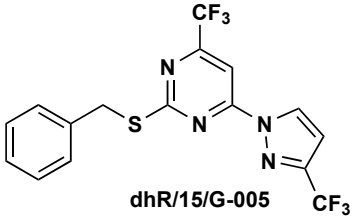
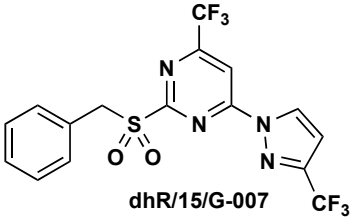
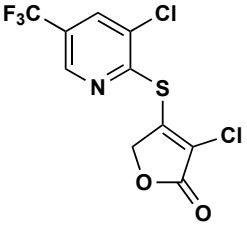
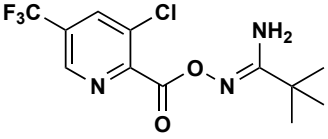
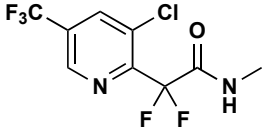
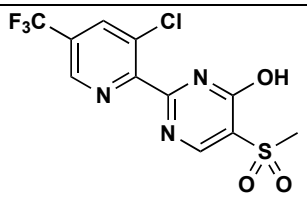
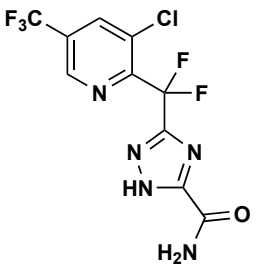
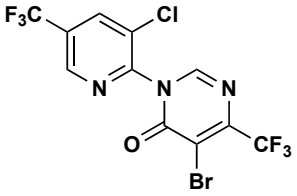
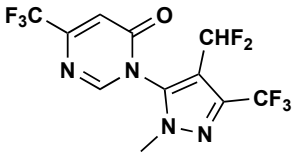
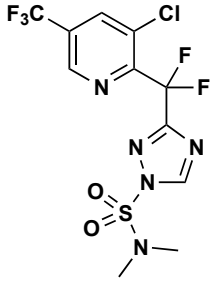
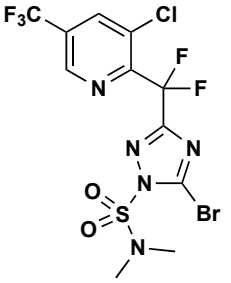
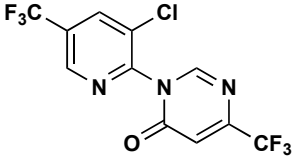
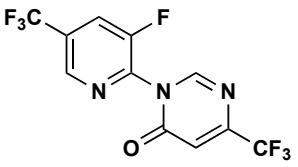
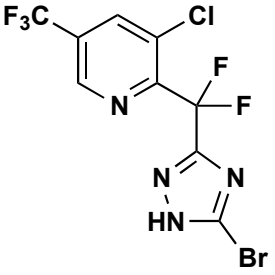
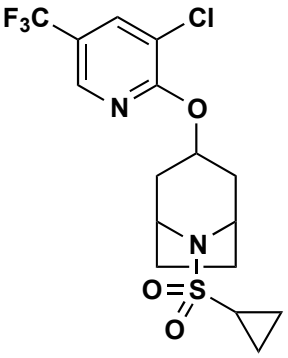
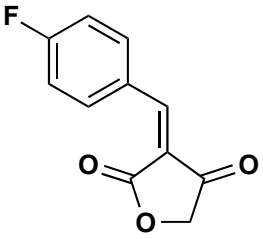
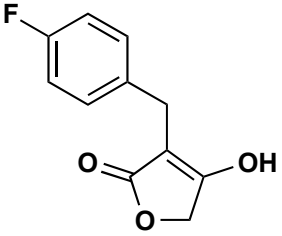
C26	dhr-15-G-006	 dhR/15/G-006
C27	dhr-15-G-005	 dhR/15/G-005
C28	dhr-15-G-007	 dhR/15/G-007
C29	dhr-18-G-022	 dhR/18/G-022
C30	dhr-11-C-013	 dhR/11/C-013
C31	dhr-10-J-143	 dhR/10/J-143
C32	dhr-09-D-071	 dhR/09/D-071

C33	dhr-13-I-004	 dhR/13/I-004
C34	dhr/12/J-002	 dhR/12/J-002
C35	dhr-0/J-170	Unknown
C36	dhr-14-J-017	 dhR/14/J-017
C37	dhr-12-I-002	 dhR/12/I-002
C38	dhr-13-D-003	 dhR/13/D-003
C39	dhr-12-J-003	 dhR/12/J-003

C40	dhr-14-G-002A	 dhR/14/G-002A
C41	dhr-13-D-002	 dhR/13/D-002
C42	dhr-18-E-025	 dhR/18/E-025
C43	dhr-18-I-001	 dhR/18/I-001
C44	dhr-18-I-002	 dhR/18/I-002

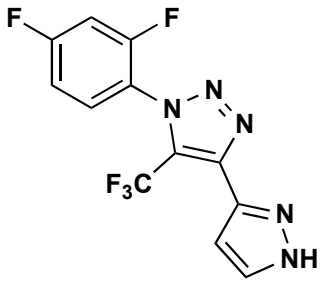
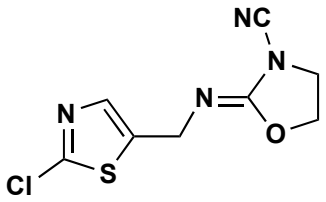
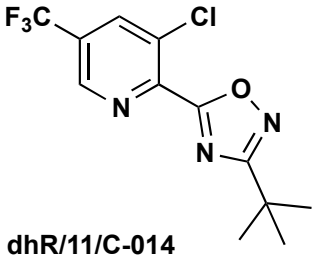
C45	dhr-18-I-024	 **dhR/18/I-024**
C46	dhr-19-C007	 **dhR/19/C-007**
C47	dhr-11-C014	 **dhR/11/C-014**

Table S.1 Structures of all synthetic compounds sent from Darr House M.I. that were tested for activity.

Primer name	**Primer sequence**
Cyp337b3 Forward 1	TTCGGTGAGCATGAGGAGTT
Cyp337b3 Reverse 1	CGGTCGGCTCCAATTTGTAG
Cyp337b3 Forward 2	ATCATCGAGAAATCTGCGCG
Cyp337b3 Reverse 2	TAGAGCCGTGGGAAAGTGTT
Cyp337b3 Forward 3	TCTGTGCAGTATCGTCCTCC
Cyp337b3 Reverse 3	GGGATAGTGGCAGAAGTCGT
Cyp6bq9 Forward 1	TCGAAACCTCATCCACCACA
Cyp6bq9 Reverse 1	CGTGGAATTATGGGCAGTGG
Cyp6bq9 Forward 2	TACCCGTCCTAGCGATTCAC
Cyp6bq9 Reverse 2	ATGGGGAACTCGGTCTTGTT
Cyp6bq23 Forward 1	TCCTGAACGGTAAGCGTCTT
Cyp6bq23 Reverse 1	GAAATCAGGGCGCGTTATGT
Cyp6bq23 Forward 2	ACAATGACGTTTGCCCTGTC
Cyp6bq23 Reverse 2	GATTGGCACTGGAGGGTACT
Cyp6bq23 Forward 3	TACCATGAGCACGAACCTGT
Cyp6bq23 Reverse 3	TGTTGAGCTCTTCCCCAGTT
Cyp6bq23 Forward 4	ACATCAGTTCGCGCACATAC
Cyp6bq23 Reverse 4	GCGTGGATCCAAATACCGTC
Cyp6bq23 Forward 5	AGTACCCTCCAGTGCCAATC

Cyp6bq23 Reverse 5	GTATGTGCGCGAACTGATGT
Cyp6cm1 Forward 1	CGCGACAAGTTCCACTACTG
Cyp6cm1 Reverse 1	CGTACTACCAGGATCGGCTT
Cyp6cm1 Forward 2	GGCACAAAGGTCTTCGTCTC
Cyp6cm1 Reverse 2	CGGCGTGGTATGAATTTCGT

Table S.2 showing qPCR primers tested to confirm overexpression of transgenes. Results all used set 1 for primers except *Cyp6bq23* where none amplified the gene.

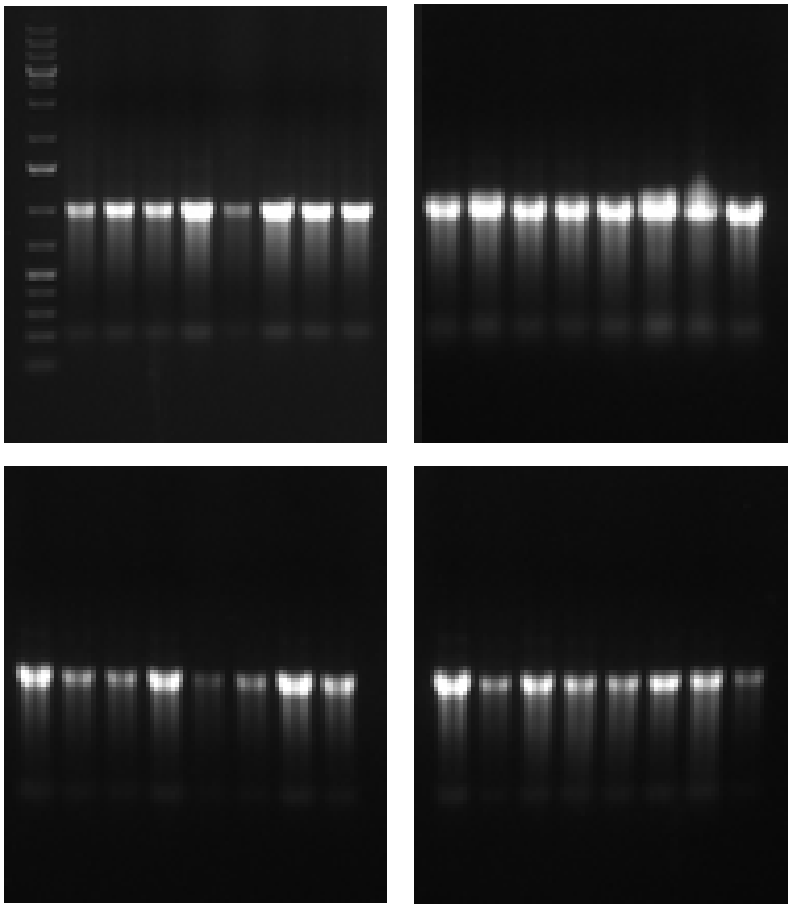


Figure S.1 Electrophoresis gel of RNA extracts from parental and offspring lines from (top left) *Cyp6cm1,* 4 x parent extracts followed by 4 x offspring extracts, (top right) *Cyp6bq9*, (bottom left) *Cyp6bq23* and (bottom right) *Cyp337b3*. Confirming presence of P450 gene insert upon arrival of lines from Cambridge.

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Or in the attached files of this thesis.

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