Insecticide resistance in Drosophila

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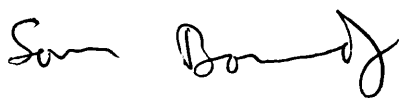
Signed 

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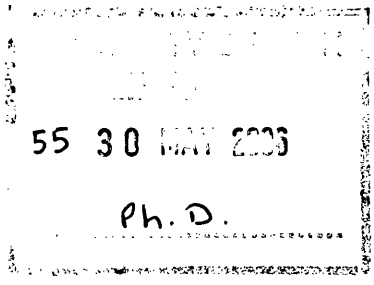
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Abstract

The aim of this study was to search for mutants that are resistant to the novel insecticide imidacloprid. All resistance was found mapped to the historic DDT-R locus at chromosome 2 position 64.5 cM and there we show that resistance is associated with a single cytochrome P450 gene, *Cyp6g1*. This gene is over-transcribed in resistant strains and confers cross-resistance to a wide range of insecticide classes. *Cyp6g1* was over-expressed throughout the entire lifecycle of the resistant insect. Micro-arrays and transgenic *Drosophila* lines were generated to prove that the up-regulation of *Cyp6g1* alone is sufficient to confer resistance to DDT and neonicotinoids. Further work was carried out on a range of strains with differing P450 expression profiles to investigate how different P450s confer differing levels of resistance to DDT, malathion and imidacloprid.

Contents

List of all figures and tables.

List of abbreviations used.

ACh	Acetylcholine
AChE	Acetylcholinesterase
AP	Alkaline Phosphatase
APS	Ammonium persulphate
BCIP	5-Bromo-4-chloro-3-indolyl Phosphate
CI	Confidence Interval
CYP/*Cyp*	Prefaces for a cytochrome P450 gene.
Cyp6g1	Refers to the gene or the mRNA transcript.
CYP6G1	Refers to the protein.
DAB	3,3'-Diaminobenzidine Tetrahydrochloride
DDT	1,1-bis(p-Chlorophenyl)-2,2,2-trichloroethane
DMC	Dosage Mortality Curve
EMS	Ethyl Methanesulfonate
HRP	Horseradish peroxidase
GABA	9-Aminobuyric Acid
GST	Glutathione S-transferase
IMI	Imidacloprid
IPTG	Isopropyl E-D-Thiogalactopyranoside
kdr	*Knockdown Resistance*
LC/MS	Liquid crystallography/mass spectroscopy
LC_{50}	Lethal Concentration at which 50% of a population will be dead
LD_{50}	Lethal Dose at which 50% of a population will be dead
MBP	Maltose Binding Protein
nAChR	Nicotinic Acetylcholine Receptor
OP	Organophosphate
P450	Cytochrome P450
pHIS	Poly His tag
Rdl	*Resistance to dieldrin*
RFLP	Restriction Fragment Length Polymorphism
RT	Reverse Transcriptase
TLC	Thin Layer Chromatography

All *Drosophila* strains used in this thesis

Strain	Origin	Abbreviation
Canton-S	Bloomington (1)	Canton-S
Hikone R	Bloomington (4267)	Hikone R
EMS1	Bath (J. Yen)	EMS1
Wis1	Wisconsin (R. ffrench-Constant)	Wis1
Wis1 lab	Wisconsin (R. ffrench-Constant)	Wis1 lab
y[1], w[1]	Bloomington (1495)	*y;w*
w; CB21/CyO[wg Egal]	K. Beckingham (Rice, Houston)	CB21
Df(2R)CB21/CyO; ry[506]	Bloomington (4960)	4960
cn[1] vg[21-3] bw[1]	Bloomington (3984)	*cn vg bw*
y[1] w[*]; P{w[+mC]=GAL4}LL7/ TM3, Sb[1]	Bloomington (5138)	Gal4
w[*]; T(2;3)ap[Xa]/CyO; TM3, Sb[1]	Bloomington (2475)	*Cy0;TM3;Xa*
UAS-Cyp6g1 3a	Bath (P. Daborn)	*UAS-Cyp6g1*^{X}
UAS-Cyp6g1 8a	Bath (P. Daborn)	*UAS-Cyp6g1*^{2}
D. simulans OV	Brazil (L. Madi-Ravazzi)	OV
D. simulans BG	Brazil (L. Madi-Ravazzi)	BG

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Ch 1. Introduction

1.1 *Drosophila melanogaster* as a model insect for studying insecticide resistance.

In a study of insecticide resistance, *Drosophila melanogaster* is not an obvious choice for a model insect. It is not a pest species, apart from on grape vines and tomatoes, and even then, it is not often directly challenged with pesticides. *Drosophila* is therefore not a target insect when a new insecticide is designed. New compounds are chosen for their activity against pest species such as Lepidoptera or Coleoptera and the results of screening on *Drosophila* are often incidental or academic (Soderlund and Bloomquist, 1990).

However, *D. melanogaster* is undoubtedly the most important model insect for the scientist. Of all the insects, *Drosophila* is the most understood and widely studied. There are a huge number of phenotypic markers for the mapping of resistance and to locate a resistant locus (Wilson, 1988). Almost a century of work has been carried out turning *Drosophila* into one of the most important genetic tools available to a scientist studying resistance to insecticides. With *Drosophila,* it is possible to use tried and tested methods to generate mutants. Either using EMS to generate point mutations for investigating receptor mutants or using x-rays to generate chromosomal rearrangements for the study of gene duplications and rearrangement (Ashburner, 1989; Daborn et al., 2001; Daborn et al., 2000; Kikkawa, 1964). Insects that are selected for resistance to a compound can then have resistance mapped to a specific locus (Roush and McKenzie, 1987; Soderlund and Bloomquist, 1990). From this information, the complete genome can be used to identify candidate genes that confer resistance (Celniker et al., 2002; Hoskins et al., 2002; Myers et al., 2000). A series of modern techniques can then be employed to identify differences in gene sequence or transcription pattern and the exact nature of a resistance mechanism can be characterised (Miyo et al., 2001). It should also be remembered that by using these techniques the gene of interest is cloned solely on the positional localisation of the resistant phenotype. Therefore, no prior assumptions about the cause of resistance can cloud experimental design (ffrench-Constant et al., 1992).

D. melanogaster has long been the model organism for geneticists from the early days of genetics at the beginning of the last century. They are easy to rear on a diet of treacle, oatmeal and a little yeast. They produce large numbers of offspring and have a simple and short lifecycle of 14 days. They are also small so large numbers of strains can be kept in a relatively small space (Ashburner, 1989). *D. melanogaster* has a small number of chromosomes and this makes the mapping of genotypes easier than for species with a large chromosome number (ffrench-Constant and Rocheleau, 1992).

Of course using *D. melanogaster* as a model species in the study of insecticide resistance relies on the existence in the pest species of homologues of the genes identified in Drosophila. A good example is the *kdr* gene. The *kdr* phenotype has now been seen in a large range if species and orders of insects and in all cases the same small number of mutations to sodium channels have been seen (Zlotkin, 1999). It is also common in the case of metabolic mutants that a series of homologues to either one gene or a series of closely related genes will confer resistance in the same way, such as *Cyp6a1* in *Musca domestica* and *Cyp6a2* in *D. melanogaster* (Dunkov et al., 1997). In this case, two homologues give the same resistance profile, and even have homologous mechanisms for the over-transcription of the gene (Sabourault et al., 2001).

1.2 Insecticide Resistance

The World Health Organisation defined resistance as: "Development of an ability in a strain of an organism to tolerate doses of a toxicant that would prove lethal to the majority of individuals in a normal (susceptible) population of the species." (Zlotkin, 1999).

Since the beginnings of agriculture mankind has been trying to contain and control pest species of insects on crops and livestock and for even longer we have tried to control those insects choosing to colonise our own body (Dent, 1991; Smith and Scott, 1997). Traditionally this involved the use of biological controls, either plant products or other animals to try to kill pests, or the use of inorganic chemicals such as arsenical compounds as a means of controlling lice. The insecticidal properties of plants such as pyrethrum have also been used as a means of controlling pests. During the industrial revolution, agriculture became more intense as more food had to be grown by fewer people but on the same area of land and support a more urban populace. This led to

introduction of early chemical mixtures such as lead arsenate as an insecticide. At the end of the 19th century, arthropods were recognised as possible vectors of human diseases. In 1939, the insecticidal properties of DDT were recognised. It took until the late 1940s for the first cases of insecticide resistance to be reported (Soderlund and Bloomquist, 1990).

Insecticide resistance is not a phenomenon that should be regarded as either novel or new. Insects and plants have been in a constant arms battle for eons. Plants have evolved to produce a range of toxic compounds (the most famous in our modern lives is probably nicotine) and insects have therefore been driven to evolve new forms of resistance to these compounds (Feyereisen, 1999). There are many examples of a plant evolving a compound and then insects evolving to be able to feed on that plant and so out-compete competitors by being able to survive in a niche environment. Examples of this include the over-expression of the cytochrome P450 CYP4M3 in *Manduca sexta* (Snyder et al., 1995). This enables the insect to survive in the presence of nicotine and therefore is possibly, why this particular Lepidoptera is capable of living on plants that produce such a potent natural pesticide. There are also cases of P450s being involved in some species of drosophilids being able to survive on cacti producing isoquinoline alkaloids (Fogleman, 2000). Similar resistance can be conferred upon *D. melanogaster,* which is normally susceptible to these chemicals, by inducing P450s with Phenobarbital. A third case of insects being able to survive on plants producing an insecticidal compound is the case of *Papilio polyxenes* which over-express CYP6B1 and CYP6B3 (Scott et al., 1999). This allows *P. polyxenes* larvae to grow in the presence of furanocoumarin, which is normally toxic to them (Li et al., 2001; Petersen et al., 2001).

There are four main general mechanisms of insecticide resistance (Soderlund and Bloomquist, 1990). First, there can be a physiological change in the insect, such as a change in the midgut wall or cuticle that makes it more difficult for the insecticide to enter the insect (Taylor and Feyereisen, 1996). This resistance mechanism has been characterised in the housefly and a number of other species (Oppenoorth, 1985). The most widely studied case being the housefly. With this mechanism a gene on chromosome III called *pen* is thought to delay the absorbance of an insecticide (Plapp and Hoyer, 1968). The exact nature of this mechanism is unknown but it delays the

penetration of DDT by approximately five fold (Sawicki and Lord, 1970). The work of Patil and Guthrie showed that there is no difference in hydrocarbon content of resistant and susceptible insect cuticle but resistance was lost upon treatment with silicic acid, which absorbs these compounds (Patil and Guthrie, 1979). A second form of resistance has been called behavioural avoidance. In this instance, an insect will not feed on areas sprayed with insecticide. This has been demonstrated with malathion in *D. melanogaster* but it seems to be a minor form of resistance (Pluthero and Threlkeld, 1984). Much more widely studied forms of resistance are changes to the target site of the insecticide, such as receptors. In these resistance mechanisms, the insecticide is unable to bind to its target and so is unable to have an effect. The fourth type of resistance is metabolic resistance where an enzyme that is capable of rendering a compound harmless to the insect is up-regulated and so the insecticide is unable to reach the target site (Taylor and Feyereisen, 1996).

1.2.1 Target site insensitivity

Insecticides act on a number of nervous pathways in insects, the main targets for each class being shown in Table 1.1. It can be seen that there are generally four major targets for insecticides. To date there are cases of resistance in three of these targets with there now being some unpublished reports of resistance being due to mutations in the nAChR. All these cases are due to point mutations and it is easy to understand the importance of single amino acid changes in target site resistance. These have the potential to cause the small non-lethal changes to the insecticide-binding site needed to facilitate resistance (Wilson, 2001). To date there has been only one case of an *Rdl*-like receptor being duplicated and this is in *Myzus persicae*, but seems to have no influence on resistance (Anthony et al., 1998b).

Table 1.1 The major classes of insecticide and there target site.

Class of Compound	Target
Organophosphates	Acetylcholinesterase
Carbamates	Acetylcholinesterase
Organochlorines	Voltage gated sodium channels
Pyrethroids	Voltage gated sodium channels
Cyclodienes	GABA receptors
Neonicotinoids	Nicotinic Acetylcholine receptors

Table 1.1 shows the major insecticide classes that have been used and their target sites in insects. Some classes such as the organochlorines and cyclodienes have now largely been removed from the market.

1.2.1.1 γ -Aminobutyric Acid Receptor based resistance

Of the three forms of target-site insensitivity the most widely distributed (at least until recently, when cyclodienes were generally withdrawn from the market) was *resistance to dieldrin* or *Rdl* (ffrench-Constant et al., 2000). The cyclodienes target the insect γ-aminobutyric acid (GABA) receptor. GABA is the major inhibitory neurotransmitter in both insects and vertebrates.

This is a classic case where the mechanism of resistance could not be determined in pest species and so *D. melanogaster* was used to try to find the mechanism of resistance. Resistance was found to be semi-dominant with the heterozygous insect's resistance falling between that of homozygous susceptible and homozygous resistant insects. It was also found that *D. melanogaster* populations in New York orchards exhibited a 1-10% frequency of resistance. Mapping was carried out and this placed resistance on chromosome 3L at 26.5 cM. This gene was then cloned and the resistance was found to be caused by a single point mutation in the GABA receptor, alanine302-serine. This point mutation was seen in all resistant *D. melanogaster* suggesting a single origin of resistance (ffrench-Constant et al., 2000). Although this may not be true as this could be the only mutation that can confer resistance and so the same mutation could occur independently. To check this hypothesis the surrounding sequence needs to be analysed to determine the origin of resistant chromosomes. So far all reported cases of *Rdl* in other species all have this same alanine to serine mutation or an alanine302-glycine substitution as seen in *M. persicae.* It is also interesting that in all species so far tested other than two *Myzus* species there are a maximum of two alleles per insect. This means that there is only one copy of the gene in the genome (Anthony et al., 1998a).

1.2.1.2 Sodium Channel based resistance

The second target of insecticides to have shown resistance is the voltage gated sodium channel. These are large transmembrane proteins present in excitable cells consisting of four domains (I-IV) each of six transmembrane elements. Where segment 4 (S4) is the most conserved in each element and consisting of a motif of positively charged amino acids (Zlotkin, 1999). These receptors open and inactivate in response to a depolarisation of the resting membrane potential. This leads to an influx of sodium ions that depolarise the membrane (Bloomquist and Soderlund, 1988; Dong, 2003; Martin et al., 2000). Inappropriate activation leads to a permanent depolarisation of the resting membrane potential which paralyses the animal. In mammals, sodium channels consist of a large α subunit and a smaller β subunit. In *D. melanogaster* the *para* gene that encodes the equivalent of the mammalian α subunit is functional when expressed alone. However, a second membrane protein, tipE, when co-expressed with the *para* gene increases the activity of the receptor suggesting that tipE may be a β subunit homologue (Vais et al., 2000).

The two major insecticide classes that target insect sodium channels are DDT and the pyrethroids. It is thought from work with mutant receptors that both these chemical classes act on the same site. DDT has now been largely withdrawn due to its ecological impact. This is largely a consequence of its extreme level of insolubility (1-2 ppb) in water when compared to lipid solubility. This strongly favours the storage and accumulation of this compound in animal membranes (Zlotkin, 1999). Pyrethroids are one of the major groups of insecticides and are developed from the natural compound pyrethrin found the flowers of certain species of chrysanthemum. Pyrethrin is a natural insecticide and is very photolabile making it unsuitable for general agricultural applications (Vais et al., 2000). The pyrethroids fall into two classes, class I pyrethroids, such as permethrin lack a α-cyano group that is present in the class II pyrethroids such as deltamethrin (Vais et al., 2000). The pyrethroids are one of the major insecticide classes used in agriculture for the control of plant and animal pests and are important in the control of human disease vectors such as mosquitoes. Pyrethroids are often the active ingredient in fly spray and are favoured due to their fast mode of action, so they knockdown pests almost immediately, and for their lack of toxicity to humans. They are also used in the control of insect vectors of malaria so studies on resistance mechanisms have strong implications for human health (Martinez-Torres et al., 1998). There have

been several theories put forward as to why pyrethroids are considerably less toxic to mammals than insects when the sodium channels are very similar (Zlotkin et al., 1999); Table 1.4 shows a comparison in toxicity to rats and aphids for a range of insecticide classes and the pyrethroid has by far the lowest toxicity factor of the insecticides tested. The first theory is based on observations that the effect of pyrethroid on insect and rat receptors expressed in *Xenopus* ooytes show no differences in the effect on the receptor (Vais et al., 2000). Thus it is thought that these compounds are less toxic to mammals due to the relative size of the animals, with the pyrethroid having to move further to reach the target site so there being more time available for the metabolism of the compound. It is also believed that as mammals have a higher body temperature than insects this leads to the increased rate of metabolism of the pyrethroid (Zlotkin, 1999). Vais however believes the difference to be due to the way the insecticide interacts with the two different receptors and the pyrethroid does not have as severe effects on the mammalian receptor (Vais et al., 2000). A further theory as to why pyrethroids are more toxic to insects and mammals is that pyrethroids have a reverse temperature sensitive response. It has been shown that at mammalian physiological temperatures pyrethroids are not as effective as they are to insects (Lees, 1998).

There have been two types of resistance reported to pyrethroids based on the sodium channel target; these are knockdown resistance (*kdr*) and super-knockdown resistance (*super-kdr*), which consist of the *kdr* mutation and a second mutation in another part of the receptor. These are amongst the earliest cases of resistance discovered (Busvine, 1951) and partly characterised (Milani 1954) (Soderlund and Bloomquist, 1990). This phenotype is called *kdr,* as the resistant insects are able to withstand the rapid "knockdown" paralysis usually observed after pyrethroid exposure (Knipple et al., 1994; Osborne and Pepper, 1992; Pittendrigh et al., 1997b). The two types of resistance cause between 10 and 500 fold resistance respectively in a range of insects including aphids, mosquitoes, Colorado potato beetle and cockroaches. These mechanisms are best characterised in the housefly (Sawicki, 1978; Soderlund and Knipple, 2003).

Knockdown resistance is caused by a single mutation of leucine to phenylalanine in (L1014F in *M. domestica*) in the S6 transmembrane segment of domain II. It is thought to disrupt the binding of DDT and pyrethroids to the receptor. The second mutation,

super-kdr, is a methionine to threonine mutation (M918T in *M. domestica*). This mutation is never seen without the *kdr* genotype and is believed to decrease the number of pyrethroid binding sites from two to one and so increase resistance (Vais et al., 2000).

1.2.1.3 Acetylcholinesterase based resistance

Acetylcholinesterase (AChE) is a serine esterase that terminates nerve impulses at the cholinergic synapse by breaking down the neurotransmitter acetylcholine (ACh) (Walsh et al., 2001). AChE is the major target of organophosphate and carbamate insecticides (combined these are the biggest selling insecticides so this mechanism is extremely important in the control of insects). They act upon the active gorge of AChE by covalently bonding with a serine residue to phosphorylate or carbamylate it. This renders the enzyme inactive and so leads to the death of the insect (Vontas et al., 2002; Walsh et al., 2001). Numerous studies have been carried out on this insecticide target in the past on a range of insects. Early work on *D. melanogaster, M. domestica* and *Lucilia cuprina* led to the discovery of a single copy of AChE in these insects, now called *Ace-2* (Weill et al., 2002). The major work on insecticide resistance has been carried out on this gene.

The work of Fournier led to two hypotheses of how resistance was mediated (Fournier et al., 1992). The first hypothesis relates to the control of AChE expression. These workers first cloned *Ace-2* (then referred to as *Ace* as the second insect AChE was then unknown.) and expressed it in a baculovirus expression system. This so called minigene increased resistance in flies with a functioning copy of *Ace-2* and was able to stop mortality in homozygous knockouts for *Ace-2*. This work showed, firstly that the minigene used was acting as an AChE as it rescued double knockouts that are normally lethal. The second observation was that over-transcription of an AChE gene leads to resistance (Fournier et al., 1992). This is the first and only case so far of insecticide resistance being caused by the up-regulation of a target protein. However, an analogous case has been described in the past in relation to drug resistance. Resistance to the drug methotrexate is conferred by the gene amplification of dihydrofolate reductase (Assaraf et al., 1989). In the case of OP or carbamate resistance, the target for the insecticides is an enzyme rather than the more common nervous receptor. Thus given that metabolic resistance can commonly be due to up-regulation of an enzyme (Feyereisen, 1999) the

up-regulation of *Ace-2* in OP and carbamate resistance is not a surprise. It may be expected that other instances where the target of an insecticide is an enzyme will be found in which a change in the expression pattern of the target confers resistance.

The second hypothesis relates to a change in the structure of *Ace* itself. Upon sequencing the *Ace-2* gene in resistant and susceptible *D. melanogaster,* it was noted that there was a non-silent point mutation of phenylalanine368 to tyrosine. This change leads to resistance. Further work by this group on *D. melanogaster* (Mutero et al., 1994), *L. cuprina* (Chen et al., 2001) and in *M. domestica* (Walsh et al., 2001), led to the discovery of a number of different point mutations that all conferred resistance alone. When combined they conferred increasing levels of resistance to a range of organophosphates and carbamates.

Two very interesting findings came from this work. Firstly, in all cases it was the same five conserved residues that were altered in all the species investigated showing how a single set of mutations can confer the same phenotype across species (Walsh et al., 2001). Secondly, in the case of *L. cuprina* after 50 years of organophosphate use in the field in Australia the only cases of resistance to these insecticides that have been isolated from the field have been changes in sequence and expression of esterases (see Section 1.2.2.1). Even when EMS mutagenesis was used in the laboratory to generate mutants to screen for resistance, no cases of *Ace-2* resistance were found (Chen et al., 2001). Nevertheless, following in-vitro site directed mutagenesis of *Ace-2* it was found that the clones that conferred resistance again had the same five different point mutations in a range of combinations (Chen et al., 2001).

Determination of the structure of AChE from the marine ray *Torpedo californica* has led to a better understanding of how this resistance mechanism works. In all the cases reported the altered amino acid residue lies within the active gorge of AChE. Interestingly in every case the R group of the substituted amino acid is larger than that of the replacement. This means that the insecticide is less capable of either binding or entering the active gorge and so is unable to react with the active serine residue (Mutero et al., 1994; Walsh et al., 2001).

Recent work on mosquitoes has added to the complexity of this form of resistance. The three previously cited references all found that there was only one copy of AChE present in their insect of choice. The publication of the *Anopheles gambiae* genome and work with *Culex pipens* and *Aedes aegyptii* has led to the discovery of a second AChE in insects, now called *Ace-1* (Weill et al., 2002; Weill et al., 2003). This gene is present in a range of insects and from the sequence of 35 AChEs from a range of organisms has led to the hypothesis that there were two copies of AChE in insects before they diversified. Only relatively recently has one copy been lost from some diptera. We know it is a relatively recent event as the mosquitoes are also dipteran so this loss happened after the split between the mosquito and fly (Weill et al., 2002). The exact cause of resistance in these strains with two copies of AChE is unknown as yet but this discovery of a second *Ace* gene highlights very well the problem of using only one insect species as a model for all other insects. The discovery of a mechanism in one species, and the discovery of that same mechanism in other species will not mean that all insects, or even closely related insects, will necessarily employ the same mechanism. In some cases, e.g. *L. cuprina* an insect may have the potential to use a resistance mechanism but it will never be seen naturally in wild-type strains and cannot be directly induced by in-vivo mutagenesis. Even though in-vitro mutagenesis of the gene has shown that the proposed mechanism is theoretically possible (Chen et al., 2001).

1.2.2 Metabolic resistance.

There are several classes of enzymes that have in the past, been implicated in insecticide resistance. These are Glutathione S-transferases (GSTs), esterases and Cytochrome P450s (P450s) (Ranson et al., 2002). In some cases, one type of enzyme is used in an insect almost exclusively for all its metabolic resistance mechanisms, such as *D. melanogaster* using P450s (Feyereisen, 1999). However, other classes of insect will make use of a wide range of mechanisms to confer resistance to a variety of insecticides such as *M. domestica.* This insect has been shown to have DDT resistance caused by over-expression of a P450 and organophosphate resistance can be conferred by amino acid substitutions in an esterase gene (Sabourault et al., 2001).

Metabolic resistance works by an enzyme being altered; either its amino acid sequence is mutated making it better able to metabolise an insecticide or an enzyme can be over-transcribed and so it takes a higher dose of insecticide to kill the resistant insect.

Alternatively, a gene may be duplicated which can change the expression level. It is also theoretically possible that resistance could be caused by the loss of gene function. For example some insecticides require activation upon entering an insect and if the enzyme causing this reaction were lost then the insecticide would remain in an inactive form. With enzymes such as P450s, it has been shown that a single amino acid substitution is sufficient to cause a large change in substrate specificity. An example of this is in the human P450 CYP2C2, which is a lauric acid hydrogenase, but a single amino acid substitution of S473V changes the substrate specificity to accept the hormone progesterone (Nelson, 1999). In this way we can see that a small change in sequence can have large consequences for substrate specificity and therefore either cause a genetic disease in mammals or as in the present case cause insecticide resistance.

1.2.2.1 Esterase based resistance

Previously published cases of insecticide resistance being due to the over-expression or mutation of metabolic enzymes are numerous. In the aphid *M. persicae* resistant insects have multiple copies of the esterase E4 and this confers organophosphate and pyrethroid resistance (Devonshire and Sawicki, 1979; Field et al., 1988; Field et al., 1993; Hemingway, 2000; Hemingway and Ranson, 2000). The susceptible aphid also expresses this gene but due to multiple replications of this gene within the esterase amplicon the resistant aphid expresses a considerably higher amount of the enzyme. This has also been seen in the mosquito *C. pipens* where the amplification of a B1 esterase is responsible for organophosphate resistance (Mouches et al., 1987; Mouches et al., 1990). It has also been characterised that a point mutation in an esterase gene can confer insecticide resistance. Two examples of this are both cases of resistance to the organophosphate malathion. In *L. cuprina* the E3 malathion carboxyesterase has a point mutation in the LcαE7 gene (McKenzie et al., 1992). This confers >130 fold resistance to malathion when the susceptible LS2 strain is compared to a strain, Woodside 5.2, which is resistant to malathion (Campbell et al., 1998). In *M. domestica* there is a point mutation in the MdαE7 (Gly137Asp) gene that confers resistance to malathion (Claudianos et al., 1999). It is interesting to note that this amino acid substitution is the same as that seen in the previous example in *L. cuprina* (Newcomb et al., 1997). This seems to be a common occurrence, for several species to all show resistance to a compound with the same mutation in a homologous gene (ffrench-Constant et al., 1999; Parker et al., 1996).

1.2.2.2 Glutathione S-Transferase based resistance

Glutathione S-transferases (GSTs) have also been widely implicated in insecticide resistance. They are a part of a protein superfamily and there are over 100 sequences currently known falling into at least 25 families (Hemingway and Ranson, 2000). They can confer resistance by conjugating a reduced glutathione (GSH) to an insecticide or its primary metabolites. This renders the compound inactive. There are several cases of GST-based insecticide resistance phenotypes including cases of organophosphate resistance in *M. domestica* (Clark et al., 1987). GSTs are also probably the cause of the most prevalent form of DDT resistance in mosquitoes (Clark and Shamaan, 1984). In this case, the GSH is believed to act as a cofactor rather than conjugate (Hemingway, 2000; Ortelli et al., 2003).

1.2.2.3 Cytochrome P450 based resistance

Although there are genes encoding for GSTs and esterases present in the *D. melanogaster* genome they have not so far been implicated in insecticide resistance. To date virtually all cases of metabolic resistance in *D. melanogaster* are caused by changes in expression profile or amino acid sequence of cytochrome P450s. For example, there are homologues to the *L. cuprina* resistance-causing esterases in *D. melanogaster* but they have not been shown to affect insecticide activity (Russell et al., 1996; Spackman et al., 1994).

The P450s are a large superfamily found in virtually all organisms from bacteria to mammals. They are also known as mixed function oxidases (MFOs) or monoxygenases (Omura, 1999). They were first described independently by Klingenberg and Garfinkel in 1958 as a carbon monoxide binding pigment in the microsomal fraction of mammalian livers (Hodgson and Tate, 1976). P450s are so called because of their absorbance peak in the optical spectrum of the carbon monoxide bound reduced form of the enzyme (Hodgson and Tate, 1976). P450s catalyse the following reaction (Feyereisen, 1999):

$$S + NADPH + H^+ + O_2 \rightarrow SO + NADP^+ + H_2O$$

They are involved in the endogenous catabolism of compounds such as hormones and in the conversion of xenobiotics into either more useful or less toxic compounds (Graham and Peterson, 1999). P450 nomenclature is based on the homology of the gene

firstly to members of the same family where the homology at the amino acid level is greater than 40%, (this is shown as the first number). The members of a subfamily have greater than 55% homology at the amino acid level (as shown by the letter). Members of each family are then classed by a second number usually dependant on when they are discovered (Feyereisen, 1999).

It would appear that all P450s arose from a precursor similar to CYP51, a lanosterol 14 α-demethylase, a P450 found in all clades. Given the presence of this P450 it has been postulated that P450s have been in existence since before the diversification of prokaryotes and eukaryotes. Virtually all P450 families fit the proposed evolutionary tree, with a few cases of parallel inheritance. Simple unicellular organisms seem to only have a small number of P450s, around 1-5 (Nelson, 1999). In more complex multicellular organisms larger numbers of P450s have evolved as more complex biochemical interactions between a wider range of molecules have evolved. The number of P450s has increased and diversified to allow the synthesis and metabolism of a wider range of molecules. It is unclear whether duplication of P450s drove the evolution of complex organisms or if they are by-products of evolution that facilitated more complex biochemical pathways (Nelson, 1999). Some species have a very large number of these genes; the genome of *Arabidosis thaliana* contains over 350 predicted P450s genes and comparison with the Rice genome sample sequence suggests that this number would appear to be about the standard for both monocotyledon and dicotyledon plants (Nelson, 1999). It is thought that plants have a very high number of P450s as they are biochemically more complex than animals. This is because they are static and so have had to evolve a range of xenobiotics in order to deter animals from feeding on them as they are unable to escape predators by moving (Feyereisen, 1999; Nelson, 1999).

The genome of *D. melanogaster* contains 87 genes predicted to encode functional P450 enzymes. This would appear to be about the average for complex animals, with humans, mice and *C. elegans* all having around 50-90 P450s (Nelson, 1999). Many of these enzymes will be involved in endogenous processes and will therefore be necessary for the development of the insect. It is almost certain that a large number will have evolved to metabolise plant xenobiotics and so increase the range of environments that the fruit flies can survive in (Stevens et al., 2000). It would appear that as many P450s are found in clusters of very similar genes it is highly likely that the diversity is from gene

duplications and if these genes are of benefit to the insect then they will not be lost and so remain functional (Tijet et al., 2001). Examples of such clusters are the two *Papilio glaucus* P450s CYP6B4 and CYP6B5 that have 99.3% homology (Feyereisen, 1999), or the cluster of three CYP6 genes and two fragments of P450s found in *M. domestica* (Cohen and Feyereisen, 1995; Feyereisen, 1995). Examples of clusters of P450s can be seen in Figure 1.2, which shows all the locations of all the predicted P450s in *D. melanogaster.*

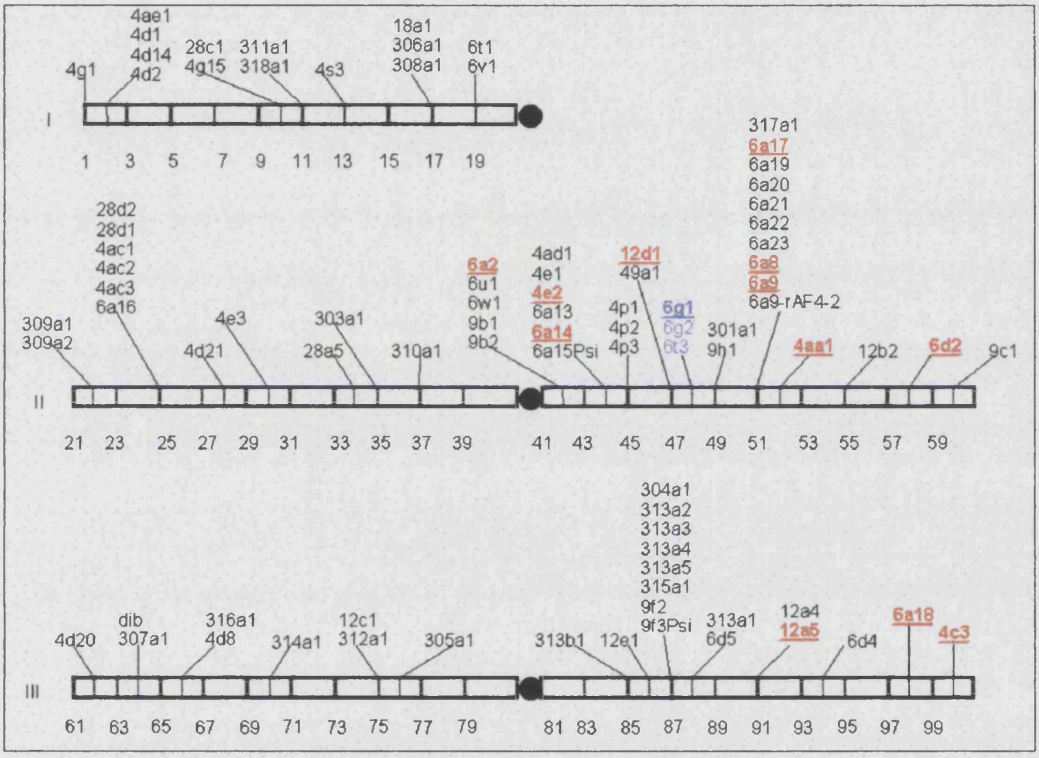


Fig. 1.2 The locations of all the Cytochrome P450 genes in *D. melanogaster*, data taken from http://flybase.net.

This figure shows the position of all the proposed *D. melanogaster* genes. Those highlighted in red and blue are of interest in Chapter 2 for Northern analysis. It also shows that in the case of *D. melanogaster* there are several large clusters of P450s, for example there is a cluster of nine of these enzymes at cytological position 51 on chromosome II, and a cluster of eight P450s at position 87 on chromosome III. It can be seen from the names of the genes in these clusters that these genes are in some cases very closely related. As can be seen in Figure 1.2 there are several clusters of homologous genes, such as the group at position 25 containing *Cyp4ac1 – 3*, or the

cluster at position 87 containing *Cyp313a2 – 5*. These clusters further show how recent the divergence of many P450s is as they are still closely clustered and very closely related, for example *Cyp12d1* and *Cyp12d2* are so closely related that it is impossible to distinguish between them on a micro-array (Le Goff et al., 2003). It will be very interesting in the future to compare the *Drosophila simulans* genome sequence to that of *D. melanogaster* and to compare the localisation and expression of P450s in these two recently separated species is.

In the brief history of insecticide resistance there have been many published cases of insecticide resistance and the majority of the examples of metabolic resistance are due to changes in the P450 profile in a resistant insect. In some cases P450s are induced in the presence of plant xenobiotics such as nicotine, and where this leads to the insect being able to feed on a new previously toxic plant, as already discussed above (Snyder et al., 1995). It is not difficult to see how insecticide resistance could evolve in an analogous way. But instead of evolution over millions of years to tolerate a plant xenobiotic we are seeing the evolution of new cases of insecticide resistance with just 50 years (or in many cases a considerably shorter period of time) of selection (Denholm et al., 2002; Oppenoorth, 1985). What we are seeing in insecticide resistance is Darwin's theory of evolution and the survival of the fittest being tested and proved in a short time period all over the world (Roush and McKenzie, 1987). We see many cases where the up-regulation of a P450 would normally be deleterious to an organism; they show a decreased fitness under normal conditions but in the presence of an insecticide it enables the insect with the mutant form of a P450 to thrive and pass on its genes (Le Goff et al., 2003). There are many laboratory cases where resistance has been induced by a gradual increase of toxin concentration over many generations and this has led to the selection of animals with a slightly higher expression level of one enzyme (Fogleman, 2000; Soderlund and Bloomquist, 1990). However, in many of these cases the up-regulation is not permanent and when the toxin is removed from the diet the enzyme profile returns to that of a susceptible insect. Subsequently the same number of generations are again needed to reselect for the up-regulation of the gene, and resistance (Roush and McKenzie, 1987). It is also the case that a simple amino acid substitution as seen with the alpha esterase of *L. cuprina* and *M. domestica* above can confer resistance (Campbell et al., 1998; Claudianos et al., 1999). However again it is a pre-existing gene being modified rather than a new gene evolving. It is also interesting to note that in

some cases a resistant phenotype is not a new mutation at all. In the case of the E4 esterase duplication in *M. persicae* as discussed above the locus of duplicated esterases is also present in the closely related aphid *M. nicotiniae.* This implies that this mutation occurred in the common ancestor to both these insects prior to the use of chemical insecticides (Anthony et al., 1998b).

Previously known cases of P450-mediated resistance are numerous. The earliest case of known P450 based resistance mechanism was reported in 1960 by Eldefrawi et al who counteracted carbaryl resistance with the methylenedioxyphenyl P450 inhibitor Sesamex (Keseru et al., 1999). Although, as we will see, other earlier forms of resistance were also P450-based but this was not known until later. More recently, there have been many other cases of P450 over-transcription conferring resistance to DDT, carbamates, organophosphates, pyrethroids and many other classes of pesticide (Scott et al., 1994). The house fly genes *Cyp6a1* and *Cyp6d1* and their homologues in *D. melanogaster*, *Cyp6a2* and *Cyp6d2* all confer DDT resistance (Berge et al., 1998). Over-transcription of *Cyp4g8* and *Cyp6b1* in *Heliothis armigera* and of *Cyp9a1* in *H. viriscens* confers pyrethroid resistance to these different insect species (Pittendrigh et al., 1997a). In the case of *H. armigera* two different strains use P450 genes from two different families to confer resistance showing how in some cases an organism has more than one enzyme that can be used to detoxify insecticides.

In some cases, the control of over-transcription is also of interest. In one case, *Cyp6a1* in the housefly is under trans-regulation and is over-transcribed by factors on *M. domestica* chromosome V. The homologue of this gene in *D. melanogaster* is *Cyp6a2*, which also confers resistance to DDT when it is over-transcribed in flies. The transcription of this P450 is also trans-regulated with the regulation machinery located on chromosome III in a region homologous to the trans-regulatory region of the housefly. This shows that not only are there cases of a gene homologue conferring the same resistant phenotype in different species but also that there are cases when it is the same control regions that affect this resistance pattern (Maitra et al., 2000).

Previously in *D. melanogaster,* three P450s have been the most strongly implicated in laboratory selected P450 based resistance. These genes are *Cyp6a2* whose properties and control have already described above. *Cyp6a8* which has been shown to be

involved in DDT resistance and is under the same or similar trans-regulation as *Cyp6a2* (Maitra et al., 2002) and *Cyp12d1* which has been implicated in DDT resistance (Brandt et al., 2002).

1.3 Historical basis of DDT resistance: past attempts to characterise DDT-R

DDT or, 1,1-bis (p-chlorophenyl)-2,2,2-trichloroethane, was first identified as an insecticide in 1939. Being described thus: "The discovery of DDT indubitably heralds a new era in man's ceaseless fight for mastery against disease" by Sir Ian M. Heilbron. DDT was the first chemical insecticide. At the time, it was hailed as a possible cure to malaria by eradicating mosquitoes (West and Campbell, 1950).

Resistance to DDT in *D. melanogaster* is one of the earliest and most studied resistance phenotypes. Since the work of King in the early 1950s a range of resistance phenotypes to DDT (DDT-R) have been seen. Resistance has been generated in two main ways. In both cases an original population of either wild strains or a mixture of wild strains and laboratory strains were collected together and exposed to insecticides. The first method has been to attempt simulate in a laboratory the selection pressure put onto insects that can increase the level of resistance in a population. This is done by exposing insects to an insecticide of choice according to a set regime. Sometimes a certain dose at set times in a generation, in other studies using doses applied at random times to attempt to further simulate field conditions (King and Somme, 1958). This method is exemplified by the work of King, Merrel, Crow and Dapkus and their co-workers who over many years constantly selected for resistance and so isolated resistant lines (Crow, 1954; Dapkus and Merrell, 1977; King and Somme, 1958). The most famous of these resistant strains is probably the 91R strain, which was later shown to over-express *Cyp6a2* (Waters et al., 1992). Other strains showing this resistance mechanism have also been shown to have *Cyp6a2* up-regulated by factors on chromosome III. Dapkus in 1977 was an early proponent of this theory that had also been suggested by Crow in 1954. In many of these cases, there are other resistance factors from other chromosomes having a secondary pleiotropic affect on resistance (Crow, 1954; Dapkus and Merrell, 1977; Groeters and Tabashnik, 2000). The second method for the selection of resistance to be used is best known from the work in the late 1950s and early 1960s from Japan. In these cases as well as selecting for resistance in wild strains, they also attempted to select for

resistance using mutagenesis (Ogita, 1960). These groups used X-rays to produce mutations and so increase the range of mutants available for screening over the more traditional natural selection for resistance used by others (Kikkawa, 1961; Shepanski et al., 1977).

Both these methods have their advantages and disadvantages. The method of Crow and his successors was aimed at creating, in a laboratory, mutations that could in theory, be seen in the field (Crow, 1954). Therefore he saw what he expected would be seen in the field after the repeated use of insecticides. This method has one major downfall; the use of a limited number of starting insects (with limited genetic variability) and the constant selection for resistance limits the phenotypes selected. The second method tries to drive evolution artificially which in theory will lead to a faster selection of a wider range of phenotypes for resistance (ffrench-Constant et al., 1992).

The Japanese group's greatest early success however was with the simple method of collecting strains from areas that had been heavily sprayed with DDT. In the case of Ogita, he collected strains from around Hikone City in Japan (Kikkawa, 1961). One such strain was found to exhibit resistance to many insecticides. The Hikone R strain as it became known was shown to be resistant to the chloro-hydrocarbons BHC and DDT, the organophosphates parathion and malathion, and to several other classes of compound including nicotine sulphate and the carbamate carbaryl (Kikkawa, 1961; Kikkawa, 1964; Ogita, 1960). In the Hikone R strain, they also noticed an interesting case of negative cross-resistance where by the Hikone R strain was susceptible to phenylthiourea, a chemical harmless to the susceptible Canton-S strain which was used as a control for these experiments.

Work done at this time mapped resistance to the now well-known 64.5 cM region of chromosome II. Although other secondary alleles were found on other chromosomes, this one region appeared to cause the cross-resistance phenotype seen in Hikone R. At the time the region and the cause of cross-resistance was named *RI* for resistance to insecticide (Kikkawa, 1961). It was not known if this phenotype was caused by pleiotropic expression of a single gene or if many genes were the cause of the phenotype seen. As well as negative cross-resistance to phenylthiourea it was also observed that there appeared to be a maternal effect, whereby the offspring of a cross

between Canton-S and Hikone R were more resistant to insecticides when the maternal lineage was derived from Hikone R. Kikkawa concluded at the time that this effect was caused by the *RI* locus although he gave no possible reasons for the effect (Kikkawa, 1961).

Some of the mutagenesis work carried out by Kikkawa led to him making the conclusion that the resistance phenotype he had discovered was polyphyletic in origin (Kikkawa, 1961; Kikkawa, 1964). This was based on him work in which a resistant strain was induced in the lab by mutagenizing Canton-S flies. He found that this mutant created an unstable resistant phenotype of intermediate resistance. These mutant strains after further crosses either reverted to a susceptible phenotype, or could be driven to a more resistant form that mapped to the same locus as resistance in Hikone R. His being able to select for resistance led to his conclusion that DDT resistance made use of the same mechanism worldwide. This led Kikkawa to conclude that the origin of resistance was not from a single mutation but from many different flies all showing a mutation causing the same effect. In fact, at the time of his earlier 1961 paper Kikkawa concluded that in all the cases of parathion resistance seen in *Drosophila* around the world, the mechanism was the same (Kikkawa, 1961). After the work of Daborn in 2002, it would appear that he is partly correct (Daborn et al., 2002). It now seems that in wild strains, only one form of DDT and organophosphate resistance has arisen and work based on the DNA sequence of many wild type strains would suggest that there is in fact a single origin of resistance rather than the pleiotropic mechanism originally proposed Kikkawa (Daborn et al., 2002).

Work on *D. melanogaster* showing the 64.5 cM *RI* phenotype has carried on until the present day. Waters and Nix (1988) showed that flies of the Hikone R strain showed two interesting bands in SDS page gels, one of which was present in the susceptible Oregon R strain, the other was not (Waters and Nix, 1988). At the time, Waters and Nix named these proteins P450A with an mw of 59.3 kDa and P450B with an mw of 55.8 kDa. These proteins have been identified by work that will be reported in this thesis. The work has made use of antibodies and micro-arrays, two methods that have been unavailable to previous groups working on this mechanism of insecticide resistance.

1.4 Neonicotinoids, a new class of compounds.

The major classes of insecticides act upon the nervous system of insects as shown in Table 1.1. Until the discovery of the neonicotinoids, insecticides targeting the nicotinic acetylcholine receptor were rare and only had a small share of the total insecticide market. The two main compounds of this type were nicotine and cartap, nicotine is a natural plant xenobiotic. Although nicotine is highly toxic to insects, it is also toxic to mammals. This toxicity has limited its use, although it is still used as a glasshouse fumigant. The second chemical is cartap, which upon entry into the insect is metabolised to the active moiety neristoxin. This chemical however has found only limited use (Tomizawa and Casida, 2003).

The neonicotinoids were originally discovered by Shell in 1970. They developed a new insecticide called nithazine. This was an extremely potent insecticide against corn earworm and slightly active against *M. domestica* but unfortunately it was not as potent against other species so it had a very limited insecticidal spectrum. The second problem with nithazine became apparent upon the commencement of field trials. It turned out that this compound was extremely unstable when exposed to sunlight, unlike imidacloprid, which is considerably more photostable (Wamhoff and Schneider, 1999). This led to a cessation of work on the compound by Shell (Kagabu et al., 2002; Matsuda et al., 2001), although nithazine has subsequently found some use in a housefly trap for use in poultry and animal husbandry (Tomizawa and Casida, 2003). The photostability of a potentially insecticidal compound is an important consideration. The more stable the compound then the longer it will remain in the field. This has both good and bad points; it is good in that the longer it is present on a crop then the more economical the insecticide as it acts for a longer period. The bad point is that the longer a population is exposed to an insecticide then the greater the chance that resistance will arise (Denholm et al., 1983; McKenzie and Batterham, 1998). One of the benefits of imidacloprid is that it has been shown to be partly metabolised by plants and many of these metabolites are as toxic to the target insects as the parent compound. This means that instead of a pest insect being exposed to one chemical it is exposed to a range of chemicals and this is hoped will make resistance, especially metabolic resistance, less likely (Nauen et al., 1998).

The work done by Shell on nithiazine was then taken up by Kagabu who recognised the similarity of this compound to the natural insecticide nicotine and the tree frog nAChR agonist epibatidine. The first step was the substitution of nithazine at the 1 position, which led, firstly to the development of PMNI then to imidacloprid. This realisation led to tests on a range of compounds using a range of different chemistries (Kagabu et al., 2002; Matsuda et al., 2001).

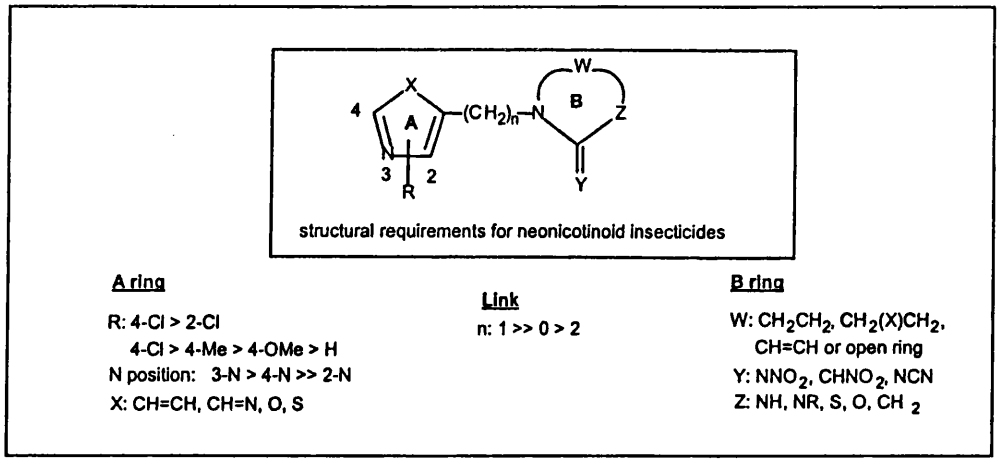


Fig. 1.3 The general structure of neonicotinoids, figure reproduced from Kagabu (2003).

This figure shows the general structure of neonicotinoids. In the case of imidacloprid in the A ring X is CH=CH, R is Cl at position 4 and there is N at position 3. The B ring has W as CH_2CH_2, Y is $CHNO_2$ and Z is NH. In the case of imidacloprid n on the linker between the two rings is 1. All neonicotinoids fall into this general scheme for structure with a great variety of different groups employed to give a range of toxicity against different species. Figure reproduced from (Kagabu, 2003).

Imidacloprid is now the worlds biggest selling insecticide with sales of US$ 455 million in 1999 (Maienfisch et al., 2001). One of the benefits of imidacloprid is its selectivity for insects over mammals. Table 1.4 shows the toxicity to rats and aphids of a range of insecticidal compounds. Two classes of closely related compounds both act on the nAChR. These classes are the neonicotinoids, such as imidacloprid and the nicotinoids such as epibatidine, nicotine and desnitro-imidacloprid. Theses classes of compound are differentiated by their structure, their action as agonists of the nAChRs of invertebrates and vertebrates and their ionisation state under physiological pH (Kagabu, 2003;

Tomizawa et al., 2000). The neonicotinoids are not ionised and are selective for insect nAChRs where as nicotinoids are ionised and are more active against vertebrate nAChRs (Tomizawa and Casida, 2003).

Table 1.4 A comparison of relative toxicity of different insecticides to rats and aphids reproduced from Leicht (1996).

	Toxicity Rat LD_{50} mg/kg	Toxicity Aphid ED_{50} mg/kg	Safety Factor LD_{50}Rat/ED_{50}Aphid
Organophosphate Oxydemeton-methyl	70	0.98	71
Carbamate Primicarb	150	0.50	300
Pyrethroid Cyfluthrin	400	0.024	17000
Neonicotinoid Imidacloprid	450	0.062	7300
Nicotinoid Nicotine	50	>5	<10

This table shows how specific for insects imidacloprid is, with a safety factor of 7300. Only one other insecticide in this table, the pyrethroid cyfluthrin, is safer and this is due to its greater activity against insects, it has a higher LD_{50} against rats than imidacloprid. Many studies have been carried out to test why this should be the case. The structure of nicotine is very close to that of imidacloprid but nicotine is more toxic to mammals than to insects (Leicht, 1996; Tomizawa et al., 2000).

Work with preparations of receptors from a range of insects has shown that imidacloprid displaces the potent nAChR binding compound α-bungarotoxin from cockroach nerve cords (Bai et al., 1991). This is also the case with honeybee and head membrane preparations of house fly heads (Tomizawa and Casida, 2001). It was also shown that imidacloprid binding to nAChRs could be displaced by a range of potent agonists for this class of receptor including nicotine and α-bungarotoxin and the muscarinic agonist atropine (Liu and Casida, 1993). It has also been possible to use imidacloprid to purify polypeptides from insects which also cross reacted with α-

bungarotoxin which would suggest that these would be candidate nAChRs (Tomizawa et al., 1996). The proposed method of action by imidacloprid is to depolarise and block synaptic transmission at the post-synaptic membrane. This action is inhibited by α-bungarotoxin (Lansdell and Millar, 2000; Matsuda et al., 2001) and is shown in Figure 1.5 from Tomizawa and Casida 2002.

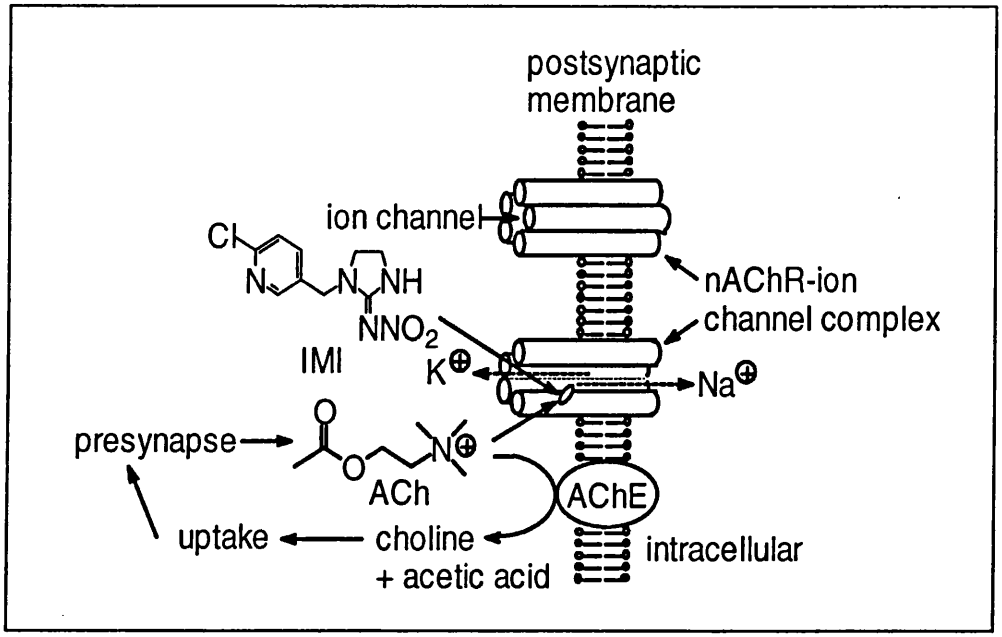


Fig. 1.5 Action of imidacloprid on the nAChR reproduced from Tomizawa and Casida (2002).

This figure, taken from Tomizawa and Casida 2002, shows cholinergic neurotransmission mediated by the nAChR on the postsynaptic membrane. The neurotransmitter acetylcholine (ACh) released presynaptically binds to the nAChR, leading to activation of the ion channel. ACh is then hydrolysed by AChE (Tomizawa and Casida, 2003).

A range of work has been done on both mammalian nAChRs and those from insects. To date a functional *D. melanogaster* nAChR has not been assembled *in-vitro*, but work has been done using a heterodimer co-expressing a *Drosophila* α-subunit with a chicken β-subunit to give a *Drosophila*-SAD-Chicken-β2 receptor. A comparison of this heteromer to a chicken α2β2 receptor showed for the first time that the presence of an insect α-subunit confers enhanced sensitivity to imidacloprid. This expression work was done using *Xenopus* oocytes (Buckingham et al., 1997; Matsuda et al., 2001).

Work has also been carried out to try to deduce why the neonicotinoids are so potent against the insect nAChR but are virtually ineffective against that of vertebrates. This is of course an extremely important ecological benefit. In the past, many insecticides have been used and then found to harm non-target organisms, including some that are actually beneficial in terms of pest control. The most famous case led to banning of the use of DDT in 1973 as, although, it is potent against insects when compared to mammals its high level of hydrophobicity leads to its bioaccumulation in cell membranes. This leads to mammals and birds eventually receiving a toxic dose, as they are unable to excrete DDT. This had a serious ecological impact that some species are still recovering from (Smith, 2001). Even today, the organophosphates are controversial insecticides with high mammalian toxicity. Work by Tomizawa in 2000 using imidacloprid and thiacloprid found that they were over 100 times more toxic to houseflies than the desnitro form of imidacloprid and descyano form of thiacloprid (Tomizawa and Casida, 2000; Zhang et al., 2000). It was also found that the exact opposite was true in mice where the modified insecticides were toxic against mice (the descyano thiacloprid was more toxic than desnitro imidacloprid) and the parent molecules virtually ineffective against mice (Tomizawa and Casida, 2000). The authors hypothesised that the nicotinic binding site differed in mammals and insects. A chemical with a negative Y group in Figure 1.3 was supposed to be better able to bind with a cluster of positively charged residues in the D loop of the insect nAChR whereas in mammals this binding site is negatively charged and so chemicals with a positive Y group are better able to bind. This theory is shown in Figure 1.6 and explains how some chemicals can be more potent against insects and some more toxic to mammals (Tomizawa and Casida, 2000; Tomizawa and Casida, 2003).

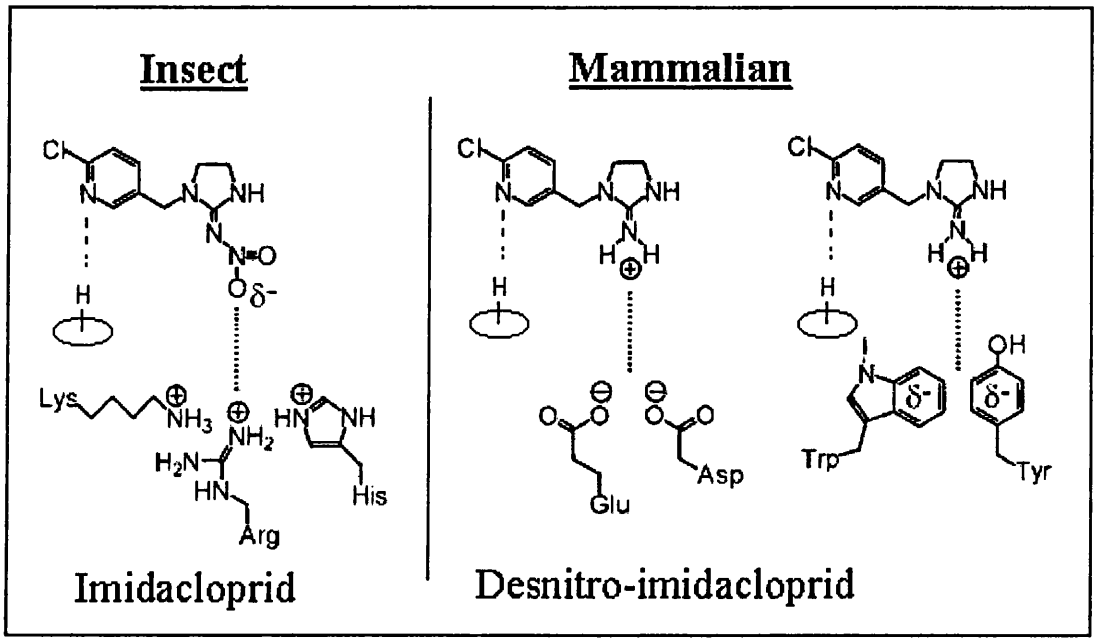


Fig. 1.6 Proposed binding of chemicals to insect and mammalian nicotinic receptors figure modified from Tomizawa (2000) and Tomizawa and Casida (2003).

Figure 1.6 shows the proposed interaction between mammalian nAChR and a sample nicotinoid, desnitro imidacloprid and an insect nAChR and the neonicotinoid imidacloprid. As can be seen in both receptors the heavily electron-negative nitrogen of the pyridol ring forms a hydrogen bond with the receptor but in the case of mammalian nAChR a group of positively charged subunits interacts with the urea of the desnitro imidacloprid and in the insect receptor it is the negative nitro group that interacts with positive residues. In both instances the distances between residues in the receptor is also believed to affect the interaction of chemicals with the nAChR (Tomizawa and Casida, 2001; Tomizawa and Casida, 2003; Tomizawa et al., 2000).

1.5 Aims of thesis.

1: To map and clone *Cyp6g1* to demonstrate that this is the gene responsible for the 64.5 DDT-R resistance phenotype.

2: Examine changes in levels of mRNA and protein in resistant and susceptible strains to show that resistant strains show a higher level of *Cyp6g1* compared to susceptible strains through out the life cycle of the insect.

3: Examine pattern of cross-resistance to the new neonicotinoid class of insecticide and to organophosphates.

4: To investigate the differing P450 expression profiles seen in a range of resistant strains and to investigate if a similar resistance mechanism is also present in the closely related *D. simulans* species.

5: To attempt to establish a screen for the analysis of the insecticide metabolites in strains over expressing CYP6G1 to see how the up-regulation of a single enzyme metabolises such a wide range of insecticides.

Ch 2. DDT and neonicotinoid resistance in strains studied.

2.1 Introduction

The aims of this chapter are: 1) to demonstrate that DDT resistance (DDT-R) also confers cross-resistance to imidacloprid, 2) to examine the effect of piperonyl butoxide (PBO) and deficiency strains on the DDT-R phenotype, 3) to map resistance using RFLP analysis and 4) to try to establish the gene responsible for resistance using northern analysis.

Work in our laboratory was originally carried out on three DDT-R strains, EMS1, Hikone R and Wis1. The strain Hikone R is the original strain used by Ogita and Kikkawa (Kikkawa, 1961; Ogita, 1960). Wis1 is a laboratory strain from Wisconsin isolated by Dr. B. Pittendrigh (Brandt et al., 2002) and EMS1 is a strain isolated by EMS mutagenesis in Bath by Janet Yen. Using the mapping scheme in Chapter 4 DDT resistance in the Hikone R, EMS1 and Wis1 strains were all mapped to the chromosome 2 64.5 cM locus. Further P-element based mapping was also carried out by Dr. Phillip Daborn as this localised resistance to five open reading frames including a cluster of three P450s (Daborn et al., 2001).

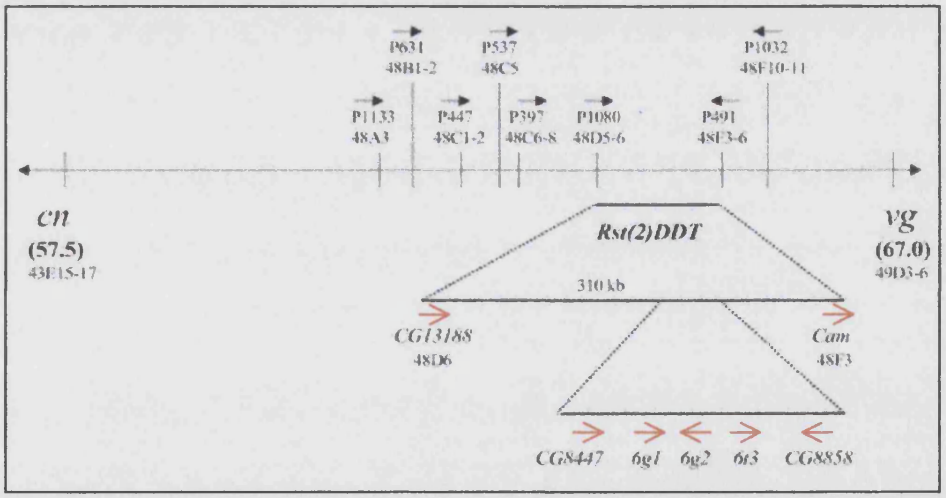


Fig 2.1 Mapping of resistance to DDT relative to P-elements Daborn et al (2002).

Figure 2.1 shows the location of DDT resistance relative to a number of P-elements. Black arrows indicate the direction that resistance maps to relative to each P-element, red arrows indicate the direction of transcription for each gene (Daborn et al., 2001).

Dosage mortality curves were generated to DDT and to imidacloprid in all the strains of interest and for crosses between resistant strains and the susceptible Canton-S strain to determine the degree of dominance (Georghiou and Taylor, 1977; Kikkawa, 1961). Crosses were also carried out between resistant strains to test if the same mechanism of resistance was present in all the strains used.

As P-element mapping had placed resistance near a cluster of three P450s, tests were carried out using the P450 inhibitor PBO. This is a synergist that has been shown to inhibit many P450s and some esterases (Feyereisen, 1999) and so can be used as a simple test to indicate if a resistance mechanism is P450 based. As PBO does not affect all P450s a positive result indicates that the mechanism is P450 based but a negative result simply shows that the mechanism is either not P450 based at all or is based on a P450 that is unaffected by PBO (Liu and Yue, 2000; Soderlund and Bloomquist, 1990).

Further mapping was also carried out using a restriction fragment length polymorphism, RFLP. An RFLP within our hypothesised resistance gene allowed the use of a PCR and digestion assay to determine if a recombinant insect had the resistant gene and either a susceptible or resistant 5' or 3' flanking region. This experiment was done to determine whether the cause of resistance was up- or downstream of our resistant gene. By using recombination to separate a resistant form of *Cyp6g1* from the resistance causing mutation we risk not discovering a mutation closely linked to the gene. This is because the closer to the gene the resistance causing mutation is, the less likely it is to find a recombinant between the gene and this locus.

The third part of this chapter experiments that used Northern analysis to test whether resistance is due to the over-transcription of a P450. We tested the three P450s in the cluster near position 64.5 cM and also selected other P450s that could be the cause of resistance but were in other locations in the *D. melanogaster* genome. We tested homologues of P450s that have been implicated in insecticide resistance in other species. This was done using a BLAST search to identify the nearest *D. melanogaster* gene at the amino acid level to any P450 previously involved in either resistance to insecticides or tolerance to plant toxins in any other insect species. We also made probes for all P450s that have been implicated in resistance in any other *D.*

melanogaster strains. The search identified 15 different P450s. These were then screened for up-regulation in the resistant strains. It was important to screen for P450s at other positions around the genome as there was a strong possibility that the large range of cross resistance previously reported in Hikone R could be caused by trans-expression of one or many genes scattered through the genome rather than the over-expression of one gene.

2.2 Methods

2.2.1 Dosage Mortality Curves to detect cross-resistance between DDT and imidacloprid.

Dosage mortality curves for imidacloprid were generated for the homozygous resistant strains EMS1, Wis1 and Hikone R and the susceptible strain Canton-S. Heterozygous crosses paired EMS1 females with Wis1, Hikone R and Canton-S males; Wis1 females were crossed with EMS1, Hikone R and Canton-S males; Hikone R females were crossed with EMS1, Wis1 and Canton-S males; while Canton-S females were crossed with EMS1, Wis1 and Hikone R males. Results were analysed using the computer program POLO (Robertson et al., 1980). Data were then plotted using a Probit scale, corrected for control mortality using Abbott's formula (Abbott, 1925; Busvine, 1971; Finney, 1952).

2.2.1.1 Contact assay for DDT resistance.

For DDT bioassays 20 adult female flies 24 h post eclosion were placed in glass vials with interior surfaces evenly coated with varying concentrations of DDT (Sigma) dissolved in 200 µl acetone (Fisher) and allowed to air dry. The vials were sealed with cotton wool soaked in 5% sucrose solution (Sigma). Mortality was scored after 24 h with flies being unable to move being scored as dead. Between 5 and 7 different insecticide concentrations were used for each cross with between 3 and 6 replicates at each concentration. Data were then analysed by POLO (Robertson et al., 1980).

2.2.1.2 Larval assay for imidacloprid resistance.

For imidacloprid bioassays 50 eggs were placed in vials containing 5g pre-mixed dry *Drosophila* food (Philip Harris Scientific), 6 ml water with varying imidacloprid (supplied by Syngenta) concentrations and the number of adults to emerge were counted. Between 5 and 7 different insecticide concentrations were used for each cross

with between 3 and 5 replicates at each concentration. Data were then analysed by POLO.

2.2.2 Test for synergisation of the response to imidacloprid and DDT using the P450 synergist PBO.

A preliminary experiment was carried out using a range of PBO (Sigma) concentration dissolved in 1 ml 1:1 acetone and ethanol diluted in 9 ml H_2O and shaken well to form an emulsion. 1ml of dilute PBO was combined with 5 ml water and 5 g dry fly food. 50 eggs were then applied to the food and the number of adults to emerge was counted. Although this method showed early results it was not repeatable, possibly due to PBO being very hydrophobic so it is very difficult to spread evenly throughout a water based fly food. Whilst it is possible to achieve an emulsion with PBO in water it is very difficult to spread the PBO evenly through the food. Because of this, the method used by Pittendrigh was used to treat adult flies with DDT (Brandt et al., 2002).

The second experiment used DDT as the test insecticide using glass vials coated with 1 µg PBO dissolved in acetone in the same method outlined above for DDT coated vials. 20 adult flies were placed in each of the vials that were then stoppered with cotton wool soaked in 5% sucrose and left at 25°C for 3 h. The flies were then transferred to vials coated with varying levels of DDT for a further 24 h. The number of flies to die from the PBO was recorded so the actual number of live flies placed onto DDT was known. Mortality was then assessed and the data entered into POLO.

2.2.3 Using DDT and imidacloprid on different life cycle stages.

In order to more fully compare the cross resistance of Hikone R to imidacloprid and DDT it was decided to use DDT as a larvicide and imidacloprid in a contact assay on adults. Varying concentrations of imidacloprid were dissolved in acetone and applied to glass vials as described in Section 2.2.1 for DDT. At up to a concentration of 200 µg imidacloprid/vial no significant and repeatable response to either of the strains used was observed so this experiment was discontinued. It was also attempted to use DDT in the larval assay described in Section 2.2.1 but no repeatable response in either resistant or susceptible *D. melanogaster* was observed at up to 100 µg DDT/vial. This is probably because the insecticide was not being evenly spread throughout the food due to the impossibility of dissolving DDT in water because of the hydrophobicity of the molecule

so the larvae were able to avoid the insecticide. This led to this series of experiments being discontinued.

2.2.4 Deficiency strains.

As it was believed that resistance was moderated by a P450, and it was known that resistance is dominant, it was necessary to know if the resistance ratio was the same when a resistant chromosome was combined with a susceptible chromosome (R/S) as it was with a chromosome deficient for the resistance locus (R/Df). It was planned to carry out a variety of dosage mortality experiments on *D. melanogaster* strains with a chromosomal deficiency over the locus containing *Cyp6g1*. Two different strains were identified as possible candidates for this deficiency, 4960 and CB21. However experiments with 4960 on DDT and imidacloprid showed that there was no observable differences with flies crossed with Hikone R with and without a deficient chromosome spanning *Cyp6g1*. This would suggest that R/S is the same as R/Df. Although it is not clear if the 4960 strain covers *Cyp6g1* as the information on the strain is unclear. This result is consistent with resistance being dominant (ffrench-Constant and Roush, 1991). The second deficiency strain, CB21, proved to be slightly resistant to both imidacloprid and DDT, although it does not contain the Accord element mentioned previously so it is probably not *Cyp6g1* mediated resistance, but as it is very difficult to distinguish resistant flies from "slightly less" resistant flies especially when working with a low resistance ratio this experiment was discontinued.

2.2.5 Restriction Mapping to localise the resistance-causing locus.

Fine scale mapping of *Cyp6g1* was carried out to localise the resistance locus. Resistance has been mapped between two chromosome 2 phenotypic markers, *cn* at 57.5 cM and *vg* at 67.0 cM as shown by Daborn (Daborn et al., 2001). From this information we can use recombination against these two markers to try to further map resistance. The following cross was used:

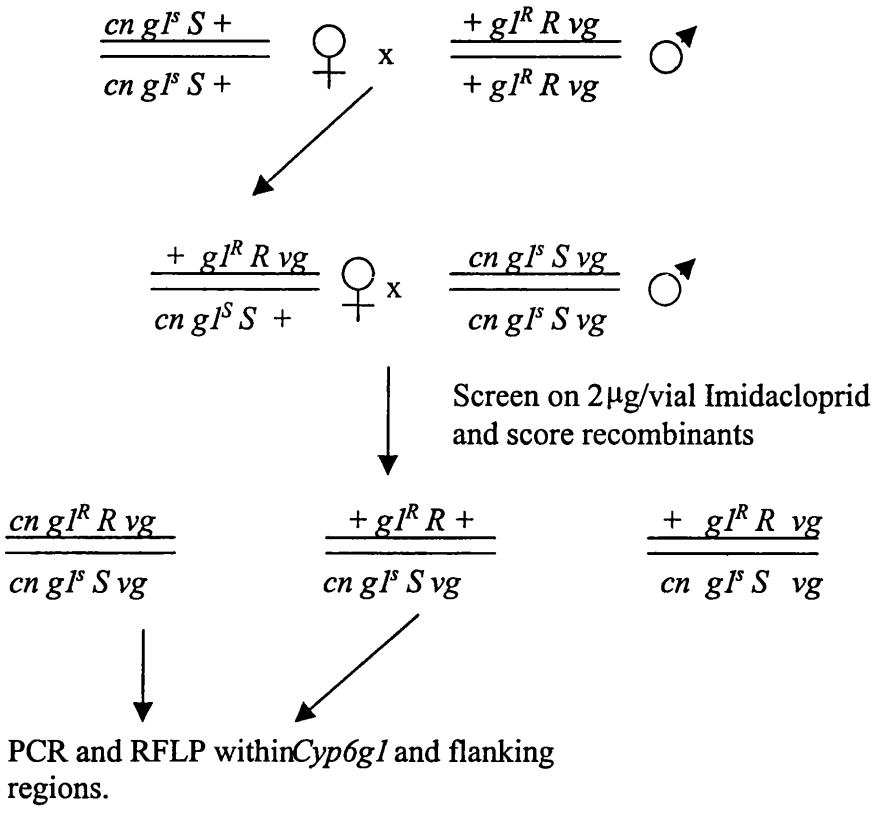


Fig. 2.2 Recombinant cross.

The fly cross shown in Figure 2.2 allows us to identify recombinants to either side of *Cyp6g1*. RFLP analysis then allows us to determine if the gene is of the resistant or susceptible genotype.

Flies were screened for resistance in a larval bioassay containing 2 µg/vial imidacloprid by allowing the F1 virgin females crossed to *cn vg bw* male flies to lay eggs on food for three days. The parents were then transferred to new vials. Emerging recombinant flies were crossed to form new lines.

Approximately 250 white eyed (*w*) vestigial (*vg*) flies and 250 flies that were wild type for eyes and wings and resistant to imidacloprid were then tested for a restriction fragment length polymorphism (RFLP) within intron 2 of *Cyp6g1*.

Discrimination between the resistant and susceptible genotypes of *Cyp6g1* was done by preparing DNA from single flies by squashing each fly with a pipette tip containing 50 µl homogenisation buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl and 200 µg/ml Proteinase K (Sigma)) which was then expelled after 10 sec. This is left at 37°C for 30 min then the Proteinase K is inactivated by heating at 95°C for 5 min. 2 µl of this DNA preparation was then used in a 20 µl PCR using 2 µl 1 x Buffer (Promega), 2.4 µl 50 mM Mg^{2+}, 2 µl 10 mM dNTPs 25 µl/ml, 2 µl forward primer CG8453F (5'-ATT CGC ACC AAG CTG ACT CCC GT –3') and 2 µl reverse primer CG8453R (5'-ATG ACC CAC CGC CCT CCA CCA -3') at 10 µg/ml, 0.2 µl *Taq* (Promega) and 7.4 µl dH_2O. This was then placed in a PCR block using the following touchdown PCR program: 95°C 3 min, 95°C 1 min, 72-65°C 1 min (dropping 1°C each cycle), 72°C 1 min, then 33 cycles with an annealing temperature of 65°C 1 min and 72°C for 6 min.

The resulting PCR product was then digested using DDE1 (NEB) for 1 h at 37°C and the reaction mixture run on a 1.5% agarose gel. A PCR product of ~360bp was scored as the susceptible *Cyp6g1* genotype and PCR products of ~550bp and ~360bp was scored as the resistant *Cyp6g1* genotype.

2.2.6 Northern analysis of putative resistance associated cytochrome P450 genes.

Northern analysis of polyA+ mRNA was carried out with PCR derived probes from several P450 genes previously reported to be involved in insecticide resistance in *Drosophila* and other insect species. TRI Reagent (Sigma) was used to isolate total RNA from adults 1-3 days post-eclosion from the resistant strains Wis1, Wis1 lab, Hikone R and EMS1 and the susceptible strains Canton-S and y[1]w[1]. PolyA+ mRNA was then isolated using the PolyATtract mRNA Isolation System (Promega). PCR based probes were made using the PCR conditions 95°C 3 min, 95°C 1 min, Annealing temperature, as shown in Table 2.3, for 1 min, 72°C 1 min and 72°C 5 min. PCR was carried out using Taq (5 U), 1 x buffer, 2 mM Magnesium Chloride and 10 mM dNTPs from Promega. Oligonucleotide sequences are shown in Table 2.3. Probes were labelled using ~25 ng DNA with a Prime-It II Random Primer Labelling Kit (Stratagene) with [α-^{32}P] dCTP. Electrophoresis of RNA and Northern blotting were performed using standard methods (Sambrook et al., 1989).

Table 2.3 PCR conditions and primers used for generation of probes.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temp. (°C)
Cyp6a2	TAC CAC CGC AAC TTC AAC TAC TG	GGC GCA TGT CCT TCC ACT T	60.9
Cyp4e2	CTT GGC TGG GAT TAG GTC TC	CCG ATT TGT GGG CTT CA	59.6
Cyp6g1	ATT CGC ACC AAG CTG ACT CCC GT	ATG ACC CAC CGC CCT CCA CCA	59.0
Cyp6g2	GCC ATG GAC GTT CTG TGT TC	GCG TGT GGA GCT TGC GAT TC	59.0
Cyp6t1	GAG CTA ATC CGC CAG GTG TTG A	AGC AGC CGG ATA GAG ACG CAG TG	62.0
Cyp6a17	ACG ACG AGG TAC ACC CGC TTT TC	GCC GCA GAT GAC GCC ACT TC	60.9
Cyp6a9	TTA CTG AAA TGG CGT CGT G	TCG TCC TCC GTG TTG TGA T	57.0
Cyp6a8	CAG CAT GGC GTT GAC TTA C	CAT TGG CCA TGA CAC TAC	57.0
Cyp6aa1	CGT TTG CTT TCG CCC CCA CAG	GCA CCC AGA TCC GCA CCA GAG A	63.7
Cyp6d2	ATC CCT AAT CGC GGG TCT GCT GTA	CCC GCG ATC GTG GAA ACT GG	64.7
Cyp12a5	GTC GCA AAA ACC CAT CGT CTT CTC	AGC CAT CCC TTC CCA TCA TTC C	63.7
Cyp6a18	CCT ACT GGC GAT CGT GAC CTA	TTT TGC CCG ACG TGA ATG T	60.9
Cyp12d1	TTA AGG AAA TCC GCG ATC CA	ATC CGT GAA TTT GAA GGG GA	55.0
Cyp4c3	CTT GGC CCG AAA TTT TTA GCA CTT	CAC TTG GCC CAC TTT TGA GAG CAG	62.4

This table shows the sequences of the primers used for generating probes for Northern blots and the optimal annealing temperature for each primer pair.

2.3 Results

The results in this chapter are an expansion, and more detailed investigation, of those shown in Daborn *et al* 2001. I shall highlight the role I played in our early investigation of imidacloprid resistance and present our early findings on P450 over-transcription, the further mapping work done using RFLP analysis and then move onto the dosage mortality curves generated for imidacloprid and DDT on a range of susceptible and resistant strains and using F1 crosses between those flies.

2.3.1 Dose mortality curves for susceptible, resistant and F1 hybrids for DDT and imidacloprid.

In this section the cross resistance between DDT and imidacloprid will be reported and discussed using the strains Hikone R, EMS1, Wis1 and Canton-S. All dosage mortality curves (DMCs) are based on data generated from adults for DDT and larvae for imidacloprid. It was attempted to use DDT as a larvicide and imidacloprid in a contact bioassay on adult *D. melanogaster*. It is hypothesised that this lack of toxic effect is due to the extremely hydrophobic properties of DDT, which means that is can not be added to dry fly food with a constant concentration throughout the food. However, no mortality was observed using up to 100 μg/vial DDT on either Hikone R and Canton-S.

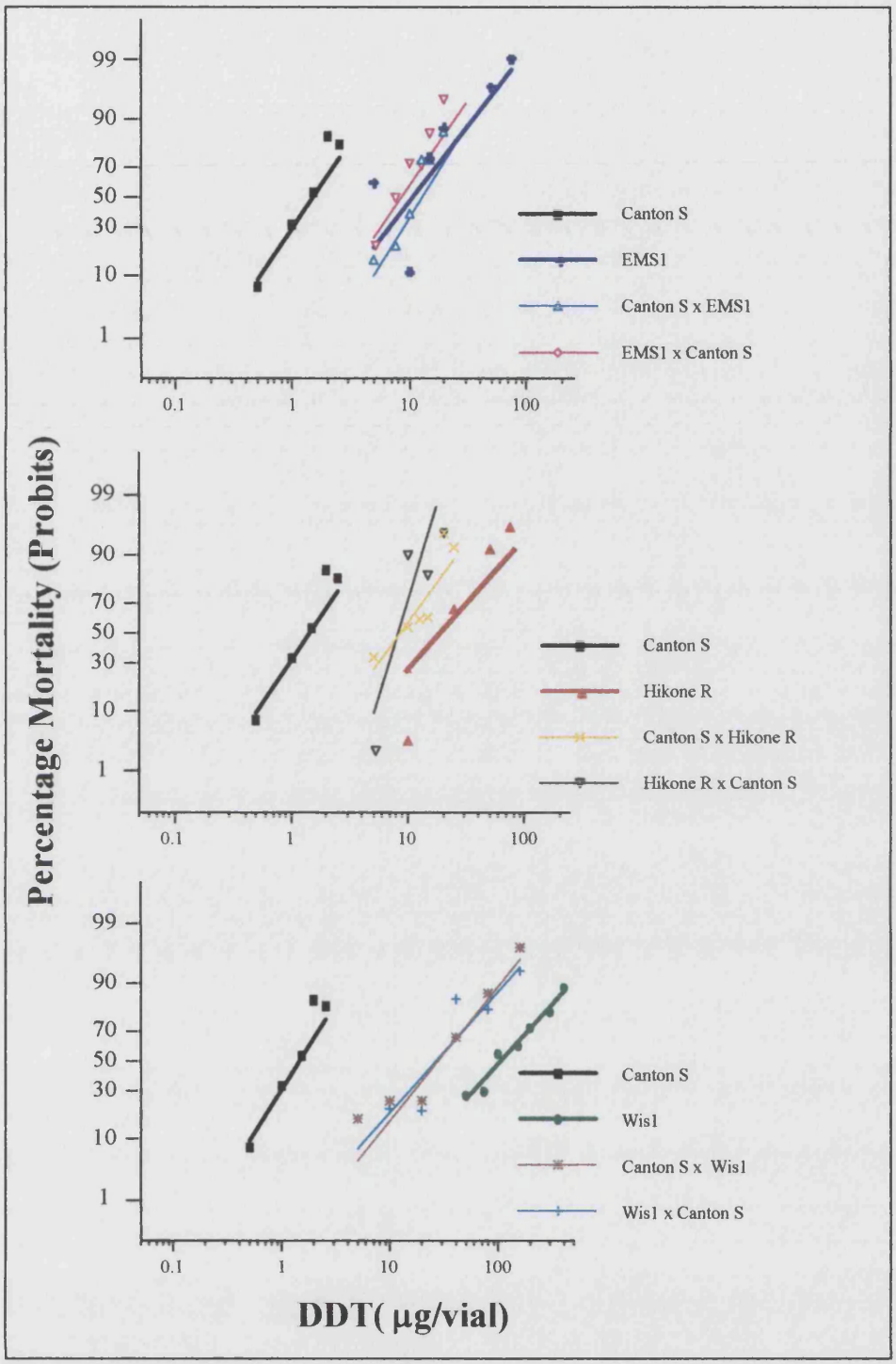


Fig. 2.4 Dosage mortality curves testing resistance to DDT in resistant and susceptible flies and their F1 progeny.

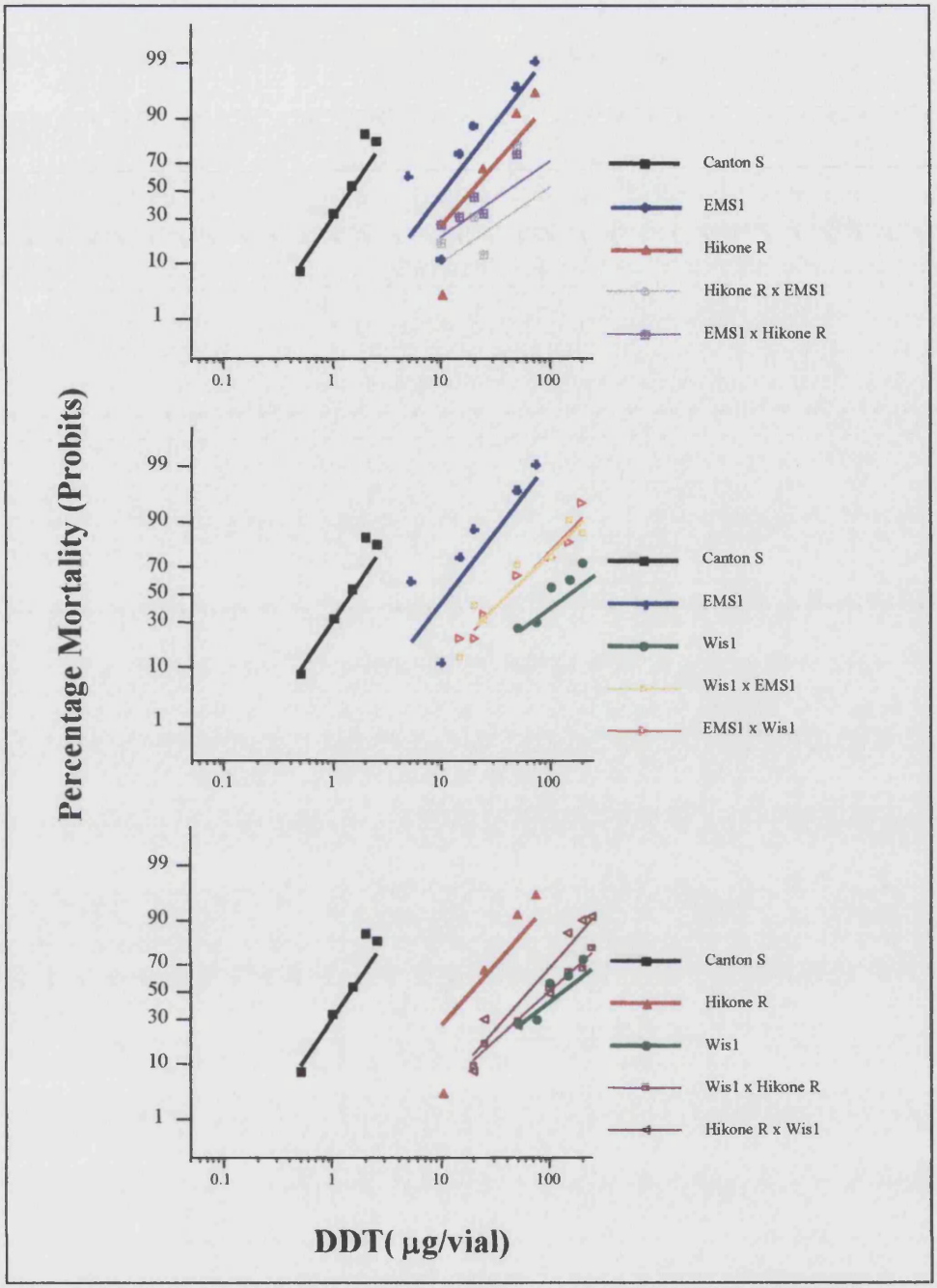


Fig. 2.5 Reciprocal crosses between different resistant strains on DDT.

These figures show a single Canton-S curve that is repeated in each DMC. Figure 2.4 shows that the strains EMS1, Hikone R and Wis1 are all resistant to DDT. From the F1 crosses with the susceptible strain Canton-S it is demonstrated that the resistance mechanism is dominant as the F1 cross shows resistance to DDT. Resistance is not fully dominant as there is a slightly lower level of resistance in the F1 progeny when compared to the resistant parent strain (Bourguet et al., 2000).

Figure 2.5 shows that the resistance mechanism employed by the resistant *Drosophila* is the similar in all the strains. Resistance appears to vary slightly between EMS1 and Hikone R, and the F1 cross shows the F1 progeny to be more resistant to DDT, this would suggest that there is a cumulative effect of crossing two slightly different mechanisms. However, when Wis1 is compared to either EMS1 there is a considerably larger shift in the resistance for Wis1 and the DMC of all the F1 progeny fall between the lines for both resistant parents. This implies that a second mechanism could be involved in the Wis1 strain and it is diluted by the addition of chromosomes from EMS1. This second factor influencing resistance could possibly be due to fitness of the *D. melanogaster* or due to varying transcription levels of different P450s or other resistance mechanisms (Groeters and Tabashnik, 2000). This theory has been tested using micro-arrays by Dr. G. Le Goff and by quantitative real time RT-PCR the results will be discussed in Chapter 3.

Tab. 2.6 Detailed results of DDT resistance in our strains.

Strain	N	LD_{50}	(90%CI)	(95% CI)	Slope	S.E.	Resistance ratio
Canton-S	780	1.45	1.22-1.72	1.17-1.80	2.91	0.25	1.00
EMS1	800	10.50	6.73-13.74	5.79-14.24	2.53	0.24	7.24
Hikone R	780	18.97	13.03-24.75	11.71-26.03	2.21	0.18	13.08
Wis1 lab	780	41.74	29.32-53.04	26.51-55.34	1.98	0.2	28.79
Wis1	800	104.41	91.4-117.4	88.7-120.1	2	0.18	72.01
Canton-S x EMS1	600	10.36	8.69-11.90	8.27-12.26	4.09	0.38	7.14
EMS1 x Canton-S	600	7.66	6.64-8.56	6.40-8.74	3.69	0.32	5.28
Canton-S x Hikone R	600	8.61	5.32-11.1	4.31-11.66	2.62	0.26	5.94
Hikone R x Canton-S	340	8.16	6.58-9.50	6.15-9.79	6.35	0.54	5.63
Canton-S x Wis1	600	13.02	8.89-17.37	7.78-18.62	1.62	0.21	8.98
Wis1 x Canton-S	600	38.46	26.0-106.0	24.51-179.7	1.13	0.22	26.52
EMS1 x Hikone R	660	26.11	21.5-32.1	20.7-33.5	1.95	0.21	18.01
Hikone R x EMS1	480	35.01	27.7-44.9	26.5-47-2	2.27	0.33	24.14
EMS1 x Wis1	760	40.78	35.4-46.7	34.3-47.9	1.99	0.15	28.12
Wis1 x EMS1	760	37.63	30.8-45.3	29.4-47.1	1.88	0.14	25.95
Hikone R x Wis1	660	101.06	88.9-115.0	86.7-118.0	1.78	0.17	69.70
Wis1 x Hikone R	720	66.04	55.9-77.3	53.9-79.9	2.22	0.16	45.54

This table gives a detailed summary of the effect of DDT on one susceptible and three resistant strains of D. melanogaster shown in the DMCs above.

The LD_{50} for the susceptible strain Canton-S is 1.45 µg/vial. This is compared to the LD_{50}'s of the resistant strains using the resistance ratio and it can be seen that there is some variance between the different resistant strains, EMS1 has a resistance ratio of 7.24 compared to 13.08 for Hikone R. The strain Wis1 shows a considerably higher level of resistance showing 72.01 fold less susceptibility to DDT than Canton-S. The variety in resistance ratios between resistant strains was originally put down to there being differing amounts of CYP6G1 in the different strains. These interesting variations in resistance ratio are discussed in detail in Chapter 4. The table also shows the results of the F1 crosses carried out between susceptible and resistant flies and shows that the resistance ratios of the F1 generation all fall between the resistant level of the parents

suggesting that resistance is partially dominant with a level of resistance being determined by both the *Cyp6g1* copies from the maternal and paternal chromosomes. This would imply that the over-transcription of one copy of the gene does not affect the transcription level of the gene on the chromosome from the other parent.

As previously noted, the differences in the F1 cross of the resistant strains show interesting results. In the case of crosses involving Wis1 it appears that the resistance ratio is between the two homozygous parent strains, and also that it is the cross where Wis1 is the paternal strain that shows the higher resistance of the two crosses. In the case of the cross between EMS1 and Hikone R we can see that in this case the cross where the paternal line is the more resistant (i.e. Hikone R is the father) the resulting progeny are less resistant than the other cross.

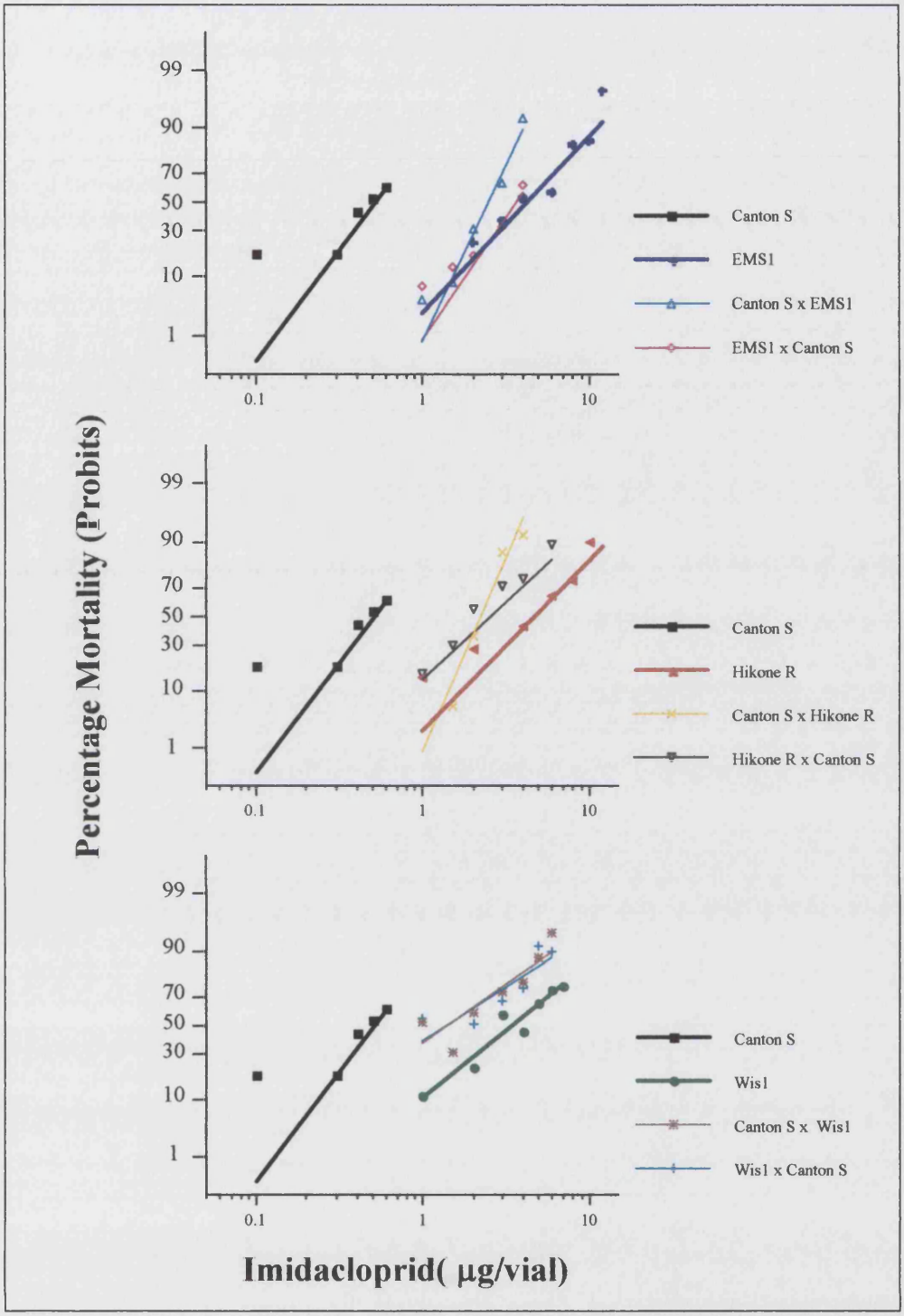


Fig. 2.7 Dosage mortality cures testing resistance to imidacloprid in resistant and susceptible strains and their F1 progeny.

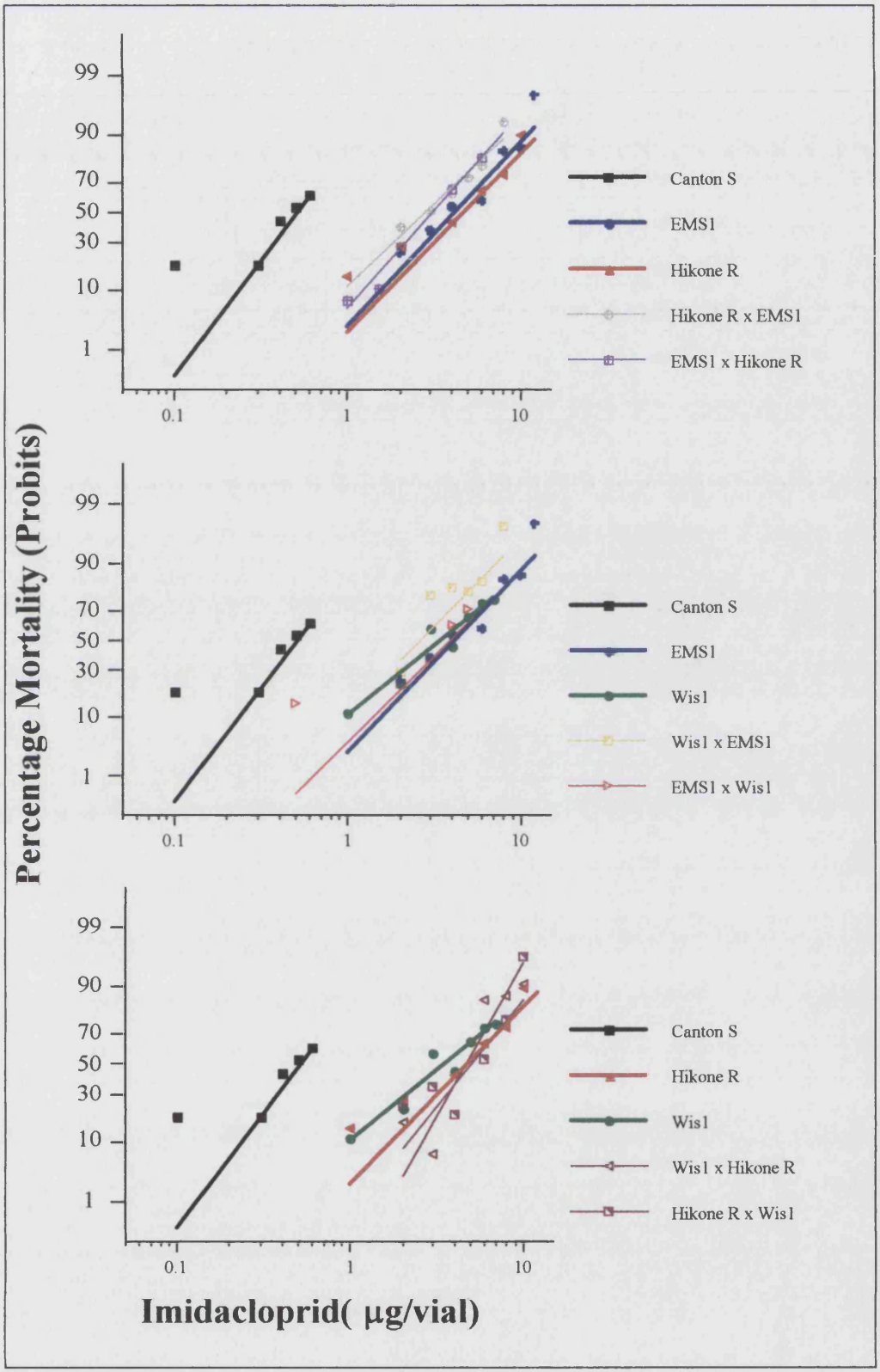


Fig. 2.8 Reciprocal crosses between different resistant strains on imidacloprid.

These figures show a single Canton-S curve that is repeated in each DMC. Figure 2.7 demonstrates that resistance to DDT confers cross-resistance to imidacloprid. As with DDT, resistance to imidacloprid is dominant over the susceptible Canton-S strain in all three resistant strains tested. The three DMCs shown in Figure 2.8 demonstrate that each strain used has a very slightly different level of resistance to imidacloprid. It is interesting to note that the differences between strains tested on DDT are not the same as the differences seen on the strains tested with imidacloprid. All the resistant strains seen, and their reciprocal crosses, show virtually identical levels of resistance. Possible reasons for this include there being a range of other P450s being over-transcribed at the differing life cycle stages used in the two different bioassays and this could affect the resistance levels. It would appear that, as suggested by Kikkawa and Ogita, in some strains a second resistance mechanism may confer DDT resistance, but that this does not affect imidacloprid resistance (Kikkawa, 1961; Ogita, 1960). This will be discussed in depth in Chapter 5.

Tab. 2.9 Detailed results of imidacloprid resistance in all our strains.

Strain	N	LD_{50}	(90%CI)	(95% CI)	Slope	S.E.	Resistance ratio
Canton-S	1500	0.53	0.42-0.68	0.40-0.72	3.86	0.97	1.00
EMS1	1850	4.11	3.19-4.97	2.99-5.13	3.11	0.36	7.75
Hikone R	1600	4.74	3.71-5.75	3.49-5.96	3.00	0.57	8.94
Wis1	4000	3.50	2.52-4.30	2.29-4.46	2.34	0.22	6.60
Wis1 lab	2250	2.18	1.76-2.53	1.67-2.59	3.25	0.47	4.11
Canton-S x EMS1	1700	2.35	2.06-2.68	2.01-2.75	6.14	0.67	4.43
EMS1 x Canton-S	1750	3.42	2.98-3.94	2.90-4.06	3.26	0.57	6.45
Canton-S x Hikone R	1750	2.16	1.92-2.41	1.88-2.47	6.86	0.48	4.08
Hikone R x Canton-S	1900	2.10	1.84-2.38	1.79-2.44	2.79	0.21	3.96
Canton-S x Wis1	4100	1.40	1.17-1.62	1.13-1.66	2.07	0.15	2.64
Wis1 x Canton-S	3850	1.47	1.20-1.73	1.15-1.78	1.86	0.17	2.77
EMS1 x Hikone R	1750	3.19	2.75-3.66	2.66-3.77	3.25	0.32	6.02
Hikone R x EMS1	1300	2.95	2.44-3.50	2.34-3.61	2.75	0.35	5.57
EMS1 x Wis1	1450	3.99	3.45-4.60	3.35-4.73	2.92	0.46	7.53
Wis1 x EMS1	1950	2.29	1.90-2.67	1.82-2.75	3.12	0.28	4.32
Hikone R x Wis1	1800	4.96	3.9-5.8	3.6-5.9	3.59	0.42	9.36
Wis1 x Hikone R	1450	4.63	3.1-5.7	2.6-5.8	5.21	0.70	8.74

This table give the detailed results from Figures 2.7 and 2.8 and clarifies the differences seen between DDT and imidacloprid resistance.

Tables 2.6 and 2.9 shows differences in resistance to DDT and imidacloprid in the same strain, for example the LD_{50} of DDT in EMS1 is 10.5 µg/vial and in Hikone R this figure is slightly higher at 18.97 µg/vial. However, this discrepancy in LD_{50} is not seen with imidacloprid with values of 4.11 and 4.74 µg/vial for each strain respectively. This would suggest that either there is a varying level of *Cyp6g1* over-transcription throughout the life of these two resistant strains. It is also possible that there are other enzymes not yet accounted for that affect resistance. The biggest discrepancy between two resistant strains can be seen when EMS1 and Wis1 are compared. LD_{50} values for imidacloprid are very similar in Wis1, (LD_{50} of 3.5 µg/vial) and in EMS1 (LD_{50} of 4.11 µg/vial), but when we look at DDT we see a considerable difference between these two

strains with EMS1 having an LD_{50} value of 10.5 µg/vial where as Wis1 shows an LD_{50} of 104.4 µg/vial. It is hard to attribute such a discrepancy between two strains as being due only to one P450 especially in the light of the results reported later in this chapter.

2.3.2 Response to PBO, a synergist capable of suppressing P450 mediated resistance.

PBO is a chemical that is capable of inhibiting many P450 enzymes. It is a standard test to determine if resistance is caused by a P450. As not all P450s are susceptible to PBO a result showing no response to PBO does not mean that resistance is not caused by a P450, but if there is a response then it is possible to say that resistance is due to a P450.

Tab. 2.10 Preliminary response to PBO in larval bioassay.

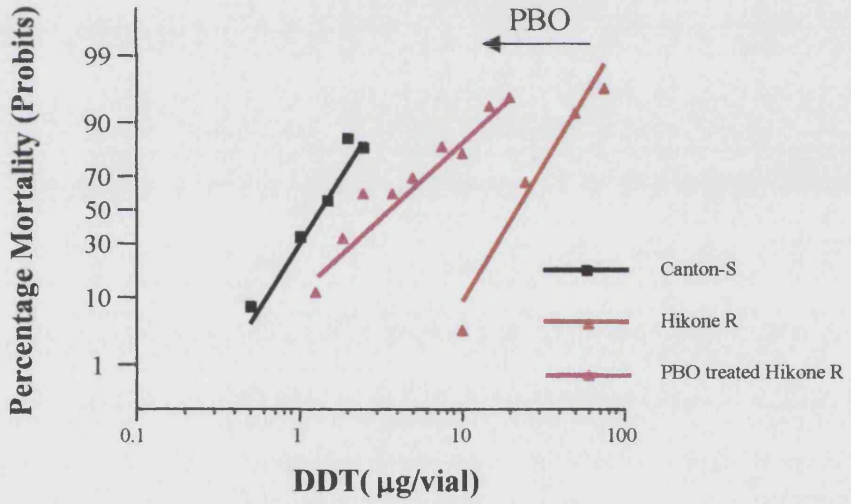
EMS1				Canton-S	
0 PBO 0 IMI	16 µl/vial PBO 0 IMI	0 PBO 2 µl/vial IMI	16 µl/vial PBO 2 µl/vial IMI	0 PBO 0 IMI	16 µl/vial PBO 0 IMI
79	39	60	1	37	2

The value shown is percentage of adults to emerge from 50 eggs with three repeats.

This table shows preliminary data using a larval screen with PBO in the food. Interesting results were seen when 16 µl/vial was applied to vials containing a discriminating dose of 2 µl/vial imidacloprid. Whilst flies survive when only either PBO or imidacloprid are added to the food when both chemicals are added the number of survivors is considerably lowered. It is interesting to note that this dose of PBO kills almost all the Canton-S control. A previous experiment had been carried out to find a dose of PBO to use and it was noted that there appeared to be a dose response to PBO with the resistant EMS1 showing resistance and Canton-S being more susceptible to the chemical. This table however does show that PBO removes imidacloprid resistance. It should also be noted that as 60% of EMS1 eggs were viable when exposed to PBO, it would imply that no PBO susceptible P450s are necessary for *D. melanogaster* to develop to the adult stage. As the flies that emerged were not grown for another generation it cannot be said whether the full life cycle can proceed in the presence of PBO. This is a somewhat unexpected result as *D. melanogaster* has 87 P450s and this result means that either any that are necessary for development are not affected by PBO, or that not one of them is actually necessary for development from the egg to an adult

or that not one of them is actually necessary for development from the egg to an adult insect. It has been commonly assumed that the diversity of P450s seen in both plants and animals is due to "chemical warfare" between the two phyla. Therefore, it is possible that many of the P450s seen in *D. melanogaster* are involved in the metabolism of plant toxins and so are not necessary for development apart from in the presence of certain chemicals (Feyereisen, 1999).

A larger experiment to generate a full dose response to imidacloprid in the presence of PBO was attempted but was not successful after several repeats. This is probably due an inability to spread the PBO evenly through the food. Because of this a second experiment was planned using a more traditional contact bioassay for PBO with the PBO dissolved in acetone and spread over the inside of glass vials, as outlined by Brandt (Brandt et al., 2002).



Strain	N	LD50	(90% CI)	Slope	S.E.	Resistance Ratio
Canton-S	780	1.45	1.22-1.72	2.91	0.25	1.00
Hikone R	780	18.97	13.03-24.75	2.21	0.18	13.08
PBO treated Hikone R	967	2.84	1.73-3.97	1.97	0.21	1.96

Fig. 2.11 PBO assay with DDT on Hikone R.

Figure 2.11 shows that the resistant phenotype of Hikone R is almost completely abolished by PBO. Since it is known that the Hikone R mutation is due to over-expression of *Cyp6g1*, this suggests that the *Cyp6g1* gene product is inhibited by PBO. It is very clear that the resistance phenotype is almost fully lost when the resistant Hikone R strain is exposed to PBO for 3 hours. The LD_{50} in this case is 2.84 µg/vial when compared to 18.97 µg/vial for Hikone R with out PBO exposure and 1.45 µg/vial for Canton-S. The resistance ratios are 13.08 with DDT and no PBO and 1.96 in the presence of both chemicals. This is conclusive proof that the resistance mechanism we are investigating is P450 based. It can be seen in this DMC that the response to DDT in the presence of PBO is relatively flat, with a slope of 1.97 compared to slopes of 2.91 for Canton-S and 2.21 for Hikone R. This shows that the response to PBO is not uniform. This could be due to the nature of the assay, as PBO is very oily although this contact bioassay means we get a complete covering of PBO over the vial some areas still have small droplets so not all the flies have an equal exposure to PBO. We also do not see a complete loss of resistance when Hikone R compared to Canton-S. Possible reasons for this are that 3 hours is insufficient time to inhibit all the P450s present, especially in a strain where there is a heavily over-transcribed P450 present, or possibly some individual insects are better able to rapidly replace the inhibited *Cyp6g1*. It is also of importance that we have no data for a susceptible form of the Hikone R strain. Such insects could very possibly show a different dose response to Canton-S so the difference we see may be the natural level of susceptibility to DDT. This is a theory that it is not possible to test as Hikone R is a 50 year old strain and there is no susceptible form if it.

2.3.3 Using recombination to map resistance.

We now had a target gene of interest to work with and it was decided to look for the actual mutation that conferred *Cyp6g1*over-transcription and so try to answer how this P450 is up-regulated. As we had the sequence of *Cyp6g1* from both Canton-S and Hikone R strains we knew that resistance was not due to a point mutation as both are identical at the amino acid level. There was, however, a silent point mutation that alters the restriction enzyme profile of the gene so we could carry out restriction fragment length polymorphism (RFLP) analysis using a PCR product covering this region.

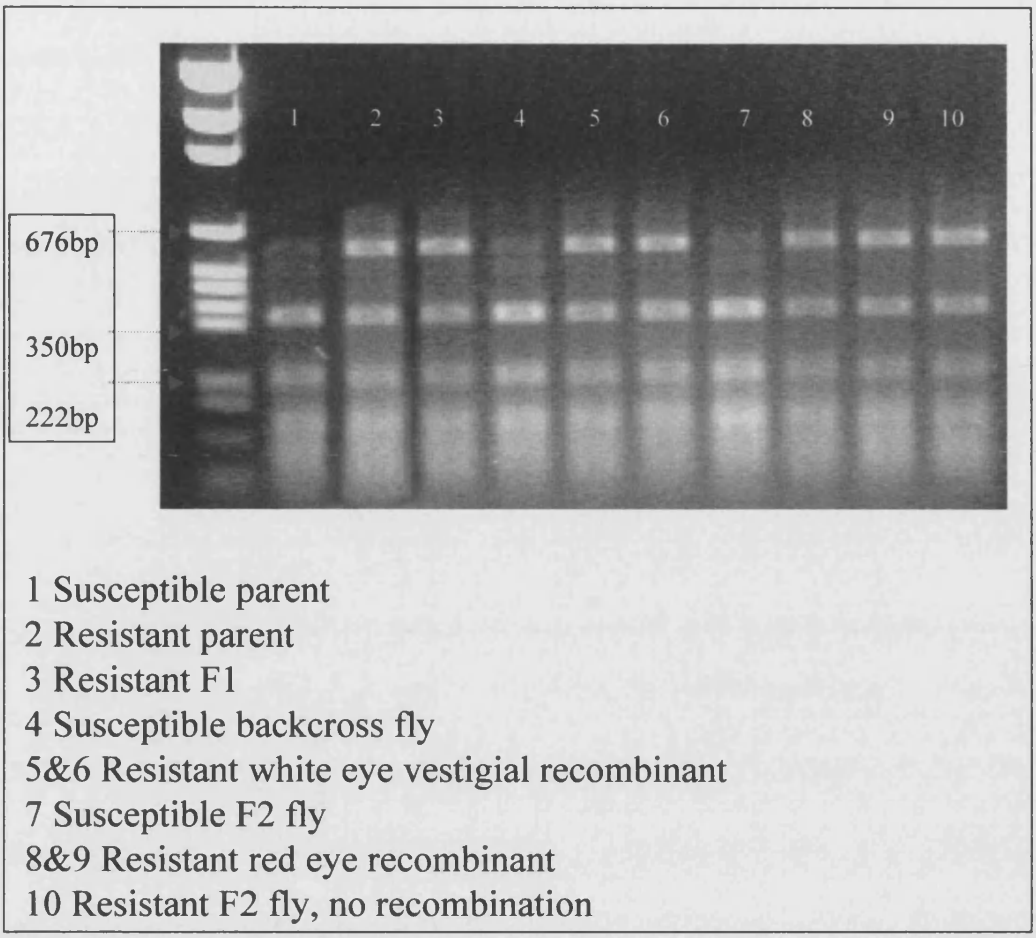


Fig. 2.12 Recombinant mapping.

Figure 2.12 shows the clear difference between the susceptible strain and progeny (lanes 1, 4 and 7) and the resistant flies showing the larger undigested band at about 500 bp as seen in lanes 2, 3, 5, 6, 8, 9 and 10. This gel shows the different progeny and parents from the cross shown in Figure 2.2 and we were searching for a recombinant fly that that showed the susceptible form of *Cyp6g1* but was resistant.

Unfortunately after screening 541 flies, 249 segregating upstream of *Cyp6g1* and 292 downstream of the *Cyp6g1*, we found no cases of the resistant phenotype and genotype being separated. This number of flies signifies a theoretical crossover event approximately every 50 bp but, in reality, this kind of experiment does not allow for that level of resolution due to the unknowable presence of hotspots for recombination. Therefore, although we found no flies showing the desired recombination event, we were still able to postulate that the mutation that causes *Cyp6g1* over-transcription was

indeed very close to the gene itself. Because no recombinants were found we are not able to say on which side of *Cyp6g1* the mutation that confers resistance is located.

2.3.4 Northern analysis and recombinant mapping of susceptible and resistant *D. melanogaster* strains

As metabolic resistance to date in *D. melanogaster* has usually been caused by changes in P450 expression or sequence it was decided to look at all the P450s in *D. melanogaster* that have been directly implicated in insecticide resistance. Those P450s with the closest homology at the amino acid level to any P450s in other insect species that have been shown to be up-regulated in resistant strains, or in some cases of being up-regulated in the presence of plant xenobiotics were examined. An example was *Cyp4c3,* which was selected because it is the closest homologue of the *M. sexta* P450 *Cyp4m3,* which has been shown to be up-regulated when the larvae are fed on a diet containing nicotine (Snyder et al., 1995). This particular P450 was tested due to the chemical similarity of nicotine and our main compound of interest, imidacloprid.

We knew there were no genes encoding nicotinic acetylcholine receptors in the 64.5 cM DDT-R region of chromosome 2 and it has long been assumed that this resistance phenotype was caused by a mutation in a transcription factor in this region. That transcription factor then up-regulates either receptor subunits or, more likely, metabolic enzymes that were capable of metabolising xenobiotics into harmless or easier to excrete compounds. As our work was carried out towards the end of 2000 we had one important and useful tool at our disposal than none of our predecessors had had access to. This was the virtually complete DNA sequence of *D. melanogaster* (Celniker et al., 2002; Hoskins et al., 2002; Myers et al., 2000). This sequence allowed us to immediately observe that there are no known transcription factors in our region of interest. However there is a cluster of three cytochrome P450s, namely *Cyp6g1, Cyp6g2* and *Cyp6t3*. The presence of these three genes led us to our early hypothesis this resistance mechanism was caused by the over-expression of a P450.

The broad range of P450s in Table 2.14 was chosen because at the time we did not think it possible for the up-regulation of a single enzyme to cause the resistance seen to so many compounds with so many different modes of actions and chemical structures. So we assumed that one P450 might be affecting the level of others, or that one of the

unknown open reading frames in that region was acting as a transcription factor and so up-regulating other P450s.

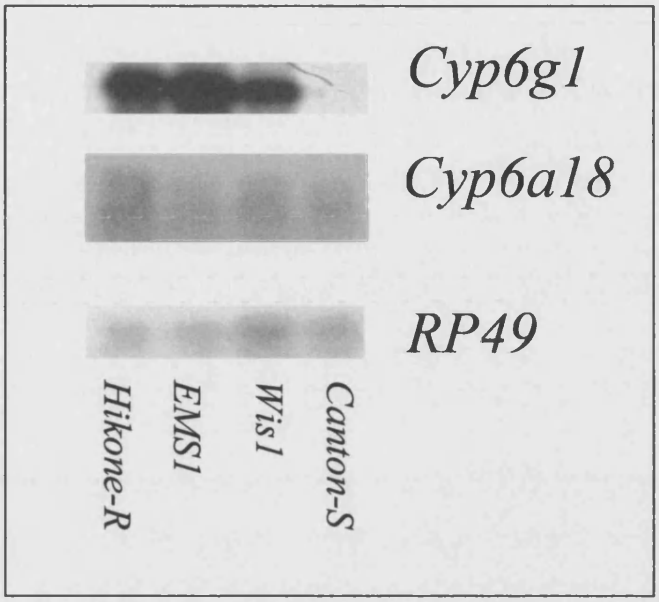


Fig. 2.13 Northern blots show Cyp6g1 over-transcription.

Figure 2.13 shows the RP49 control with transcription at an approximately equal level in all strains. Also shown is the result for *Cyp6a18,* this gene is a homologue of *Cyp6a1* which has been implicated in insecticide resistance in the Rutgers strain of *M. domestica* (Guzov et al., 1998). As can be seen there was no difference in the signal seen between resistant and susceptible strains. This shows that this gene is not up-regulated and so not involved in this resistance phenotype. The other result shown is for the P450 *Cyp6g1*, this is one of the three P450s clustered at the 64.5 cM locus and appears to be very highly over-transcribed, with there being a very clear and strong signal shown in all the resistant strains and no detectable signal at all in the susceptible strain.

Tab. 2.14 Northern results.

Gene	Northern Analysis[a]	Documented Expression in other strains and species[b]	Reference
Cyp6a2	- / -	Over expressed in *D. melanogaster* DDT^R and 91-R strains	(Brun et al., 1996); (Waters et al., 1992)
Cyp4e2	- / -	Over expressed in *D. melanogaster* Raleigh DDT^R strain	(Amichot et al., 1994)
Cyp6a14	n.d. / n.d.	*Cyp9a1* over-expressed in carbamate resistant *H. viriscens*	(Rose et al., 1997)
Cyp6g1	- / +	-	
Cyp6g2	- / -	-	
Cyp6t3	- / -	-	
Cyp6a17	- / -	*Cyp4b4* and *Cyp4b5* induced by xanthotoxin in *P. glaucus*	(Berenbaum and Zangerl, 1992)
Cyp6a9	n.d. / n.d.	Over expressed in *D. melanogaster* 91-R strain	(Maitra et al., 1996)
Cyp6a8	- / -	Over expressed in *D. melanogaster* 91-R and MHIII-D23 strains	(Maitra et al., 2000)
Cyp4aa1	- / -	*Cyp4m1* induced by nicotine in *M. sexta*	(Snyder et al., 1995)
Cyp6d2	- / -	*Cyp6d1* over-expressed in *M. domestica* Learn-PyrR strain	(Tomita et al., 1995) (Tomita et al., 1995)
Cyp12a5	n.d. / n.d.	*Cyp12a1* over expressed in *M. domestica* Rutgers strain	(Guzov et al., 1998)
Cyp6a18	- / -	*Cyp6a1* over expressed in *M. domestica* Rutgers strain	(Carino et al., 1994)
Cyp12d1	n.d/n.d	Over expressed in Wis1	(Brandt et al., 2002)
Cyp4c3	- / -	*Cyp4m3* induced by nicotine in *M. sexta*	(Snyder et al., 1995)

[a] *n.d./ n.d. not detectable in susceptible or resistant strains, - / - equally expressed in susceptible and resistant strains, - / + over-expressed in resistant strains*

[b] *difference in protein and/or RNA levels between resistant and susceptible strains or P450 involved in toxin metabolism.*

This table (previously shown in part in Daborn *et al* 2001) shows all the P450s examined by Northern analysis. It shows the level of transcription seen in the resistant and susceptible strains and the homologue and reference is made as to why it was decided to include each P450 in the Northern screen. Of the 15 P450s tested four showed no detectable signal in either resistant or susceptible strains implying that either the P450 was being transcribed at a very low level in these strains or was not transcribed at all in the adult flies used for the Northern blot. However, these genes could be transcribed at different life cycle stages. Although we know that DDT resistance is a phenotype of our adult resistant flies we can say that none of these P450s are likely to be involved in this resistance mechanism. Ten of the P450s tested show some transcription, however, there is no discernable difference between the resistant and susceptible phenotype. Therefore these enzymes are not involved in this form of insecticide resistance, unless the mechanism was due to a point mutation within the gene as in Berge (1998). However, this is not a possibility unless it was either *Cyp6g2* or *Cyp6t3* as they are the only P450s that map to the region of interest and we know the amino acid sequence in Canton-S and Hikone R is the same. Included in this group is *Cyp6a18* as shown in Figure 2.13. Only one P450 is over-expressed in resistant strains, and that is *Cyp6g1,* also shown in Figure 2.13.

From these results we developed the hypothesis that *Cyp6g1* over-transcription was the sole cause of the resistance phenotype reported since the 1950s. This hypothesis was tested further by characterising this resistance form using a range of classical and new methods to try to learn more about this enzyme. It is also important to note that over-transcription of *Cyp6a8* and *Cyp12d1* was seen (see Chapter 4 and published in Le Goff *et al*, 2003).

2.4 Discussion.

This chapter demonstrates that insects resistant to DDT, where that resistance maps to the 64.5 cM region of chromosome II are also resistant to the modern insecticide imidacloprid. This result gives some interesting implications for cross-resistance. This resistance mechanism was originally worked on by Ogita and Kikkawa as an investigation of DDT and organophosphate resistance (Kikkawa, 1961; Ogita, 1960). It was shown by these authors that a mutation in this region caused cross-resistance to a range of diverse compounds. Now, it has been shown here that this resistance genotype also confers cross resistance to imidacloprid, a new insecticide from a novel class that is directed to a receptor that very few other insecticide classes also target (see Table 1.1) (Leicht, 1996). When this work was begun it was thought that as very few insecticides target nAChR, neonicotinoid resistance was unlikely to be caused by a previously characterised mechanism, for example both pyrethroid and DDT resistance is conferred by the *kdr* mechanism (Zlotkin, 1999).

It is also possible to say that resistance to imidacloprid is probably directly linked to the level of expression of the P450 gene product *Cyp6g1*. We know the amount of RNA of this gene to be of approximately the same level in all the resistant strains tested; at the adult level, the level of RNA in other life cycle stages is shown in Figure 3.3 and the LD_{50}'s are all very similar with regard to imidacloprid, Table 2.9. The same cannot be said of DDT resistance however as the level of resistance varies between strains, Table 2.6.

It can be seen that the resistance mechanism would not appear to be the same in the three resistance strains used in this chapter. EMS1 and Hikone R show very similar resistance levels to DDT implying that a similar mechanism is conferring resistance in both strains. Figure 2.5 shows that while both these strains would appear to have a similar mechanism the large difference seen on the DMC between Wis1 and either EMS1 or Hikone R shows that the Wis1 strain could well have another factor affecting resistance. It is shown in Figure 2.13 that the level of *Cyp6g1* over-transcription is not at a visibly higher level in Wis1 compared to the other resistant strains and in fact given the relative *RP49* levels it would appear that the level of *Cyp6g1* may actually be slightly lower than in Hikone R. The Northern blots shown in this figure would suggest that none of the P450s previously implicated in resistance in other strains and the

homologues of those seen in other species are over-transcribed. However, it is possible that the Wis1 strain has one or more point mutations within *Cyp6g1* or another P450 that confer a superior ability to metabolise DDT.

The story for imidacloprid is clearer than that for DDT. As with DDT resistance, in all the three strains investigated imidacloprid resistance is dominant, but whereas with DDT the LD_{50}s differed greatly between strains, in the case of imidacloprid the LD_{50}s were all very similar. Table 2.9 shows that the LD_{50} of imidacloprid for EMS1, Hikone R and Wis1 only varies by 2 μg/vial implying a similar mechanism for all three strains. Figure 2.8 also shows that in all three strains the mechanism is similar with the reciprocal crosses between resistant strains all having very similar values with the major differences being due to the slope of the lines rather than their LD_{50}. The slope of a line on a DMC shows how homozygous a population is. The steeper the line then the smaller range of insecticide concentration that affects the strain and so the smaller the range of alleles conferring resistance that are present (Busvine, 1971).

When comparing the results from Figures 2.5 and 2.8 it can be seen that the resistance mechanisms conferring resistance to DDT and imidacloprid are probably slightly different. It would appear that resistance to imidacloprid is the more simple mechanism. It could be that DDT resistance is polygenic as suggested by Ogita with different factors on other chromosomes having an effect on resistance (Ogita, 1960). The three different DDT-R strains all show very similar LD_{50} values for imidacloprid. However, DDT resistance would appear to be more complex. Hikone R and EMS1 have similar values for their LD_{50}'s and the reciprocal cross between these two strains gives similar F1 progeny, whereas Wis1 appears to utilize a different mechanism (Brandt et al., 2002).

Further work was carried out to investigate resistance. Two deficiency strains were used to try to create an insect that had a R/- phenotype rather than a R/S phenotype. This would reveal if one resistant copy of a gene was sufficient to confer the same level of resistance as having a resistant and a susceptible copy of the gene. This would also answer if there is any trans-regulation on the chromosome containing the susceptible gene by the resistant copy. This method is generally more helpful when working with a target site mutant where a deficiency strain would eliminate all susceptible copies of a receptor and so might show a higher level of resistance that a susceptible and resistant

heterozygous insect (ffrench-Constant et al., 2000). Two deficiency strains were identified and used in this thesis and Section 2.2.4 describes the strains and methods used. Strain 4960 was found to have no effect on the response to DDT when crossed to Hikone R, but the deficiency in this strain possibly does not cover the region containing *Cyp6g1*. The second strain used, CB21, shows a moderate level of resistance to DDT and imidacloprid so it was decided not to continue with these experiments as the resistance ratios seen to imidacloprid are not large, so that it would be difficult to distinguish between susceptible (Canton-S), resistant (Hikone R) and slightly less resistant (CB21) insects.

PBO is a synergist that inactivates many P450s and some esterases (Feyereisen, 1999). Because of this PBO can be used as a synergist for insecticides to overcome P450 based resistance. Because PBO is equally effective against mammalian and insect P450s, its addition to insecticide mixtures must be carefully considered for environmental and health reasons. In Figure 2.11 we can see that the resistance mechanism in Hikone R is clearly PBO suppressible, suggesting it is P450 based.

A three hour exposure to PBO before a 24 hour exposure to DDT was sufficient to decrease the resistance ratio from 13 to less than 2, or from 19 µg/vial to 2.8 µg/vial. Originally a 24 hour exposure to PBO was attempted but this resulted in a high level of control mortality so the method of Brandt was used (Brandt et al., 2002). In his work on the Wis1 strain Brandt found that PBO decreased the DDT resistance ratio of this strain from approximately 100 to 30 fold resistance. There are two interesting points to note from this paper, firstly Brandt reports the LC50 of Wis1 to be either 87.8 or 196 µg/vial, or 32.5 and 98 fold resistance ratios respectively, (the latter figure is possibly higher due to the PBO assay having exposure to DDT for 21 hours as opposed to 24 hours. Although the resistance ratio would be expected to be the same over 21 hours or 24 hours when compared to Canton-S exposed for the same time spans). Secondly, Brandt found that PBO decreased the resistance ratio of Wis1 when compared to untreated Canton-S to 31 fold. This implies that not all the P450 is being inactivated by PBO, at least not to as great an extent to that seen in Figure 2.11. Therefore it is possible that *Cyp12d1*, shown by Brandt *et al* and Le Goff *et al* (Le Goff *et al,* 2003 and Chapter 5) to be over-transcribed in the Wis1 strain, is not affected by PBO and the relatively small

decrease in resistance seen by Brandt is due to the almost complete inhibition of *Cyp6g1*.

At this stage we had only worked with two insecticides, DDT and imidacloprid, and as imidacloprid acts as a larvicide and DDT kills adults attempts were made to use imidacloprid in an adult bioassay and DDT in the larval bioassay. DDT is an extremely hydrophobic molecule, being soluble in water in the region of 1-2 parts per billion (Zlotkin, 1999). This is too small to dissolve an effective dose of DDT and so irreproducible results were generated.

Imidacloprid is an insecticide used mainly on sucking pests (such as aphids and whiteflies), which is possibly why it has no effect on resistant or susceptible *D. melanogaster* at up to 200 μg/vial in a 24 hour contact bioassay. This could either be because it has no means of entering through the exoskeleton of the adult insects or because its target, the nAChRs, are in a different isoform in adult *Drosophila* which is immune to imidacloprid. Due to the impossibility of using the same stage of the *D. melanogaster,* comparisons have to be made between two different life cycle stages where differences in the physiological structure of the insects and the temporal control of important genes that could be involved in resistance to these insecticides must be allowed for. There have been recent reports of groups using imidacloprid in a modified contact bioassay on adult flies where imidacloprid solution was applied to sugar agar food and the adult flies ingest the insecticide as they feed. From personal communication with Dr. D. Greenhow (Syngenta, Jealotts Hill, UK), this method works as a means of getting flies to ingest imidacloprid (the aim of the experiment) but cannot be used as a method for the generation of DMCs as the results are not reproducible.

The work of Daborn mapped DDT and imidacloprid resistance to the 64.5 cM region of chromosome II (Daborn et al., 2001). This placed resistance in a region containing five genes (Figure 2.1). At this time we did not know what caused resistance and the over transcription of *Cyp6g1* seen in Figure 2.13 and Table 2.14. The RFLP analysis of Section 2.3.3 was an attempt to disassociate the resistant form of the gene (at the amino acid level the sequence of Cyp6g1 in Canton-S and Hikone R are identical but there is a silent point mutation allowing RFLP analysis) from the actual mutation conferring resistance. Over 500 recombinant flies that were resistant to imidacloprid were analysed

and none of the desired recombinants were identified. This number of recombination events over approximately 10 cM would theoretically equal one crossing over event every 50 bases.

Further work by Dr. P. Daborn (Daborn et al., 2002) led to the theory that resistance is caused by the insertion of a part of an *Accord* element upstream of *Cyp6g1* and this leads to the over-transcription of this gene and so confers the resistant phenotype first reported by Ogita (Ogita, 1960). It has been suggested that transposable elements, such as *Accord* elements could cause resistance by affecting the expression of a gene (Wilson, 1993). In the case of DDT-R, it would appear that this hypothesis has held true and this is the cause of resistance. Analysis of intron 1 also showed that as all resistant strains from around the world, ranging from those collected in the 1950s and 1960s and to modern recently collected strains have an identical sequence implying that there was a single point of origin for all the strains tested and this spread around the world (Daborn et al., 2002). It is also of some interest that the over-transcription of *Cyp6g1* apparently has no deleterious fitness effect on these strains. Hikone R for example has been in stock centres for 40 years and is still resistant.

This single point of origin theory also has two other points to note, firstly Kikkawa reported having used X-rays to mutagenise susceptible *D. melanogaster* (Kikkawa, 1961). The resistant mutant generated mapped to the 64.5 cM region. Although possible, it is unlikely that this fly line actually came from the mutagenised line as only one point of origin for resistance is thought to have occurred in the world. The chances of it being induced a second time in a laboratory after screening only 20000 insects are very low. It would however, be an interesting experiment to test this line to discover if it is in fact a second origin of *Cyp6g1* mediated resistance. Our experience has proved very similar. It may be noted that the *Accord* element is a chromosomal rearrangement and this will not be induced by EMS mutagenesis (selected as our mutagen because we were originally looking for receptor mutants, in which point mutations are generally the cause of resistance) and so our EMS1 strain is unlikely to actually be an EMS induced strain. However, to confuse matters further *Cyp6g1* sequencing shows that EMS1 is not one of the strains in our laboratory. Therefore, it is not a contaminant from our own stocks. It is either a line we isolated from the background of the susceptible strain used in the screen that was already present, or is a contaminant from outside the laboratory.

Given the chances of a DDT and imidacloprid resistant insect flying into our lab and getting into our cages is extremely unlikely it suggests that the EMS1 strain was naturally present in the susceptible strain we were mutating.

The early molecular work shown in this chapter shows that *Cyp6g1* is the only P450 probed to be over-transcribed in our strains of interest. This leads to the early hypothesis that *Cyp6g1* over-transcription confers resistance to DDT at the adult stage. Although imidacloprid resistance maps to the same region (Daborn et al., 2001) at this stage we were not sure if resistance is due to the same mechanism. A least two further experiments are needed to test this theory, that it is indeed *Cyp6g1* over-transcription through out the lifecycle that confers the resistance phenotypes seen. Firstly, we need to show that *Cyp6g1* is over-transcribed through out the lifecycle of resistant strains and to observe if, and when, *Cyp6g1* is naturally transcribed. This question is addressed in Chapter 3. The second question to answer has already been addressed and proved so far impossible to answer, namely does resistance at the larval stage mean that resistance is also seen at the adult stage to the same chemical. This is not possible to answer with DDT or imidacloprid but will be investigated in Chapter 5 with the organophosphate malathion.

Other points that need to be addressed are that only 15 of 87 P450s were tested by Northern analysis. It is not an economical use of resources to carry out Northern analysis of all 87 P450s, especially as all should be tested at both the larval and adult stages. It later became possible to use a micro-array with all 87 *D. melanogaster* P450s and the results of experiments using this micro-array are shown in Chapters 4 and 5 (Le Goff et al., 2003).

Future experiments using DMCs could be improved in a number of ways. The adult bioassay for DDT and PBO with DDT both worked extremely well and do not need modification. However some slight changes to how the actual insecticide doses are chosen could improve the assay, namely doing a preliminary experiment using a slightly larger range of doses then carry out a second experiment using a larger number of doses across a smaller range. This improvement would also apply to the larval bioassay but this second method also has some problems with how it is carried out. The larval bioassay has high control mortality, not all eggs will successfully develop into adults.

This leads to problems with analysing data (Abbott, 1925). It is however very difficult to remedy this problem. The obvious option is to use 1st instar larvae in the assay instead of eggs as they have already emerged so this will decrease the control mortality. It should be considered however that this is a very time consuming method and it takes a lot more time to collect larvae than eggs. The dry fly food can also be a problem as it is prone to drying out and fungal infections. The fungal infections were stopped by the addition of tegosept (5 μg/vial) to the food but the drying out of the food is a big problem. The dry food however is the most desirable to use in these bioassays as it allows the most accurate addition of insecticide concentrations. In addition, as the food is cold there is no danger of the insecticide being damaged by addition to overly hot agar based food.

Ch 3. Quantifying *Cyp6g1* message and protein in different strains and life-stages

3.1 Introduction

The aim of this chapter is to test if *Cyp6g1* is over-transcribed at all life cycle stages, and to create an anti-CYP6G1 antibody with which to test if the protein is present as well as the mRNA transcript.

In Chapter 2 and Daborn *et al*, 2001 it has been shown that resistance to DDT and cross-resistance to imidacloprid maps to position 64.5 cM on chromosome II of three different resistant *D. melanogaster* strains. It has been shown using Northern blots that there is an increased level of *Cyp6g1* transcript in three day old adult insects of all three strains but we did not know if this gene was expressed throughout the lifecycle of resistant insects. It was also not known whether the *Cyp6g1* transcript is successfully translated and so is there an increased level of CYP6G1 protein in the resistant insects. Determining the natural expression profile of this P450 would perhaps give some idea of its native function.

In the past, there have been a number of publications on the functional expression of P450s (Berge et al., 1998). From the point of view of the questions listed above, a functional gene was not a necessity, the main requirement was for a large amount of soluble CYP6G1 that could then be injected into rabbits for the production of polyclonal antibodies. P450s have been expressed in four systems, in mammalian and insect cells using a baculovirus to deliver the P450 gene into the cell, using a yeast expression system or in bacteria (Berge et al., 1998). The bacterial expression system was used as it is the simplest system to use when expressing an over-transcribed gene especially when the sequence of the gene is known as primers can be designed to the ends of the gene. By including restriction sites at the ends of the primers and a reverse transcriptase reaction followed by PCR can be carried out and the PCR product can be excised and cloned. This method can result in a clone of *Cyp6g1* that can then be over-expressed to create sufficient protein. This method requires the P450 to be purified and, to facilitate this, the protein is tagged with either maltose binding protein (MBP), a poly His tag (pHIS) or GST. Because P450s are either microsomal or mitochondrial, and because the proteins are membrane bound (the N-terminal has an ~20 residue transmembrane

anchor) solubility can be a problem when expressing eukaryotic P450s (Tijet et al., 2001). Work by Doray has shown the pHIS tagging of a human P450 led to the bacteria not growing unless the N-terminal transmembrane anchor was removed prior to cloning (Doray et al., 2001). They surmised that this is because the combination of the pHIS tag and the transmembrane anchor constitutes a bacterial export signal. The rest of the P450 protein then interferes with this mechanism causing a blocking of pores and so an intracellular increase in extracellular proteins that could harm the bacteria. It was also found that a P450 lacking its transmembrane N-terminal sequence was expressed at a relatively low level. By coincidence however they found that the removal of the transmembrane region combined with a pHIS tag increased expression (Doray et al., 2001).

3.2 Methods

3.2.1 Life cycle stage collection

Different lifecycle stages were required for Quantitative-Real Time-PCR (Q-RT-PCR) experiments to demonstrate *Cyp6g1* transcript levels at different lifecycle stages. These stages were collected using cages with several hundred *D. melanogaster* of the strains of interest. Sugar agar was made (9 g plant agar and 300 ml water are combined and 100 ml blackcurrant cordial, 0.6 g tegosept (Sigma) and 10 g sucrose are combined and boiled separately. Both are cooled to 60°C then mixed and poured) in small petri dishes and allowed to set. A thick yeast paste was placed in the centre of each plate with an un-yeasted area around the edge for egg laying. The eggs were collected at different times, incubated to the desired age, washed into a sieve to remove yeast and then snap frozen in liquid nitrogen. 1st and 3rd instar larvae were collected by allowing flies to lay on the agar plates then the plates were kept at 25°C and the larvae allowed to develop to the desired age. The larvae were then collected by washing through a sieve and snap frozen in liquid nitrogen. Pupae were collected 2 days post pupation by removing them from the vial and snap freezing. Adults were collected less than 3 days post emergence (for the micro-arrays flies of exactly 3 days old were used) and snap frozen in liquid nitrogen.

3.2.2 Quantitative Real-Time polymerase chain reaction (Q-RT-PCR) on different life cycle stages.

The relative abundance of *Cyp6g1* transcripts in the resistant strains EMS1 and Hikone R and the susceptible strain Canton-S at different life cycle stages was determined relative to the housekeeping gene *RP49* (O'Connell and Rosbash, 1984).

Total RNA was extracted from EMS1, Hikone R and Canton-S at different stages in the life cycle; 3 day old adults, pupae, 3rd instar larvae, 1st instar larvae and eggs (~50 mg tissue) using TRI reagent (Sigma), digested with 1.0 U of DnaseRQ1 (Promega)/µl, and re-extracted using TRI reagent. Lack of DNA contamination was checked in each sample via PCR using RNA as the template. Oligonucleotide primers were used within *Cyp6g1* gene spanning intron 2. CG8453F (5'-ATT CGC ACC AAG CTG ACT CCC GT-3') and CG8453R (5'-ATG ACC CAC CGC CCT CCA CCA-3') using the PCR protocol 95°C 3 min, 40 cycles of 95°C 1 min, 59°C 1 min, 72°C 1 min, then 72°C 5 min using Promega *Taq*, 1x Promega buffer 1, 10 mM dNTPs (Promega) and 3 mM Magnesium Chloride (Promega).

Single-step reverse transcriptase RT-PCR was performed following the protocols of the LightCycler-RNA Amplification Kit SYBR GreenI (Roche). PCR conditions were RT 55°C, 30 min, PCR 45 cycles of 95°C 5 sec, 50°C 10 sec, 72°C 25 sec with a step at 83°C 0 sec to read fluorescence. Oligonucleotide primers were synthesised for a ~300bp region within the *Cyp6g*1 gene (6g1F 5'-CGG CTG AAG GAC GAG GCT GTG-3', 6g1R 5'-GCT ATG CTG TCC GTG GAG AAC TGA-3') and for the housekeeping gene *rp49* (RP49F 5'-ATC CGC CCA GCA TAC AG-3', RP49R 5'-TCC GAC CAG GTT ACA AGA A-3'). A standard curve was generated using known amounts of genomic *D. melanogaster* DNA (from 100 ng – 10 pg). This was then used to quantify the abundance of each transcript in the RNA sample.

3.2.3 Cloning of *Cyp6g1*

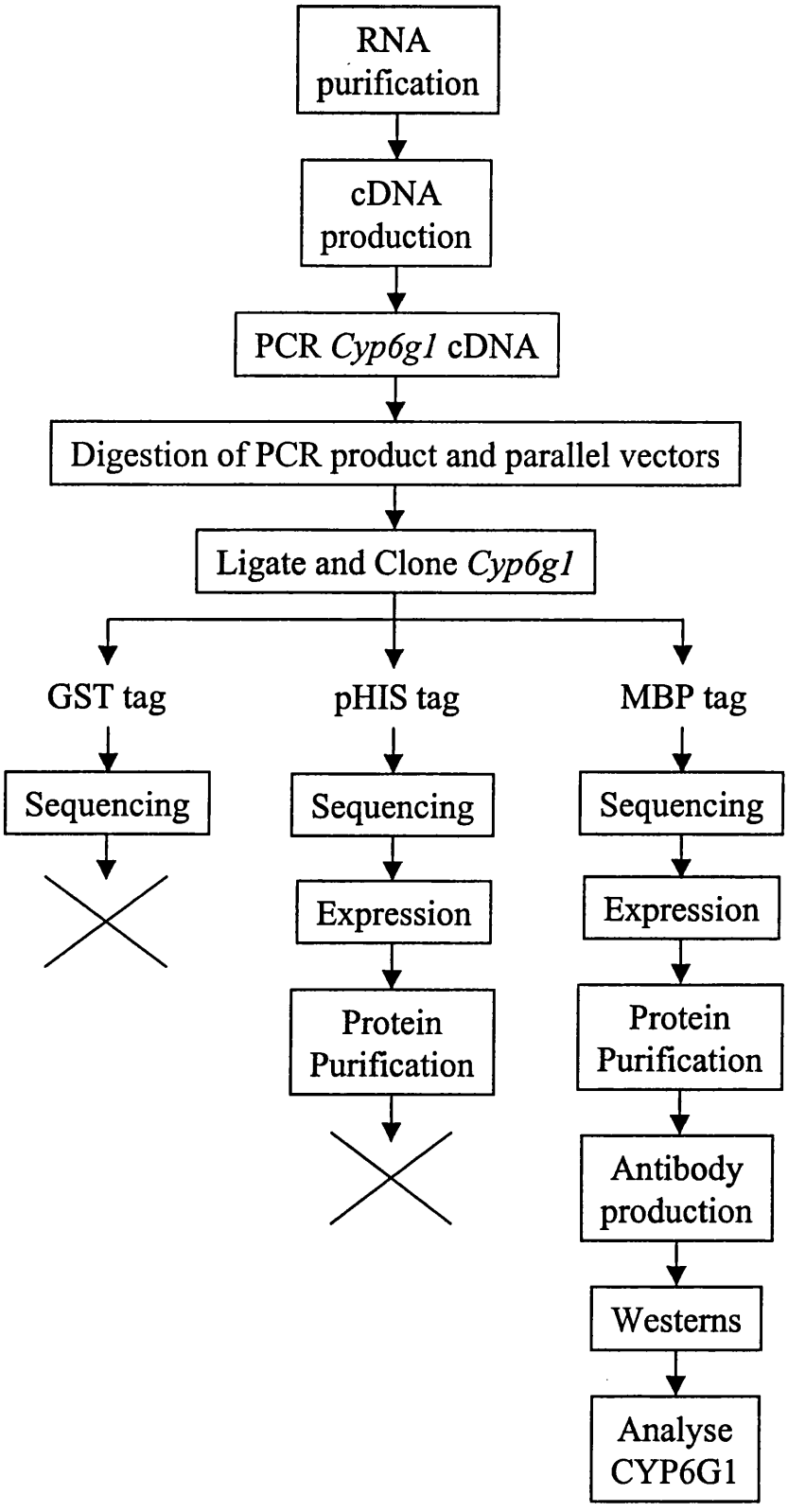


Fig. 3.1 Cloning strategy for making CYP6G1 fusion protein.

Total RNA was purified from ~50 mg Hikone R 1 - 3 day old adults using TRI reagent (Sigma) digested with 1.0 U of DNaseRQ1 (Promega)/µl, and re-extracted using TRI reagent. Lack of DNA contamination was checked in each sample via PCR using the purified RNA as the template in a PCR spanning intron 2 using primers CG8453F (5'-ATT CGC ACC AAG CTG ACT CCC GT-3') and CG8453R (5'-ATG ACC CAC CGC CCT CCA CCA-3'). Using the PCR protocol 95°C 3 min, 40 cycles of 95°C 1 min, 59°C 1 min, 72°C 1 min, then 72°C 5 min using Promega *Taq*, 1 x buffer, 3 mM Magnesium Chloride and 10 mM dNTPs. In samples where a PCR product was seen a further DNase digest and TRI reagent extraction were carried out as described above and the PCR repeated.

Cyp6g1 cDNA was then generated from the pure total RNA using M-MLV Reverse Transcriptase, RNase H minus, point mutant (Promega) (M-MLV RT). 1 µl RNA, 1µl primer ORF6g1R (5'-GCG ATT CTA GAT CAT TGG AGC GAT GGA GC-3'), 12µl DEPC treated water was combined with a 11 µl pre-mix containing 2.5 µl M-MLV RT 1 x buffer, 5 µl 10 mM dNTPs (Promega), 1 µl (200 U) M-MLV RT and 2.5 µl DEPC treated water (mixed in that order as described in the instruction manual). The reaction mixture was incubated at 50°C for 60 min.

The resulting cDNA was then used as a template in a PCR to generate a *Cyp6g1* DNA product using Immolase DNA polymerase (BIOLINE). The oligonucleotide primers ORF6g1F (5'-CGA CAG CGG CCG CAT GGT GTT GAC CGA GGT C-3') and ORF6g1R were used, these primers contain a NotI and XBA1 restriction site respectively, with 10 mM dNTPs (Promega), 2 mM magnesium Chloride, 2 µl cDNA (in a 50 µl reaction), 1 x Immolase buffer (BIOLINE) and 1 µl (5 U) Immolase (BIOLINE). The following PCR protocol was used: 95°C 7 min hot-start, then 40 cycles of 95°C 1 min, 64°C 1 min, 72°C 2 min, and 72°C for 10 min.

This PCR product was then purified using low melting temperature agarose (Sigma) (LMT agarose) using the following protocol: A 0.7% LMT agarose gel loaded with the PCR product was run at 60 volts for 1 h The bands were then cut out of the gel under ultra violet (UV) illumination. 3 times the melted gel volume of 1 X TE (Sambrook et al., 1989) was added and the sample incubated at 65°C for 10 min. Then one phenol extraction using an equal volume of phenol to the gel–TE mixture was carried out. The

sample was vortex mixed then centrifuged at 13 K RPM for 2 min at room temperature and the aqueous layer transferred to a new tube. Then one phenol chloroform extraction was carried out using an equal volume of phenol chloroform to the gel–TE mixture. The sample was vortex mixed then centrifuged at 13 K RPM for 2 min at room temperature and the aqueous layer transferred to a new tube. Next, one chloroform extraction was carried out using an equal volume of chloroform to gel–TE mixture. The sample was vortex mixed then centrifuged at 13 K RPM for 2 min at room temperature and the aqueous layer transferred to a new tube. $1/10^{th}$ volume of 3 M Sodium Acetate was added to the sample and 2 times the volume of ice-cold 100% ethanol. The sample was left overnight at -20°C. Next the sample was centrifuged at 4°C, 13 K RPM for 30 min the sample was washed with ice-cold 70% ethanol and centrifuged at 4°C 13 K RPM for 30 min. The ethanol was then removed by pipetting and the sample was air dried at 37°C and resuspended in 30 µl water.

E. coli strain XL1 blue containing the vector pUAST was grown overnight at 37°C in 50 µg/ml ampicillin (Sigma) and DNA was extracted and purified using Miniprep columns (QIAgen) following the protocol, using the optional wash step and leaving the column to stand for 2 min after adding water to elute the DNA prior to the final centrifuge step. The purified plasmid was resuspended in 50 µl water at 70°C.

The vector and PCR product were then digested using 1 U/µl Not1 and XBA1 (NEB) simultaneously and 1x buffer 3 (NEB) at 37°C for 2 h. The digested vector and PCR product insert were run on a 0.7% agarose gel (Sigma) and the corresponding bands cut out under UV illumination and the bands purified using the QIAquick Gel Extraction Kit (QIAgen) following the protocol and resuspending the digested DNA in 25 µl water at 70°C.

The digested *Cyp6g1* PCR product insert and pUAST vector were then ligated together using 40 U/µl T4 DNA Ligase (NEB). A 1:3 molar ratio of vector to insert (calculated from the intensity of the band on an agarose gel and the length of each fragment) were incubated with 1 x T4 DNA Ligase buffer (NEB) at 16°C for 12 h. 5 µl of the resulting construct was then electroporated into 40 µl concentrated *E. coli* XL1 Blue cells and 950 µl LB broth (12.5 g LB (SLS) in 500ml H_2O), and incubated at 37°C for 1 h. The culture was then plated onto LB agar plates (400ml H_2O, 10g LB, 6 g Agar (Difco)

containing 50 µl/ml ampicillin.) The plates were incubated overnight at 37°C and colonies picked into LB broth containing 50 µl/ml ampicillin and the cultures were incubated overnight at 37°C. The pUAST-*Cyp6g1* construct containing cultures were then purified using the QIAgen Miniprep protocol with the optional wash step and the vector was resuspended in 30 µl water at 70°C.

The purified pUAST-*Cyp6g1* construct was sequenced using an ABI3700 automatic sequencer with BIGDYE (ABI) and 2 µl construct per reaction using the primers seq2 (5'-CTT TTG CCC TGT ACG AGA TGG-3'), seq5 (5'-TAT ACA ATG ATC CGC GGC TGA AGG-3'), pUASTF (5'-AGA ATC TGA ATA GGG AAT TGG G-3'), pUASTR (5'-CCT CAT TAA AGG CAT TCC ACC-3') and CG8453R (see above).

A construct with the correct *Cyp6g1* sequence was then chosen for insertion into the parallel expression vectors for fusion with a N-terminal pHIS tag, Maltose Binding Protein (MBP) or Glutathione S-Transferase (GST) fusion proteins. The parallel 2 vectors were chosen to keep the fusion protein and *Cyp6g1* in the same open reading frame (ORF) (Sheffield et al., 1999). PCR primers were designed with a BamH1 restriction site on the forward primer, 6G1HISTagF (5'-CGC GGA TCC TGG TTC CAG CGC AAC CAT-3'), and a XBA1 restriction site and extra stop codon on the reverse primer, 6G1HISTagR (5'-CTA GTC TAG ACT ATC ATT GGA GCG ATG GAG C-3'). The forward primer is designed to remove the 20bp trans-membrane region at the N terminus of CYP6G1 as is done in (Doray et al., 2001) to try to improve the solubility of the fusion protein.

The purified *Cyp6g1*-pUAST construct was used as the template for PCR to generate a product to be ligated into the parallel 2 vectors. The following conditions were used, 10 mM dNTPs, 2 mM Magnesium Chloride (BIOLINE), 1 µl (5 U) Immolase, 6G1HISTagF and 6G1HISTagR primers with the PCR protocol 95°C 7 min, 40 cycles of 95°C 1 min, 55°C 1 min, 72°C 2 min then 72°C 15 min. The resulting PCR product was purified using LMT agarose extraction (see above) and the DNA was resuspended in 30 µl water.

The parallel 2 vectors were grown overnight at 37°C in LB with 50 µl/ml ampicillin and purified using the QIAgen Miniprep protocol. The purified vectors were resuspended in

30 µl 70°C water. Both the purified pUAST-*Cyp6g1* construct and parallel 2 vectors were digested with BamH1 1 U/µl (NEB) and XBA1 1 U/µl using the specific BamH1 buffer (NEB) for 2 h at 37°C. The desired DNA fragments were then purified from a 0.7% agarose gel using the QIAquick gel extraction protocol from QIAgen. The DNA fragments were ligated overnight and electroporated as described above. Colonies were then picked and grown overnight in LB broth containing 50 µl/ml ampicillin. The cultures showing ampicillin resistance were purified using the Miniprep protocol and sequenced using seq2, seq5, CG8453F and pGEX 5' and pGEX 3' (Amersham Biosciences) for the GST constructs, MAL E (NEB) and M13 universal (NEB) for the MBP construct, or T7 Promoter (NEB) and T7 Terminator primers (NEB) for HIS tagged constructs.

One MBP-*Cyp6g1* construct was of the correct sequence and in the correct ORF after 6 months work, so it was decided to attempt to express this construct.

3.2.4 Expression of a MBP-CYP6G1 fusion protein.

For improved protein expression the purified *MBP-Cyp6g1* construct was electroporated into BL21(DE) cells, (see protocol in Section 4.2.1.2). An overnight culture of the BL21(DE) containing the *MBP-Cyp6g1* construct was used to seed 1 l LB containing 50 µl/ml ampicillin. This culture was grown to a optical density (OD) of 0.5 at 37°C then 5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) was added and the culture grown for a further 3 h at 37°C. The bacterial cell pellet was then purified by centrifugation, 20 min at 10 K RPM in a Sorval centrifuge. The MBP-CYP6G1 fusion protein was then purified using the inclusion body purification method from (Sambrook et al., 1989). It had been attempted to purify the fusion protein using the method described by Sambrook for purification of a MBP construct but unfortunately the protein is insoluble so this method did not work (Sambrook et al., 1989).

It was found that the fusion protein was insoluble so inclusion bodies were purified from the bacterial cell pellet using the following method: the samples were centrifuged at 7000 g for 5 min. The pellet resuspended in Inclusion Body Buffer1 (100 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 8.0)) to a final concentration of 10% weight/vol (i.e. 10 g pellet resuspended in 100 ml buffer). Lysozyme (Sigma) was added to each sample at a concentration of 1 mg/ml. The sample was incubated at room temperature for 20 min

and then centrifuged at 5000 g for 10 min at room temperature. The supernatant was discarded and the pellet stored on ice and resuspended in ice cold Inclusion Body Buffer2 (100 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 50 mM Tris (pH8.0)) and incubated on ice for 10 min $MgCl_2$ was added to a final concentration of 8 mM and DNase (NEB) at a concentration of 10 μg/ml. The sample was incubated at 4°C with gentle stirring until the viscosity disappeared with fresh DNase being added as necessary. The inclusion bodies were then removed by centrifugation at 10000 g for 10 min at 4°C. The pellet was then washed with Inclusion Body Buffer3 (1% NP-40, 100 mM NaCl, 1 mM EDTA, 50 mM Tris (pH.8.0)) then resuspended in Inclusion Body Buffer3 (Sambrook et al., 1989).

These inclusion bodies were then further purified by running the sample on an 8% denaturing SDS polyacrylamide gel (Sambrook et al., 1989). The desired band was then purified by excision from the gel and grinding in a pestle and mortar with liquid nitrogen. The inclusion bodies were resuspended in inclusion body buffer1 (see above) and centrifuged at 13 K RPM for 10 min at room temperature and the supernatant was removed to a new tube and diluted to a concentration of 30 μg/ml total protein. An equal volume of Imject Alum (Pierce) was added to the purified MBP-CYP6G1 fusion protein and this was injected into rabbits for production of a CYP6G1 antibody. The following booster and test bleed protocol was used to ensure that the rabbits had a sufficient level of MBP-CYP6G1 construct present:

Day 1 1st injection

Day 21 Booster injection 1

Test ear bleed

Day 42 Booster injection 2

Test ear bleed

Day 63 Booster injection 3

Test ear bleed

Bleed every 2 weeks for 6 weeks

Day 105 Booster injection 4

Day 119 humanely kill rabbits and collect blood.

3.2.5 Purification of polyclonal antibodies.

It was initially intended to purify the polyclonal antibody by using a pHIS tagged CYP6G1 construct bound to a nickel column and then passing the rabbit sera over this and eluting off the specific antibodies bound to the CYP6G1 in the column.

3.2.5.1 poly HIS tagging N terminal *Cyp6g1*.

A poly HIS tagged CYP6G1 construct is needed to purify the rabbit serum from the previous section. The first method involved the use of the pHIS-Parallel 2 vector. A PCR product was generated using 6g1HisF and 6g1HisR2 (5'-ATA AGA ATG CGG CCG CTC ATT GGA GCG ATG GAG C-3') using the PCR protocol outlined in 4.2.1.2. The resulting PCR product was then purified using Qiagen Gel Extraction Kit following the instructions provided. The PCR product was made up to 300 µl with water. After numerous failures using both digestion enzymes together in the digest (as in Section 3.2.1) it was decided to use two single digests and risk losing some of the DNA in the extra purification step that this method entailed. The purified PCR product and mini prepped pHIS-Parallel 2 vector were then digested for 2 h 37°C with 2 U/µl Not1 (NEB) using 1 x buffer 2 (NEB) and 1/100 dilution of BSA (NEB). The digested products were then purified using Qiagen Gel Extraction kit and digested for 2 h 37°C with 2 U/µl BamH1, 1x BamH1 buffer and 1/100 dilution BSA. The products were then purified using Qiagen Gel extraction kit and resulting products ligated together in a molar ratio of 3 digested *Cyp6g1* to 1 digested pHIS-parallel 2 vector overnight at 16°C using 40 U/µl ligase (NEB) and 1 x ligase buffer (NEB).

The resulting colonies were then sequenced using the primers and conditions outlined above and a CYP6G1-pHIS-parallel 2 construct with the correct sequence was identified. This was then grown under various conditions to produce a soluble protein. The clone was grown at 37°C until OD 0.3 was reached, when protein expression was induced by the addition of varying amounts of IPTG at 0, 0.125, 0.25 and 0.5 mM. The samples were then either induced at 37°C for 3 h or 16°C overnight. The samples were then solubilized using the following procedure.

Cells were pelleted by centrifuging at 10 K RPM in a Sorval centrifuge for 10 min and resuspended in lysis buffer (20 mM Tris pH.7.5, 10% glycerol, 500 mM NaCl, 10 mM E-mercaptoethanol, 0.2% Triton, 1 mM PMSF (Sambrook et al., 1989)). The samples

were lysed via sonication using the following procedure; Pulse on = 0.3sec, Pulse off = 1.0 sec, Total time = 8 sec, amplitude = 50-60%. This was repeated twice. Sonicates were then centrifuged at 15 K RPM, 20 min, 4°C. The supernatant (containing soluble protein) was decanted and retained and the insoluble pellet was resuspended in 5 ml dH_2O and retained. 10 µl of each sample was added to 10 µl of 2 x loading buffer (Sambrook et al., 1989) and run on a 10% SDS-PAGE. No over-expression of the HIS-CYP6G1 was seen under any conditions tested.

At this stage it was suggested that a N-terminal pHIS tag can inhibit the solubility of P450s (Rene Feyereisen pers. com.). After further analysis it was decided to place the pHIS tag at the C terminal of CYP6G1 as a collaborator had expressed a desire to try to purify and crystallize CYP6G1. By removing the first 20 amino acids to improve solubility I had not only removed the desired trans-membrane region but also inadvertently a part of a possibly important structural region of the gene.

3.2.5.2 poly HIS tagging C terminal of Cyp6g1.

A new vector, pET24a(+) (Novagen) was used for this procedure. New PCR primers were designed to keep *Cyp6g1* in frame and to include the whole of the gene, including the N terminal trans-membrane region removed in the MBP fusion protein. The primers 6g1HisF3 (5'- CGC GGA TCC ATG GTG TTG ACC GAG GTC CTC-3') and 6g1HisR4 (5'-ATA GAG ATG CGG CCG CTT GGA GCG ATG GAG CGC T-3') were used in a PCR containing 1 x PFU buffer (Promega), 1.5 mM dNTPs, 1 U/µl PFU (Promega), 1.6 mmol forward and reverse primers. The PCR protocol of 95°C 3 min, then 40 cycles of 95°C 1 min, 70°C 1 min and 72°C 2 min then 72°C 10 min.

The resulting PCR product and mini prepped pET24a(+)vector that had been grown overnight at 37°C with 100 µ/ml kanamycin (Sigma) were individually digested with Not1 and BamH1 according to the procedure outlined in Section 3.2.3.1. Purification at each step was carried out using 100 KDa Microcon spin columns (Microcon) instead of LMT gel extraction or Qiagen gel extraction as the spin columns were found to give a higher and cleaner yield, following the manufacturer's procedure for DNA purification. The resulting double digested products were then ligated in a molar ratio of 3 *Cyp6g1* insert to 1 pET24a(+) vector using Promega "QUICK LIGATION" 1 x buffer (Promega) 0.3 U/µl Ligase (Promega) and incubated at room temperature for 10 min.

The resulting construct was then electroporated into DH5α electrocompetent cells and incubated in 1 ml LB broth and then plated on LB agar plates containing 100 μg/ml kanamycin. Colonies were then picked and grown overnight at 37°C in LB broth containing 100 μg/ml kanamycin then mini prepped and a test digest using BamH1 was carried out. Constructs containing the *Cyp6g1* insert were then sequenced as outlined above. A construct with the correct *Cyp6g1* sequence was identified and purification of the CYP6G1-pHIS protein construct was attempted.

3.2.5.3 Expression of a C terminal HIS tagged CYP6G1.

Initial tests were carried out on the C terminal tagged CYP6G1-HIS construct to assess protein expression and solubility. The constructs were transferred to BL21 and BL21* cells to facilitate better expression. Primary experiments were carried out using IPTG to induce protein expression. A small amount of protein was seen of the correct size after 3 h induction at 37°C of a 0.5 OD culture with 0.5 mM IPTG. Further analysis found that only very small quantities of the new construct were expressed under a variety of different conditions so it was decided to use an unpurified form of the antibody.

3.2.6 Analysis of anti-CYP6G1 antibody using Western blotting.

A variety of methods were used to allow for the successful production of Western blots. A range of primary antibody concentrations, secondary conjugates and concentrations and substrate systems were used to allow a visualization of the amount of CYP6G1 in different samples.

3.2.6.1 Extraction of protein for Western blots.

All the following work was carried out with the samples kept on ice whenever possible. Protein was extracted from insects by adding 50 μl 1% SDS to ~100 μl insects and homogenising the mix using a disposable hand held homogeniser. A further 200 μl of 1% SDS was added and the large remaining body parts removed by sieving the sample through a fine cloth mesh by centrifugation at 500 RPM for 30 sec in a desktop centrifuge. The resulting mixture was heated at 100°C for 5 min to lessen the effect of proteases present in the flies. The samples were then centrifuged 5 K RPM for 5 min and the central aqueous layer taken, thus avoiding some smaller body parts, membranes and fat.

A Bradford assay was then carried out on these samples to find the total protein concentration. A standard curve was generated using known amounts of BSA (NEB). 20 µl sample, 80 µl water and 900 µl Bradford reagent (Sigma) were combined in a 1 ml polyacrylamide curvette and left to stand for 10 min. The samples were then inverted once to mix and the absorbance at 595 nm read using a spectrophotometer. A Microsoft Excel worksheet was then used to calculate the sample concentration based on the standard curve. The samples were then diluted to the desired concentration based on this result. This sample was then diluted with 2 x protein running buffer (Sambrook et al., 1989) and loaded onto an 8% SDS PAGE gel (Sambrook et al., 1989).

3.2.6.2 Method for Western blotting, secondary antibodies and substrates used.
The SDS PAGE gel was run for 2 h at 100volts and blotted using a Bio-Rad Semi Dry Transblotter. The gel was trimmed of the wells and equilibrated in Western buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 10 min. At the same time the membrane (Immun-Blot PVDF Membrane (Bio-Rad)) was soaked in methanol for 3 min then soaked in Western buffer for 10 min. Six pieces of 3 MM chromatography paper were cut to the size of the gel (8 cm x 9 cm). These were soaked briefly in Western buffer and 3 pieces placed on the trans blotter. The membrane was added on top of this followed by the gel then 3 more pieces of blotting paper. Air bubbles were removed by rolling a 50 ml tube over the top layer of blotting paper being careful not to dislodge the gel or membrane. The membrane was then blotted at 15 volts for 15 min.

The membrane was transferred to PBS containing 5% skimmed milk powder (Marvel) and left at 4°C overnight. The membrane was then washed three times in 1 x TBS (2.9 g NaCl, 0.24 g Tris Base to pH7.5 in 1 l dH_2O) containing 0.05% Tween 20 (Sigma) for 20 min. The primary antibody was then added in 20 ml TBS Tween containing 5% milk powder and shaken at room temperature for 2 h, a 1 in 1/1000 dilution of primary antibody was found to give the best Western results. The membrane was then washed 4 times for 20 min in TBS Tween and the secondary antibody added to 20 ml TBS Tween containing 5% milk powder. The membrane was then washed three times for 20 min in TBS Tween and the substrate added. Substrates and secondary antibodies are summarised in the table below.

Table 3.2 Secondary antibodies and substrates used.

Secondary	Company	Concentration Range	Substrate	Company	Visualisation Method
Anti-rabbit IgG Alkaline phosphatase from goat.	Sigma	1/1000 - 1/20000	BCIP	Sigma	Too faint for scanning
VECTASTAIN ABC-HRP kit Rabbit IgG	Vector Labs	As instructions	ECL Plus Western Blotting Detection System	Amersham Biosciences	X-Ray film
			SuperSignal West Femto	Pierce	X-ray film
			DAB substrate system with nickel enhancement.	Vector Labs	Scanner
Anti-rabbit IgG HRP from goat	Sigma	1/1000 - 1/10000	As VECTASTAIN ABC		

This table shows the secondary antibodies and substrates used.

The VECTASTAIN kit was found to give the best results and the DAB substrate the clearest image. The BCIP system originally used yielded clear results but the colour was too faint to be scanned and so different conjugated antibodies and substrates were tested. The two chemiluminescent substrates although giving very clear results were too intense and gave too bright a signal which led to trouble with the exposure of the X-ray film, an exposure of about 3 sec was the maximum possible without too high a signal.

3.3 Results

The results in this section are unpublished and are an investigation of the temporal expression and translation of the protein CYP6G1. Results are shown using a variety of methods for calculating the level of *Cyp6g1* mRNA transcript present and for visualising the presence of CYP6G1 protein.

3.3.1 Quantitative RT-PCR of *Cyp6g1* transcript in different susceptible and resistant life stages.

It has already been shown in Chapter 2 that flies over-transcribing *Cyp6g1* at the adult life cycle stage are resistant to both DDT when tested on adults and to imidacloprid when tested on larvae. This would suggest that *Cyp6g1* is over-transcribed at both these life cycle stages. The next series of results are presented to show when the *Cyp6g1* transcript and CYP6G1 protein are being produced naturally in susceptible strains and if the resistant stains show a higher level of protein throughout their life cycle.

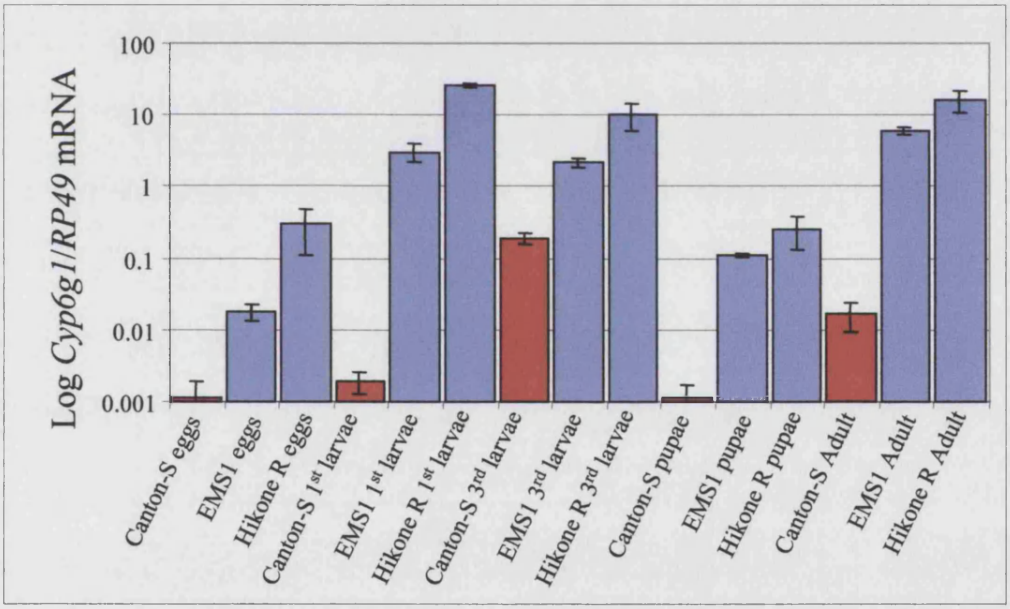


Fig. 3.3 Q-RT-PCR results for resistant and susceptible flies at different life cycle stages.

Figure 3.3 shows the relative quantity of Cyp6g1 transcript compared to the ribosomal protein RP49 for Canton-S (red) and two resistant strains (blue). It can be seen that when susceptible *D. melanogaster* are compared to the resistant flies *Cyp6g1* is very clearly over-transcribed at every life cycle stage. One of the most interesting points to note is the relatively high level of *Cyp6g1* in 3rd instar Canton-S larvae. It is possible that this is the stage at which the transcript is naturally transcribed. Nevertheless, it is still clear that the resistant flies still show a considerably higher level of transcript. It is also unlikely that the increased level of *Cyp6g1* in the Canton-S insects at this life cycle stage would confer resistance, at least in the bioassays used in this study. This is because in the larval bioassay described in Ch.2, the insects raised from eggs in the continuous presence of insecticides. Therefore, both eggs and 1st instar larvae (where there is a very low level of *Cyp6g1* transcript) will already have been exposed to imidacloprid and are probably dead before the 3rd instar is reached.

It should be noted though that the standard error bars in this experiment are very close together in most cases. As each bar on the chart is based on three separate RNA preparations this shows how there is a similar level of *Cyp6g1* transcript in each replicate sample from each strain and lifecycle stage.

3.3.2 Using the CYP6G1 antibody to calculate levels of protein and to investigate when in the life cycle CYP6G1 is present.

With a working polyclonal antibody it is possible to test part of our original hypothesis, that resistance is conferred by over-expression of CYP6G1 in our resistant strains. Until this stage, we could not be certain that the transcript was actually translated and so resistance was due to an increase in CYP6G1 levels. Even at this late stage, there was a possibility (although somewhat remote) that resistance was caused by the mRNA *Cyp6g1* transcript acting in some way on another gene.

The first experiment therefore was to carry out a Western using a range of total protein concentrations as calculated by Bradford assay with pairs of susceptible and resistant samples run in adjacent lanes as this would allow an estimate of the relative expression of CYP6G1 in Hikone R and Canton-S.

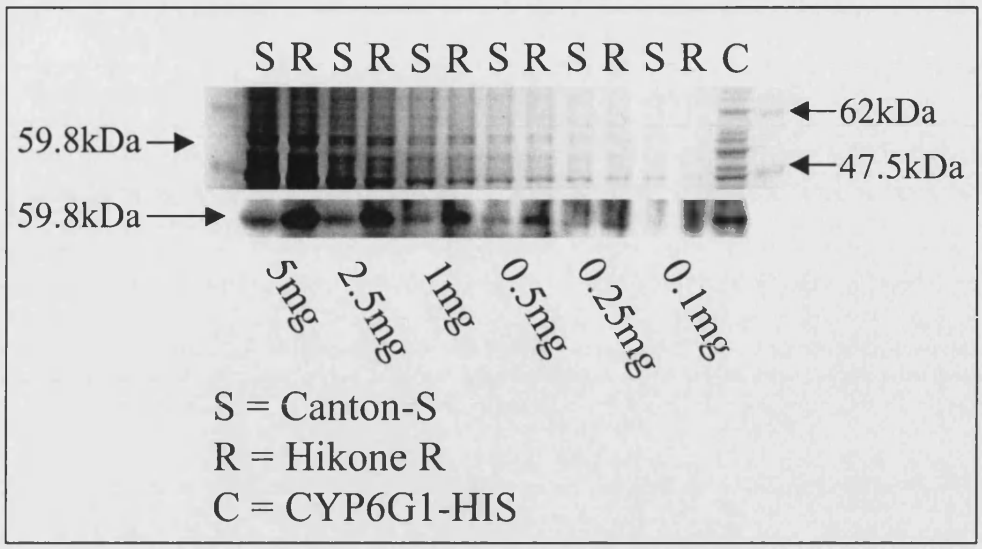


Fig. 3.4 Western blot analysis of polyclonal antibody.

This figure shows a coomassie stained gel showing the same amount of protein is present in strain at each concentration and a western showing *CYP6G1* amount at each concentration. From this experiment, it is very clear that there is more CYP6G1 present in the resistant strain than the susceptible strain. Moreover, although there does appear to be some CYP6G1 present in Canton-S it is at a significantly lower level than that seen in Hikone R. It is possible from this western to estimate that there is between 10 and 20 times more CYP6G1 present in Hikone R. The bands seen when 0.5 and 0.25 mg total Hikone R protein are compared to the band of Canton-S at 5 mg total protein are of a comparable intensity. Therefore, it is possible to estimate that the intensity of the susceptible band would appear to be between the intensity these two resistant bands so therefore there is approximately between 10 and 20 fold more CYP6G1 in the resistant strains.

This experiment was carried out using a 1:1000 dilution of whole rabbit sera for the primary antibody and 1:5000 Anti rabbit goat HRP conjugated secondary antibody (Sigma). It was visualised using SuperSignal West Femto substrate (Pierce) and X-ray film. The X-ray film was exposed to the western for 3 seconds. This experiment had previously been tried using an alkaline phosphatase conjugated anti rabbit goat secondary (Sigma) using BCIP substrate (Sigma). Unfortunately although it was clear to the naked eye that this experiment had worked it was not possible to transfer the

western blots to a digital format as the bands were of a very faint blue and so would not scan or show up in digital photographs so the HRP secondary was suggested and gave a very clear result.

These westerns also appear to show that CYP6G1 is an unstable protein as in all lanes where there is a signal of the correct size there is a ladder of smaller bands that are not present in lanes where the band of the correct size is not seen. This suggests that the protein is unstable and so we see a ladder of degradation products of CYP6G1 on the western. We can safely say that this is not random binding of the primary or secondary antibody as it is only ever seen in lanes with a band of the correct size, and it is seen in both resistant and susceptible lanes.

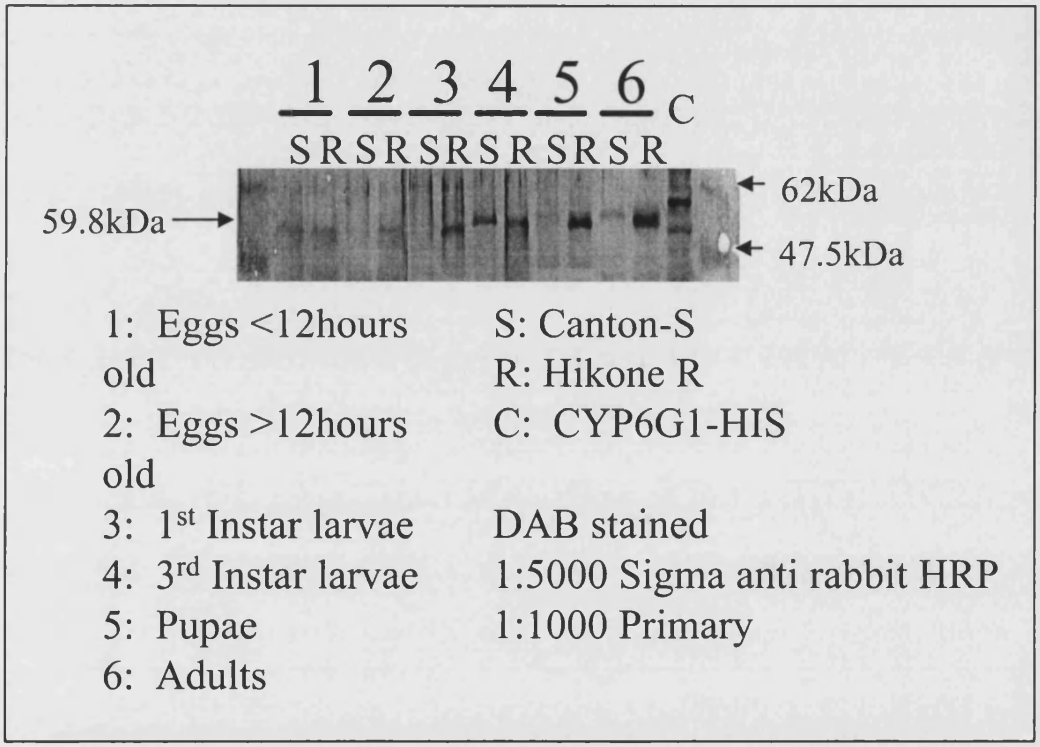


Fig. 3.5 Western analysis life cycle stages.

This figure now tells the story of the actual level of CYP6G1 present in each separate life cycle stages. It was decided to separate eggs into those older and those younger than 12 hours as the insitu-hybidisation work published on http://flybase.bio.indiana.edu/ suggest that in eggs less than 12 hours old there is no *Cyp6g1* present. Outlined below are some of the possible flaws and errors with just looking at the level of transcript.

This Western gives some interesting results in that it would appear to show that in eggs less than 12 h old there is an equal level of CYP6G1 which is not the result shown in the previous figure or demonstrated in insitu-hybidisation experiments from http://flybase.bio.indiana.edu. However, as this work is based on a large-scale screen it may not be as fully accurate as individually assessed samples. It would appear that there is no, or virtually no CYP6G1 present in later eggs and 1st instar susceptible larvae but there is a detectible amount present in their resistant counterparts. Also of interest is the band shown by 3rd instar Canton-S which agrees with the data shown in the previous graph where it was postulated that this is the life cycle stage at which CYP6G1 is normally produced. In both the pupal and adult stages only very small amounts of CYP6G1 are seen in the susceptible flies and it would appear that these are the stages that in the resistant flies the most CYP6G1 is present, with there being more present in the adult than pupa.

Staining on this Western was done in a slightly different way to Figure 3.3. In this case, the same combination of primary and secondary antibody were used but instead of a chemiluminescent substrate DAB (VECTOR LABS) was used according to the instructions. It was found that this gave a very good result and the substrate was dark enough to be scanned into a digital format using a flat bed scanner.

3.3.3 Western of different Resistant strains and transgenic flies.

A Western was carried out using 5 mg total protein in each lane. This Western has all the major strains used through out this thesis on it, including the transgenic lines and *D. simulans* strains that will be discussed in later chapters. It was stained using DAB with the VECTASTAIN ABC secondary. The primary antibody was diluted 1000 fold.

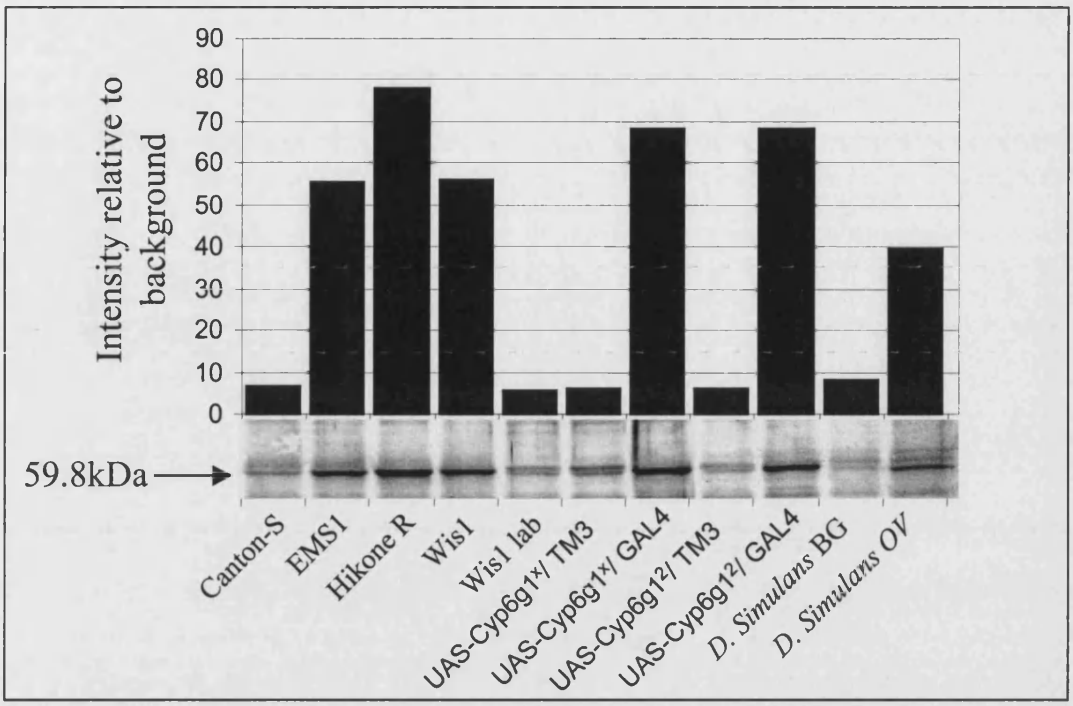


Fig. 3.6 Western of resistant and susceptible strains used in thesis, and transgenic lines and *D. simulans* strains.

This figure shows the differing levels of CYP6G1 present in different strains. It is clear from the western blot that CYP6G1 is present in all the strains used but is at a higher level in those strains resistant to imidacloprid. The lanes showing the resistant OV and susceptible BG *D. simulans* also have a homologue of CYP6G1, which is over expressed in the resistant strain. The detailed responses to insecticides for these two strains are detailed in Figure 5.5 and Table 5.6. The relative quantity of protein was calculated using the Quantity1 software from BioRad using their Chemidoc camera system.

The *D. melanogaster* strains on this western show that there is 8.3 times more CYP6G1 present in EMS1 and Wis1 as compared to Canton-S and 11.7 times more protein in Hikone R. This level of over-expression corresponds closely to that seen in pyrethroid resistant *M. domestica* over-expressing CYP6D1 (Smith and Scott, 1997). These relative amounts correspond to the resistance ratios seen in Table 2.9 where the results for imidacloprid are detailed. In that table Hikone R shows the largest resistance ratio and Wis1 and EMS1 both have a lower value, although in that table EMS1 has a higher

resistance ratio value than Wis1 implying that there is a possibility that other factors might be lowering the resistance in the Wis1 strain. Without this figure, it would have been assumed that these differences were due entirely to the amount of CYP6G1 present in the strain.

3.4 Discussion.

In this chapter, it has been shown that *Cyp6g1* is over-transcribed though out the life cycle of two resistant strains of *D. melanogaster*. The production of an anti-CYP6G1 antibody allowed us to show that the protein is also over-expressed throughout the lifecycle of resistant insects. It is very interesting to note the Westerns in Figures 3.4, 3.5 and 3.6 all show that there is a detectable level of CYP6G1 present in most of the susceptible life cycle stages and from Figure 3.4 it would appear that Canton-S has approximately 10-20 fold less CYP6G1 than the resistant Hikone R strain. This result is supported by the Western shown in Figure 3.6. This figure uses the Quantity One software from Bio-Rad to produce a semi-quantitative result for the relative amount of CYP6G1 present in each strain used in the thesis. However, a semi-quantitative method of this form precludes accurate comparisons between either strains or to compare the Western analysis to the three modes of RNA quantification used in this thesis. As two semi-quantification methods have been used (Figures 3.4 and 3.6) and give similar results we can be relatively certain that there is in the order of ten fold more CYP6G1 in adult Hikone R than adult Canton-S. This figure does not coincide with the result seen for Q-RT-PCR where there was apparently around 1000 fold more *Cyp6g1* transcript in Hikone R adults. Clearly, there is a large discrepancy between the two methods, it appears that there is a strong difference in the level of transcript seen when compared to the level of protein. This is also supported by Figure 2.13 which shows the Northern result for *Cyp6g1* transcript amount. In this case it would appear that the result seen in the Q-RT-PCR is the correct value as there is so little Canton-S *Cyp6g1* signal that it is not visible on the Northern blot where as there is a very strong signal for the three resistant strains. However, again it needs remembering that the Northern blot is also only semi-quantitative and so does not allow the calculation of accurate mRNA levels. It should be noted as well that this Northern blot shows that we would expect to see less CYP6G1 in Wis1 than in Hikone R as the signal for the transcript is weaker and this is supported by Figure 3.6 where there is slightly less CYP6G1 visible in Wis1 CYP6G1 level compared Hikone R. This pattern is mirrored in EMS1 and where there would

appear to be a slightly lower signal in the Northern when compared to Hikone R. However it should be remembered that this slight difference could equally be due to a number of other factors including the different stages being of slightly different ages or from the loading or running of the SDS-PAGE gel.

There are several possible reasons for this apparent discrepancy between protein and transcript levels. These include the stability of the mRNA transcript. We cannot know how stable this is in-vivo. It could be that there is indeed 1000 fold more mRNA present in the resistant Hikone R compared to Canton-S but it may be easily degraded and so a small amount may actually be translated. It is an equal possibility that the CYP6G1 protein is also very easily degraded. Therefore, although there may be a very large excess of mRNA transcripts they only serve to maintain CYP6G1 protein levels at approximately 10 fold above the normal susceptible level. Neither of these hypotheses are easily tested so this adds a further reason why the direct comparison of mRNA and protein levels should be carefully considered.

Therefore, it would appear that although the result we see when comparing relative levels of transcript or protein between our resistant strains the same pattern is repeated with Hikone R having a higher transcript and protein level than either Wis1 or EMS1. However this trend can not be accurately quantified as it would appear that the level of *Cyp6g1* transcript is not detectable by either Northern blot or Q-RT-PCR but we can clearly see a signal for the protein in all the Western blots shown. There are a number of reasons for this. It is possible that as the CYP6G1 antibody was not purified that there could be some cross reactivity to another P450. Therefore we see that P450 in the Western blots giving a low signal in the susceptible strain and as the probe for the Northern is to a unique region of *Cyp6g1* and specific primers are used in the Q-RT-PCR we do not see the same cross reactivity at the mRNA level.

There has also been a report in Science suggesting that the expression of many *D. melanogaster* genes is linked between certain life cycle stages. This report hypothesises that a similar expression profile is shown by eggs and pupae, whilst adults and larvae also have very similar expression profiles (Arbeitman et al., 2002). In this case it would appear to partially true when using Q-RT-PCR (the paper reports using insitu-hybridisation so it was looking at the mRNA level not the protein level) with Canton-S

eggs and pupae having a low level of transcript and 3rd instar larvae and adults showing a higher copy number. The level of transcript in 1st instar larvae however does not follow this reported pattern being at a similar level to eggs and pupae.

It is clear that this discrepancy in the relative mRNA and protein levels needs to be resolved. The most obvious method would be to use a more robust technique for assaying the level of CYP6G1 in susceptible and resistant insects. This would require purified CYP6G1 with a non-MBP tag for both antibody purification and for use in a competitive ELISA. An ELISA could assay the level of protein and enable us to assay the correct relative amounts of protein at different life cycle stages and between strains. A non-competitive ELISA was attempted and although it gave a result for adult flies of an approximate 10-fold increase in protein when Hikone R was compared to Canton-S the result was irreproducible. Therefore, a competitive ELISA was seen as a more promising method to obtain the desired result. However, there is a problem with using a competitive ELISA. It is necessary to generate a large amount of purified CYP6G1 to compete the samples against and this has not been possible.

This problem of generating pure CYP6G1 is common throughout the expression work with *Cyp6g1* constructs. Although some protein was generated from the MBP-*Cyp6g1* construct it was relatively insoluble and very difficult to purify in large amounts. For injections for antibody production a very small quantity is needed (approximately 30 μg/ml of MBP-CYP6G1 construct) but this is about 100 to 1000 fold too low an amount for either antibody purification or an ELISA. The pHIS tagged constructs were also made but both N and C terminal tagged proteins were expressed at a very low concentration and as would be expected of a P450 was always found in the insoluble fraction after sonication under a range of conditions. This made purification very difficult and there was not enough protein to use in a competitive ELISA.

Other approaches to developing an ELISA might include; first using a life cycle stage that does not have CYP6G1 present to purify the antibody as any non-anti-CYP6G1 antibodies in the rabbit sera that cross react to *D. melanogaster* proteins would be removed using this method. However as Figures 3.5 and 3.6 show, there is a possibility that CYP6G1 is present in all the life stage of the susceptible strain. A second possible method would be the creation of an RNAi knock-out for *Cyp6g1*. There is a third

method that that might allow the purification of a pure antibody and a soluble protein to be used in a competitive ELISA. Instead of trying to express a soluble whole CYP6G1 protein to generate clones of parts of the protein and so try to express CYP6G1 fragments. If this could be done with a unique part of the protein then there would be a good possibility that this could be used to purify the antibody. An antibody purified in this way could then be used in a competitive ELISA with the insect preparation being competed against the pure fragment so we would be expected to get a result in this way. This method would also make a sandwich ELISA possible, as two unique regions could be cloned and then both used in an ELISA which would increase the specificity of the experiment.

Ch 4. Cross resistance to new neonicotinoids.

4.1 Introduction.

The aims of this chapter are to investigate whether resistance to imidacloprid confers cross-resistance to new neonicotinoid compounds. We also now had access to a transgenic *Drosophila* strain (Daborn et al., 2002) that would allow us to test if it is *Cyp6g1* over transcription that confers resistance to all these insecticides. At this stage, a micro-array also became available to the laboratory so we were able to demonstrate that it was only *Cyp6g1* over-transcribed in the transgenic line. The majority of these results have been published in part in Le Goff *et al,* 2003.

Since the discovery of imidacloprid in the late 1980s a series of new neonicotinoid compounds have been developed. All are based on the general structure shown in Figure 1.3 and some examples of the actual structures are shown in Figure 4.1. Cross resistance to the new active ingredients reaching the market by resistance mechanisms that are already present in the populations of wild insects is an important applied question. The design aims for new compounds are to vary the properties of the basic compound in order to produce new insecticides with new features such as increased penetrability or slightly different binding features for the nAChR (Kagabu, 2003). These changes can lead to compounds with a new insecticidal profile that can increase sales by targeting new species. Imidacloprid is most effective against sucking pests so changes in the basic structure may reveal a compound that is active against insects with different feeding habits. Thus although imidacloprid has been used with some success against the Colorado potato beetle (Olson et al., 2000) it is hoped that new compounds would be more active against a different range of insects. This has the benefit of a new compound not being in direct competition with one already established on the market. If the new compound targets a different range of insect species then there is a decreased likelihood of encountering cross-resistance from other neonicotinoids. This is because the new target species will not have been directly targeted by this class of insecticide in the past. Changes to neonicotinoid structure might also affect their binding to the target receptor. Thus, it is also hoped to decrease the effects of cross-resistance if target site resistance were to evolve, as the new compound would bind in a slightly different way. This should negate the target site resistance seen to the original compound. This is however theoretical until resistant insects are isolated with demonstratable target site resistance.

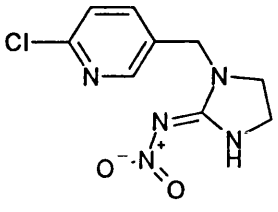
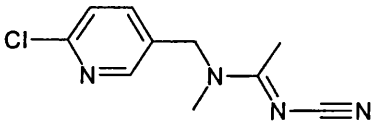
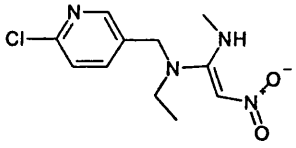
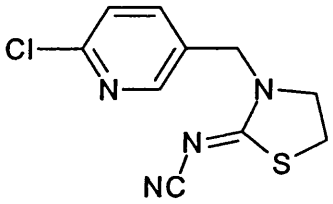
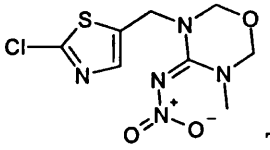
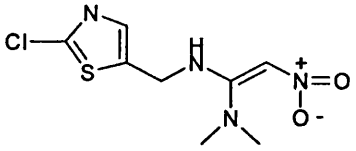
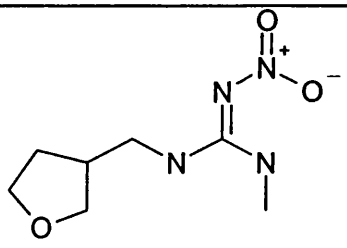
Neonicotinoid generation	*Examples*
First	 Imidacloprid (Bayer)  Acetamiprid (Nippon Soda)  Nitenpyram (Takeda)  Thiacloprid (Bayer)
Second	 Thiamethoxam (Syngenta)  Clothinidin (Takeda/Bayer)
Third	 Dinotefurum (Mitsui)

Fig. 4.1 New neonicotinoid compounds.

Cross-resistance can have both biological and economic consequences as there is a stage when the cost of applying sufficient chemical to kill the insect is greater than the profit made from selling a decreased yield from a crop that has been attacked by a pest species (Broadhurst, 1998; Denholm et al., 1998; Dent, 1991). There are also environmental considerations, as many chemicals used as insecticides are also toxic to other animals and so there is a maximum dose that can be applied regardless of whether the dose will kill the pest species (Smith, 2001). The increased awareness of the environment and an increased consideration of the cost of insecticides has led to a closer interaction between the agrochemical industry and farmers in an attempt to save the farmers money by applying either less insecticide or a cocktail of insecticides to control a pest. This, at first glance, is not in a company's best interest as they want to sell the maximum amount of their product but a closer look reveals how this might benefit the company. The more carefully a compound is applied in the field, the smaller are chances of resistance developing, which in turn increases the life span of a product and this therefore increases the long term profits.

Two transgenic strains with a *UAS-Cyp6g1* construct were generated in our laboratory (Daborn et al., 2002; Korytko et al., 2000), and these strains were used to investigate DDT resistance in adults so it was possible to use a strain with GAL4 expression driven by a heat shock promoter. This led to the possibility of generating a cross where all the F1 generation were heterozygous for both the heat shock apparatus and *UAS-Cyp6g1* so upon heating all insects would become resistant. Unfortunately, it is not possible to use this method with neonicotinoids because they are used in a larval bioassay so would require rounds of heating and cooling for the full developmental time, which would kill the larvae. A second GAL4 driving strain was used where GAL4 expression is linked to tubulin expression and is thus constitutive. This strain however is not homozygous so only half the F1 cross in this case constitutively over-express CYP6G1. This however allows for easy controls for the experiment as we have flies emerge from the same cross that are susceptible and so act as the control for those insects over-expressing *Cyp6g1* (Le Goff et al., 2003).

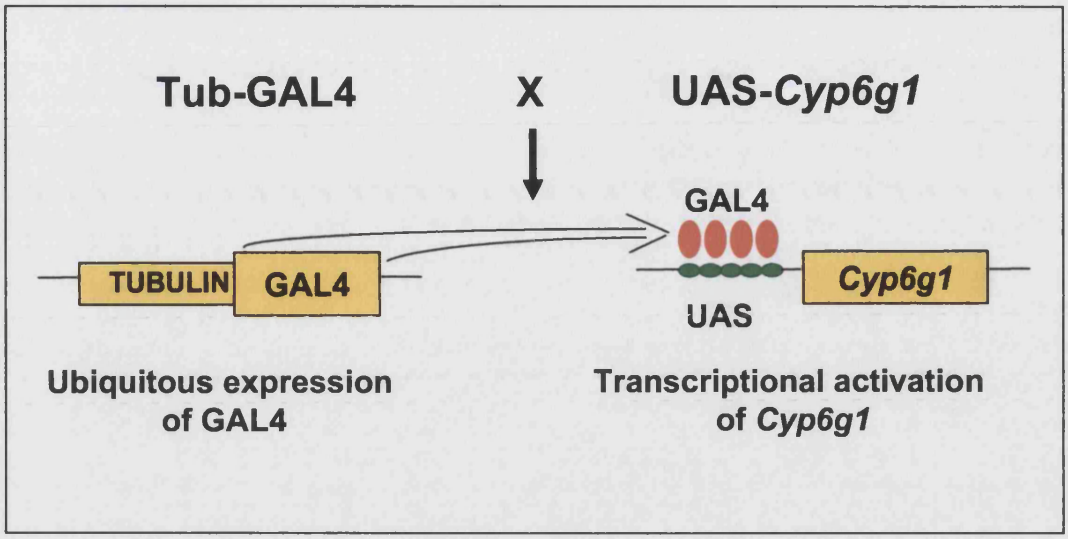


Fig. 4.2 Expression of transgenic *Cyp6g1*

This chapter also sees the use of a micro-array. In the conclusion to Chapter 1 it was stated that our work investigating the over-transcription of *D. melanogaster* P450s using Northern blots was not the best method to use. It is too time consuming to generate a result for all the P450s in the *D. melanogaster* genome and the nature of the result limits the quantitative accuracy of this method. It can also be difficult to resolve a result when only a slight level of over-transcription is seen, although at the time it was the best method available to us. It would be better to use a micro-array that consisted of all the *D. melanogaster* P450s. This micro-array was first used in Daborn *et al*, 2002 and was provided by the laboratory of Dr. R. Feyereisen. This micro-array allowed the investigation of P450 expression profiles in the transgenic *D. melanogaster* lines and Chapter 5 of a range of different resistant strains (Le Goff et al., 2003).

4.2 Methods

4.2.1 Generation of dosage mortality curves with new neonicotinoids.

To generate DMCs for the neonicotinoids acetamiprid, nitenpyram and thiamethoxam the procedure for larval assays was used as described on Section 2.2.1.2. The homozygous strains Hikone R, EMS1 and Canton-S were used with between 5 and 7 insecticide concentrations with between 4 and 10 replicates at each insecticide concentration.

4.2.2 Mapping neonicotinoid resistance.

Resistance to the neonicotinoids acetamiprid, nitenpyram and thiamethoxam was carried out using the following crosses to localise resistance firstly to a specific chromosome then to a specific site on chromosome II.

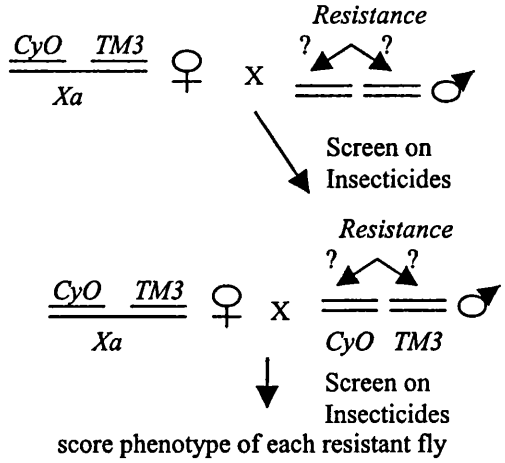


Fig 4.3 Mapping cross to locate resistance to one autosome.

This cross was used to map neonicotinoid resistance to a specific autosome. Xa/CyO; TM3 is a multiply marked balancer strain where chromosomes II and III carrying ap^{Xa} co-segregate due to a chromosome transposition. The other copies of chromosomes II and III (CyO and TM3) assort independently. Males of $Rst(2)DDT^{Hikone\ R}$ are crossed to Xa/CyO;TM3 females and progeny are reared on 20 µg/vial acetamiprid, nitenpyram or thiamethoxam. Emerging resistant males are crossed to Xa/CyO;TM3 females and progeny again reared on 20 µg/vial acetamiprid, nitenpyram or thiamethoxam and the phenotype of the offspring scored. The use of male resistant flies in the parent cross and

only using the male F1 flies means that we can be sure that resistance is not located on chromosome I as this chromosome is not passed to the males of the F1 generation.

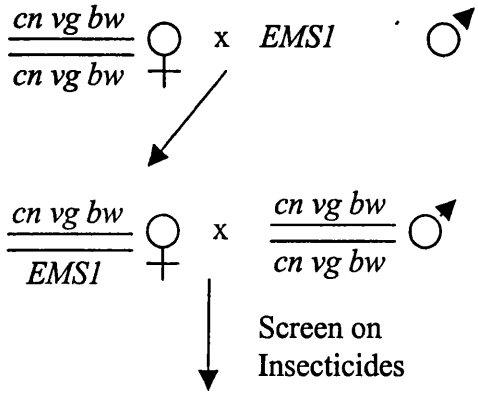


Fig 4.4 Mapping cross to localise resistance on Chromosome II.

Neonicotinoid resistance in strain Rst(2)DDT^{EMS1} was mapped against visible mutants on chromosome II. The same insecticide concentrations were used as in the cross to locate resistance to one autosome. This allows the calculation of the position of insecticide resistance relative to the known visual markers cinnabar and vestigial.

4.2.3 Transgenic flies.

Transgenic *D. melanogaster* were created by Dr. P. Daborn (Daborn et al., 2002; Le Goff et al., 2003). The Canton-S *Cyp6g1* ORF was amplified by PCR from Canton-S genomic DNA and cloned into the PUAS vector. This construct was injected into *y; w* embryos. Transformed flies were raised as individual. Two lines were found with the *UAS-Cyp6g1* construct, *UAS-Cyp6g1*^{X} and *UAS-Cyp6g1*^{2} mapping to chromosomes I and II respectively.

Female transgenic flies, homozygous for *UAS-Cyp6g1*, were crossed to y[1] w[*]; P{w[+mC]=GAL4}LL7/ TM3, Sb[1] which are heterozygous for tubulin driven Gal4 expression. The F1 progeny either over-transcribe *Cyp6g1* or have the *Stubble* phenotype so flies for the DDT bioassay can be separated into those over-transcribing *Cyp6g1* and those not over-transcribing Cyp6g1 and assayed separately. Adults to emerge from the larval screen were scored for *Stubble* or wildtype thoracic bristles.

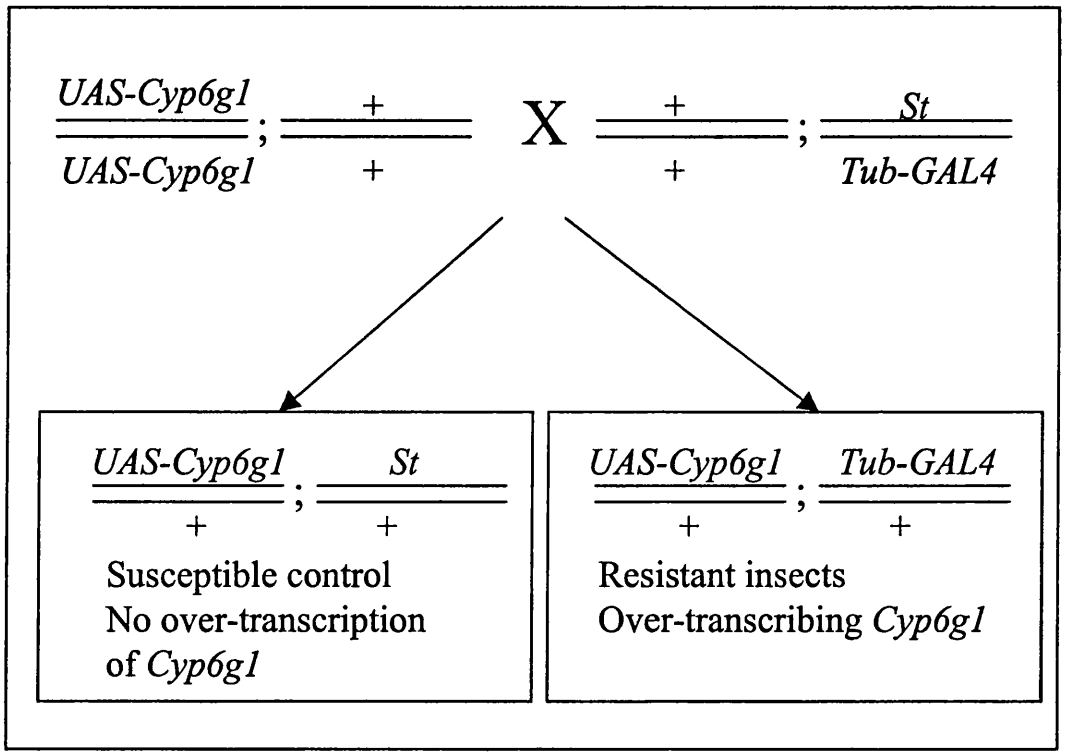


Fig. 4.5 Transgenic fly cross

This figure shows the cross between the UAS-Cyp6g1 transgenic line and the tubulin driven constitutive GAL4 over-transcribing strain to give progeny that segregate for Stubble or Cyp6g1 over-transcription.

4.2.4 Micro-array work

4.2.4.1 RNA extraction

Three day old adult *Drosophila* were flash frozen in liquid nitrogen and homogenized with a pestle and mortar. The resultant powder was then re-suspended in 600 µl of 4 M guanidine thiocyanate, 25 mM Na citrate, 0.5% sodium N-lauryl-sarcosine and 1% 2-mercaptoethanol and following the addition of 500 µl of phenol, 120 µl of chloroform and 36 µl of Na acetate 3 M pH 5.2, the mixture was incubated on the ice for 15 min. After phenol extraction, the aqueous phase was precipitated with one volume of isopropanol by incubation on ice for 1 h. The resultant pellet obtained after centrifugation was washed with 70% ethanol, dried and re-suspended in RNase free water. DNA was removed from the sample by adding 1 µl of DNase (Promega) and incubating for 30 min. at 37°C. A further phenol-chloroform extraction and isopropanol

precipitation was then repeated as described above and the resulting RNA used directly for labeling.

4.2.4.2 Micro-array construction and hybridization

A micro-array of PCR products from 132 genes of *D. melanogaster* was constructed. These genes represent all 90 cytochrome P450 genes predicted from the full genome sequence, several other genes encoding metabolic enzymes, such as esterases and glutathione-S-transferases (GSTs) and several 'housekeeping' genes included as controls. A fragment of each gene was amplified via the polymerase chain reaction (PCR) and spotted on the array in four independent locations. To label sample RNAs for hybridization, 10 μg RNA was labeled with the CyScribe First-Strand DNA labeling kit (Amersham), according to the manufacturer's instructions. Unlabelled RNA was removed by digestion with 100 μg/ml of RNase A at 37°C for 1 h. The array was hybridized to cDNA from both resistant and susceptible strains simultaneously using Cy3 and Cy5 labels. Experiments were repeated in triplicate in which the Cy3 and Cy5 labels were swapped between strains to account for potential differences in labeling efficiency. The Cy3 and Cy5 labeled samples were purified separately using a QIAquick (Qiagen) nucleotide removal kit, according to the manufacturer's instructions. The samples were then dried under vacuum and resuspended into 13 μl of DIG easy Hyb solution. The Cy3 and Cy5 labeled samples were then combined immediately prior to hybridization. This hybridization mixture was then placed on the micro-array and covered by a 22 x 40 mm glass cover slip. Arrays were then placed into a hybridization chamber and submerged in a water bath at 50°C for 16–20 h., in the dark. Following hybridization, arrays were washed in 1 x SSC, 0.03% SDS for 5 min, taking care to float the cover slip gently off the array. Then two additional washes in 0.2 x SCC and 0.05 x SCC at room temperature, each for 5 min, were carried out before the slides were dried by centrifugation.

Labeled arrays were scanned with a GMS 418 array scanner (Genetic Microsystems). The best dynamic range of the data was achieved by adjusting the gain so that the highest signal was just under saturation. Quantification of the signal from each spot, and superimposition of both dye channels, was performed using the ImaGene software, version 4.2 (Biodiscovery). Data files generated by ImaGene were downloaded into GeneSpring version 5.0 (Silicon Genetics) where data normalization and statistical

analysis was performed. Values of less than 0 were set to 0, and the data normalized using an intensity dependent algorithm (Lowess). The statistical significance of the over-transcription of specific genes was tested using individual t-tests comparing the mean value for a specific P450 gene against the mean of all other P450 genes combined (GeneSpring).

4.2.5 Neonicotinoid resistance in transgenic flies over-transcribing *Cyp6g1.*

Data for resistance to neonicotinoids in transgenic over-transcription of *Cyp6g1* was generated by placing 50 eggs on agar based fly food (1350 ml water, 80 g oatmeal, 138 g black treacle, 7.5 g plant agar, 16 g deactivated yeast powder, 10 ml per vial) containing a discriminating concentration of insecticide, either 2 µg/vial imidacloprid, 12.5 µg/vial acetamiprid, 20 µg/vial nitenpyram or 10 µg/vial thiamethoxam per vial. The number and phenotype of emerging adults was observed. 10 repeats were used for each insecticide and results were adjusted using controls of 50 eggs placed on wet fly food with no insecticide. UAS-*Cyp6g1* females were crossed with tub-GAL4 males to over-transcribe *Cyp6g1*. Controls used were y[1]w[1] females crossed with tub-GAL4/TM3 males, y[1]w[1] females crossed with UAS-*Cyp6g1* males and Canton-S. Flies with the *Stubble* (*Sb*) phenotype were scored as not over-transcribing *Cyp6g1* and flies that were wild type for *Stubble* were scored as over-transcribing *Cyp6g1*. The vials containing the transgenic cross that was expected to over-transcribe *Cyp6g1* had 100 eggs placed in them as with 50 eggs, of which only 50% were expected to over-transcribe *Cyp6g1,* too few flies emerged for statistical analysis. In these vials, the flies to emerge in the controls were scored, and the ratio of *St* to *wt* was calculated and this was used to calculate the number of eggs of each phenotype to be placed on the food. Unfortunately, this method makes the unavoidable assumption that both genotypes will be equally fit and the same number of eggs will develop successfully into adults. The ratio of *St* to *wt* eggs was then assumed true in those vials containing insecticide so this value was used as the control when calculating the number of eggs of each phenotype to be placed on the diet and so used in calculations of percentage survival plotted in Figure 4.9.

This experiment had originally been planned as a series of DMCs using the dry fly food used in the previous DMCs but after several failed attempts at this method where all the transgenic flies died even in controls it was decided to use the wet fly food. A single

dose was used, as it proved difficult to get sufficient eggs, especially when using 100 eggs from each transgenic cross in every vial, without the adults used ageing too much and so laying less healthy eggs. It is also difficult to make the food with insecticide using this method, as it has to be done when the food is still liquid, but not so hot it would degrade the insecticide. Doing the 8 repeats necessary for each of four insecticides was too much work for a relatively small figure and it was decided that time was better spent working on other parts of the project.

4.3 Results.

The results in this chapter have been largely published by Le Goff (Le Goff et al., 2003). I shall outline my role in this work which is a study of further cross resistance to new classes of neonicotinoids, and of resistance to malathion and also introduces the results of using transgenic flies over-transcribing *Cyp6g1* in a controlled way.

4.3.1 Cross Resistance to new neonicotinoids.

Due to the commercial importance of the neonicotinoids, it was decided to carry out further DMCs on three other members of this new class of insecticide. They are acetamiprid, nitenpyram and thiamethoxam, all supplied by Syngenta, UK. The structures of these chemicals are shown in Figure 4.1.

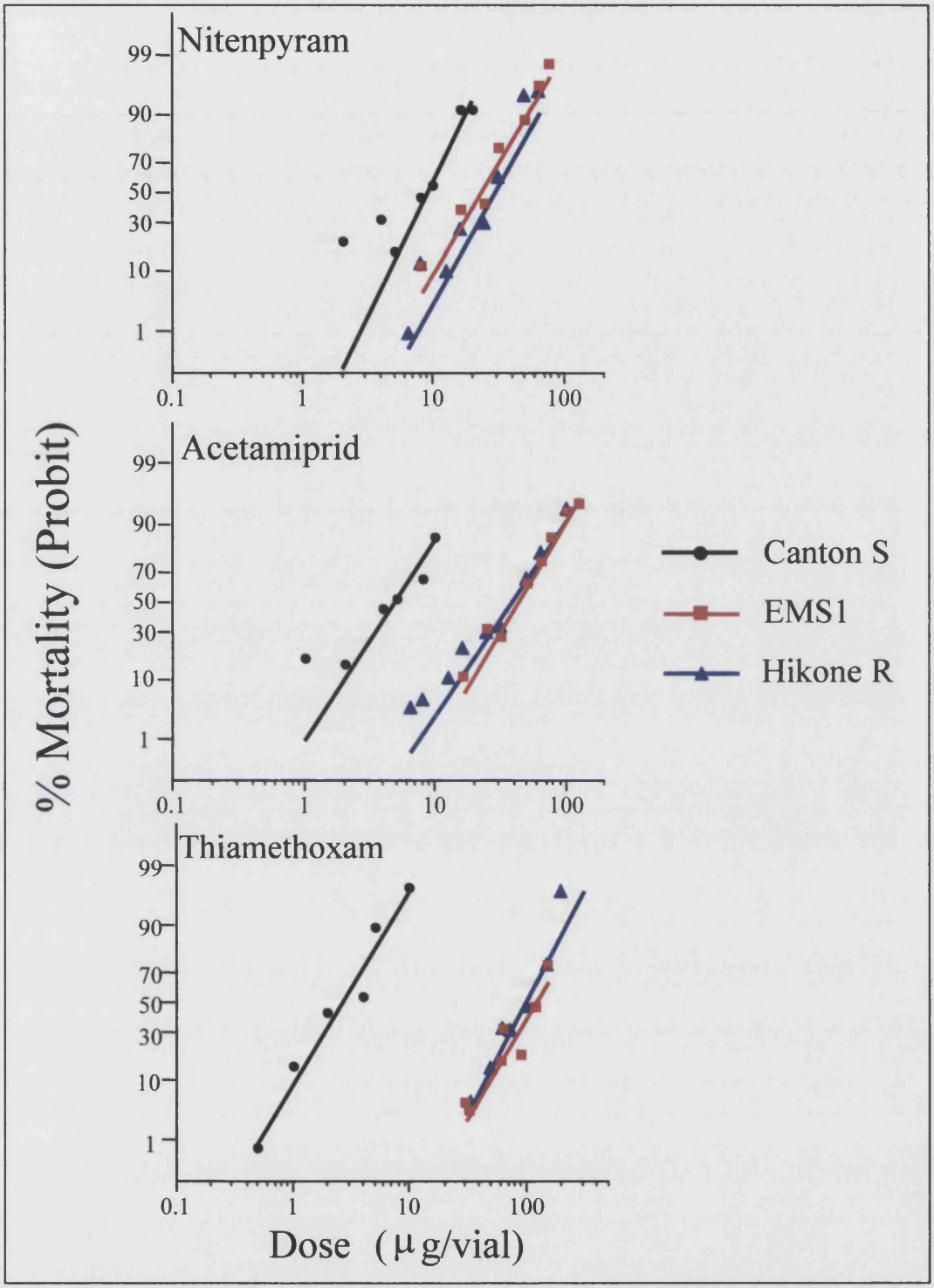


Fig. 4.6 Dosage mortality curves for new neonicotinoids on Canton-S, EMS1 and Hikone R.

Table 4.7 A detailed comparison of the potency of the different insecticides used.

Strain	N	LD_{50}	(90%CI)	Slope	+-S.E.	Resistance ratio
Imidacloprid						
Canton-S	1500	0.53	(0.42-0.68)	3.86	0.97	1.00
EMS1	1850	4.11	(3.19-4.97)	3.11	0.36	7.75
Hikone R	1600	4.74	(3.71-5.75)	3.00	0.57	8.94
DDT						
Canton-S	780	1.45	(1.22-1.72)	2.91	0.25	1.00
EMS1	800	10.50	(6.73-13.74)	2.53	0.24	7.24
Hikone R	780	18.97	(13.03-24.75)	2.21	0.18	13.08
Acetamiprid						
Canton-S	5400	4.99	(4.36-5.55)	3.35	0.27	1.00
EMS1	5400	41.60	(38.90-44.15)	3.64	0.16	8.34
Hikone R	2450	36.97	(32.56-40.88)	3.26	0.29	7.41
Nitenpyram						
Canton-S	5200	8.99	(7.85-9.93)	4.46	0.37	1.00
EMS1	5350	21.89	(19.84-23.78)	3.71	0.17	2.43
Hikone R	2500	27.98	(24.87-30.72)	3.90	0.34	3.11
Thiamethoxam						
Canton-S	5400	2.73	(2.30-3.11)	3.26	0.26	1.00
EMS1	5400	115.98	(106.3-127.2)	3.31	0.31	42.48
Hikone R	7500	94.38	(86.7-101.8)	3.75	0.17	34.57

It is interesting to note that of the differing resistance ratios seen nitenpyram has the smallest resistance ratio suggesting that it would be the most effective neonicotinoid to use in an attempt to combat resistance of this mechanism as it can be used at a slightly higher dose and this will kill the resistant insects. Thiamethoxam has a relatively large resistance ratio in comparison to the other neonicotinoids and this could have implications if resistance were to arise in a pest species using the same mechanism reported in this thesis. However, after imidacloprid it is the most effective insecticide to use on susceptible insects which would mean a lower dose of thiamethoxam than either

nitenpyram or acetamiprid could be used in the field which would be more ecologically sound. This does not however take into account the stability and other environmental properties of these compounds. Of the neonicotinoids tested, imidacloprid is the most effective, with a LD_{50} of 0.53 μg/vial for susceptible *D. melanogaster* and 4.11 and 4.74 μg/vial for the EMS1 and Hikone R respectively. Acetamiprid demonstrates an approximately 10 fold lower efficacy against both susceptible and resistant *D. melanogaster* giving a similar resistance ratio of between 7 and 9 fold over Canton-S.

The resistance to each of these compounds was also mapped using the crosses shown in Figures 4.3 and 4.4. Resistance in EMS1 was mapped to 65.5 ± 1.3 cM, chromosome 2 for acetamiprid, 64.6 ± 4.1 cM for nitenpyram and position 64.9 ± 1.2 cM for thiamethoxam. All these values are in the region of 65.0, which is the region of interest for EMS1 and Hikone R and corresponds favourably to the values published for DDT resistance in EMS1 where Daborn *et al* (2001) mapped DDT resistance to 65.1 ± 0.25. From this we can conclude that the resistance mechanism is likely to be the same for all the neonicotinoids tested on these strains.

4.3.2 Using Transgenic flies to test our *Cyp6g1* hypothesis.

In the previous chapter, it was shown that *Cyp6g1* is over-transcribed throughout the life cycle of resistant *D. melanogaster* strains, and Western blots were used to show that this is also true of the expressed protein. In this section, transgenic flies are used to show that when *Cyp6g1* is the only P450 over-transcribed in a previously susceptible strain then resistance is induced.

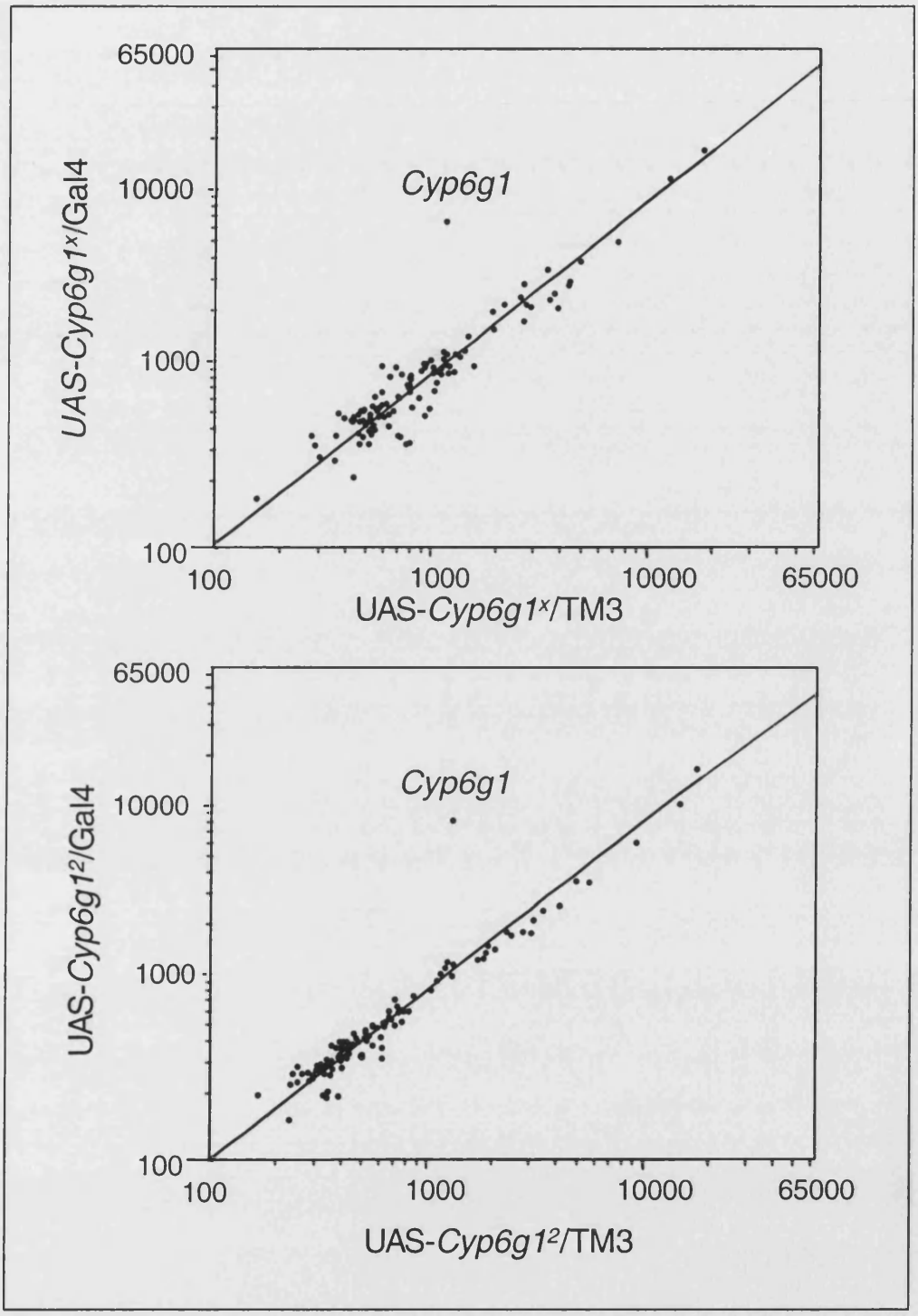


Fig. 4.8 Micro-array results comparing transgenic flies transcribing and not transcribing *Cyp6g1*.

Figure 4.8 shows the micro-array result for the two different transgenic UAS-*Cyp6g1* lines giving a comparison of the F1 offspring with and without tubulin linked GAL4 expression. These two micro-array results show that the only P450 over-transcribed in this cross is *Cyp6g1*. This shows that the transgene works properly and that it is under control as the control strain shows a lower level of transcription of *Cyp6g1*. It should be noted that the transgene would appear to be more up regulated in the strain where it is on the X chromosome than the strain where it was inserted into chromosome II. This could give an interesting result when the strains are exposed to insecticide.

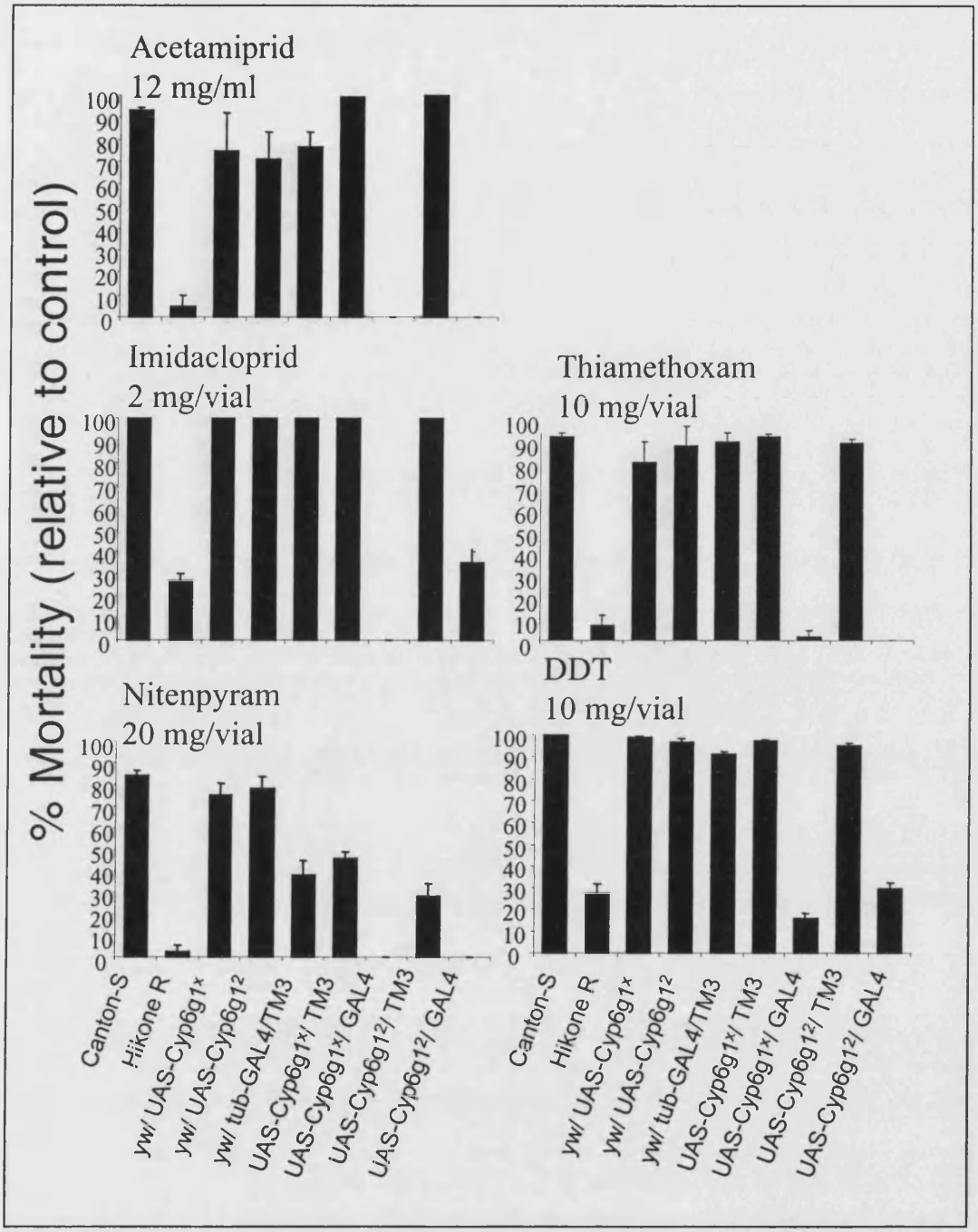


Fig. 4.9 Transgenic over transcription of *Cyp6g1* confers resistance to insecticides.

The five panels of this figure show the mortality of each transgenic line and controls when exposed to the discriminating dose of each insecticide (as worked out from the DMCs for Canton-S and Hikone R.) The results are calculated as percentage mortality relative to controls run simultaneously with no insecticide in the food. This allows for differential mortality between replicates.

Figure 4.9 shows that the over-transcription of *Cyp6g1* does indeed confer resistance to a range of compounds. In all cases, there was considerably more mortality in the cross with the transgenic fly over *St* that had no *Cyp6g1* over-transcription when compared to the transgenic fly with tubulin linked gal4 over-transcription. It should be noted that with imidacloprid the transgenic chromosome II flies showed more mortality than flies with the transgene on the X chromosome. This could be due to the higher transcription of the X linked *Cyp6g1* as seen in Figure 4.8. This effect was also seen in a more limited way with DDT where slightly fewer flies with a chromosome II linked transgene survived exposure to the insecticide than those with an X-linked transgene. It is possible that this effect was only seen in these two parts of the experiment as this is where a higher relative dose of insecticide was used. The result for nitenpyram shows the compromise made when selecting the discriminating dose, as although all the expected transgenic flies emerged many flies without the tubulin driven gal4 over-expression and hence no *Cyp6g1* over-transcription also survived the screen.

4.4 Discussion.

The results of this chapter show that resistance to DDT and imidacloprid conferred by *Cyp6g1* over-transcription confers cross-resistance to all the other neonicotinoid compounds tested in this chapter. It is interesting to note that as with DDT and imidacloprid the EMS1 and Hikone R strains showed very similar levels of resistance compared to Canton-S, as shown in Table 4.7. This adds further evidence for the hypothesis that *Cyp6g1* is solely responsible for insecticide resistance in these two strains and similar levels of resistance are seen as both have similar levels of CYP6G1 (Figures 3.3 and 3.6). It is also important to observe the differences in the LD_{50}'s and resistance ratios seen for the four neonicotinoids tested in this thesis. DDT is not included in this comparison as it is tested on a different life cycle stage to the neonicotinoids. This means that other factors, which vary between adults and larvae, may also affect resistance. The wide range of LD_{50}'s seen when comparing the four neonicotinoids used is an interesting finding of this thesis. As the 64.5 cM resistance mechanism has proved to be metabolic in its mode of action it is not a surprise that resistance to imidacloprid confers cross-resistance to other molecules with a similar structure (Kikkawa, 1964). However, a comparison of LD_{50}'s for the four neonicotinoids tested shows firstly a large range of LD_{50}'s for Canton-S. These results

show that the different compounds have a range of effectiveness against *D. melanogaster*. Table 4.7 shows imidacloprid to be the most effective compound and nitenpyram to be least effective against Canton-S. Possible reasons for this range of LD_{50}'s are that each compound is aimed at a different pest species and so will show differing abilities to enter *D. melanogaster* and interact with its nAChR. Alternatively, the chemical structures of the different compounds could affect how efficiently they are able to reach the target receptors.

Figures 4.6 and 4.9 and Table 4.7 show that resistance to DDT and imidacloprid from the over-transcription of *Cyp6g1* confers cross resistance to three newer neonicotinoids. This is a possibly worrying trend that this enzyme is capable of metabolising all the neonicotinoids so far tested on the resistant strains. If any pest species were to evolve resistance based on a homologue of CYP6G1 being over-expressed then there would be a serious problem in combating that insect with the current range of chemical insecticides. The only good thing about these resistance mechanisms is that they generally confer a small resistance ratio so an increase in topical dose should not be necessary to kill the resistant insects. It has previously been reported that although the over-expression of this enzyme does confer cross-resistance to many classes of compound it has one interesting effect. That, this enzyme appears to metabolise phenylthiourea a compound harmless to insects to phenylurea so the addition of this synergist to insecticide preparations would go some way to combat this resistance type (Ogita, 1960).

Another interesting result from this chapter is that the resistance ratios vary greatly between neonicotinoids. We know that both the EMS1 and Hikone R strains show similar expression patterns for *Cyp6g1* and that they always have a similar resistance ratio to each other with the insecticides tested so far is to be expected. The combined result for both these strains gives extra evidence to the hypothesis that CYP6G1 is not equally capable of metabolising all neonicotinoid compounds. It would appear that although these four compounds are very similar in their structure the subtle differences are enough to cause a wide range of resistance ratios. A simple explanation for the range in LD_{50}'s for EMS1 and Hikone R could be that the enzyme has a differing affinity for each compound and so is best able to metabolise thiamethoxam (as this has the highest resistance ratios) and is least capable of metabolising nitenpyram (the lowest resistance

ratios). However, it is also possible that some of the metabolites produced by CYP6G1 are toxic. It has been shown by Nauen that some of the plant metabolites of imidacloprid are almost as toxic, and in some cases more toxic than the parent molecule to aphids (Nauen et al., 1998). So it is possible that CYP6G1 is equally capable of metabolising all the neonicotinoids, and in fact all the insecticides that this resistance isoform has been linked to, but that the differences seen in resistance ratios are caused by the varying toxicity of the different metabolites. In this case the metabolite generated from nitenpyram would need to be almost as toxic as the parent compound whereas the metabolite of thiamethoxam produced by CYP6G1 would need to be approximately 40 times less toxic than the parent molecule. Such hypothetical differences in toxicity could be due to a number of reasons. It could be that the metabolites are less able to interact with the insect nAChR. Figure 1.6 shows a cartoon of the postulated interaction of neonicotinoids with the receptor. A change in the charge of either of the groups interacting with the receptor, or a change in the distance between them could alter the affinity for the receptor and so have and effect on the toxicity of the compound (Tomizawa and Casida, 2001). It is also a possibility that a change in the ionic charge of the molecule would inhibit the ability of the molecule to enter the insect nervous system. This would mean the molecule would not be able to affect the nAChR. Kagabu has speculated that the charge of a neonicotinoid may affect its ability to cross the ion barrier to the insect nervous system (Kagabu, 2003). A third possibility of why we see such a range of resistance ratios between the neonicotinoids could be that the different metabolites having differing degrees of ease of excretion. Thus, the metabolites of nitenpyram might remain as relatively insoluble molecules and so not a lot easier to excrete than the parent molecule. Alternatively, thiamethoxam could be metabolised to a compound that is more hydrophilic than the parent molecule and so is easier to excrete. This theory could also be closely related to the second hypothesis, as a change in the ionic state of the compound would affect the ability to cross the ion barrier as well as affect solubility. These various theories are not mutually exclusive.

The use of the transgenic *Drosophila* line supplied by Dr. P. Daborn proves our early hypothesis form Chapter 2 that *Cyp6g1* over-transcription confers resistance to a range of compounds. The micro-array results show that only *Cyp6g1* is over-transcribed in these two lines in the presence of GAL4. This tells us that the resistance seen in Figure 4.9 is due to an increased level of *Cyp6g1*, and not any other P450 on the micro-array.

These results do not however exclude the possibility that other (non-P450) genes may be involved. At this point Figure 3.6 should also be considered. The two transgenic strains shown on this Western both have very similar amounts of CYP6G1 present. However, it should be noted that the X-linked trans gene appears to be the more resistant strain from Figure 4.9 and appears to have a higher level of the mRNA transcript from Figure 4.8. The Western in Figure 3.6 also agrees with that observation, as there appears to be a relatively higher amount of the CYP6G1 protein present in the more resistant X-linked line.

The results for the Canton-S and Hikone R controls show that the correct dose was selected in most cases. It can be seen than a slightly higher dose of thiamethoxam could have been used as only 90% of the Canton-S died. The same is true of acetamiprid. Nitenpyram posed an interesting problem as although the LD_{50}'s for Hikone R and Canton-S are quite different (27.98 and 8.99 μg/vial respectively), the DMCs shown in Figure 4.6 overlap considerably and so choosing a dose that would kill all the susceptible flies would result in the killing of a considerable percentage of the resistant insects. Because of this situation, a slightly lower dose was chosen where although some susceptible insects would survive the majority would die and most resistant flies would survive. The control in this case worked very well with only 17% of Canton-S surviving compared to 97% of Hikone R. It might also have been possible to use slightly lower doses of DDT and imidacloprid as we saw about 20% mortality in the resistant control strain but the doses used were those used in mapping experiments where it was important that no susceptible flies emerged and the same doses were used in these experiments.

Improvements could be made to the work carried out in this chapter in several ways. Firstly a full micro-array (i.e. one covering the whole genome) could be used instead of the limited one we have access to but this would be a considerably more expensive option as a full *D. melanogaster* micro-array is considerably more expensive than the type we used. A complete micro-array would however answer whether it is solely *Cyp6g1* over-transcription conferring the resistance profile seen of if *Cyp6g1* acts on other genes. For example, it is still a remote possibility that the over-expression of CYP6G1 affects the level of other enzymes or even receptors although this is extremely unlikely, as we know that no other P450 is over-transcribed. CYP6G1 is very unlikely

to affect the targets of the insecticides tested, as virtually all classes of insecticide are included in the DDT-R resistance profile. This means that all insecticide targets would also have to be affected by CYP6G1 over-expression, which is unlikely.

The improvements suggested in Chapter 2 were used in the generation of the DMCs in this chapter and given the broad range of effective insecticide doses on both resistant and susceptible insects they worked very well. It was possible to get a good approximation of the range of effective doses using an initial log10 concentration range. Then a more refined range was used in a second DMC experiment. It is interesting to note the number of eggs counted for each insecticide in Table 4.7 (N) shows that it would appear that approximately two to five fold more eggs were required using this second method, which would appear at first to imply the new method required more work. However, this difference is due to many of the original DMC experiments on imidacloprid not being usable as a completely incorrect dose was applied to the vial. It can also be seen in the number of eggs used for Hikone R is approximately half the number used to generate the EMS1 DMCs for acetamiprid, nitenpyram and thiamethoxam. This is because the original DMC for each of these compounds was generated using Canton-S and EMS1 therefore when Hikone R was assayed a suitable range of concentrations was already known. From these results, it appears that the changes to the bioassay suggested in Chapter 2 improve this experiment.

The other suggested improvement from Chapter 2 for the larval bioassay was to use the agar based food for the medium instead of dry fly food. This was used to generate Figure 4.9 as several traditional DMC attempts using dry fly food had resulted in almost 100% mortality in all the vials including the transgenic controls. Because Canton-S and Hikone R flies did not die, we can be sure that this was a problem with the transgenic strains so a second method had to be used. As predicted this method did lead to lowered control mortality as the food was not as prone to drying out. However, also as predicted, it was an extremely difficult procedure to use and it is not possible to get a precise insecticide concentration. This is because the thickness of the molten food does not allow accurate measuring and makes a thorough mixing of the food to distribute the insecticide evenly difficult. Thus although a discriminating dose could be used a true DMC would not be obtainable. Possible ways around these problems would be to make a smaller quantity of food at a time so it would not need measuring before addition of

the insecticide but this would still not alleviate the potential problems with thorough mixing and possible damage to the insecticide form adding it to food that is too hot.

The experiments in this chapter raise important ideas for further work. First, there is the need to identify the metabolites being produced by CYP6G1 in resistant strains. Work done trying to create an assay for this purpose is shown in Appendix 1. Second, we need to know whether it is the affinity of CYP6G1 for different compounds that causes the range of resistance ratios seen to the neonicotinoids used in this chapter, or if that range is due to the differing toxicity of the metabolites. This question could be answered in part by metabolism studies as it would show if there were differing amounts of metabolite present for each compound. This could suggest whether CYP6G1 has a differing affinity for each compound. Alternatively there could be an equal amount of each metabolite present supporting the hypothesis that the toxicity of the metabolite varies. Following on from that experiment it would be interesting to synthesize the predicted metabolites and then use them in a DMC. This would test their toxicity to both resistant and susceptible strains. It could also prove possible to expose other species to the new compounds to test if a *Cyp6g1* homologue were to cause resistance in a pest species if the probable metabolites would be toxic to the pest insect. This last experiment could allow possible alterations to a new insecticide to make resistance less likely or would answer which of several compounds would be the most effective to use if this type of resistance were selected for in the wild.

Ch 5. Other insecticides, P450s and species

5.1 Introduction

So far, in this thesis, the resistance of *D. melanogaster* to DDT and imidacloprid has been investigated in three different resistant strains and the resistance to three new neonicotinoids has been tested in two resistant strains. This chapter is an expansion of this work investigating the resistance profiles of three different strains and also concerns the search for a *Cyp6g1* homologue in *D. simulans*. The work of this chapter is published in part in Le Goff *et al*, 2003.

Previous publications have implicated a variety of *D. melanogaster* genes to be involved in P450 based metabolic resistance. The work of Maitra investigated DDT resistance conferred by *Cyp6a8* (Maitra et al., 2000). Brandt has worked with the Wis1 strain where they found *Cyp12d1* to be over-transcribed and linked to DDT resistance (Brandt et al., 2002). Many other groups have also worked on *Cyp6a2,* which, with *Cyp6g1* is the most investigated *D. melanogaster* P450. *Cyp6a2* is over expressed in the 91R strain first selected for by King (King and Somme, 1958), but as we have found no examples of this gene being involved in resistance in any of the strains we have so far tested it is not used in this thesis.

Maitra and many other groups including Berge and others have found that the 91R strain has shown a higher level of *Cyp6a2* expression than the 91C strain isolated at the same time and not exposed to DDT (Berge et al., 1998; Maitra et al., 2000). Maitra also looked for other P450s being over-transcribed and found that some strains also over transcribe *Cyp6a8*. They found that some strains do not transcribe this gene at all, some including 91R transcribe a small level of this gene and some such as MH-III over-transcribe a considerable amount of mRNA for *Cyp6a8*. Unfortunately this paper contains no data for DDT resistance so the resistance profile conferred by *Cyp6a8* is unknown (Maitra et al., 2000).

We also wanted to investigate if other insects also have the same resistance mechanism as the Hikone R strain. Brazilian *D. simulans* strains were supplied by Dr. L. Madi-Ravazzi. These insects were then screened for DDT and imidacloprid resistance and then a micro-array used to discover if these closely related drosophilids use a homologue of *Cyp6g1* to confer the wide resistance profile seen in *D. melanogaster*. If

it were shown that the same gene was over-transcribed it would also be of interest to discover if the same mutation was responsible for this in both species, since this could indicate that the *Cyp6g1* resistance mechanism has probably been in the *D. melanogaster* population since the two species separated. Work has been done by Catania carrying out micro-satellite analysis of a range of resistant *D. melanogaster* with resistance conferred by the 5' insertion of an *Accord* element (Catania *et al*, unpublished data), (Daborn et al., 2002). An analysis of strains collected from around the world found that evidence was inconclusive as to whether exposure of *D. melanogaster* to DDT and other insecticides from the late 1940s onwards selected for a fly with the *Accord* element insertion or if this is a historical mutation that has always been present in *D. melanogaster*. If the same *Accord* element were found in a *D. simulans* strain showing a similar resistance profile then it would indicate than this transposable element jumped into the 5' region of *Cyp6g1* prior to speciation.

Finally the use of the organophosphate malathion allowed us to confirm a result first discussed in Chapter 2. Early in the thesis it became clear that DDT was too hydrophobic to be used in the water based larval bioassay and imidacloprid and the neonicotinoids are ineffective against adult *D. melanogaster*. Malathion however has been shown to be active in an adult bioassay and is also soluble enough to be used in the larval screen. This means that it is now possible to test if the same insecticide shows a similar resistance ratio when used against different stages in the lifecycle.

5.2 Methods

5.2.1 Generation of DMCs for malathion in *D. melanogaster*, and for imidacloprid, DDT and malathion for *D. simulans*.

To generate DMCs for the organophosphate malathion the procedure for larval assays was used as described on Section 2.2.1.2. The homozygous strains Hikone R, Canton-S, Wis1 and Wis1 Lab were used with malathion using between 5 and 7 insecticide concentrations with between 4 and 10 replicates at each insecticide concentration. Malathion was also used in an adult contact bioassay as described in Section 2.2.2.1. The strains Canton-S and Hikone R were used in this assay with between 4 and 10 replicates at each concentration used.

When using *D. simulans* in the larval bioassay for testing resistance to imidacloprid and malathion it was found that the eggs hatch at a earlier time than those of *D. melanogaster* so it was necessary to set up the cage to collect eggs late in the afternoon and collect the eggs the following morning. If they were left for longer than 20 hours the eggs would begin to hatch. Further problems encountered with *D. simulans* included the resistant and susceptible strain not appearing to be homozygous so a broad range of insecticide concentrations were needed. It is also of note that these flies appear to prefer to pupate on the food rather than the sides of the vials or bottles as it the case of *D. melanogaster.* This could have led to the slightly higher control mortality as the pupae would be more susceptible to bacteria and fungi in the dry fly food, and possibly to desiccation, as they will be affected more by the evaporation of water from the food.

The DDT bioassays for *D. simulans* were carried out exactly as described in Section 2.2.3.1 although more bottles were required to produce an equivalent number of *D. simulans* to *D. melanogaster.* Again, this could be due to the *D. simulans* pupating on the food, which would give a smaller surface area for pupae to occupy.

5.3 Results

The results in this section are published in part, by Le Goff et al (Le Goff et al., 2003). They are a more detailed investigation of that work into the differing P450 expression profiles of the different strains used in this thesis and how this affects the resistance phenotypes seen.

5.3.1 Resistance to DDT and imidacloprid confers resistance to malathion in some strains.

In the past, organophosphate resistance has been reported in the literature in the strains now shown to over-express CYP6G1. It was decided to show that these strains still show resistance to these compounds and to expand upon the survey of cross-resistance so far carried out in our laboratory. In a recent paper, Pittendrigh claimed that the strain Wis1 also over-transcribed a different P450, *Cyp12d1* (Brandt et al., 2002) . When this was announced a Northern blot was carried out in our laboratory using a *Cyp12d1* probe and this found no over-transcription of *Cyp12d1* in our Wis1 lab strain, which at the time was thought to be the same as Wis1. The observation was also made that resistance to DDT differed considerably between Wis1 lab and Wis1, but this was originally explained by Wis1 lab being a laboratory strain with the marker vestigial. This marker causes flies to be smaller than wild type. Further as wings are used as a part of the courtship ritual not as fecund as their wild type relatives. At this time we had no data with which to make comparisons of imidacloprid resistance as all experiments using Wis1 lab eggs placed on the ready mixed dry fly food led to virtually no survivors in the control and no survivors at all in the resistant flies. Again, this was put down to the reduced fitness of the vestigial Wis1 lab strain, it was not even considered to carry out the imidacloprid DMC at a lower concentration range. As we shall see in the following results hindsight is a wonderful thing!

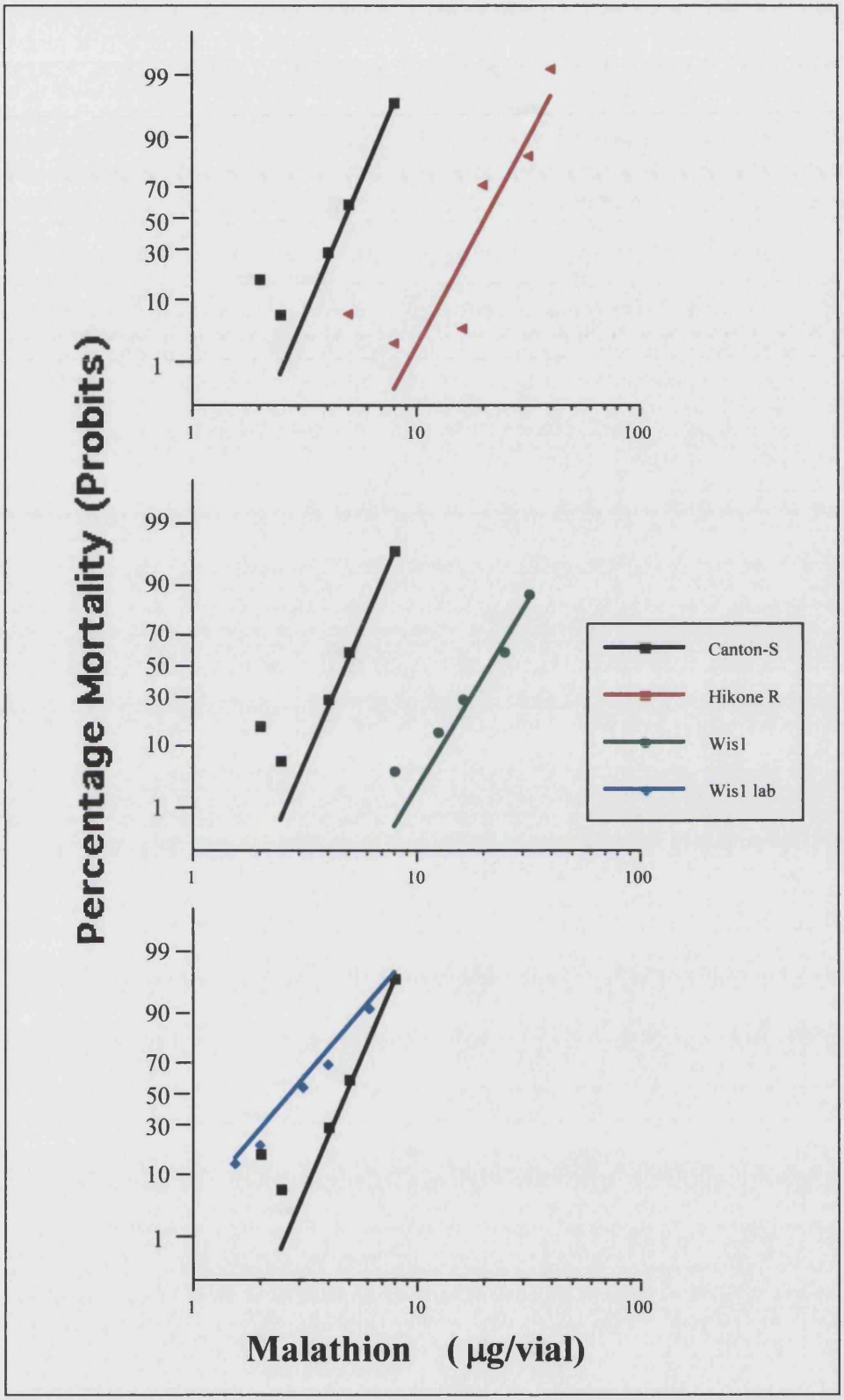


Fig. 5.1 Dosage mortality curve for malathion tested on Canton-S, Hikone R, Wis1 and Wis1 lab in the larval bioassay.

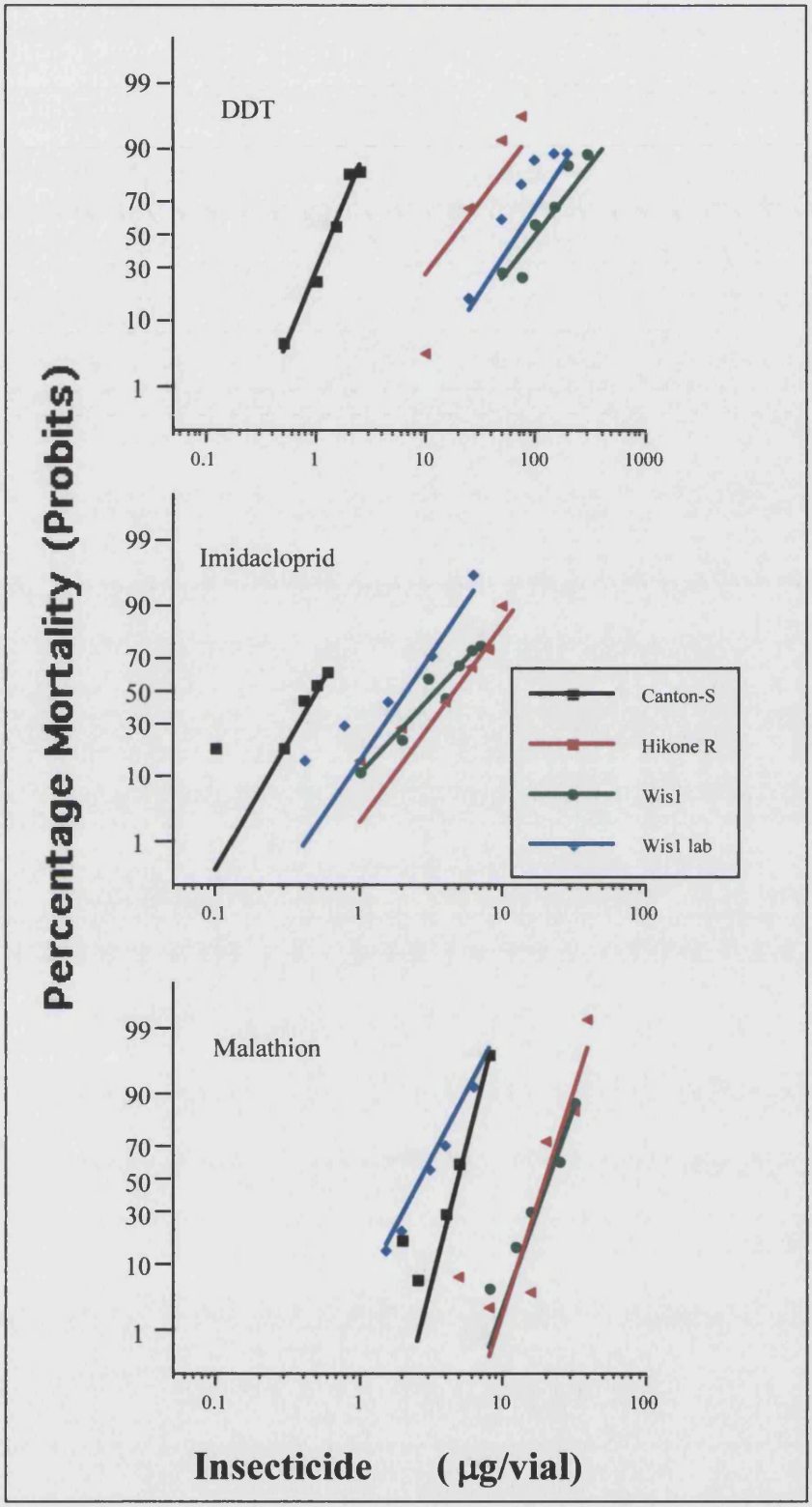


Fig. 5.2 Dosage mortality curves for imidacloprid, DDT and malathion tested on Canton-S, Hikone R, Wis1 and Wis1 lab.

As can be seen in Figure 5.1 two of the strains used show resistance to malathion whereas the Wis1 lab strain shows no resistance to malathion, and indeed seems to be more susceptible to malathion at lower concentrations.

The panel of DMCs shown in Figure 5.2, (already seen in part in Figures 2.4 and 2.7) show clear differences in the cross-resistance profiles of these three strains. It can be seen in graph A that Hikone R is the least resistant fly to DDT, and that Wis1 is more resistant than Wis1 lab. Graph B shows that although all strains are resistant to imidacloprid Wis1 lab is clearly less resistant that either of the other two resistant strains tested. This gives an explanation as to why was not possible to generate dosage mortality curves for Wis1 lab when the original DMCs were carried out. At the time, the high control mortality masked the fact that the wrong insecticide concentrations had been used. It was this high control mortality that stopped us getting a DMC for Wis1 lab and so having two differing results between what were, assumed to be, the same strain simply with different markers. In the final graph, C, we can again see the result from the previous figure showing how similar Wis1 and Hikone R are in their resistance profiles to malathion and it can be seen just how susceptible Wis1 lab is in comparison to Canton-S and the two resistant strains.

Tab. 5.3 A detailed comparison of cross resistance to DDT, imidacloprid and malathion in our strains of interest.

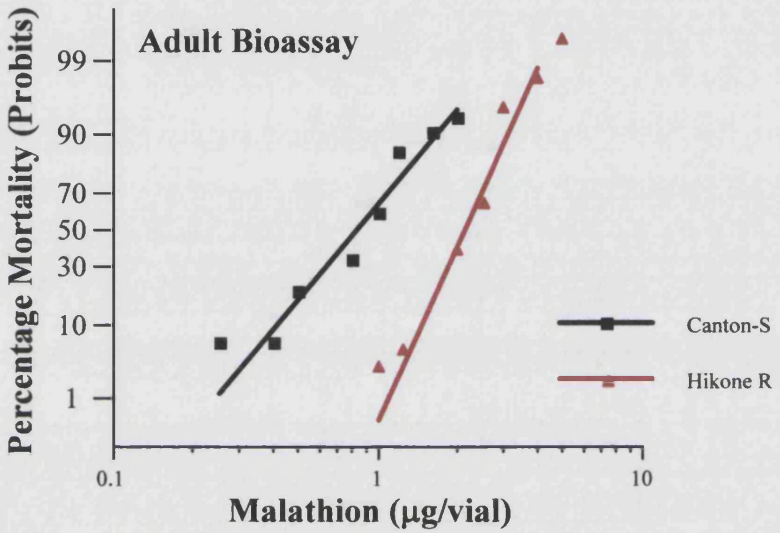
Strain	N	LD_{50}	(90%CI)	Slope	+-S.E.	Resistance ratio
Imidacloprid Mix						
Canton-S	1500	0.53	(0.42-0.68)	3.86	0.97	1.00
Hikone R	1600	4.74	(3.71-5.75)	3.00	0.57	8.94
Wis1	4000	3.50	(2.52-4.30)	2.34	0.22	6.60
Wis1 lab	2250	2.18	(1.76-2.53)	3.25	0.47	4.11
DDT						
Canton-S	780	1.45	(1.22-1.72)	2.91	0.25	1.00
Hikone R	780	18.97	(13.03-24.75)	2.21	0.18	13.08
Wis1	740	104.34	(90.99-113.70)	2.14	0.19	71.96
Wis1 lab	680	44.39	(36.10-51.93)	2.57	0.25	30.61
Malathion						
Canton-S	2500	4.88	(4.26-5.41)	8.59	1.00	1.00
Hikone R	2000	20.26	(16.73-23.17)	6.71	0.59	4.15
Wis1	2000	21.23	(18.75-23.36)	6.09	0.53	4.35
Wis1 lab	2500	2.72	(2.19-3.16)	4.22	0.50	0.56

This table gives the detailed information cross resistance in the strains of interest.

The variety of resistance ratios seen for each strain to the different insecticides shows how each strain has a different resistance profile. Canton-S is susceptible to all the chemicals used. Hikone R is resistant to all the insecticides tested, and although not as resistant as either Wis1 lab or Wis1 to DDT, it is very slightly more resistant than Wis1 and considerably more resistant to imidacloprid when compared to Wis1 lab. Wis1 shows an almost identical level of resistance to malathion as Hikone R and is considerably more resistant to DDT than either of the other two strains. Wis1 lab shows the most striking resistance profile. This strain is the most resistant to DDT with a resistance ratio of 30.61 compared to that of Hikone R with a resistance ratio of 13.08. However, this strain shows only about half as much resistance to imidacloprid as Hikone R, and is in fact almost twice as susceptible to malathion as the susceptible control Canton-S.

5.3.2 Does resistance at one lifecycle stage confer resistance to the same insecticide at different stages of the lifecycle.

When we originally tested resistance to DDT and imidacloprid it had proved impossible to devise a reliable protocol for DDT at the larval stage as DDT is too hydrophobic, and imidacloprid had no effect on adult *D. melanogaster*. It has been reported that malathion is active against adult *Drosophila* so it was decided to generate a dosage mortality curve for this compound to compare to the larval assay shown in Figure 5.1.



Strain	N	LD50	(90%CI)	Slope	+-S.E.	Resistance ratio
Malathion Larval Assay						
Canton S	2500	4.88	(4.26-5.41)	8.59	1.00	1.00
Hikone R	2000	20.26	(16.73-23.17)	6.71	0.59	4.15
Malathion Adult Assay						
Canton S	1400	0.84	(0.77-0.90)	4.29	0.35	1.00
Hikone R	1040	2.12	(1.98-2.25)	7.95	0.87	2.52

Fig. 5.4 Resistance to malathion in an adult bioassay.

Figures 5.1 and 5.4 show DMCs for malathion on both the larval and adult stages of Hikone R and Canton-S. It can be seen that there is resistance in Hikone R to this compound at both life cycle stage. This provides evidence that the same mechanism has a similar effect throughout the life of the insect. The differences in LD_{50}'s in adults and larvae are explained by the method of application of the insecticide. In the two assays two very different methods are used, including the mode of entry into the insect and

they would not be expected to give the same LD_{50}. However, it is interesting to see how the resistance ratio varies. If the level of CYP6G1 were equal throughout the lifecycle then we would expect similar resistance ratios from both experiments. As can be seen this is not the case and the resistance ratio of the adult bioassay is approximately half that of the larval bioassay. This suggests that the ratio of CYP6G1 at the adult stage is lower than at the larval stage, and this is in accord with the result seen in Figures 3.3 and 3.5 where it has been shown that there is an increase in the level of both protein and mRNA in Canton-S larvae from the 3rd instar onwards.

5.3.3 Resistance to DDT also confers IMI and malathion resistance in *D. simulans.*

As *D. melanogaster* is very similar to *D. simulans* it was decided to look for the same form of cross-resistance seen in *D. melanogaster*. Wild type *D. simulans* strains were collected in Brazil by L. Madi-Ravazzi. The strains were then screened by exposing the larvae to increasing doses of imidacloprid and flies to emerge were placed on DDT and survivors collected. In this way, a DDT resistant strain showing cross-resistance to imidacloprid was identified. The resistant strain OV was compared to the susceptible strain BG and bioassays were carried out on imidacloprid, DDT and malathion. These results are shown in Figure 5.5 where it can be seen that the resistant OV strain shows a similar resistance pattern as to Hikone R.

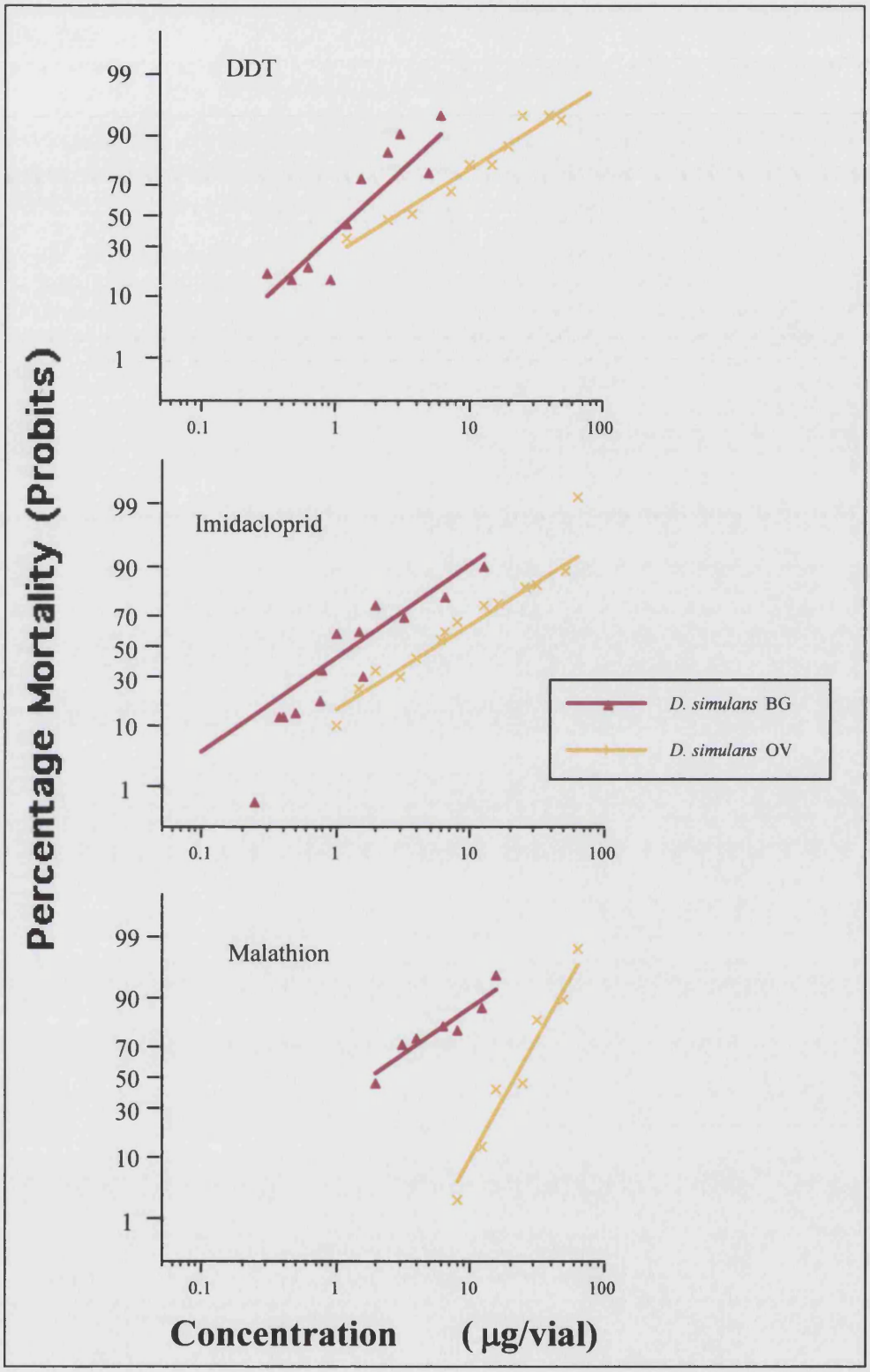


Fig. 5.5 Dosage mortality curves showing resistance of *D. simulans* to imidacloprid, DDT and malathion.

Tab. 5.6 Table giving details of resistance in *D. simulans* to imidacloprid, DDT and malathion.

Strain	N	LD_{50}	(90%CI)	Slope	+-S.E.	Resistance ratio
Imidacloprid Mix						
Canton-S	1500	0.53	(0.42-0.68)	3.86	0.97	1.00
Hikone R	1600	4.74	(3.71-5.75)	3.00	0.57	8.94
BG	3500	1.39	(0.90-1.99)	1.54	0.11	1.00
OV	4000	5.94	(4.36-7.67)	1.38	0.09	4.27
DDT						
Canton-S	780	1.45	(1.22-1.72)	2.91	0.25	1.00
Hikone R	780	18.97	(13.03-24.75)	2.21	0.18	13.08
BG	1280	1.37	(1.03-1.76)	2.01	0.12	1.00
OV	1300	2.92	(2.11-3.76)	1.40	0.10	2.13
Malathion						
Canton-S	2500	4.88	(4.26-5.41)	8.59	1.00	1.00
Hikone R	2000	20.26	(16.73-23.17)	6.71	0.59	4.15
BG	2000	1.79	(0.89-2.62)	1.79	0.14	1.00
OV	2000	21.42	(18.26-24.23)	3.92	0.30	11.97

This table gives a detailed summary of results for two D. simulans strains and the results of Canton-S and Hikone R as a comparison to the resistance levels seen in D. melanogaster.

This table gives the detailed data for the graphs in the previous figure. Here we see that the resistance ratios for *D. simulans*, whilst low in comparison to the *D. melanogaster* strains shown in the table are however significant with the 90% confidence intervals of the resistant and susceptible strain not overlapping. The column showing the number of flies or eggs used in each bioassay is also interesting. In this set of experiments as it can be seen that whilst with malathion a similar number of eggs were used in comparison to *D. melanogaster*. However, with both DDT and imidacloprid relatively more eggs and adults were needed in the bioassays for *D. simulans* (in actual fact around twice the number of adults or eggs were needed in each bioassay). This is due to the non-

homozygous nature of the *D. simulans* populations requiring considerably more data points on each dosage mortality curve to cover the full range of these two insecticides.

5.4 Discussion

The results in this chapter show that different strains have different resistance profiles. Hikone R and Wis1 are both resistant to the three insecticides used in this chapter where as Wis1 lab is resistant to DDT and slightly resistant to imidacloprid but is not resistant to malathion. In fact, Wis1 lab is actually slightly more susceptible than the susceptible control Canton-S to malathion. Figure 5.7 shows the micro-array data generated by Dr. G. Le Goff that shows that each strain has a different P450 over-transcription profile when compared to Canton-S. We can see that in Hikone R only *Cyp6g1* is over transcribed, as is shown in Table 2.14 where the original Northern blot results are shown. As has been demonstrated in Chapters 2, 3 and 4 *Cyp6g1* over-transcription and CYP6G1 over-expression is sufficient to confer resistance to a range of neonicotinoids, DDT and an organophosphate. This micro-array also shows that no other P450s are over-transcribed in this strain. This allows us to disregard the possible hypothesis suggested in Chapter 2 that *Cyp6g1* over-transcription caused the up regulation of a number of P450s. Equally we may discard the possibility that the Accord element insertion downstream of *Cyp6g1* caused the over-transcription of other P450s by trans-regulation as we only see one P450 to be over-transcribed compared to Canton-S. This hypothesis has already been disproved by the transgenic fly lines used in Chapter 4 that showed that the over-transcription of *Cyp6g1* alone is sufficient to confer resistance to a range of compounds but now it has also been demonstrated that this is true of the wild type Hikone R strain.

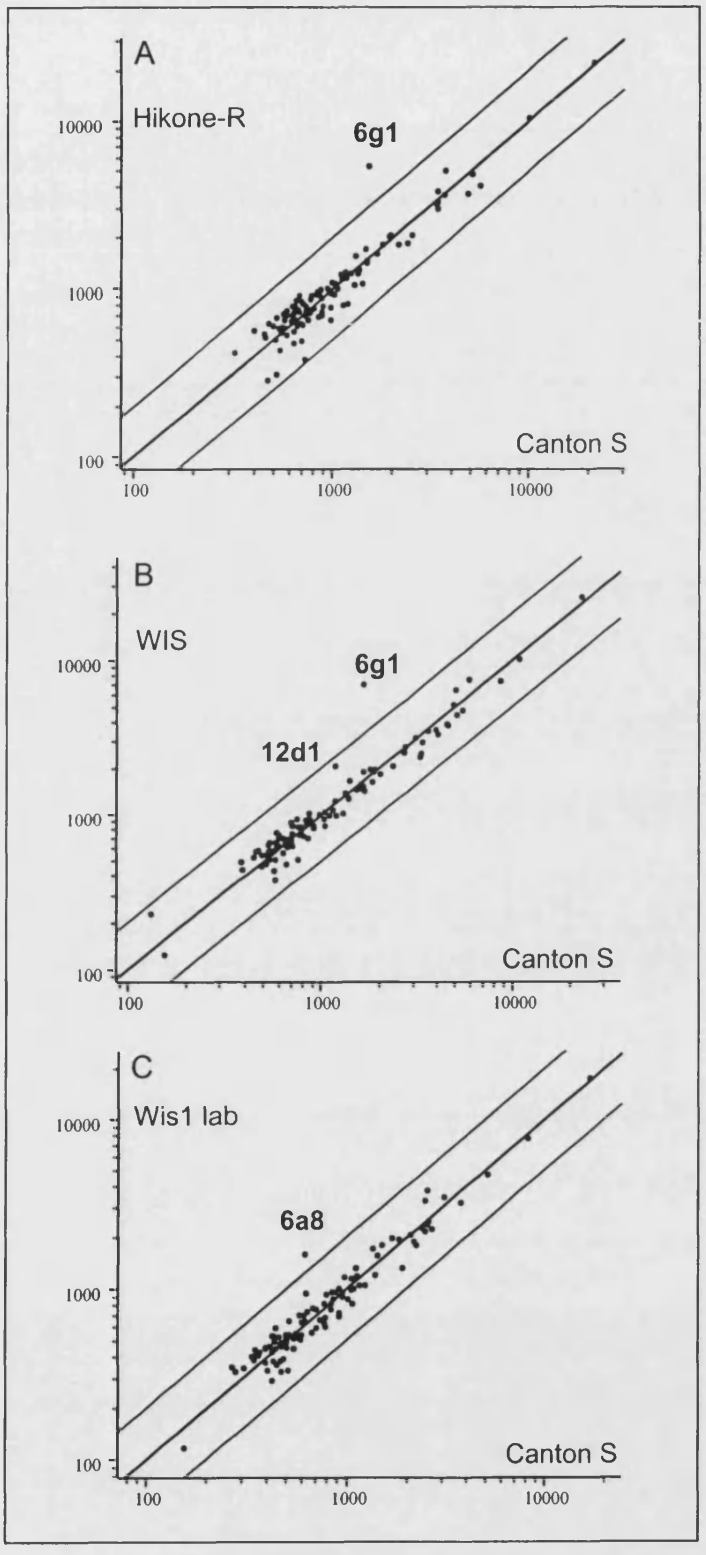


Fig. 5.7 Micro-arrays analysis of DDT-R strains, originally from Le Goff et al, 2003.

Wis1 has previously been shown to over-transcribe both *Cyp6g1* and *Cyp12d1* by Brandt (Brandt et al., 2002). In that paper, it was shown that *Cyp12d1* over-transcription was induced by exposure to DDT. This might explain why we see such a relatively low level of transcription in the micro-array, as the flies used for this protocol are not exposed to DDT. From the results seen here and in Brandt et al, it would appear that *Cyp12d1* over transcription only confers extra DDT resistance over *Cyp6g1*. We can see identical malathion resistance for both Hikone R and Wis1 suggesting that the presence of an extra P450 does not increase resistance to this compound. However, it should be remembered that the DDT bioassay is carried out on adult insects and malathion for this experiment was used as a larvicide so there is a possibility that *Cyp12d1* is not inducible in the larval stages of the lifecycle. There is also a possibility that *Cyp12d1* does confer a very slight level of resistance to malathion at the larval stage. In Table 2.9, we can see that whilst the resistance ratios for Hikone R and Wis1 are similar for imidacloprid Wis1 has a slightly lower ratio (8.94 fold for Hikone R compared to 6.60 fold for Wis1). In Figure 3.6 where the relative amounts of CYP6G1 is assayed by a Western blot we again see that there is slightly less CYP6G1 present in Wis1 adults when compared to Hikone R. This would suggest that *Cyp6g1* over-transcription is lower at the larval stage and at the adult stage in Wis1 so to see the result in Table 5.3 where we see virtually identical resistance ratios for Wis1 and Hikone R when looking at malathion it would suggest that there is a second factor present raising the level of resistance to malathion. It is possible that this second factor is *Cyp12d1* and the very slight increase in resistance factor from what would be predicted is due to the low level of basal *Cyp12d1* over-transcription seen. If this were the case then it is possible that while *Cyp12d1* may be able to metabolise malathion the presence of this organophosphate does not induce over-transcription as Brandt showed was the case when Wis1 was exposed to DDT (Brandt et al., 2002).

The third *D. melanogaster* strain considered in this chapter is Wis1 lab. Originally it was thought that this strain and Wis1had the same resistance profile as it was believed that Wis1 lab was a line derived from Wis1 but in a *w; vg* background. As it turns out this selection for DDT resistance in a vestigial mutant led to the loss of *Cyp6g1* over-transcription and the laboratory based selection of *Cyp6a8* over-transcription in the same method used by Maitra (Maitra et al., 2002). This early misconception caused us some confusion and early setbacks. It should be noted that the two new P450s we are

investigating in this chapter, *Cyp12d1* and *Cyp6a8* were both tested for over expression on "Wis1" as part of the Northern screen in Chapter 2 (Table 2.14), and neither was seen to be over-transcribed. The most likely reason for this is that at the time we used Wis1 and Wis1 lab interchangeably and so by chance a Northern membrane with Wis1 RNA was used for the *Cyp6a8* screen and a membrane with Wis1 lab RNA was used with the *Cyp12d1* probe. This resulted in the correct expression pattern being missed at an early stage. It was also noted that Wis1 lab seemed to be more susceptible to imidacloprid than was expected, the original bioassays were carried out over the same concentration range used for Hikone R and EMS1 and this resulted in almost 100 percent mortality. At the time we assumed that this was due to the vestigial background as these mutants seem to be smaller and so would be more susceptible to dehydration which is common in the water based dry fly food used in these bioassays. There is also a possibility that the vestigial mutation also affects general fitness, as it is a developmental mutant. Also, as a fly uses its wings as a part of the courtship ritual a fly with no wings may be less likely to mate and so there might be a number of virgin female flies present laying unfertilised eggs. These would be counted into the vial so could account for some of the control mortality. At the time we did not consider that Wis1 lab had a different P450 expression profile and simply used the Wis1 in Daborn *et al*, 2001 instead of Wis1 lab as was originally intended.

The reason for this error is now clear, firstly the Western blot shown in Figure 3.6 shows clearly that there is no *Cyp6g1* over-expressed in this strain as the level of protein seen is identical to the Canton-S strain and secondly Figure 5.7 shows very clearly that the only P450 over-transcribed in this strain is *Cyp6a8*. Therefore, from the results of this chapter we concluded that *Cyp6a8* confers resistance to DDT at a higher level than *Cyp6g1* but less than that of both *Cyp6g1* and *Cyp12d1* combined. Imidacloprid resistance is also seen but at only 4.11 fold higher than Canton-S compared to 8.94 with Hikone R, this suggests that while *Cyp6a8* is capable of metabolising imidacloprid it is either not as efficient as *Cyp6g1* or is not present in as great a quantity. It is also of interest to note that the over-transcription of *Cyp6a8* does not confer any kind of malathion resistance. In fact, it appears that the presence of increased copies of *Cyp6a8* makes the strain more susceptible to malathion. This is a possibility and it has been shown that the over-expression of a P450 can make a compound more toxic, for example Hikone R is more susceptible to phenylthiourea than Canton-S (Ogita, 1960),

so *Cyp6a8* could activate malathion or create a metabolite that is more toxic than maloxon, the normal activated form of malathion. This hypothesis might also explain why *Cyp6a8* mediated resistance has never been seen in a wild type strain as *D. melanogaster* are exposed to a range of insecticides a mutation that confers resistance to one insecticide but increased susceptibility to a second active ingredient will only be able to survive when not exposed to the toxic chemical. But this may not be the case at all, as suggested earlier the vestigial mutation appears to cause smaller less fit offspring so this slight decrease in LD_{50} seen between Canton-S and Wis1 lab could be due to the size difference or fitness cost of the vestigial mutation.

Tab. 5.8 Summary of P450 over-expression and resistance profiles.

Strain	P450 profile	Insecticide profile
Canton-S	None	None
Hikone R	*Cyp6g1*	DDT Imidacloprid Malathion
Wis1	*Cyp6g1* *Cyp12d1*	(Increased) DDT Imidacloprid Malathion
Wis1 Lab	*Cyp6a8*	(Increased) DDT (Slight) imidacloprid

This table summarises the P450 expression profile for the four D. melanogaster strains of interest and the insecticides to which they confer resistance.

The three DMCs seen in Figure 5.5 show an interesting result. It is clear from the DMCs that the OV strain is more resistant to all the insecticides tested when compared to the susceptible BG strain. However, it is clear from the DDT and imidacloprid graphs that this is not a high level of resistance and the slopes of the lines are relatively flat in comparison to the equivalent *D. melanogaster* DMCs. The more vertical a line on a DMC then the smaller range at which the insecticide of interest works and therefore the smaller the range of alleles for the resistant gene in the population being tested. It should be noted that in this case, the highest level of resistance is to malathion, and this is also the most vertical line of the three DMCs. This may suggest that this *D. simulans* strain derives from a population that was exposed to malathion or another

organophosphate at one time in the past, and so this is the class of compound that now has the highest resistance ratio. If this argument is then applied to our *D. melanogaster* strains then they show the highest resistance to DDT suggesting that they were originally selected by exposure to DDT. This is likely as DDT was the prevalent insecticide in the 1950s when this phenotype was first seen.

The two *D. simulans* strains used in this thesis demonstrate that the over-transcription of an apparent *Cyp6g1* homologue (Figure 5.9) confers resistance to DDT, imidacloprid and malathion. The *D. simulans Cyp6g1* like gene is evidently closely homologous to the *D. melanogaster* gene at both the mRNA and protein level as it was detectable by an antibody specific for *D. melanogaster* and also the probe on the micro-array slide. In this case there would appear to be about 4.9 times more protein present in the resistant than the susceptible line and this would lead to a hypothesis that the BG strain will not have as high a resistance ratio as the resistant *D. melanogaster* strains I have assayed. Figure 5.5 and Table 5.7 show this to be the case for imidacloprid with OV having a resistance ratio of 4.27, about half of that of the Hikone R. It would again, however, appear that the level of CYP6G1 or its homologue is only closely linked to imidacloprid resistance, and the resistance factors for DDT and malathion indicate that other factors must also be involved in resistance to these compounds.

Several future experiments arise from this chapter. In it, we conclude that different P450s show different resistance profiles. It would be useful to increase the range of insecticides screened on each strain. However, that was outside the time scale of this thesis. It would also be desirable to have transgenic lines that over-transcribe *Cyp6g1* (this strain has already been created by Dr. P. Daborn), *Cyp6a8, Cyp12d1* and it would also be desirable to have a *Cyp6a2* transgenic strain that could be tested with a range of insecticides. A Wis1 lab strain not in the *w; vg* background is also desirable to rule out the health costs of the vestigial mutation and a strain where *Cyp12d1* is not co-transcribed with *Cyp6g1* is also necessary to finalise the resistance profile for *Cyp12d1*. The use of transgenic lines would remove fitness costs of the different strains by having all the lines in the same background strain, and thus, being able to use good controls. Further to this, it would also be worthwhile to carry out micro-arrays to compare the different strains in the presence and absence of different insecticides to test the effect of induction of P450s on the resistance profile.

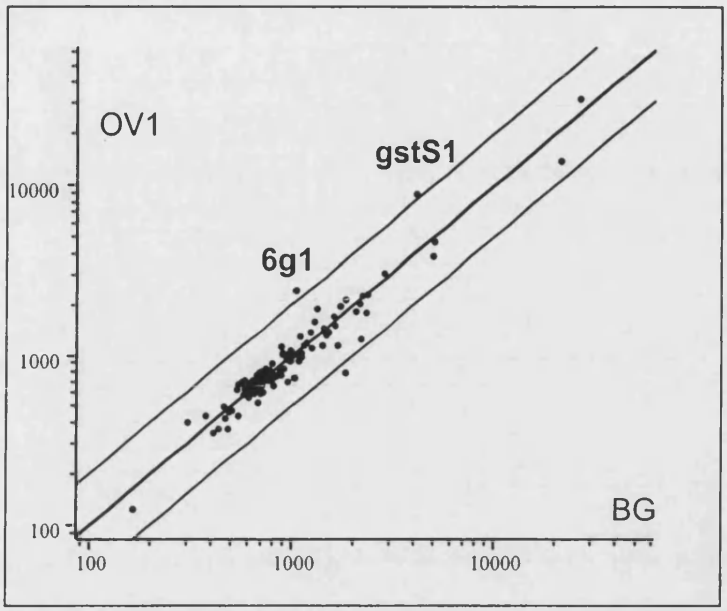


Fig. 5.9 Micro-array of resistant (OV) and susceptible (BG) *D. simulans*, originally from Le Goff *et al* (2003).

Following on from the experiments on the *D. simulans* strains, several experiments are suggested. Firstly, another screen should be carried out on the resistant OV strain as the angle of the slopes in the DMCs shown in Figure 5.5 shows that this may not be a homozygous population (Busvine, 1971). Therefore, further selection could remove susceptible insects from the strain. A further possibility could be that heterogeneous conditions in the bioassay might give the same result. Although this evidently was not the case for *D.* melanogaster, it could have been true for *D. simulans.* A new susceptible control is also desirable as the BG strain is a wild type strain and it appears that there is some background resistance. This is possibly due to seeding the original population with too high a number of individuals and so having a large genotypic range in the line. A better control would be a susceptible strain from a stock centre. A second set of experiments to carry out now that a similar transposable element has been found to confer resistance in *D. simulans* is to start looking at other species. Personal communication with Dr. R. Feyereisen has indicated that *Cyp6g1* is a gene unique to drosophilids but it would still be an interesting series of experiments to screen a more diverse range if insects, from drosophilids to other diptera and so on to look for a *Cyp6g1* homologue conferring resistance in other species.

The final experiment suggested by this chapter ties into the future suggestions of Chapter 4 for the creation of a screen to look for metabolites of different compounds and for different P450s. We now have three P450s conferring resistance, so it is important to know if they all generate the same metabolites. It is already clear that Cyp6a8 will either not metabolise malathion, or possibly metabolise it to a more harmful compound, while there is evidence that Cyp12d1 might confer a small level of malathion resistance. This implies that malathion metabolism differs between these strains, so it would be interesting to discover how the respective malathion metabolites relate to each other. It is also clear that all three P450s confer differing levels of resistance to DDT. Is this because they produce three different metabolites of varying toxicity? Alternatively is the range of resistance ratios due to all three P450s of interest generating the same metabolites but having slightly different affinity for DDT and so having differing efficiency of metabolism? During the course of this thesis, attempts were made to investigate neonicotinoid metabolism by Hikone R and Canton-S but no successful assay was developed. This work is outlined in Appendix A.

Chapter 6. Conclusions and future work.

6.1 Conclusions

The work presented in this thesis shows that the over-expression of a single P450 confers resistance to a broad range of insecticides. Since the DDT-R 64.5 cM phenotype was first isolated in the 1950s in Japan (Ogita, 1960) many groups from around the world have worked to elucidate how it could be possible that one single locus can confer such a broad resistance range. Work in the 1980s discovered the possible over-expression of an as then unknown P450, called P450-B in Hikone R (Waters and Nix, 1988). Since this publication, a number of new techniques have become available and this has allowed us to further analyse the DDT-R locus. The original mapping work localised resistance to DDT and malathion to the 64.5 cM region of chromosome II (Ogita, 1960). Subsequent work has placed imidacloprid resistance at the same locus and P-element mapping reduced the candidate DDT-R gene to 6 open reading frames (Daborn et al., 2001).

Further work using the synergist PBO showed that resistance was probably P450 mediated. Following on from this, Northern blots of the three candidate P450s in the 64.5 cM region showed that one gene, *Cyp6g1* was over-expressed. At the same time, all the other P450s in the *D. melanogaster* genome that had previously been linked to insecticide resistance were also tested, and only this single P450 was over-transcribed (Daborn et al., 2001). Subsequent work using a limited micro-array containing all the *D. melanogaster* P450s showed that *Cyp6g1* is indeed the only P450 over-transcribed in Hikone R when compared to Canton-S (Le Goff et al., 2003). This demonstrated that the over-expression of the single P450 gene is sufficient to confer resistance to a broad range of insecticides (Daborn et al., 2001).

My work also shows that the DDT-R locus is also capable of conferring resistance to a new class of insecticides, the neonicotinoids (Le Goff et al., 2003). This is a result that could possibly have large economic implications for agrochemical companies. Neonicotinoids are already in widespread use and it was hoped that cross-resistance from previously encountered chemicals was unlikely as very few chemicals in the past have targeted the insect nAChR (Tomizawa and Casida, 2001). The cross-resistance shown by *Cyp6g1* over-transcription shows that it is possible for there to be resistance in field populations before the release of a new chemical due to previous exposure to

unrelated chemicals. It is also interesting to note that the over-expression of *Cyp6g1* appears to have very little, if any, fitness cost. The Hikone R strain has been kept in stock centres for 40 years and still exhibits DDT resistance, although, no selection with insecticides takes place in the stock centre. It has been demonstrated that this is true of many DDT resistant strains over-transcribing *Cyp6g1* that have been collected over 40 years (Daborn et al., 2002). This means that if any pest species has developed resistance via this mechanism then it is likely that even if that insect has not been exposed to insecticides the resistant alleles will persist in the population. Work by Schlenke and Begun has shown that *D. simulans* also has a similar mutation to *D. melanogaster* that results in the over-transcription of a *Cyp6g1* homologue. This gene is over-transcribed by the insertion of Doc element in approximately the same position as the *Accord* element in Hikone R. The *D. simulans Cyp6g1* homologue is similar enough to cross-react to an anti-CYP6G1 antibody and to a *D. melanogaster* micro-array (Le Goff et al., 2003; Schlenke and Begun, 2003).

The *D. simulans* strains over-transcribing a *Cyp6g1* homologue also show a very similar resistance profile to the DDT-R strains, including resistance to imidacloprid. This means that other pest species that have evolved insecticide resistance via a *Cyp6g1* homologue will probably also be resistant to the neonicotinoids. However, it has been observed that *Cyp6g1* appears to be unique to diptera and possibly the drosophilids (R. Feyereisen pers. com.). It should be noted though that whilst *Cyp6g1* homologues are not known to exist in any pest species, our work and the work of our predecessors has shown that over-transcription of a single P450 can confer resistance to a broad range of insecticides. This means that any future insecticide will be subject to the risk that it may already face resistance in the field even when first released.

The work of Dunkov, Maitra and Brandt has shown that three other *D. melanogaster* P450s can also be involved in insecticide resistance (Brandt et al., 2002; Dunkov et al., 1996; Maitra et al., 2000). This work showed that *Cyp6a2, Cyp6a8* and *Cyp12d1* are all over-expressed in resistant strains. All these three genes confer DDT resistance but as yet none appear to confer the same broad resistance profile seen from *Cyp6g1* over-transcription (Le Goff et al., 2003).

6.2 Future work.

The work carried out in our laboratory has now answered which gene is responsible for the DDT-R phenotype. However, in answering this question it has asked a number of new ones. We now know that one gene confers resistance to a broad range of chemicals but we do not know how it does it. Work to investigate possible metabolites is an important experiment, as it will show how one single enzyme can confer resistance to so many chemicals. Some work towards this is shown in Appendix A but this work failed to yield results.

Another area where work can continue is to analyse the *Cy6a2, Cyp6a8* and *Cyp12d1* phenotypes. Transgenic lines would facilitate a more controlled analysis of these other resistance causing P450s as to date we have had no chance to work with *Cyp6a2* at all. *Cyp6a8* is in a *vestigial* background, which could have caused uncontrollable problems with fitness. The *Cyp12d1* over-transcribing strain, Wis1, over-transcribes *Cyp6g1* as well. Transgenic lines with each of these genes would allow controlled comparisons to investigate the different resistance phenotypes seen with these different P450s. This work could also involve the production of antibodies to each P450 to investigate the expression of the proteins.

One last experiment to carry out is the purification of the *Cyp6g1* antibody. This would allow a more accurate analysis of protein levels. It could also be used to investigate expression patterns throughout the lifecycle of resistant and susceptible insects. This experiment might discover the native purpose of *Cyp6g1*, although it should be remembered that given the evolutionary history of P450s there may be no native function and it evolved to metabolise plant xenobiotic (Feyereisen, 1999).

Appendix A: Metabolism of neonicotinoids in *D. melanogaster.*

A1 Introduction

The aims of this work described in this chapter were, firstly to create a robust protocol for Syngenta as a part of my CASE scholarship to use for analysis of novel insecticidal compounds and secondly to answer questions about the specific metabolites of imidacloprid and other neonicotinoids from susceptible and resistant *Drosophila* strains. A series of experiments were carried out to attempt to identify and analyse the metabolites of the neonicotinoids from resistant and susceptible *Drosophila*. Several previous groups have analysed the metabolites of imidacloprid and have categorised the major metabolites and analysed their toxicity to *M. persicae* (Nauen et al., 1998). Metabolites have also been reported for *M. domestica* in a injection screen (Miyagawa et al., 2002). We are especially interested in creating a protocol, as this would allow a comparison of all the different compounds apparently metabolised by CYP6G1. The range of insecticide structures that increased expression of this enzyme confers resistance to means that it could carry out a series of reactions or have a very non-specific active site that can catalyse many diverse compounds.

Several other groups have investigated the metabolism of imidacloprid in both resistant and susceptible insects. It has been previously thought extremely unlikely that P450 based imidacloprid resistance would be found (Byrne et al., 2003). This is because the proposed metabolites of imidacloprid generated by a P450 are as toxic or more toxic to some species than the parent compound (Nauen et al., 1998). Nauen found that the 4- and 5-hydroxy metabolites and imidazoline (or olefin) derivatives were as toxic or more toxic to *M. persicae* and *Aphis gossypii* than the parent compound (Nauen et al., 1998). Byrne and his colleagues have investigated imidacloprid metabolism in *Bemisia tabaci* and found a very low level of imidacloprid metabolites (Byrne et al., 2003). Additionally work by Kagabu on *M. domestica* shows that many of the possible imidacloprid metabolites are also toxic (Kagabu et al., 2002). However the work of Schulz-Jander demonstrates that whilst P450s are capable of metabolising imidacloprid P450 enzymes in mammalian liver microsomes and the human P450 3A4 show the major metabolites to be the 5-hydroxy and imidazoline derivatives (Schulz-Jander and Casida, 2002).

A2 Methods

A2.1 Metabolism of imidacloprid and the other neonicotinoids

Five different methods were used to try to generate neonicotinoid metabolites in resistant and susceptible *D. melanogaster*. These were then analysed using either thin layer chromatography (TLC) or liquid chromatography/mass spectroscopy (LC/MS). The two radio-chemicals used, [^{14}C] imidacloprid and [^{14}C] thiamethoxam, both labelled on the B ring at the carbon atom linking to the Y group (see Figure 1.3), were supplied by Syngenta, UK.

A2.1.1 Contact screen.

This was a screen based on the DDT assay used in Chapter 2. In this case [^{14}C] imidacloprid was dissolved in acetone and vials were coated with 100, 200,400, 800 and 1600 µg of the chemical. Twenty flies were then placed in each of 4 vials at each imidacloprid concentration and stoppered with cotton wool soaked in 5% sucrose solution for 24 h at room temperature and then frozen at -20°C to kill the flies.

Flies were then decanted into 2ml Q Biogene Lysing matrix A FastPrep vials (Bio 101 Systems [North America]) vials and washed 5 times with 1 ml of methanol. 1 ml 75:25 acetonitrile:water (MeCN:water) was then added to each vial and macerated for 2 x 20 sec. The vials were then centrifuged at 13 K RPM in a micro-centrifuge for 5 min and the resulting supernatant decanted directly into glass vials and dried down and resuspended in 200 µl acetone and 100 µl was loaded onto TLC plates.

A2.1.2 Topical application.

A 1:10 dilution of the radiolabelled stock was prepared containing 0.1 mBq in 10 ml. Resistant and susceptible insects were rendered unconscious using carbon dioxide and 0.5 µl was applied to the abdomen of each using a microdroplet applicator. Insects were then kept on sugar agar overnight and killed by freezing -20°C 1 h and prepared for TLC analysis as in Section A2.1.1.

A2.1.3 Feeding bioassay.

A 24 well plate was prepared with 750 µl sugar-agar (see Section 2.3.1) per well allowed to dry for 4 h 20,000 dps (2.6 µg) of radiolabelled imidacloprid was added to each well (an aliquot of stock solution was diluted with HFE to required volume,

roughly half and half). 10 flies were distributed into each well using a paintbrush and the wells then sealed with breathable tape. The plate was left for 48 h in an incubator at 25°C. Flies were then killed by freezing -20°C 1 h and prepared for TLC analysis as in Section A2.1.1.

A2.1.4 Abdominal screen.

Flies were dissected under CO_2 and the abdomen removed at the junction with the thorax. 10 male and 10 female abdomens of each strain were placed in 200 µl PBS buffer pH 7.2. Then 1 mM NADPH and either 20 µM imidacloprid or 20 µM thiamethoxam was added to each tube. The samples were incubated for 2 h at 25°C and the reaction was quenched with 200 µl acetonitrile. A control was also run in parallel that contained no abdomens to show that the insecticide did not degrade under the reaction conditions. 200 µl of each solution was then run on a TLC plate as in Section A2.1.6.

A2.1.5 Larval screen.

This method was carried out using cold insecticides. Dry fly food was prepared in petri dishes containing 0.05 µg/ml imidacloprid, 0.5 µg/ml acetamiprid, 1.0 µg/ml nitenpyram and 0.3 µg/ml thiamethoxam. These dishes were placed in cages containing either Hikone R or Canton-S flies and the flies were allowed to lay eggs on the dish for 24 h. The dishes were then kept at 25°C until the eggs had reached 3rd instar larvae. The larvae were then collected and snap frozen in liquid nitrogen. They were then prepared for LC/MS following the method outlined in Section A2.1.1.

A2.1.6 Thin layer chromatography method.

A glass TLC tank with 1 cm 5% methanol, 95% dichloromethanol had a piece of chromatography paper (20 cm x 20 cm) placed and allowed to saturate the air in the tank. A 20 cm x 20 cm Silica gel 60 F254 normal phase plates was used in all experiments.

5 µl of 100 µg/200 µl treatment solution [^{14}C] imidacloprid or thiamethoxam (125 dps) was loaded at each end of the plate. 100 µl of each sample was then loaded using a Hamilton micro syringe, applying small quantities and allowing it to dry to keep the spot to the minimum size. Samples were loaded 3 cm onto the plate and the solvent

front was allowed to proceed to 3 cm from the top of the plate. The plates were then dried for 30 min and placed on a phosphor imager plate overnight (Fuji film). Plates were imaged using a Fuji film Bas-1500 phosphor imager and the plate analysed using Aida 3.1 software.

A2.1.7 LC/MS method.

The samples from Section A2.1.5 were analysed by LC/MS as they used non-radiolabelled insecticides. A HPLC Waters 2795 was used for analysis under the following conditions:

Detector Quattro Micro

Ionisation Electrospray +ve ion

Mode Single ion monitoring

Cone voltage 30 V

Nebuliser gas Nitrogen

Column ACE 3C18 50 mm x 3.0 mm i.d.

Mobile Phase Acetonitrile : 0.2% Acetic acid in ultrapure water (Fisher)

Flow rate 1 ml/min

Gradient Time min %MeCN %Aqueous Slope

0 - 0.5 5 95

0.5 - 4.5 95 5

linear gradient

4.5 - 5.7 95 5

5.7 - 5.8 5 95

linear gradient

5.8 - 6.0 5 95

A3 Results

The results in this section outline the work carried out to investigate the enzymatic action of CYP6G1. It was hoped to discover the metabolites produced by this gene to a range of insecticides and so be able to propose a hypothesis about the mode of action of this enzyme and how it is capable of metabolising a wide range of molecules.

A3.1 Metabolism.

Five different methods were tested, a topical screen on adults, a contact bioassay on adults, a larval feeding screen, a modified contact bioassay with the flies feeding on the insecticide on food and a screen based on using dissected abdomens. None of these methods showed significant differences between resistant and susceptible insects and no metabolites were detectable when using LC/MS. Although, some of the TLCs appeared to show increased metabolism of the parent compound, no metabolites were seen. TLCs were carried out using both [^{14}C] labelled imidacloprid and thiamethoxam and although some metabolites were seen in the adult feeding bioassay, there was no discernable difference in the intensity of these bands between the resistant and susceptible flies and the metabolites were at too low a concentration to be analysed by LC/MS. The contact bioassay, topical bioassay and abdominal screen were also run on TLC and showed no clear metabolites but showed some differences in the level of parent compound between resistant and susceptible flies. However, again there were no metabolites to be analysed by LC/MS. The larval screen was deemed to be too unsafe to use with a radiolabelled compound due to having to dispose of the increased amount of radioactive material as it is heavily diluted in ready mixed fly food and all this would need careful handling and disposal. Therefore, it was decided to do the larval screen on a variety of non-radiolabelled neonicotinoids as this might have a higher chance of one working. Unfortunately, again no metabolites could be seen and as this method was not done with a radiolabelled insecticide, so it was not possible to run these samples on TLC.

The TLC results shown bellow are all phosphor imager pictures, where blue represents a low signal, through green, yellow, and then red, a high signal.

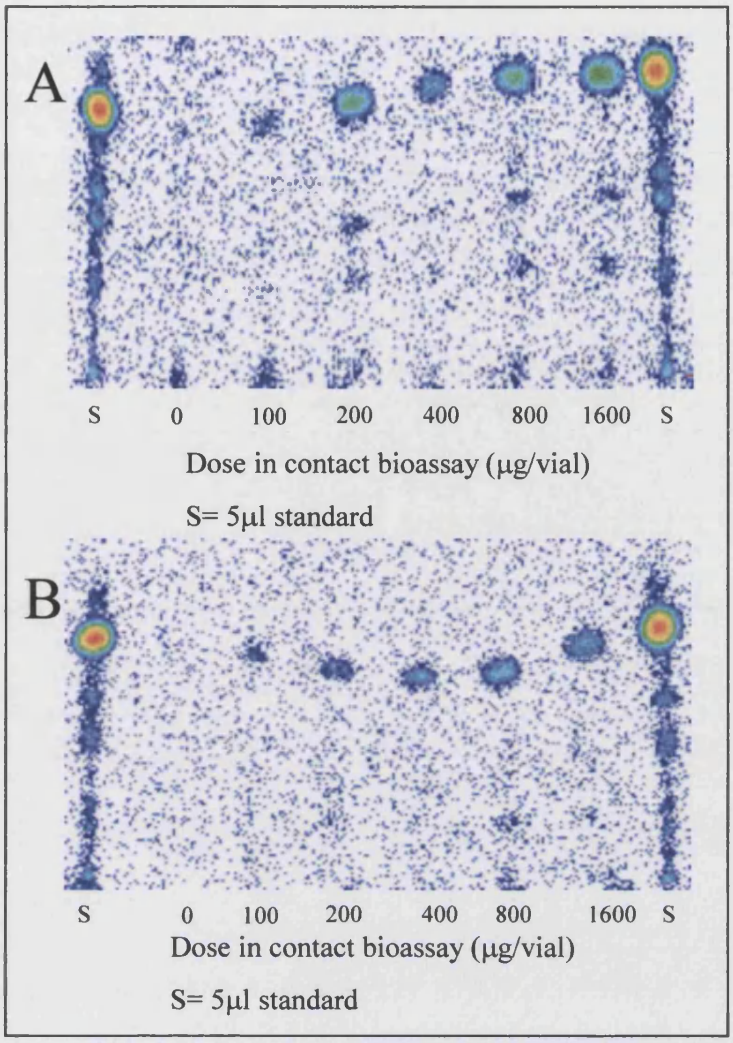


Fig. A1 TLC of Canton-S and EMS1 in contact bioassay.

The two TLCs shown in Figure A1 correspond to a contact bioassay carried out on Canton-S (A) and EMS1 (B). Both strains were exposed to differing levels of insecticide for 24 h. No metabolites were visible in on this TLC, but it there is a higher signal for imidacloprid in Canton-S at each susceptible concentration than the corresponding EMS1 concentration. This suggests that the imidacloprid is being metabolised at a higher rate in the resistant fly than the susceptible fly. This would agree with the results seen in the western Figure 3.6 where there is a clear signal for CYP6G1 in both the resistant and susceptible strain with resistance being caused by an increase in the protein. Therefore, we would expect to see similar metabolites in both

strains with their being more metabolite and less parent compound in the resistant strain.

The problem with this assay apart from not showing the presence of any metabolites is that we do not know how much imidacloprid was taken up by the fly. It is possible that EMS1 shows a decreased amount of parent molecule because it is less capable of taking up the insecticide than Canton-S. This is extremely unlikely to be the case given that the evidence suggests that imidacloprid resistance is due to the level of CYP6G1 rather than the penetrability of the insect cuticle but it must still be considered. As the previous method did not yield the expected answers about the metabolites produced by CYP6G1 it was next decided to use a topical bioassay where 0.5 µl of insecticide dissolved in acetone was applied directly to the abdomen of each individual fly. With this method, it was hoped that we would have a more controlled dose of insecticide directly onto the insect. This is similar to the work done by Miyagawa injecting imidacloprid directly into the abdomen of *M. domestica* (Miyagawa et al., 2002). Due to the size of *D. melanogaster,* it proved impossible to inject a controlled amount of insecticide into the fly without killing the fly from the injection or the solvent the insecticide is dissolved in.

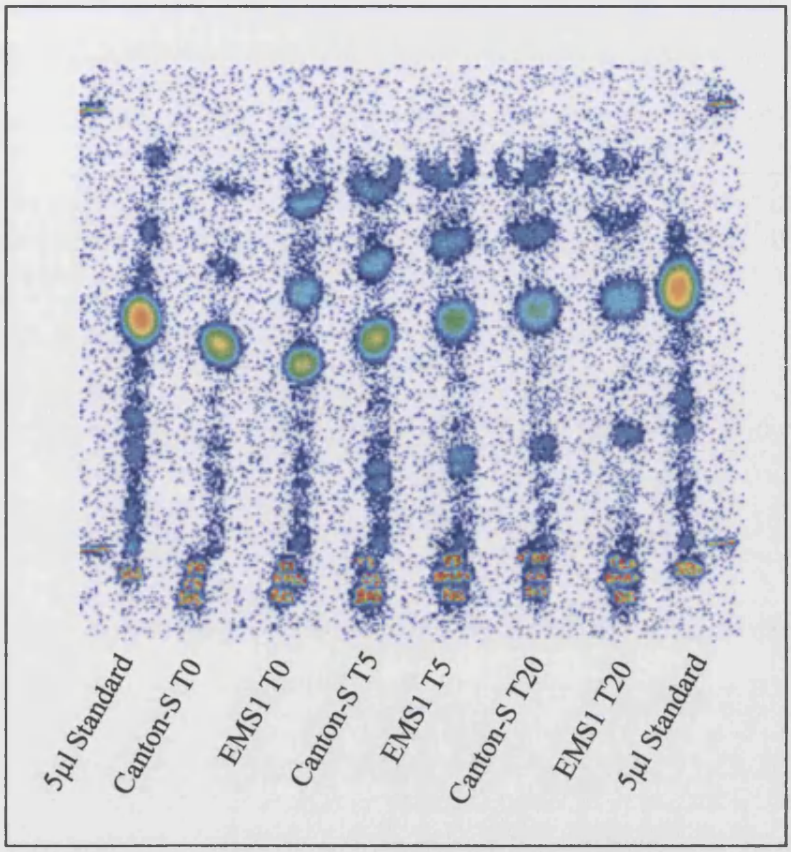


Fig. A2 TLC of Canton-S and EMS1 in topical bioassay.

Figure A2 shows the TLC results for the topical application of imidacloprid to resistant and susceptible insects. Insects were homogenised 0, 5 and 20 h after initial application of insecticide. As can be seen this method again yielded no significant differences in metabolites between EMS1 and Canton-S at any of the time points tested. The slight variety in products above the parent molecule, are, possibly due to variations in the loading of the TLC plate or in slight variation in the amount applied to the flies. In this case, there are some possible metabolites seen in all the flies at 5 and 20 hours below the parent compound. However, this amount was too low to be seen when the samples were run through LC/MS. In this case, when the two strains are compared there is slightly less parent in the EMS1 sample than Canton-S at both 5 and 20 hours. This again implies that there is more of the parent compound being broken down by the insect but again it has proved impossible to see any metabolites. Either, they are at too low a concentration to be visible using the phosphorimager, or they are not running up the TLC plate due to the solvent. However as the metabolites expected are a mono hydroxylated or a urea form of the parent molecule both would be expected to run in the

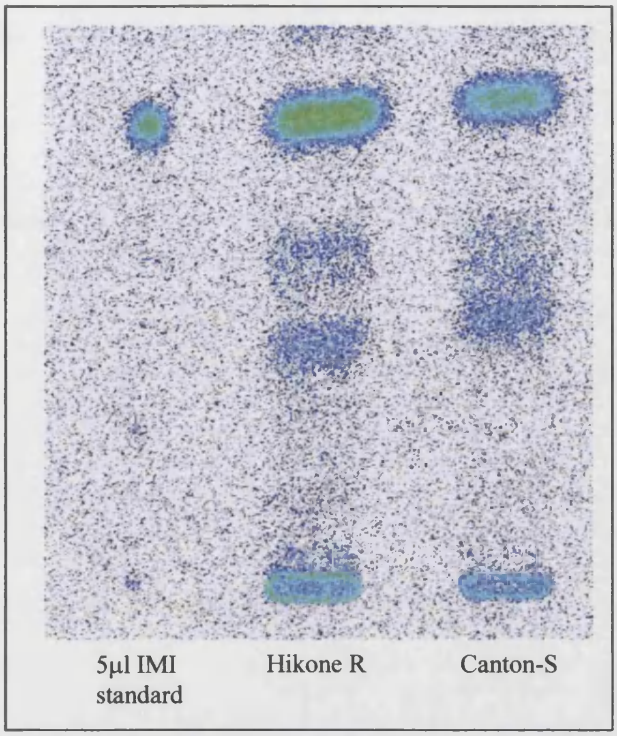


Fig. A3 TLC of Canton-S and Hikone R in adult feeding bioassay. (Result supplied by B. Cheek, Syngenta UK).

This figure shows the possible presence of metabolites in both resistant and susceptible strains. However again, they were at too low a concentration for detection using LC/MS. This method whilst working in part is currently being improved to attempt to increase the yield of metabolite. It is interesting to note that in this case there would appear to be less parent molecule in the Canton-S strain. This is the opposite result to that seen in Figures A1 and A2. It is likely that this difference is due to mortality of the Canton-S flies, so that these insects had less time to ingest the insecticide than the resistant strain.

The next method to be used was an abdominal bioassay. It was hoped that this method would give a more controlled insecticide dose and so give a better comparison between the resistant and susceptible strain. This method could also remove possible problems with insecticide uptake, as there was no longer a need to cross the cuticle or for digestion.

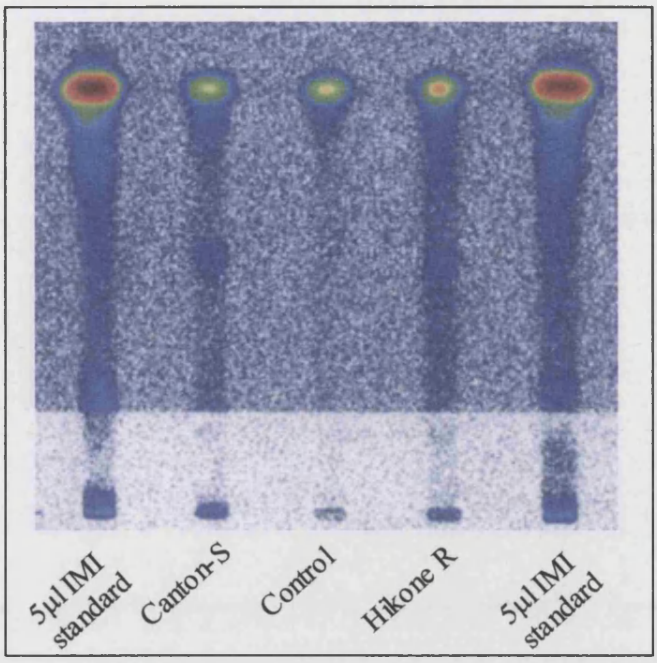


Fig. A4 TLC of imidacloprid in abdominal bioassay.

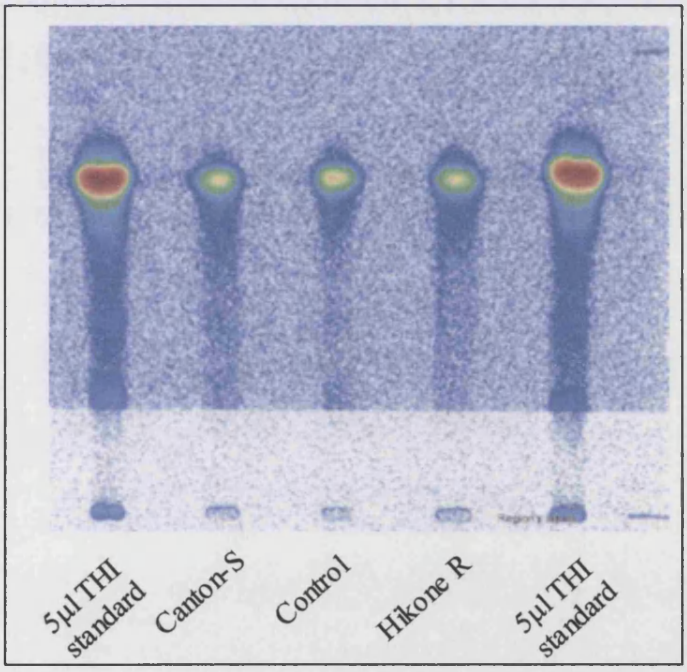
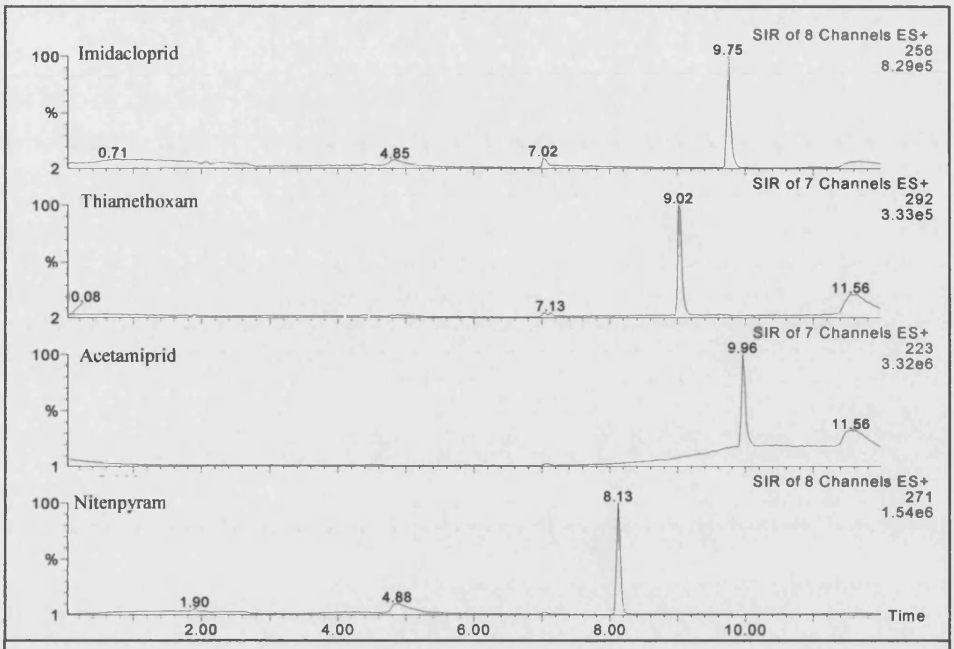
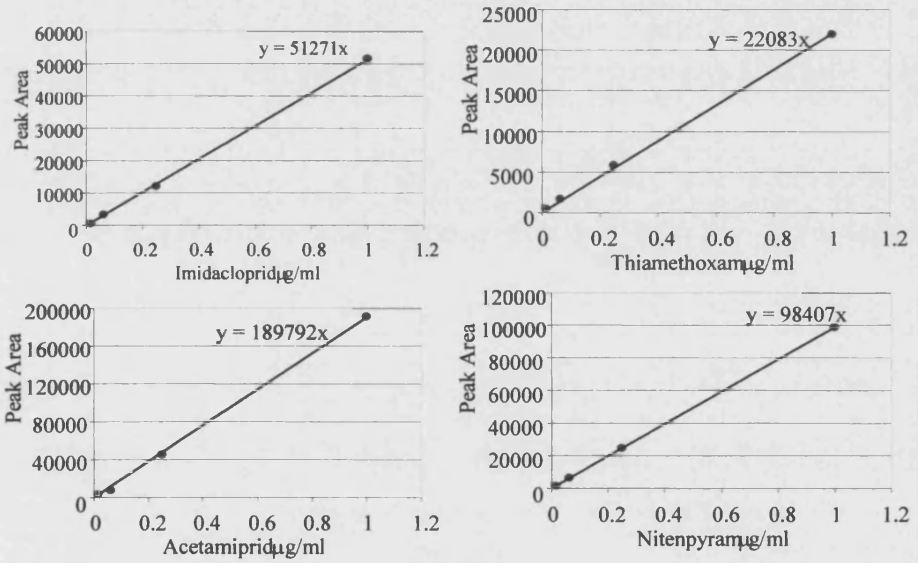


Fig. A5 TLC of thiamethoxam in abdominal bioassay.

These figures show a TLC of an abdominal bioassay carried out using Hikone R and Canton-S. Insects were exposed to imidacloprid (IMI) and thiamethoxam (THI). This method also yielded no possible metabolites with insufficient metabolism of the insecticides to yield detectable levels of the metabolites either on TLC or by LC/MS.

The final assay tried was on larvae. This, is the life stage at which we know the insect to be susceptible to neonicotinoids, and so it was thought that this life stage would yield the most metabolites. Eggs were placed on to ready mixed dry fly food containing a dose of imidacloprid, thiamethoxam, nitenpyram, or acetamiprid that was equal to the LD_{50} of Canton-S. As this experiment was done using non-radiolabelled compounds it was decided to use the same dose on both susceptible and resistant insects as it would not be possible to quantify the actual amount of insecticide taken up by the larvae. The eggs were allowed to develop until the reached the 3rd instar and were then harvested, this stage was chosen as larvae empty their gut contents before pupation so wandering larvae and pupae would not contain as high a level of insecticide in their gut so this is the largest feeding stage. The larvae were then homogenised and resuspended in 75%/25% acetonitrile water and run through the LC/MS.





Strain	Volume	Insecticide Detected (μg/ml)			
	Loaded (μl)	Imidacloprid	Thiamethoxam	Acetamiprid	Nitenpyram
Canton-S	5	0.009	0.000	0.005	0.045
Canton-S	10	0.051	0.000	0.008	0.048
Hikone R	5	0.000	0.000	0.016	0.010
Hikone R	10	0.000	0.000	0.000	0.017
Control	5	2.301	2.825	2.227	2.278

Fig. A6 Larval screen using new neonicotinoids.

Figure A6 is split into three panels. Panel A shows the LC/MS peaks at 1 ppm for of the neonicotinoids used in this experiment. It is clear that in all cases the parent molecule can be detected by the apparatus at 1 ppm. The second panel shows the calibration curve for each insecticide. This curve was then used to calculate the total amount of insecticide present in each sample. Again, any possible metabolites were at too low a concentration to be detected. Panel C shows the average amount of each parent compound present in each sample when 5 µl and 10 µl were loaded onto the LC/MS. In each case, the result is an average of three different samples. The result for imidacloprid shows there to be imidacloprid present in Canton-S but not in Hikone R. This would suggest that the compound has been metabolised and the metabolites are at too low a concentration to be detected or were excreted from the larvae prior to collection. The result for thiamethoxam shows that no parent molecule was detectable in any of the samples. This would suggest that the insecticide is at too low a concentration for detection or the compound has been metabolised by both strains or that the uptake of thiamethoxam is very low. The result for acetamiprid shows a similar result to that of imidacloprid, although there is a surprisingly large amount of parent molecule present in the 5 µl Hikone R sample. This is probably an erroneous result. In this experiment, nitenpyram allowed the highest discriminating dose as it has a high LD_{50} for Canton-S and the LD_{50} of resistant and susceptible flies are very similar. From Figure 4.6 and Table 4.7 we can see that the LD_{50} of Canton-S is 8.99 and 27.98 µg/vial for Hikone R. The relatively high LD_{50} for Canton-S allows a higher dose of insecticide to be applied to the food and so gives the possibility of a there being more parent molecule and metabolite present so allowing easier detection. In this table, we can see that when 5 µl are loaded, there is 4.5 times more insecticide present in the susceptible insect and when 10 µl is loaded the value is 2.8 fold. This figure corresponds closely to the resistance ratio seen in the nitenpyram DMC (Figure 4.6) where the resistance ratio for Hikone R when compared to Canton-S is 3.11. This result would seem to imply that the resistance ratio is a direct link to the relative amount of insecticide metabolised when the resistant and susceptible strains are compared. Unfortunately as this experiment was done using "cold" rather than radiolabelled insecticides, there is no way to know the total amount of parent metabolised.

A4 Discussion

None of the methods used give us a robust method to investigate the mode of action of CYP6G1 and so we have been unable to answer how it would appear that one enzyme is capable of metabolising a broad range of chemical structure. These methods have in places, worked but they do not appear repeatable. None of the methods used give sufficient metabolites to be detected by LC/MS. The decreased amount of parent molecule can be detected in some experiments but the possible metabolites are not at a high enough concentration. There are a number of possible reasons for this. The metabolites could simply be at too low a concentration to be detected by the phosophor imager in the case of the TLC plates or by the LCMS. It is also a possibility that the metabolites are in an ionic state that will not run on the TLC and so cannot be seen or were undetectable by the LC/MS. It is also possible that in some bioassays the metabolite is excreted quickly and so is not present in the insect when tested. We also cannot be sure in the case of the contact bioassays that the insecticide is taken up by the insect. The insecticide detected could be due to insecticide on the cuticle of the insect.

There are a number of possible improvements to be made to create a screen to test D. melanogaster for insecticide metabolites. Two major methods suggest themselves. Firstly, an in-vitro assay could be devised using a bacterial or yeast system and cloned Cyp6g1. This method has been used in the past to express other P450s but is beyond the remit of this thesis as it is a considerable undertaking to express active P450 (Feyereisen, 1999). It was also undesirable for Syngenta to use an in-vitro P450 expression system, as this does not mimic all the interactions between an insecticide and the complete biochemical pathways of an insect. A second method would be to use a microsomal preparation. This would dispense with problems of the entry into the insect by the insecticide and would allow for a known concentration of insecticide to be run on a TLC or LC/MS, as the rate of uptake would no longer be a factor. This method has been used to investigate insecticide metabolism in both mammalian and insect microsomes (Byrne et al., 2003; Schulz-Jander et al., 2002).

References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*. 18:265-267.

Amichot, M., A. Brun, A. Cuany, C. Helvig, J.P. Salaun, F. Durst, and J.B. Berge. 1994. Expression study of CYP genes in drosophila strains resistant or sensitive to insecticides. *In* Cytochrome P450. 8th International Conference. M.C. Lechner, editor. John LIbbey Eurotext, Paris. 689-692.

Anthony, N., T. Unruh, D. Ganser, and R.H. ffrench-Constant. 1998a. Duplication of the *Rdl* GABA receptor in an insecticide-resistant aphid, *Myzus persicae*. *Molecular and General Genetics*. 260:165-177.

Anthony, N.M., J.K. Brown, R. Feyereisen, and R.H. ffrench-Constant. 1998b. Diagnosis and characterization of insecticide-insensitive acetylcholinesterase in three populations of the sweet potato whitefly *Bemisia tabaci*. *Pesticide Science*. 52:39-46.

Arbeitman, M.N., E.E. Furlong, F. Imam, E. Johnson, B.H. Null, B.S. Baker, M.A. Krasnow, M.P. Scott, R.W. Davis, and K.P. White. 2002. Gene expression during the life cycle of Drosophila melanogaster. *Science*. 297:2270-5.

Ashburner, M. 1989. Drosophila: a Laboratory Handbook. Cold Spring Harbor Laboratory Press. 434 pp.

Assaraf, Y.G., A. Molina, and R.T. Schimke. 1989. Sequential amplification of dihydrofolate reductase and multidrug resistance genes in Chinese hamster

ovary cells selected for stepwise resistance to the lipid-soluble antifolate trimetrexate. *J Biol Chem.* 264:18326-34.

Bai, D., S.C.R. Lummis, W. Leicht, H. Breer, and D. Sattelle. 1991. Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neurone. *Pesticide Science.* 33:197-204.

Berenbaum, M.R., and A.R. Zangerl. 1992. Genetics of physiological and behavioral resistance to host furanocoumarins in the parsnip webworm. *Evolution.* 46:1373-1384.

Berge, J.B., R. Feyereisen, and M. Amichot. 1998. Cytochrome P450 monooxygenases and insecticide resistance in insects. *Philos Trans R Soc Lond B Biol Sci.* 353:1701-5.

Bloomquist, J.R., and D.M. Soderlund. 1988. Pyrethroid insecticides and DDT modify alkaloid-dependent sodium channel activation and its enhancement by sea anemone toxin. *Molecular Pharmacology.* 33:543-50.

Bourguet, D., A. Genissel, and M. Raymond. 2000. Insecticide resistance and dominance levels. *J Econ Entomol.* 93:1588-95.

Brandt, A., M. Scharf, J.H. Pedra, G. Holmes, A. Dean, M. Kreitman, and B.R. Pittendrigh. 2002. Differential expression and induction of two *Drosophila* cytochrome P450 genes near the *Rst(2)DDT* locus. *Insect Mol Biol.* 11:337-341.

Broadhurst, M.D. 1998. The influence of the molecular basis of resistance on insecticide discovery. *Philosophical Transactions of the Royal Society of London, Series B.* 353:1723-1728.

Brun, A., A. Cuany, T. Le Mouel, J. Berge, and M. Amichot. 1996. Inducibility of the *Drosophila melanogaster* cytochrome P450 gene, *CYP6A2*, by phenobarbital in insecticide susceptible or resistant strains. *Insect Biochemistry and Molecular Biology.* 26:697-703.

Buckingham, S., B. Lapied, H. Corronc, and F. Sattelle. 1997. Imidacloprid actions on insect neuronal acetylcholine receptors. *J Exp Biol.* 200:2685-92.

Busvine, J. 1971. Techniques for testing insecticdes. Commonwealth Agricultural Bureaux, Slough.

Busvine, J.R. 1951. Mechanism of resistance to insecticide in houseflies. *Nature.* 168:193-195.

Byrne, F.J., S. Castle, N. Prabhaker, and N.C. Toscano. 2003. Biochemical study of resistance to imidacloprid in B biotype Bemisia tabaci from Guatemala. *Pest Manag Sci.* 59:347-52.

Campbell, P.M., J.L. Yen, A. Masoumi, R.J. Russell, P. Batterham, J.A. McKenzie, and J.G. Oakeshott. 1998. Cross-resistance patterns among Australian sheep blowfly, *Lucilia cuprina* (Diptera: Calliphoridae), resistant to organophosphorus insecticides. *Journal of Economic Entomology.* 91:367-375.

Carino, F.A., J.F. Koener, F.W. Plapp, Jr., and R. Feyereisen. 1994. Constitutive overexpression of the cytochrome P450 gene CYP6A1 in a house fly strain with metabolic resistance to insecticides. *Insect Biochem Mol Biol.* 24:411-418.

Celniker, S.E., D.A. Wheeler, B. Kronmiller, J.W. Carlson, A. Halpern, S. Patel, M. Adams, M. Champe, S.P. Dugan, E. Frise, A. Hodgson, R.A. George, R.A. Hoskins, T. Laverty, D.M. Muzny, C.R. Nelson, J.M. Pacleb, S. Park, B.D. Pfeiffer, S. Richards, E.J. Sodergren, R. Svirskas, P.E. Tabor, K. Wan, M. Stapleton, G.G. Sutton, C. Venter, G. Weinstock, S.E. Scherer, E.W. Myers, R.A. Gibbs, and G.M. Rubin. 2002. Finishing a whole-genome shotgun: release 3 of the Drosophila melanogaster euchromatic genome sequence. *Genome Biol.* 3:RESEARCH0079.

Chen, Z., R. Newcomb, E. Forbes, J. McKenzie, and P. Batterham. 2001. The acetylcholinesterase gene and organophosphorus resistance in the Australian sheep blowfly, Lucilia cuprina. *Insect Biochem Mol Biol.* 31:805-16.

Clark, A.G., and N.A. Shamaan. 1984. Evidence that DDT-dehydrochlorinase from the house fly is a glutathione S-transferase. *Pestic Biochem Physiol.* 22:249-261.

Clark, A.G., N.A. Shamaan, M.D. Sinclair, and W.C. Dauterman. 1987. Insecticide metabolism by multiple glutathione S-transferases in two strains of the house fly, *Musca domestica* (L.). *Pestic Biochem Physiol.* 25:169-175.

Claudianos, C., R.J. Russell, and J.G. Oakeshott. 1999. The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. *Insect Biochem Mol Biol.* 29:675-86.

Cohen, M.B., and R. Feyereisen. 1995. A cluster of cytochrome P450 genes of the CYP6 family in the house fly. *DNA and Cell Biology*. 14:73-82.

Crow, J.F. 1954. Analysis of a DDT-resistant strain of *Drosophila*. *Journal of Economic Entomology*. 47:393-398.

Daborn, P., S. Boundy, J. Yen, B. Pittendrigh, and R. ffrench-Constant. 2001. DDT resistance in *Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Mol. Genet. Genomics*. 266:556-563.

Daborn, P.J., J.A. McKenzie, and P. Batterham. 2000. A genetic analysis of cyromazine resistance in *Drosophila melanogaster* (Diptera: Drosophilidae). *Journal of Economic Entomology*. 93:911-919.

Daborn, P.J., J.L. Yen, M. Bogwitz, G. LeGoff, E. Feil, S. Jeffers, N. Tijet, T. Perry, D. Heckel, P. Batterham, R. Feyereisen, T. Wilson, and R.H. ffrench-Constant. 2002. A single P450 allele associated with insecticide resistance in global populations of *Drosophila*. *Science*. 297:2253-2256.

Dapkus, D.C., and D.J. Merrell. 1977. Chromosomal analysis of DDT-resistance in a long-term selected population of *Drosophila melanogaster*. *Genetics*. 87:685-697.

Denholm, I., M. Cahill, T.J. Dennehy, and A.R. Horowitz. 1998. Challenges with managing insecticide resistance in agricultural pests, exemplified by the whitefly *Bemisia tabaci*. *Philosophical Transactions of the Royal Society of London*. 353:1757-1767.

Denholm, I., G.J. Devine, and M.S. Williamson. 2002. Evolutionary genetics. Insecticide resistance on the move. *Science*. 297:2222-3.

Denholm, I., A.W. Farnham, K. O'Dell, and R.M. Sawicki. 1983. Factors affecting resistance to insecticides in house-flies *Musca domestica* L. (Diptera: Muscidae). 1. Long-term control with bioresmethrin of flies with strong pyrethroid resistance potential. *Bulletin of Entomological Research*. 73:481-489.

Dent, D. 1991. Insect Pest Management. C.A.B. International, Wallingford.

Devonshire, A.L., and R.M. Sawicki. 1979. Insecticide-resistant Myzus persicae as an example of evolution by gene duplication. *Nature*. 280:140-141.

Dong, K. 2003. Voltage-gated Sodium channels as insecticide targets. *In* Chemistry of Crop Protection. G. Voss and G. Ramos, editors. WILEY-VCH, Weinhein. 167-176.

Doray, B., C.D. Chen, and B. Kemper. 2001. N-terminal deletions and His-tag fusions dramatically affect expression of cytochrome p450 2C2 in bacteria. *Arch Biochem Biophys*. 393:143-53.

Dunkov, B.C., G. Mocelin, F. Shotkoski, R.H. ffrench-Constant, and R. Feyereisen. 1997. The *Drosophila* cytochrome P450 gene *Cyp6a2*: structure, chromosomal localization, heterologous expression and induction by phenobarbital. *DNA and Cell Biology*:1345-1356.

Dunkov, B.C., R. Rodriguez-Arnaiz, B. Pittendrigh, R.H. ffrench-Constant, and R. Feyereisen. 1996. Cytochrome P450 gene clusters in *Drosophila melanogaster*. *Molecular and General Genetics*. 251:290-297.

Feyereisen, R. 1995. Molecular biology of insecticide resistance. *Toxicology Letters*. 82/83:83-90.

Feyereisen, R. 1999. Insect P450 Enzymes. *Ann. Rev. Entomol.* 44:507-533.

ffrench-Constant, R.H., N. Anthony, K. Aronstein, T. Rocheleau, and G. Stilwell. 2000. Cyclodiene insecticide resistance: from molecular to population genetics. *Annual Review of Entomology*. 45:449-466.

ffrench-Constant, R.H., B. Pittendrigh, A. Vaughan, and N. Anthony. 1999. Why are there so few resistance-associated mutations in insecticide target genes? *In* Insecticide Resistance: From Mechanisms to Management. I. Denholm, J.A. Pickett, and A.L. Devonshire, editors. CABI, The Royal Society, Wallingford, Oxon, UK. 9-18.

ffrench-Constant, R.H., and T. Rocheleau. 1992. Drosophila cyclodiene resistance gene shows conserved genomic organization with vertebrate ϑ-aminobutyric $acid_A$ receptors. *Journal of Neurochemistry*. 59:1562-1565.

ffrench-Constant, R.H., and R.T. Roush. 1991. Gene mapping and cross-resistance in cyclodiene insecticide-resistant *Drosophila melanogaster* (Mg.). *Genetical Research, Cambridge*. 57:17-21.

ffrench-Constant, R.H., R.T. Roush, and F. Carino. 1992. *Drosophila* as a tool for investigating the molecular genetics of insecticide resistance. *In* Molecular Approaches to Pure and Applied Entomology. M.J. Whitten and J.G. Oakeshott, editors. Springer-Verlag, Berlin. 1-37.

Field, L.M., A.L. Devonshire, and B.G. Forde. 1988. Molecular evidence that insecticide resistance in peach potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochemical Journal.* 251:309-312.

Field, L.M., M.S. Williamson, G.D. Moores, and A.L. Devonshire. 1993. Cloning and analysis of the esterase gene conferring insecticide resistance in the peach-potato aphid, *Myzus persicae* (Sulzer). *Biochemical Journal.* 294:569-574.

Finney, D.J. 1952. Probit Analysis. Cambridge University Press, Cambridge, U.K. 318 pp.

Fogleman, J.C. 2000. Response of Drosophila melanogaster to selection for P450-mediated resistance to isoquinoline alkaloids. *Chem Biol Interact.* 125:93-105.

Fournier, D., J.M. Bride, F. Hoffmann, and F. Karch. 1992. Acetylcholinesterase. Two types of modifications confer resistance to insecticide. *J Biol Chem.* 267:14270-4.

Georghiou, G.P., and C.E. Taylor. 1977. Genetic and biological influences in the evolution of insecticide resistance. *Journal of Economic Entomology.* 70:319-323.

Graham, S.E., and J.A. Peterson. 1999. How similar are P450s and what can their differences teach us? *Arch Biochem Biophys*. 369:24-9.

Groeters, F.R., and B.E. Tabashnik. 2000. Roles of selection intensity, major genes, and minor genes in evolution of insecticide resistance. *J Econ Entomol*. 93:1580-7.

Guzov, V.M., G.C. Unnithan, A.A. Chernogolov, and R. Feyereisen. 1998. CYP12A1, a mitochondrial cytochrome P450 from the house fly. *Archives of Biochemistry and Biophysics*. 359:231-240.

Hemingway, J. 2000. The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. *Insect Biochem Mol Biol*. 30:1009-15.

Hemingway, J., and H. Ranson. 2000. Insecticide resistance in insect vectors of human disease. *Annu Rev Entomol*. 45:371-91.

Hodgson, E., and L. Tate. 1976. Cytochrome P450 Interactions. *In* Insecticide biochemistry and physiology. C. Wilkinson, editor. Plenum Press, New York. 115-148.

Hoskins, R.A., C.D. Smith, J.W. Carlson, A.B. Carvalho, A. Halpern, J.S. Kaminker, C. Kennedy, C.J. Mungall, B.A. Sullivan, G.G. Sutton, J.C. Yasuhara, B.T. Wakimoto, E.W. Myers, S.E. Celniker, G.M. Rubin, and G.H. Karpen. 2002. Heterochromatic sequences in a Drosophila whole-genome shotgun assembly. *Genome Biol*. 3:RESEARCH0085.

Kagabu, S. 2003. Molecular design of neonicotinoids: Past, present and future. *In* Chemistry of Crop Protection. G. Voss and G. Ramos, editors. WILEY-VCH, Weinhein. 193-212.

Kagabu, S., H. Nishiwaki, K. Sato, M. Hibi, N. Yamaoka, and Y. Nakagawa. 2002. Nicotinic acetylcholine receptor binding of imidacloprid-related diaza compounds with various ring sizes and their insecticidal activity against Musca domestica. *Pest Manag Sci.* 58:483-90.

Keseru, G.M., G. Balogh, I. Czudor, T. Karancsi, A. Feher, and B. Bertok. 1999. Chemical models of cytochrome P450 catalyzed insecticide metabolism. Application to the oxidative metabolism of carbamate insecticides. *J Agric Food Chem.* 47:762-9.

Kikkawa, H. 1961. Genetical studies on the resistance to parathion in *Drosophila melanogaster* . I. Gene analyses. *Ann. Rep. Sci. Works Fac. Sci. Osaka Univ.* 9:1-20.

Kikkawa, H. 1964. Genetical studies on the resistance to parathion in *Drosophila melanogaster.* II. Induction of a resistance gene from its susceptible allele. *Botyu-Kagaku.* 29:37-42.

King, J.C., and L. Somme. 1958. Chromosomal analysis of the genetic factors for resistance to DDT in two resistant lines of *Drosophila melanogaster. Genetics.* 43:577-593.

Knipple, D.C., K.E. Doyle, P.A. Marsella-Herrick, and D.M. Soderlund. 1994. Tight genetic linkage between the kdr insecticide resistance trait and a voltage-

sensitive sodium channel gene in the house fly. *Proceedings of the National Academy of Sciences*. 91:2483-7.

Korytko, P.J., R.J. MacLntyre, and J.G. Scott. 2000. Expression and activity of a house-fly cytochrome P450, CYP6D1, in *Drosophila melanogaster*. *Insect Mol Biol*. 9:441-449.

Lansdell, S.J., and N.S. Millar. 2000. The influence of nicotinic receptor subunit composition upon agonist, alpha-bungarotoxin and insecticide (imidacloprid) binding affinity. *Neuropharmacology*. 39:671-9.

Le Goff, G., S. Boundy, P.J. Daborn, J.L. Yen, L. Sofer, R. Lind, C. Sabourault, L. Madi-Ravazzi, and R.H. ffrench-Constant. 2003. Microarray analysis of cytochrome P450 mediated insecticide resistance in Drosophila. *Insect Biochem Mol Biol*. 33:701-8.

Lees, G. 1998. Effects of pyrethroid molecules on rat nerves in vitro: potential to reverse temperature-sensitive conduction block of demyelinated peripheral axons. *Br J Pharmacol*. 123:487-96.

Leicht, W. 1996. Imidacloprid - a chloronicotinyl insecticide biological activity and agricultural significance. *Pflanzenschutz-Nachrichten Bayer*. 49:79-81.

Li, W., M.R. Berenbaum, and M.A. Schuler. 2001. Molecular analysis of multiple CYP6B genes from polyphagous Papilio species. *Insect Biochem Mol Biol*. 31:999-1011.

Liu, M.Y., and J.E. Casida. 1993. High affinity binding of [^{3}H]-imidacloprid in the insect acetylcholine receptor. *Pesticide Biochemistry and Physiology*. 46:40-46.

Liu, N., and X. Yue. 2000. Insecticide resistance and cross-resistance in the house fly (Diptera: Muscidae). *J Econ Entomol*. 93:1269-75.

Maienfisch, P., M. Angst, F. Brandl, W. Fischer, D. Hofer, H. Kayser, W. Kobel, A. Rindlisbacher, R. Senn, A. Steinemann, and H. Widmer. 2001. Chemistry and biology of thiamethoxam: a second generation neonicotinoid. *Pest Manag Sci*. 57:906-13.

Maitra, S., S.M. Dombrowski, M. Basu, O. Raustol, L.C. Waters, and R. Ganguly. 2000. Factors on the third chromosome affect the level of *Cyp6a2* and *Cyp6a8* expression in *Drosophila melanogaster*. *Gene*. 248:147-56.

Maitra, S., S.M. Dombrowski, L.C. Waters, and R. Ganguly. 1996. Three second chromosome-linked clustered *Cyp6* genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*. *Gene*. 180:165-171.

Maitra, S., C. Price, and R. Ganguly. 2002. Cyp6a8 of Drosophila melanogaster: gene structure, and sequence and functional analysis of the upstream DNA. *Insect Biochem Mol Biol*. 32:859-70.

Martin, R.L., B. Pittendrigh, J. Liu, R. Reenan, R. ffrench-Constant, and D.A. Hanck. 2000. Point mutations in domain III of a Drosophila neuronal Na channel confer resistance to allethrin. *Insect Biochem Mol Biol*. 30:1051-9.

Martinez-Torres, D., F. Chandre, M.S. Williamson, F. Darriet, J.B. Berge, A.L. Devonshire, P. Guillet, N. Pasteur, and D. Pauron. 1998. Molecular characterization of pyrethroid knockdown resistance *(kdr)* in the major malaria vector *Anopheles gambiae ss. Insect Molecular Biology*. 7:179-184.

Matsuda, K., S.D. Buckingham, D. Kleier, J.J. Rauh, M. Grauso, and D.B. Sattelle. 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences*. 22:573-580.

McKenzie, J.A., and P. Batterham. 1998. Predicting insecticide resistance: mutagenesis, selection and response. *Philosophical Transactions of the Royal Society of London, Series B*. 353:1729-1734.

McKenzie, J.A., A.G. Parker, and J.L. Yen. 1992. Polygenic and single gene responses to selection for resistance to diazinon in Lucilia cuprina. *Genetics*. 130:613-20.

Miyagawa, H., K. Sato, H. Nishiwaki, Y. Nakagawa, and M. Miyashita. 2002. Metabolism of imidacloprid in houseflies and effect of synergists. *In* 10th IUPAC International Congress of the Chemistry of Crop Protection, Basel, Ch.

Miyo, T., H. Takamori, Y. Kono, and Y. Oguma. 2001. Genetic variation and correlations among responses to five insecticides within natural populations of Drosophila melanogaster (Diptera: Drosophilidae). *J Econ Entomol*. 94:223-32.

Mouches, C., M. Magnin, J.-B. Berge, M. de Silvestri, V. Beyssat, N. Pasteur, and G.P. Georghiou. 1987. Overproduction of detoxifying esterases in organophosphate-resistant *Culex* mosquitoes and their presence in other insects. *Proceedings of the National Academy of Sciences*. 84:2113-2116.

Mouches, C., Y. Pauplin, M. Agarwal, L. Lemieux, M. Herzog, M. Abadon, A.V. Beyssat, O. Hyrien, S.V.B.R. de, G.P. Georghiou, and a.l. et. 1990. Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in Culex. *Proceedings of the National Academy of Sciences*. 87:2574-8.

Mutero, A., M. Pralavorio, J.-M. Bride, and D. Fournier. 1994. Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proceedings of the National Academy of Sciences*. 91:5922-5926.

Myers, E.W., G.G. Sutton, A.L. Delcher, I.M. Dew, D.P. Fasulo, M.J. Flanigan, S.A. Kravitz, C.M. Mobarry, K.H. Reinert, K.A. Remington, E.L. Anson, R.A. Bolanos, H.H. Chou, C.M. Jordan, A.L. Halpern, S. Lonardi, E.M. Beasley, R.C. Brandon, L. Chen, P.J. Dunn, Z. Lai, Y. Liang, D.R. Nusskern, M. Zhan, Q. Zhang, X. Zheng, G.M. Rubin, M.D. Adams, and J.C. Venter. 2000. A whole-genome assembly of Drosophila. *Science*. 287:2196-204.

Nauen, R., K. Tietjen, K. Wagner, and A. Elbert. 1998. Efficacy of plant metabolites of imidacloprid against Myzus persicae and Aphis gossypii (Homoptera: Aphididae). *Pesticide Science*. 52:53-57.

Nelson, D.R. 1999. Cytochrome P450 and the individuality of species. *Arch Biochem Biophys*. 369:1-10.

Newcomb, R.D., P.M. Campbell, D.L. Ollis, E. Cheah, R.J. Russell, and J.G. Oakeshott. 1997. A single amino acid substitution converts a carboxylesterase to an

organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proceedings of the National Academy of Sciences.* 94:7464-7468.

O'Connell, P., and M. Rosbash. 1984. Sequence, structure and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Research.* 12:5495-5513.

Ogita, Z. 1960. Genetical and biochemical studies on negatively correlated cross-resistance in *Drosophila melanogaster.* I. An attempt to reduce and increase insecticide-resistance in *D. melanogaster* by selection pressure. *Botyu-Kagaku.* 26:7-18.

Olson, E.R., G.P. Dively, and J.O. Nelson. 2000. Baseline susceptibility to imidacloprid and cross resistance patterns in Colorado potato beetle (Coleoptera: Chrysomelidae) populations. *J Econ Entomol.* 93:447-58.

Omura, T. 1999. Forty years of cytochrome P450. *Biochem Biophys Res Commun.* 266:690-8.

Oppenoorth, F.J. 1985. Biochemistry and genetics of insecticide resistance. *In* Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 12. G.A. Kerkut and L.I. Gilbert, editors. Pergamon, New York. 731-773.

Ortelli, F., L.C. Rossiter, J. Vontas, H. Ranson, and J. Hemingway. 2003. Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide resistance locus, from the malaria vector Anopheles gambiae. *Biochem J.* 28.

Osborne, M.P., and D.R. Pepper. 1992. Mechanisms of *kdr* and *super-kdr* resistance. *In* Molecular mechanisms of insecticide resistance. American Chemical Society. 71-89.

Parker, A.G., P.M. Campbell, M.E. Spackman, R.J. Russell, and J.G. Oakeshott. 1996. Comparison of an Esterase Associated with Organophosphate Resistance in Lucilia cuprina with an Orthologue Not Associated with Resistance in Drosophila melanogaster. *Pestic Biochem Physiol.* 55:85-99.

Patil, V., and F. Guthrie. 1979. Cuticular lipids in two resistant and a susceptible strain of houseflies. *Pesticide Science.* 10:399-406.

Petersen, R.A., A.R. Zangerl, M.R. Berenbaum, and M.A. Schuler. 2001. Expression of CYP6B1 and CYP6B3 cytochrome P450 monooxygenases and furanocoumarin metabolism in different tissues of Papilio polyxenes (Lepidoptera: Papilionidae). *Insect Biochem Mol Biol.* 31:679-90.

Pittendrigh, B., K. Aronstein, E. Zinkovsky, O. Andreev, B. Campbell, J. Daly, S. Trowell, and R.H. ffrench-Constant. 1997a. Cytochrome P450 genes from *Helicoverpa armigera*: expression in a pyrethroid-susceptible and -resistant strain. *Insect Biochemistry and Molecular Biology.* 1997:507-512.

Pittendrigh, B., R. Reenan, R.H. ffrench-Constant, and B. Ganetzy. 1997b. Point mutations in the *Drosophila* sodium channel gene *para* associated with resistance to DDT and pyrethroid insecticides. *Molecular General Genetics.* 256:602-610.

Plapp, F., and R. Hoyer. 1968. Insecticide resistance in the housefly: decreased rate of absorption that acts as an intensifier of resistance. *J Econ Entomol.* 61:1298-1303.

Pluthero, F.G., and S.F.H. Threlkeld. 1984. Mutations in *Drosophila melanogaster* affecting physiological and behavioral response to malathion. *Canadian Entomologist.* 116:411-418.

Ranson, H., C. Claudianos, F. Ortelli, C. Abgrall, J. Hemingway, M.V. Sharakhova, M.F. Unger, F.H. Collins, and R. Feyereisen. 2002. Evolution of supergene families associated with insecticide resistance. *Science.* 298:179-181.

Robertson, J.L., R.M. Russell, and N.E. Savin. 1980. POLO: a user's guide to probit or logic analysis. *United States Forestry Service Technical Report PSW.* United States Forestry Service Technical Report PSW.

Rose, R.L., D. Goh, D.M. Thompson, K.D. Verma, D.G. Heckel, L.J. Gahan, R.M. Roe, and E. Hodgson. 1997. Cytochrome P450 (CYP)9A1 in *Heliothis virescens*: the first member of a new CYP family. *Insect Biochemistry and Molecular Biology.* 27:605-615.

Roush, R.T., and J.A. McKenzie. 1987. Ecological genetics of insecticide and acaricide resistance. *Annu Rev Entomol.* 32:361-80.

Russell, R.J., G.C. Robin, P. Kostakos, R.D. Newcomb, T.M. Boyce, K.M. Medveczky, and J.G. Oakeshott. 1996. Molecular cloning of an x-esterase gene cluster on chromosome 3R of *Drosophila melanogaster. Insect Biochemical Molecular Biology.* 26:235-247.

Sabourault, C., V.M. Guzov, J.F. Koener, C. Claudianos, F.W. Plapp, Jr., and R. Feyereisen. 2001. Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase (MdalphaE7) gene in resistant house flies. *Insect Mol Biol.* 10:609-18.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab., Cold Spring Harbor, NY.

Sawicki, R., and K. Lord. 1970. Some properties of a mechanism delaying penetration of insecticides into houseflies. *Pesticide Science.* 1:213-217.

Sawicki, R.M. 1978. Unusual response of DDT-resistant houseflies to carbinol analogues of DDT. *Nature.* 275:443-444.

Schlenke, T.A., and D.J. Begun. 2003. Natural selection drives Drosophila immune system evolution. *Genetics.* 164:1471-80.

Schulz-Jander, D.A., and J.E. Casida. 2002. Imidacloprid insecticide metabolism: human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. *Toxicol Lett.* 132:65-70.

Schulz-Jander, D.A., W.M. Leimkuehler, and J.E. Casida. 2002. Neonicotinoid insecticides: reduction and cleavage of imidacloprid nitroimine substituent by liver microsomal and cytosolic enzymes. *Chem Res Toxicol.* 15:1158-65.

Scott, J.A., F.H. Collins, and R. Fegereisen. 1994. Diversity of cytochrome P450 genes in the mosquito, *Anopheles albimanus. Biochemical and Biophysical Research Communications.* 205:1452-1459.

Scott, J.G., N. Liu, Z. Wen, F.F. Smith, S. Kasai, and C.E. Horak. 1999. House-fly cytochrome P450 CYP6D1: 5' flanking sequences and comparison of alleles. *Gene.* 226:347-53.

Sheffield, P., S. Garrard, and Z. Derewenda. 1999. Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors. *Protein Expr Purif.* 15:34-9.

Shepanski, M.M.C., T.J. Glover, and R.J. Kuhr. 1977. Resistance of *Drosophila melanogaster* to DDT. *Journal of Economic Entomology.* 70:539-543.

Smith, F.F., and J.G. Scott. 1997. Functional expression of house fly (Musca domestica) cytochrome P450 CYP6D1 in yeast (Saccharomyces cerevisiae). *Insect Biochem Mol Biol.* 27:999-1006.

Smith, L.L. 2001. Key challenges for toxicologists in the 21st century. *Trends Pharmacol Sci.* 22:281-5.

Snyder, M.J., J.L. Stevens, J.F. Andersen, and R. Feyereisen. 1995. Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, Manduca sexta. *Arch Biochem Biophys.* 321:13-20.

Soderlund, D.M., and J.R. Bloomquist. 1990. Molecular mechanisms of resistance. *In* Pesticide Resistance in Arthropods. R.T. Roush and B.E. Tabashnik, editors. Chapman and Hall, New York. 58 - 96.

Soderlund, D.M., and D.C. Knipple. 2003. The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochem. Mol. Biol.* 33:563-577.

Spackman, M.E., J.G. Oakeshott, K.A. Smyth, K.M. Medveczky, and R.J. Russell. 1994. A cluster of esterase genes on chromosome 3R of *Drosophila melanogaster* includes homologues of esterase genes conferring insecticide resistance in *Lucilia cuprina*. *Biochemical Genetics*. 32:39-62.

Stevens, J.L., M.J. Snyder, J.F. Koener, and R. Feyereisen. 2000. Inducible P450s of the CYP9 family from larval Manduca sexta midgut. *Insect Biochem Mol Biol.* 30:559-68.

Taylor, M., and R. Feyereisen. 1996. Molecular biology and evolution of resistance to toxicants. *Molecular Biological Evolution*. 13:719-734.

Tijet, N., C. Helvig, and R. Feyereisen. 2001. The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron-exon organization and phylogeny. *Gene*. 262:189-198.

Tomita, T., N. Liu, F.F. Smith, P. Sridhar, and J.G. Scott. 1995. Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica*. *Insect Molecular Biology*. 4:135-140.

Tomizawa, M., and J.E. Casida. 2000. Imidacloprid, thiacloprid, and their imine derivatives up-regulate the alpha 4 beta 2 nicotinic acetylcholine receptor in M10 cells. *Toxicol Appl Pharmacol.* 169:114-20.

Tomizawa, M., and J.E. Casida. 2001. Structure and diversity of insect nicotinic acetylcholine receptors. *Pest. Manag. Sci.* 57:914-922.

Tomizawa, M., and J.E. Casida. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu Rev Entomol.* 48:339-364.

Tomizawa, M., B. Latli, and J.E. Casida. 1996. Novel neonicotinoid-agarose affinity column for Drosophila and Musca nicotinic acetylcholine receptors. *J Neurochem.* 67:1669-76.

Tomizawa, M., D.L. Lee, and J.E. Casida. 2000. Neonicotinoid insecticides: molecular features conferring selectivity for insect versus mammalian nicotinic receptors. *Journal of Agricultural Food Chemistry.* 48:6016-6024.

Vais, H., M.S. Williamson, S.J. Goodson, A.L. Devonshire, J.W. Warmke, P.N. Usherwood, and C.J. Cohen. 2000. Activation of Drosophila sodium channels promotes modification by deltamethrin. Reductions in affinity caused by knock-down resistance mutations. *J Gen Physiol.* 115:305-18.

Vontas, J.G., M.J. Hejazi, N.J. Hawkes, N. Cosmidis, M. Loukas, and J. Hemingway. 2002. Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly Bactrocera oleae. *Insect Mol Biol.* 11:329-36.

Walsh, S.B., T.A. Dolden, G.D. Moores, M. Kristensen, T. Lewis, A.L. Devonshire, and M.S. Williamson. 2001. Identification and characterization of mutations in housefly (Musca domestica) acetylcholinesterase involved in insecticide resistance. *Biochem J.* 359:175-81.

Wamhoff, H., and V. Schneider. 1999. Photodegradation of imidacloprid. *J Agric Food Chem.* 47:1730-4.

Waters, L.C., and C.E. Nix. 1988. Regulation of insecticide resistance-related cytochrome P-450 expression in *Drosophila melanogaster. Pesticide Biochemistry and Physiology.* 30:214-227.

Waters, L.C., A.C. Zelhof, B.J. Shaw, and L.Y. Ch'ang. 1992. Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated P450 gene in *Drosophila. Proceedings of the National Academy of Sciences.* 89:4855-4859.

Weill, M., P. Fort, A. Berthomieu, M.P. Dubois, N. Pasteur, and M. Raymond. 2002. A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the *ace* gene in *Drosophila. Proc R Soc Lond B Biol Sci.* 269:2007-2016.

Weill, M., G. Lutfalla, K. Mogensen, F. Chandre, A. Berthomieu, C. Berticat, N. Pasteur, A. Philips, P. Fort, and M. Raymond. 2003. Comparative genomics: insecticide resistance in mosquito vectors. *Nature.* 423:136-137.

West, T., and G. Campbell. 1950. DDT and newer persistent insecticides. Chapman and Hall Ltd., London.

Wilson, T.G. 1988. *Drosophila melanogaster* (Diptera: Drosophilidae): a model insect for insecticide resistance studies. *Journal of Economic Entomology.* 81:22-27.

Wilson, T.G. 1993. Transposable elements as initiators of insecticide resistance. *Journal of Economic Entomology*. 86:645-651.

Wilson, T.G. 2001. Resistance of Drosophila to toxins. *Annu Rev Entomol*. 46:545-71.

Zhang, A., H. Kayser, P. Maienfisch, and J.E. Casida. 2000. Insect nicotinic acetylcholine receptor: conserved neonicotinoid specificity of [(3)H]imidacloprid binding site. *J Neurochem*. 75:1294-303.

Zlotkin, E. 1999. The insect voltage-gated sodium channel as target of insecticides. *Annu Rev Entomol*. 44:429-55.

Zlotkin, E., A.L. Devonshire, and J.W. Warmke. 1999. The pharmacological flexibility of the insect voltage gated sodium channel: toxicity of AaIT to knockdown resistant (kdr) flies. *Insect Biochem Mol Biol*. 29:849-53.

Publications arising from this thesis.

DDT resistance in Drosophila correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid.

Daborn P, Boundy S, Yen J, Pittendrigh B, ffrench-Constant R.
Mol Genet Genomics. 2001 Dec;266(4):556-63.

Micro-array analysis of cytochrome P450 mediated insecticide resistance in Drosophila.

Le Goff G, Boundy S, Daborn PJ, Yen JL, Sofer L, Lind R, Sabourault C, Madi-Ravazzi L, ffrench-Constant RH.
Insect Biochem Mol Biol. 2003 Jul;33(7):701-8.

Mol Genet Genomics (2001) 266: 556–563
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ORIGINAL PAPER

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DDT resistance in *Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid

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Abstract Mutagenesis can be used as a means of predicting likely mechanisms of resistance to novel classes of insecticides. We used chemical mutagenesis in *Drosophila* to screen for mutants that had become resistant to imidacloprid, a neonicotinoid insecticide. Here we report the isolation of two new dominant imidacloprid-resistant mutants. By recombinational mapping we show that these map to the same location as *Rst(2)DDT*. Furthermore, we show that pre-existing *Rst(2)DDT* alleles in turn confer cross-resistance to imidacloprid. In order to localize the *Rst(2)DDT* gene more precisely, we mapped resistance to both DDT and imidacloprid with respect to P-element markers whose genomic location is known. By screening for recombinants between these P-elements and resistance we localized the gene between 48D5–6 and 48F3–6 on the polytene chromosome map. The genomic sequence in this interval shows a cluster of cytochrome P450 genes, one of which, *Cyp6g1*, is over-expressed in all resistant strains examined. We are now testing the hypothesis that resistance to both compounds is associated with over-expression of this P450 gene.

Keywords Insecticide resistance · DDT · Imidacloprid · Cytochrome P450 · *Drosophila melanogaster*

Introduction

Chemical mutagenesis is a useful way of predicting likely mechanisms of resistance to novel insecticide classes. Mutagenesis has previously been used to predict mechanisms of resistance to the insect growth regulators cyromazine and methoprene in *Drosophila melanogaster* (Wilson and Ashok 1998; Daborn et al. 2000) and to isolate dieldrin-, diazinon-, malathion- and cyromazine-resistant mutants of *Lucilia cuprina* (McKenzie and Batterham 1998). The neonicotinoids, including imidacloprid, are a successful class of new insecticides which act on the nicotinic acetylcholine receptor as nicotine mimics or "agonists" (Bai et al. 1991; Liu and Casida 1993; Buckingham et al. 1997). To date, few cases of field-associated resistance to imidacloprid have been reported (Zhao et al. 2000). Therefore, in order to predict likely resistance mechanisms to these novel insecticides we have carried out a chemical mutagenesis in *D. melanogaster* and screened for mutants that are resistant to imidacloprid.

Here we report the isolation of two new imidacloprid-resistant mutants that map to the same chromosomal location as *Rst(2)DDT*, a gene responsible for resistance to DDT. Furthermore, we show that individuals bearing existing *Rst(2)DDT* alleles, such as $Rst(2)DDT^{1Uk5neR}$, display pre-existing cross-resistance to imidacloprid. Resistance to both compounds maps to the same location in the *D. melanogaster* genome. This region contains a cluster of P450 genes, which is consistent with the hypothesis (derived from the analysis of other DDT-resistant strains) that cytochrome P450s may be involved in the molecular basis of resistance. In order to provide background for the discussion of these results, previous attempts to map *Rst(2)DDT* will be briefly reviewed here alongside evidence for the involvement of P450s in DDT metabolism.

DDT resistance in *D. melanogaster* has been mapped by numerous investigators. Crow (1954) concluded that resistance was polygenic in the strain that he studied. Later work by Ogita (1960, 1961) showed that DDT resistance mapped to a single, dominant, locus at 65 cM on the right arm of chromosome 2, and that new alleles could be generated with X-rays. Since then,

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several other investigators have derived similar map locations (King and Somme 1958; Dapkus and Merrell 1977; Shepanski et al. 1977; Dapkus 1992), but none of these has been sufficiently accurate to allow for cloning of the gene responsible. However, given the margins of error associated with the recombinational mapping of an insecticide resistance phenotype, all previous estimates are consistent with the presence of a major locus for DDT resistance at ~64.5 ± 2 cM.

There is considerable circumstantial evidence to support the involvement of cytochrome P450-based metabolism in insecticide resistance in *D. melanogaster*. Ogita (1960) recognized that his DDT resistance alleles also conferred negative cross-resistance to phenylthiourea, presumably because the products of the resistance alleles preferentially metabolize phenylthiourea to the toxic phenylurea. The map position 65 on chromosome 2 was also found to be linked to increased P450 enzymatic activity in the Hikone-R resistant strain (Hallstrom 1985).

Much of the further work on putatively P450-mediated resistance has been performed on different strains, notably 91-R, which shows less than 10-fold multi-factorial resistance to DDT, with resistance being associated with each of the three large chromosomes (Dapkus and Merrell 1977). It is therefore difficult to directly relate this work to the DDT resistance locus on chromosome 2 discussed here. However, some recombinant P450s such as CYP6A2 have been shown to be capable of metabolizing insecticides including DDT (Dunkov et al. 1997; Berge et al. 1998). Furthermore, resistance can involve both changes in regulation as well as point mutations that increase the efficiency of DDT metabolism (Berge et al. 1998). Regulatory loci may also reside at genomic locations other than the sites of the structural P450 genes responsible for the metabolism of the insecticide (Berge et al. 1998; Feyereisen 1999; Maitra et al. 2000).

In order to predict the likely mechanism of resistance to the neonicotinoid insecticide imidacloprid and to clarify the relationship between imidacloprid resistance and DDT resistance, we have isolated two new imidacloprid resistance mutants by chemical mutagenesis. Here we show that these new mutations map to the same location as previously described *Rst(2)DDT* alleles and also confer cross-resistance to DDT. Furthermore, we show that flies bearing these pre-existing *Rst(2)DDT* alleles are themselves cross-resistant to imidacloprid. Via recombinational mapping relative to P-elements of known genomic location we localized the *Rst(2)DDT* gene to a specific region of the polytene chromosome map, 48D5–6 to 48F3–6. This region contains a cluster of P450 genes, one of which (*Cyp6g1*) is over-expressed in all the resistant strains. The potential involvement of this locus in DDT resistance is discussed.

Materials and methods

Drosophila strains

As our standard insecticide-susceptible strain of *D. melanogaster*, and for chemical mutagenesis, we used Canton-S. We compared our two new mutants [$Rst(2)DDT^{EMS1}$ and $Rst(2)DDT^{EMS2}$] to two field-derived DDT resistance alleles: Hikone-R [$Rst(2)DDT^{Hikone-R}$], a strain collected in Japan, and Wisconsin-1 ($Rst(2)DDT^{Wis1}$), which was collected in Door County, Wis., USA. Strains used for mapping were obtained from the Drosophila Stock Center in Bloomington, Ind., USA.

Mutagenesis and screening

We mutagenized Canton-S males with ethyl methanesulfonate (EMS) as described elsewhere (Grigliatti 1986). Mutagenized males were outcrossed to Canton-S females and their progeny screened for imidacloprid resistance. Briefly, up to 200 eggs were placed in vials with 1.5 g of instant fly food (Carolina) and 6 ml of water containing 1.2 µg of imidacloprid. This dose is above the LC_{100} for Canton-S. Any emerging flies surviving this dose were backcrossed to Canton-S and re-screened.

Dose-response curves and cross-resistance

We derived dose-response curves with both imidacloprid and DDT for the EMS mutants using the computer program POLO (Robertson et al. 1980). We also bioassayed heterozygotes generated by backcrossing resistant strains to Canton-S. In addition, we bioassayed the existing DDT-resistant strains with imidacloprid to check for cross-resistance. For tests with imidacloprid, 50 eggs were added per vial and the number of emerging adults counted. For DDT, females 1–3 days post-eclosion were used in a contact assay. DDT was coated on the inside of glass scintillation vials by applying 200 µl of acetone containing varying concentrations of DDT and rolling the vial until the acetone had evaporated. Vials were plugged with cotton wool soaked in 5% sucrose. Then 20 flies were placed in each vial and mortality was scored after 24 h. For both assays, five replicates at each concentration were used, and each dose-response curve is constructed from at least five concentrations. Control mortality in the absence of insecticide was taken into account in deriving dose response curves.

Resistance mapping and Northern analysis

We mapped insecticide resistance in the different strains in three stages. First, we crossed each resistant strain to the multiply marked balancer strain *w; T(2;3) ap^{Xa}/CyO; TM3,Sb* in order to determine with which chromosome resistance was associated (Fig. 1). Survival of both males and females in screened progeny of a cross between attached-X females and resistant males indicated that resistance was not sex-linked (data not shown).

Having established that resistance mapped to chromosome 2 in each case, we then performed three-point mapping relative to the visible markers *cinnabar* (*cn*) and *vestigial* (*vg*) (Fig. 2a). We then crossed $Rst(2)DDT^{Wis1}$ to stocks containing P-elements at known chromosomal locations (Fig. 3a), to allow for more precise localization of resistance by recombinational mapping against each P-element.

Northern analysis of poly(A)$^+$ mRNA was carried out with PCR-derived probes from several P450 genes previously reported to be involved in insecticide resistance. TRI Reagent (Sigma) was used to isolate total RNA from adults 1–3 days post-eclosion, and mRNA was then isolated using the PolyATtract mRNA Isolation System (Promega). Probes were made using the Prime-It II

Random Primer Labeling Kit (Stratagene) with [α-^{32}P]dCTP. Fractionation of RNA and Northern hybridizations were performed using standard methods.

Results

Dominance and cross-resistance

Resistance to imidacloprid in the new EMS mutants was ~8-fold higher than in the wild-type stock [the LC_{50} for Canton-S was 0.53 µg/vial, with 90% confidence limits of 0.42 and 0.68 µg/vial; and for EMS1 was 4.11 (3.19–4.97) µg/vial). The imidacloprid-resistant mutants also show ~7-fold higher cross-resistance to DDT [LC_{50} for Canton-S is 1.45 (1.22–1.72) µg/vial and for EMS1 is 10.5 (6.73–13.74) µg/vial] (Table 1). The dose-response curves for the new mutants show that resistance to both imidacloprid and DDT is dominant, as the response of the heterozygotes is similar to that of the resistant homozygotes (Fig. 4). Conversely, the field-isolated DDT-resistant strain Wisconsin-1 shows cross-resistance to imidacloprid and levels of dominance for resistance to both compounds are similar (Fig. 4, Table 1).

Resistance mapping and Northern analysis

In both of the new EMS-induced imidacloprid-resistant mutants, and also in both of the field-derived DDT-resistant strains, resistance to imidacloprid and DDT maps to the chromosome 2 (Table 2). Furthermore, resistance in both EMS mutants and also the Wisconsin-1 field strain maps to ~65 cM by three-point mapping with respect to *cn* and *vg* (Table 3, Fig. 2b). More precise localization by recombinational mapping of *Rst(2)DDT*^{Wis1} relative to P-elements whose chromosomal location is known shows that the resistance locus lies between P1080 and P491, defining a region of the chromosome extending from 48D5–6 to 48F3–6 (Table 4, Fig. 3b). Examination of predicted ORFs within this region of the genome shows that there is a cluster of P450 genes present (Fig. 3b), which is consistent with the hypothesis that resistance may be associated with a P450-encoding locus.

The expression of thirteen P450 genes were examined by Northern analysis. These were chosen from the estimated 72 P450 genes in *D. melanogaster* on the basis of (1) their documented over-expression in other resistant strains of *D. melanogaster*, or (2) because their putative orthologs have been reported to be over-expressed in resistant strains of other species and/or (3) because putative orthologs are known to be induced by chemicals or to map near position 65 on chromosome 2. Of the thirteen P450 genes examined by Northern analysis only the *Cyp6g1* transcript was significantly over-expressed. Furthermore, the transcript of this gene was over-expressed in all the resistant strains examined (Fig. 5). In contrast, *Cyp6g2* and *Cyp6i3*, two genes in the same cluster, showed similar levels of expression in the resistant and susceptible strains.

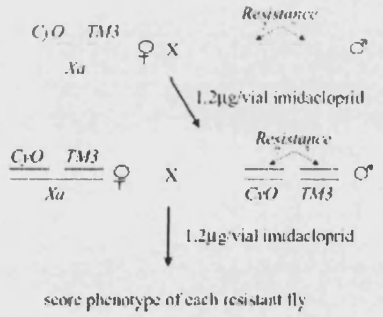

Fig. 1 Crossing scheme used to map imidacloprid resistance to a specific autosome. *Xa/CyO; TM3* is a multiply marked balancer strain in which chromosomes *2* and *3* (marked with ap^{Xa}) co-segregate due to a chromosome transposition. The other copies of chromosomes *2* and *3* (*CyO* and *TM3*, respectively) assort independently. Males of *Rst(2)DDT*^{EMS1} and *Rst(2)DDT*^{EMS2} are crossed to *Xa/CyO;TM3* females and progeny are reared on medium containing imidacloprid (1.2 µg/vial). Emerging resistant males are crossed to *Xa/CyO;TM3* females and their progeny are again reared on 1.2 µg/vial imidacloprid. The numbers of emerging flies of each phenotypic class are recorded in Table 2. The absence of the *CyO* marker in the resistant progeny indicates that imidacloprid resistance maps on chromosome *2* in both *Rst(2)DDT*^{EMS1} and *Rst(2)DDT*^{EMS2}

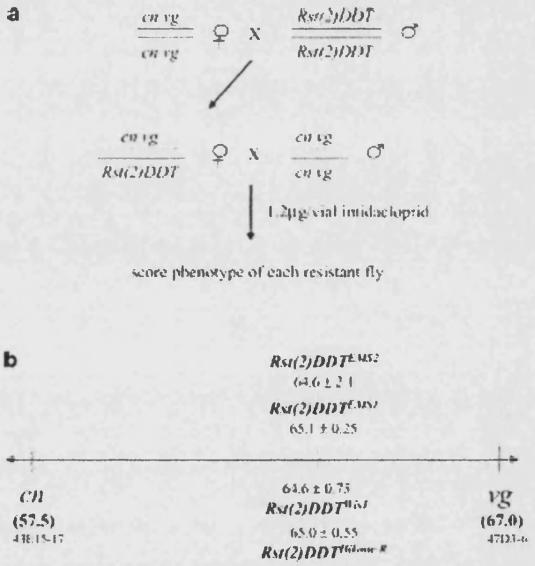

Fig. 2a, b Mapping of imidacloprid resistance relative to visible mutants on chromosome *2*. The mapping cross (**a**) and the resulting map position of imidacloprid resistance (**b**) in the strains *Rst(2)DDT*$^{Hakone-R}$, *Rst(2)DDT*^{Wis1}, *Rst(2)DDT*^{EMS1} and *Rst(2)DDT*^{EMS2} are depicted. Imidacloprid resistance maps to approximately 65 cM in all four strains, between the visible markers *cinnabar* (*cn*) and *vestigial* (*vg*). Mapping data are shown in Table 3

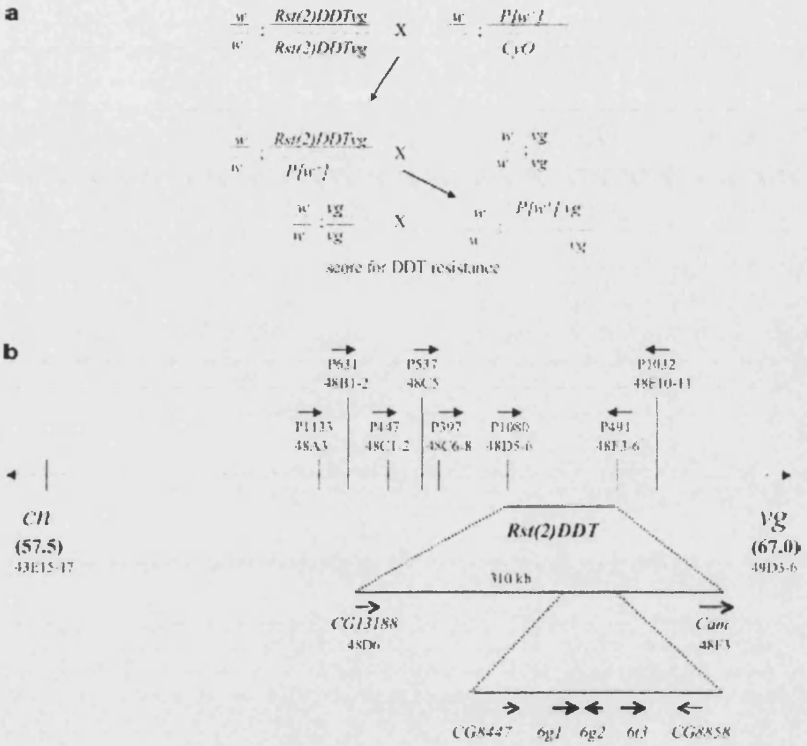

Fig. 3a, b Mapping of DDT resistance relative to P-elements of known chromosomal location. Each P-element insert carries a *white*$^+$ (w^+) gene within the P-element construct. This gene is expressed, serving as a visible marker for the P-element. Crosses were performed in a white mutant (w) eye background. **a** Mapping cross in which females are generated which are heterozygous for *Rst(2)DDT* and a P-element whose chromosomal location is known. Recombinants are then scored for the presence or absence of the resistance gene, thus localizing the gene to one side or the other of the P-element. **b** Location of the DDT resistance gene relative to a number of different P-elements. The *arrows* indicate the direction of the resistance gene relative to the different P-elements. The numbers of recombinants scored for resistance to both DDT and imidacloprid is given in Table 4. The *inset* shows genes in the 48D3–48F3 region to which resistance maps

Table 1 Dose-response data for DDT and imidacloprid-treated *D. melanogaster*

Strain	DDT				Imidacloprid			
	n[a]	LC_{50} (90% CL)[b]	Slope ± S.E.	RR[c]	n[a]	LC_{50} (90% CL)[b]	Slope ± S.E.	RR[c]
Canton-S	780	1.45 (1.22–1.72)	2.91 ± 0.25	1.00	1500	0.53 (0.42–0.68)	3.86 ± 0.97	1.00
EMS1	800	10.50 (6.73–13.74)	2.53 ± 0.24	7.24	1850	4.11 (3.19–4.97)	3.11 ± 0.36	7.75
EMS1 × Canton-S	600	7.66 (6.64–8.56)	3.69 ± 0.32	5.28	1750	3.42 (2.98–3.94)	3.26 ± 0.57	6.45
Canton-S × EMS1	600	10.36 (8.69–11.90)	4.09 ± 0.38	7.14	1700	2.35 (2.06–2.68)	6.14 ± 0.67	4.43
Wis1	780	41.74 (29.32–53.04)	1.98 ± 0.20	28.79	4000	3.50 (2.52–4.30)	2.34 ± 0.22	6.60
Wis1 × Canton-S	600	38.46 (26.0–106.0)	1.13 ± 0.22	26.52	3850	1.47 (1.20–1.73)	1.86 ± 0.17	2.77
Canton-S × Wis1	600	13.02 (8.89–17.37)	1.62 ± 0.21	8.98	4100	1.40 (1.17–1.62)	2.07 ± 0.15	2.64
Hikone-R	780	18.97 (13.03–24.75)	2.21 ± 0.18	13.08	1600	4.74 (3.71–5.75)	3.00 ± 0.57	8.94
Hikone-R × Canton-S	340	8.16 (6.58–9.50)	6.35 ± 0.54	5.63	1900	2.10 (1.84–2.38)	2.79 ± 0.21	3.96
Canton-S × Hikone-R	600	8.61 (5.32–11.1)	2.62 ± 0.26	5.94	1750	2.16 (1.92–2.41)	6.86 ± 0.48	4.08

[a] Total number of samples tested
[b] Concentration of test compound (in µg/vial)
[c] Resistance ratio (RR), i.e. level of resistance relative to Canton-S

as did the ten other P450 genes examined from different locations throughout the genome (Table 5).

Discussion

Implications for insecticide resistance

The neonicotinoids are an increasingly successful class of new insecticides which act as agonists upon the nicotinic acetylcholine receptor of insects (Bai et al. 1991; Liu and Casida 1993; Buckingham et al. 1997). In this study we have used mutagenesis and screening in Drosophila to isolate imidacloprid-resistant mutants and therefore to predict likely mechanisms of resistance to these new compounds. Theoretically, resistance could be conferred either by mutations that result in more rapid metabolism of imidacloprid or make its target receptor insensitive to the compound. Both of the new mutants isolated here map to positions that do not

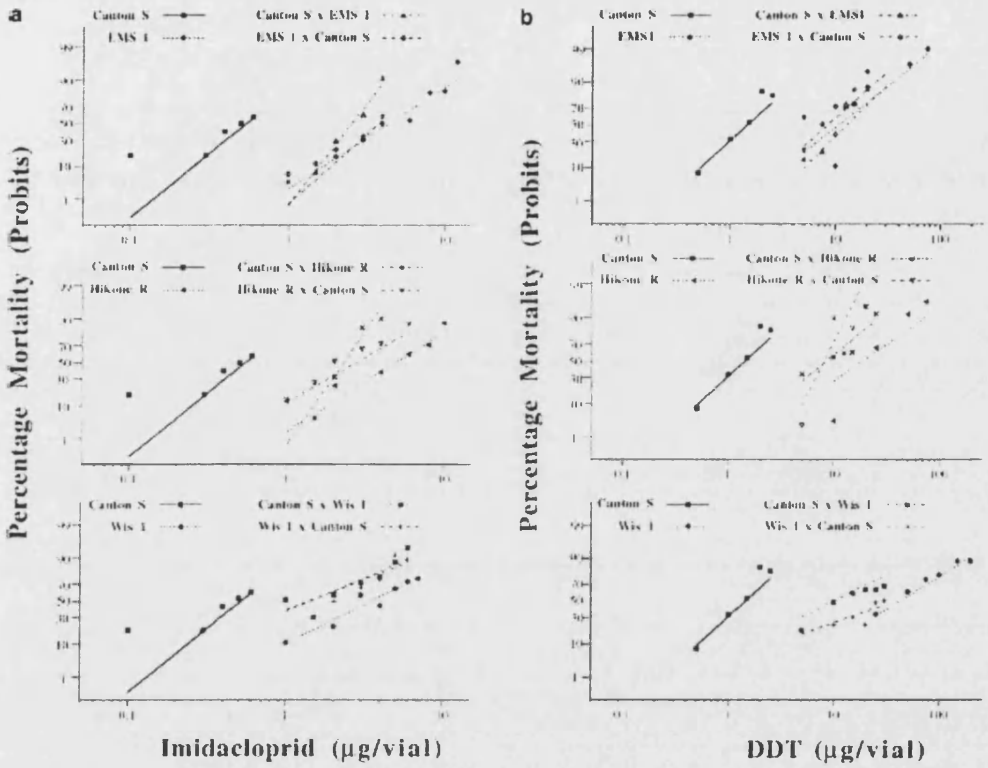

Fig. 4a, b Dose-response curves for the mutants that are resistant to both imidacloprid (a) and DDT (b). Note how the response of the respective heterozygotes is similar to that for the resistant homozygotes, showing that the resistance to both compounds is largely dominant (see Table 1 for statistical analysis)

correspond to known locations of genes for subunits of the nicotinic acetylcholine receptor, but appear to carry new alleles of a gene, *Rst(2)DDT*, that is involved in a known resistance mechanism that confers resistance to DDT. The new EMS-induced imidacloprid-resistant mutants [$Rst(2)DDT^{EMS1}$ and $Rst(2)DDT^{EMS2}$] therefore also show cross-resistance to DDT. Correspondingly, pre-existing alleles that confer DDT resistance, such as $Rst(2)DDT^{Hikone\ R}$ and $Rst(2)DDT^{Wis1}$, also show cross-resistance to imidacloprid.

Assuming that this phenomenon is conserved across other insect species, as in some other cases of resistance (ffrench-Constant et al. 1998), then we can predict that initial resistance to imidacloprid may be conferred by pre-existing DDT resistance alleles in pest populations. Given that *Rst(2)DDT* also confers broad cross-resistance to a

Table 2 Imidacloprid-resistance in $Rst(2)DDT^{EMS1}$ and $Rst(2)DDT^{EMS2}$ maps to chromosome 2

Genotype	EMS1[a]		EMS2[a]	
	Control[b]	Screened[c]	Control	Screened
Tf 2;3) ap^{Xa}/CyO; TM3,Sb males	9	0	7	0
Tf 2;3) ap^{Xa}/CyO; TM3,Sb females	15	0	5	0
Tf 2;3) ap^{Xa}/ CyO; + males	11	0	15	0
Tf 2;3) ap^{Xa}/ CyO; + females	15	0	19	0
Tf 2;3) ap^{Xa}/R; TM3,Sb males	8	12	6	4
Tf 2;3) ap^{Xa}/R; TM3,Sb females	11	14	8	4
Tf 2;3) ap^{Xa}/R; + males	21	10	7	9
Tf 2;3) ap^{Xa}/R; + females	14	15	7	12
R/CyO; +/TM3, Sb males	8	19	8	10
R/CyO; +/TM3, Sb females	5	16	7	9

[a]Numbers refer to progeny of each genotype from the cross in Fig. 1
[b]Control flies were reared on medium that contained no imidacloprid
[c]Screened flies were reared on medium containing imidacloprid (1.2 µg/vial)

Table 3 Recombination mapping of imidacloprid resistance relative to the markers *or* and *vg*

Genotype[a]	Selected	Control	Fitness[b]	Map position[c]
Rst(2)DDT^{EMS1}				
+ +	566	222	1.08	5.98 (*or*)
or +	36	23	1.06	
+ *vg*	9	18	0.83	1.90 (*vg*)
or vg	0	192	0.93	
Rst(2)DDT^{Wis1}				
+ +	302	300	1.11	
or +	16	32	1.12	4.97 (*or*)
+ *vg*	1	24	0.84	
or vg	0	243	0.90	0.41 (*vg*)
Rst(2)DDT$^{Hikone-R}$				
+ +	337	174	1.14	7.98 (*or*)
or +	33	20	1.25	
+ *vg*	8	14	0.87	2.56 (*vg*)
or vg	0	140	0.92	
Rst(2)DDT^{Wis1}				
+ +	0	184	1.19	7.82 (*or*)
or +	7	19	1.05	
+ *vg*	19	17	1.17	3.21 (*vg*)
or vg	157	132	0.85	

[a] *Rst(2)DDT*^{Wis1} was mapped using a *or Rst(2)DDT*^{Wis1} *vg* strain
[b] Fitness is calculated from unselected lines using numbers predicted with the map position of markers
[c] The genetic distance between the resistance gene and the indicated marker was calculated, following adjustment for fitness, as recombinants/total × 100

Table 4 Mapping of DDT resistance in *Rst(2)DDT*^{Wis1} relative to P-element insertions of known location

P-element strain	Cytological position of P-element insertion	Number of DDT resistant lines[a]	Number of imidacloprid resistant lines[a]	Total number of lines tested
P1133	48A3–5	5	5	22
P631	48B1–2	4	4	32
P447	48C1–2	2	2	21
P537	48C5–6	5	5	31
P397	48C6–8	4	4	24
P1080	48D5–6	2	2	28
P491	48F3–6	0	0	33
P1032	48F10–11	0	0	12

[a] Every line resistant to DDT was also resistant to imidacloprid

range of organophosphorus and carbamate insecticides (Ogita 1960, 1961), it is also likely that resistant alleles at this locus will be maintained in insect populations by selection with these compounds, long after the withdrawal of DDT itself. As there have been few reported cases of resistance to imidacloprid in the field to date, it is difficult to test this hypothesis. However, imidacloprid resistance has been detected in populations of Colorado potato beetle on Long Island, and has been inferred to involve P450-mediated metabolism based on synergism studies with piperonyl butoxide (Zhao et al. 2000). This observation would be consistent with our prediction that P450-mediated mechanisms will play a role in imidacloprid resistance. However, at present we can not be certain if this involves cross-resistance associated with an existing resistance gene [such as that described here with *Rst(2)DDT*] or whether it represents a novel mechanism.

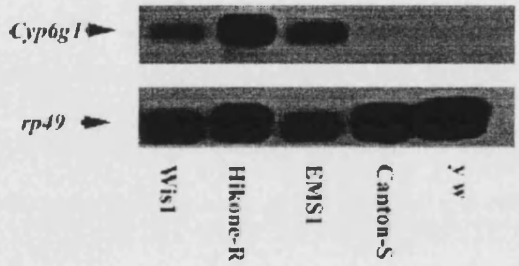

Fig. 5 Northern analysis of poly(A)$^+$ mRNA from resistant and susceptible fly strains, probed with *Cyp6g1*. The same lanes were also probed with rp49 (O'Connell and Rosbash 1984) to illustrate the amount of mRNA loaded. Note that *Cyp6g1* is over-expressed in both laboratory-generated (EMS1) and field-derived (Hikone-R and Wis1) resistant strains but not in susceptible strains (y w and Canton S)

Table 5 *D. melanogaster* P450 genes examined for over-expression

Gene	Cytological position	Northern analysis[a]	Documented expression in other strains and species[b]	Reference
Cyp6a2	2R: 42C4–5	=	Over expressed in *D. melanogaster* DDT^R and 91-R strains	Waters et al. (1992); Brun et al. (1996)
Cyp4e2	2R: 44C1–3	=	Over expressed in *D. melanogaster* Raleigh DDT^R strain	Amichot et al. (1994)
Cyp6g1	2R: 48F1	+		
Cyp6g2	2R: 48F1	=		
Cyp6t3	2R: 48F1	=		
Cyp6a17	2R: 51D2	=	*Cyp6b4* and *Cyp6b5* induced by xanthotoxin in *Papilio glaucus*	Berenbaum et al. (1990)
Cyp6a9	2R: 51D2	n.d./n.d.	Over-expressed in *D. melanogaster* 91-R strain	Maitra et al. (1996)
Cyp6a8	2R: 51D2–4	=	Over-expressed in *D. melanogaster* 91-R and MHH-D23 strains	Maitra et al. (2000)
Cyp4aa1	2R: 51D11–14	=	*Cyp4m1* induced by nicotine in *Manduca sexta*	Snyder et al. (1993)
Cyp6d2	2R: 58F2–3	=	*Cyp6d1* over-expressed in *Musca domestica* Learn-PyrR strain	Tomita et al. (1995)
Cyp12a5	3R: 98A1–2	n.d./n.d.	*Cyp12a1* over expressed in *Musca domestica* Rutgers strain	Guzov et al. (1998)
Cyp6a18	3R: 98A1–2	=	*Cyp6a1* over expressed in *Musca domestica Rutgers* strain	Cariño et al. (1994)
Cyp4c3	3R: 100B1–2	=	Cyp4m3 induced by nicotine in *Manduca sexta*	Snyder et al. (1993)

[a]n.d./n.d. not detectable in susceptible or resistant strains. = equally expressed in susceptible and resistant strains. + over-expressed in resistant strains

[b]Difference in protein and/or RNA levels between resistant and susceptible strains or P450 involved in toxin metabolism

Implications for molecular mechanisms of resistance

Given the considerable evidence suggesting that P450s are involved in metabolic resistance in *D. melanogaster*, we have examined the predicted ORFs in the 48D5–6 to 48F3–6 chromosomal region. This region does contain a cluster of P450 genes, comprising *Cyp6g1*, *Cyp6g2* and *Cyp6t3*. Of these only *Cyp6g1* is over-expressed in the resistant strains examined here. Significantly, this one gene is over-expressed both in the two field-derived strains and also in the mutants generated in the laboratory. Furthermore, the two other P450 genes in this cluster show no differences in their expression levels between resistant and susceptible strains. Although not quantified in the current Northern analysis, the level of *Cyp6g1* mRNA transcription in the EMS1 strain appears higher than that in the Wis1 strain. This contrasts with levels of resistance to DDT, which are 7.2-fold higher (relative to Canton-S) in EMS1 and 28.3-fold higher in Wis1. It is therefore still a formal possibility that *Cyp6g1* transcript levels are not the only factor contributing to resistance. Thus, amino acid substitutions within CYP6G1, as found in CYP6A2 (Berge et al. 1998), may also increase the efficiency of insecticide metabolism.

Several alternatives therefore remain for the possible molecular basis of DDT/imidacloprid resistance. First, resistance may be associated with over-expression of *Cyp6g1* itself; i.e. elevated CYP6G1 is capable of metabolizing both DDT and imidacloprid. Such over-expression may result either from mutations within the *Cyp6g1* locus itself, or at another regulatory locus some distance away from *Cyp6g1* itself. Second, several studies of both insecticide resistance and the expression of specific P450 genes have shown that P450 genes can be up-regulated by *trans*-acting factors. Alternatively, therefore, CYP6G1 may metabolize a *trans*-acting factor which then up-regulates presently uncharacterized P450 genes, as postulated for the ali-esterase gene in houseflies (Feyereisen 1999). Finally, linkage of elevated *Cyp6g1* transcription with resistance may be coincidental, and the resistance gene could correspond to another ORF in the identified region. At this stage we postulate that the association between elevated *Cyp6g1* expression and resistance is causal, but the precise mechanism, whether direct or *trans*-acting, remains uncertain. To differentiate between these alternative hypotheses we will test which of the ORFs in the region corresponds to the resistance gene via P element-mediated germline transformation of a susceptible *Drosophila* strain. Furthermore, we will examine the potential of recombinant CYP6G1 itself to metabolize insecticides.

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References

Amichot M, Brun A, Cuany A, Helvig C, Salaun JP, Durst F, Berge JB (1994) Expression study of *CYP* genes in *Drosophila* strains resistant or sensitive to insecticides. In: Lechner MC (ed) Cytochrome P450. John Libbey Eurotext, Paris, pp 689–692

Bai D, Lummis SCR, Leicht W, Breer H, Sattelle D (1991) Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neurone. Pestic Sci 33:197–204

Berenbaum MR, Cohen MB, Schuler MA (1989) Cytochrome P450 in plant-insect interactions: inductions and deductions. In: Hagedorn HH, Hildebrand JG, Kidwell MG, Law JH (eds) Molecular insect science. Plenum, New York, pp 257–262

Berge J-B, Feyereisen R, Amichot M (1998) Cytochrome P450 monooxygenases and insecticide resistance in insects. Phil Trans R Soc Lond B 353:1701–1705

Brun A, Cuany A, Le Mouel T, Berge J, Amichot M (1996) Inducibility of the *Drosophila melanogaster* cytochrome P450 gene, *CYP6A2*, by phenobarbital in insecticide susceptible or resistant strains. Insect Biochem Mol Biol 26:697–703

Buckingham S, Lapied B, Corrone H, Sattelle F (1997) Imidacloprid actions on insect neuronal acetylcholine receptors. J Exp Biol 200:2685–2692

Cariño FA, Koener JF, Plapp FW Jr, Feyereisen R (1994) Constitutive overexpression of the cytochrome P450 gene *CYP6A1* in a house fly strain with metabolic resistance to insecticides. Insect Biochem Mol Biol 24:411–418

Crow JF (1954) Analysis of a DDT-resistant strain of *Drosophila*. J Econ Entomol 47:393–398

Daborn PJ, McKenzie JA, Batterham P (2000) A genetic analysis of cyromazine resistance in *Drosophila melanogaster* (Diptera: Drosophilidae). J Econ Entomol 93:911–919

Dapkus D (1992) Genetic localization of DDT resistance in *Drosophila melanogaster* (Diptera: Drosophilidae). J Econ Entom 85:340–347

Dapkus DC, Merrell DJ (1977) Chromosomal analysis of DDT-resistance in a long-term selected population of *Drosophila melanogaster*. Genetics 87:685–697

Dunkov BC, Mocelin G, Shotkoski F, ffrench-Constant RH, Feyereisen R (1997) The *Drosophila* cytochrome P450 gene Cyp6a2: structure, chromosomal localization, heterologous expression and induction by phenobarbital. DNA Cell Biol :1345–1356

Feyereisen R (1999) Insect P450 enzymes. Annu Rev Entomol 44:507–533

ffrench-Constant RH, Park Y, Feyereisen R (1998) Molecular biology of insecticide resistance. In: Puga A, Wallace KB (eds) Molecular biology of the toxic response. Taylor and Francis, Philadelphia, pp 533–551

Grigliatti T (1986) Mutagenesis. In: Roberts DB (eds) *Drosophila*: a practical approach. IRL Press, Oxford, pp 39–58

Guzov VM, Unnithan GC, Chernogolov AA, Feyereisen R (1998) CYP12A1, a mitochondrial cytochrome P450 from the house fly. Arch Biochem Biophys 359:231–240

Hallstrom I (1985) Genetic regulation of the cytochrome P450 system in *Drosophila melanogaster*. II. Localization of some genes regulating cytochrome P-450 activity. Chem Biol Interact 56:173–184

King JC, Somme L (1958) Chromosomal analysis of the genetic factors for resistance to DDT in two resistant lines of *Drosophila melanogaster*. Genetics 43:577–593

Liu MY, Casida JE (1993) High affinity binding of [^{3}H]-imidacloprid in the insect acetylcholine receptor. Pestic Biochem Physiol 46:40–46

Maitra S, Dombrowski SM, Waters LC, Ganguly R (1996) Three second chromosome-linked clustered Cyp6 genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*. Gene 180:165–171

Maitra S, Dombrowski SM, Basu M, Raustol O, Waters LC, Ganguly R (2000) Factors on the third chromosome affect the level of Cyp6a2 and Cyp6a8 expression in *Drosophila melanogaster*. Gene 248:147–156

McKenzie JA, Batterham P (1998) Predicting insecticide resistance: mutagenesis, selection and response. Phil Trans R Soc Lond B 353:1729–1734

O'Connell P, Rosbash M (1984) Sequence, structure and codon preference of the *Drosophila* ribosomal protein 49 gene. Nucleic Acids Res 12:5495–5513

Ogita Z (1960) Genetical and biochemical studies on negatively correlated cross-resistance in *Drosophila melanogaster*. I. An attempt to reduce and increase insecticide-resistance in *D. melanogaster* by selection pressure. Botyu-Kagaku 26:7–18

Ogita Z (1961) Genetical and biochemical studies on negatively correlated cross-resistance in *Drosophila melanogaster*. III. Genetical studies on actions of mixed insecticides with negatively correlated substances. Botyu-Kagaku 26:88–93

Robertson JL, Russell RM, Savin NE (1980) POLO: a user's guide to probit or logic analysis. United States Forestry Service Technical Report PSW

Shepanski MMC, Glover TJ, Kuhr RJ (1977) Resistance of *Drosophila melanogaster* to DDT. J Econ Entomol 70:539–543

Snyder MJ, Stevens JL, Andersen JF, Feyereisen R (1995) Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, *Manduca sexta*. Arch Biochem Biophys 321:13–20

Tomita T, Liu N, Smith FF, Sridhar P, Scott JG (1995) Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica*. Insect Mol Biol 4:135–140

Waters LC, Zelhof AC, Shaw BJ, Ch'ang LY (1992) Possible involvement of the long terminal repeat of transposable element *17.6* in regulating expression of an insecticide resistance-associated P450 gene in Drosophila. Proc Natl Acad Sci USA 89:4855–4859

Wilson TG, Ashok M (1998) Insecticide resistance resulting from an absence of target-site gene product. Proc Natl Acad Sci USA 95:14040–14044

Zhao JZ, Bishop BA, Grafius EJ (2000) Inheritance and synergism of resistance to imidacloprid in the Colorado potato beetle (Coleoptera: Chrysomelidae). J Econ Entomol 93:1508–1514

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Microarray analysis of cytochrome P450 mediated insecticide resistance in *Drosophila*

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Abstract

Insecticide resistance in laboratory selected *Drosophila* strains has been associated with upregulation of a range of different cytochrome P450s, however in recent field isolates of *D. melanogaster* resistance to DDT and other compounds is conferred by one P450 gene, *Cyp6g1*. Using microarray analysis of all *Drosophila* P450 genes, here we show that different P450 genes such as *Cyp12d1* and *Cyp6a8* can also be selected using DDT in the laboratory. We also show, however, that a homolog of *Cyp6g1* is over-expressed in a field resistant strain of *D. simulans*. In order to determine why *Cyp6g1* is so widely selected in the field we examine the pattern of cross-resistance of both resistant strains and transgenic flies over-expressing *Cyp6g1* alone. We show that all three DDT selected P450s can confer resistance to the neonicotinoid imidacloprid but that *Cyp6a8* confers no cross-resistance to malathion. Transgenic flies over-expressing *Cyp6g1* also show cross-resistance to other neonicotinoids such as acetamiprid and nitenpyram. We suggest that the broad level of cross-resistance shown by *Cyp6g1* may have facilitated its selection as a resistance gene in natural *Drosophila* populations.

Keywords: Insecticide resistance; DDT resistance; Cytochrome P450; Neonicotinoid; *Drosophila melanogaster*

1. Introduction

With the advent of complete, annotated, genome sequences for different insects such as *Drosophila* (Misra et al., 2002) and *Anopheles* (Holt et al., 2002; Ranson et al., 2002; Zdobnov et al., 2002), the study of insecticide resistance is entering a genomic era (Oakeshott et al., 2003). Genomic approaches are particularly attractive where resistance is controlled by individual members of large multigene enzyme families, such as the cytochrome P450s and glutathione-S-transferases (Ranson et al., 2002). Here, the role of individual family members in resistance may be unclear due the large number of candidate genes. By using microarrays comprising all known family members the role of specific genes in resistance can be more readily dissected (Daborn et al., 2002). We are using this approach to facilitate a genetic dissection of P450 mediated resistance in *Drosophila*.

The genome of *Drosophila melanogaster* contains approximately 90 recognized cytochrome P450 genes (Tijet et al., 2001), several of which have been implicated in insecticide resistance associated with different strains. Early genetic studies mapped resistance to different locations on chromosome II and chromosome III. Later studies then implicated specific chromosomal regions, notably map position 64.5 cM on the right arm of chromosome II, specific genes such as *Cyp4e2*, *Cyp6a2*, *Cyp6a9* and *Cyp6a8* (Amichot et al., 1994; Maitra et al., 1996; Dunkov et al., 1997; Maitra et al., 2002) or even specific mutations (Bergé et al., 1998). In some cases the P450s implicated, such as CYP6A2, have been functionally expressed and shown to be able to met-

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abolize various insecticides (Dunkov et al., 1997). The diversity of P450s implicated in insecticide resistance in *Drosophila* has been reviewed elsewhere (Bergé et al., 1998; Feyereisen, 1999), however, many of the different studies examined were carried out on strains selected for long periods in the laboratory and the relevance of any one specific P450 to field associated resistance often remains unclear. Recent work on DDT resistant field isolates has shown that *Cyp6g1* is over-transcribed in 20 resistant strains of diverse global origin (Daborn et al., 2002). Moreover, transgenic over-transcription of *Cyp6g1* in susceptible flies is both necessary and sufficient to restore DDT resistance (Daborn et al., 2002). The resistant allele of *Cyp6g1* contains an *Accord* transposable element in the 5′ end of the gene and this allele is identical in flanking nucleotide sequence regardless of its global origin (Daborn et al., 2002). This suggests that field associated resistance is associated with the single origin of a single resistant allele and that the presence of the transposon up-regulates *Cyp6g1* transcription.

These results, whereby a single allele of a single gene confers resistance in the field, stand in marked contrast to the handful of genes implicated in resistance by laboratory studies. Several, not mutually exclusive, explanations may account for this apparent disparity. First, individual genes such as *Cyp6g1* may be particularly prone to resistance-associated mutations in field populations. Second, particular P450s may show unusually broad cross-resistance, potentially facilitating their selection by the wide range of alternative insecticides used in the field. In this paper we begin to investigate the second hypothesis by comparing which P450 genes are upregulated in laboratory selected and field isolated strains and subsequently examining the breadth of cross-resistance to other insecticides that they confer.

2. Materials and methods

2.1. Fly strains, insecticide selection and mapping

The origins of the DDT-resistant strains Wisconsin-1 (WIS1) and Hikone-R have been described previously (Daborn et al., 2001). Canton-S was used as a standard susceptible strain for comparative purposes. Methods of larval (neonicotinoids and malathion) and adult (DDT) bioassay and dose mortality analysis have been described elsewhere (Daborn et al., 2001). Prior to this study, adult flies from WIS1 were repeatedly selected on 10μg/vial DDT at each of five generations, whereas Hikone-R was not subjected to further DDT selection in the laboratory. WIS1lab was generated by crossing WIS1 to the *w;vg* mapping strain, backcrossing progeny to *w;vg* and then selecting for DDT resistance for five generations in progeny with vestigial wings. Strains used for mapping DDT resistance were obtained from the *Drosophila* Stock Center, Bloomington, IN and from the Szeged *Drosophila* Stock Center, Szeged, Hungary. The susceptible, BG, and DDT resistant, OV1, *D. sinvulans* strains were collected from Bento Gonçalves and Onda Verde in Brazil, respectively. DDT resistance in Hikone-R and WIS1lab was mapped using male recombination at the site of *P*-elements (Chen et al., 1998). Recombination events in males were detected using the flanking markers *cn* and *vg*, and adults of each recombinant line were tested for DDT resistance. Classical recombination between the markers *cn* and *vg* was used to map WIS1. Forty recombinant lines were isolated, and each line was screened for DDT resistance and genotyped for 12 Restriction Fragment Length Polymorphisms (RFLPs) between *cn* and *vg*. Transgenic flies over-transcribing *Cyp6g1* driven by the *GAL4/UAS* system were derived as described previously (Daborn et al., 2002) except that over-transcription of *Cyp6g1* was driven by GAL4 under the regulation of a tubulin driver in the y[1]w[1];P{w[+mC]=tubP-GAL4}LL7/TM3,Sb[1] (Bloomington) strain.

2.2. RNA extraction

Three day old adult *Drosophila* were flash frozen in liquid nitrogen and homogenised with a pestle and mortar. The resultant powder was then re-suspended in 600 μl of 4 M guanidine thiocyanate, 25 mM Na citrate, 0.5% sodium N-lauryl-sarcosine and 1% 2-mercaptoethanol and following the addition of 500 μl of phenol, 120 μl of chloroform and 36 μl of Na acetate 3M pH5.2, the mixture was incubated on the ice for 15 min. After phenol extraction, the aqueous phase was precipitated with one volume of isopropanol by incubation on ice for 1 h. The resultant pellet obtained after centrifugation was washed with 70% ethanol, dried and re-suspended in RNAse free water. DNA was removed from the sample by adding 1 μl of DNAse (Promega) and incubating for 30 min at 37 °C. A further phenol-chloroform extraction and isopropanol precipitation was then repeated as described above and the resulting RNA used directly for labelling.

2.3. Microarray construction and hybridization

A microarray of PCR products from 132 genes of *D. melanogaster* was constructed. These genes represent all 90 cytochrome P450 genes predicted from the full genome sequence, several other genes encoding metabolic enzymes, such as esterases and glutathione-S-transferases (GSTs) and several 'housekeeping' genes included as controls. A fragment of each gene was amplified via the polymerase chain reaction (PCR) and spotted on the array in four independent locations. To label sample RNAs for hybridization, 10 μg RNA was labelled with the CyScribe First-Strand cDNA labelling kit (Amersham), according to the manufacturer's instruc-

tions. Unlabelled RNA was removed by digestion with 100 μg/ml of RNAse A at 37 °C for1h. The array was hybridised to cDNA from both resistant and susceptible strains simultaneously using Cy3 and Cy5 labels. Experiments were repeated in triplicate in which the Cy3 and Cy5 labels were swapped between strains to account for potential differences in labelling efficiency.

The Cy3 and Cy5 labelled samples were purified separately using a QIAquick (Qiagen) nucleotide removal kit, according to the manufacturer's instructions. The samples were then dried under vacuum and resuspended into 13 μl of DIG easy Hyb solution. The Cy3 and Cy5 labelled samples were then combined immediately prior to hybridisation. This hybridization mixture was then placed on the microarray and covered by a 22 × 40 mm glass coverslip. Arrays were then placed in a hybridization chamber and submerged in a water bath at 50 °C for 16–20 h, in the dark. Following hybridisation, arrays were washed in 1 x SSC, 0.03% SDS for 5 min., taking care to float the coverslip gently off the array. Then two additional washes in 0.2 x SCC and 0.05 x SCC at room temperature, each for 5 min, were carried out before the slides were dried by centrifugation.

Labelled arrays were scanned with a GMS 418 array scanner (Genetic Microsystems). The best dynamic range of the data was achieved by adjusting the gain so that the highest signal was just under saturation. Quantification of the signal from each spot, and superimposition of both dye channels, was performed using the ImaGene software, version 4.2 (Biodiscovery). Data files generated by ImaGene were downloaded into GeneSpring version 5.0 (Silicon Genetics) where data normalization and statistical analysis was performed. Values of less than 0 were set to 0, and the data normalized using an intensity dependent algorithm (Lowess). The statistical significance of the over-transcription of specific genes was tested using individual T-tests comparing the mean value for a specific P450 gene against the mean of all other P450 genes combined (GeneSpring).

3. Results

3.1. DDT resistance maps to different chromosomal locations

We compared two *D. melanogaster* strains extensively selected with DDT in the laboratory, WIS1 and WIS1-lab, with the field derived strain Hikone-R. WIS1 and WIS1lab were derived from the same field collection in Wisconsin but were subjected to different selection regimes in the laboratory. WIS1 was repeatedly selected with DDT with no genetic intervention. Whereas in WIS1lab, the region encompassing *Cyp6g1* was removed from the strain via recombination and DDT selection then continued. The resulting levels of DDT resistance displayed by each strain are shown in Table 1. Following these different selection regimes, we subsequently mapped DDT resistance in these three strains using *P*-element markers and RFLPs of known genomic location. In Hikone-R, DDT resistance maps to the physical location of *Cyp6g1*, whereas resistance in the other two strains maps either to the left (WIS1) or the right (WIS1lab) of *Cyp6g1* (Fig. 1). These results are consistent with the previously observed over-transcription of *Cyp6g1* in the field derived Hikone-R but suggest that different P450s may be over-transcribed in the strains selected with DDT in the laboratory, either alone or in combination. We also examined a recently field collected strain of *D. simulans* from Brazil for resistance. This strain shows resistance to DDT, malathion and imidacloprid (Table 1).

3.2. DDT resistance is associated with different P450s

We then made RNA from each of the resistant strains and hybridized it to a microarray of all the *D. melanogaster* P450 genes in the presence of RNA from Canton-S, a standard susceptible strain. This analysis confirms that *Cyp6g1* alone is over-transcribed in Hikone-R (Fig. 2a). However, both *Cyp6g1* and *Cyp12d1* are up-regulated in WIS1 (Fig. 2b) but only *Cyp6a8* in Wis1lab (Fig. 2c). Hybridization of the array with a DDT resistant *D. simulans* strain, a different but closely related species, shows that a gene probably orthologous to *Cyp6g1* is over-transcribed, alongside a glutathione-S-transferase gene, termed *DsGSTS1* (Chelvanayagam et al., 2001). The relative levels of over-transcription for each of these genes and their statistical significance are given in Table 2.

3.3. Cross-resistance in resistant strains

All three DDT resistant *D. melanogaster* strains showed cross-resistance to the neonicotinoid imidacloprid (Table 1). Two of the resistant strains also showed cross-resistance to the organophosphorus compound malathion. However Wis1lab, which only over-transcribes *Cyp6a8*, was not cross-resistant to malathion. To determine the extent of cross-resistance to different neonicotinoids conferred by *Cyp6g1* we examined a transgenic strain over-transcribing *Cyp6g1* under *GAL4/UAS* control. Two different transgenic strains, each with a single UAS-*Cyp6g1* transgene inserted either on the X- or the second-chromosome, both over-transcribed only *Cyp6g1* when driven by a tubulin promoter as shown by microarray analysis (Fig. 3). Levels of transgenic over-expression were not affected by chromosomal location or sex of progeny tested. Progeny expressing the *Cyp6g1* transgenes showed cross-resistance to malathion and all three neonicotinoid insecticides tested, imidacloprid,

Table 1
LD_{50} estimates and resistance ratios for *Drosophila* strains

Strain	P450[c]	DDT[a] N[d]	LD50 (90%CI)	Slope ±S.E.	RR	Imidacloprid[b] N[d]	LD50 (90%CI)	Slope ±S.E.	RR	Malathion[b] N[d]	LD50 (90%CI)	Slope ±S.E.	RR
D. melanogaster													
Canton-S	–	780	1.45 (1.22-1.72)	2.91±0.25	1.00	1500	0.53 (0.42-0.68)	3.86±0.97	1.00	2500	4.88 (4.26-5.41)	8.59±1.00	1.00
Hikone-R	*Cyp6g1*	780	18.97 (13.03-34.75)	2.21±0.18	13.08	1600	4.74 (3.71-5.75)	3.00±0.57	8.94	2000	20.26 (16.73-23.17)	6.71±0.59	4.15
Wis1	*Cyp6g1*, *Cyp12d1*	740	104.34 (90.99-113.70)	2.14±0.19	71.96	4000	3.50 (2.52-4.30)	2.34±0.22	6.60	2000	21.23 (18.75-23.36)	6.09±0.53	4.35
Wis1 lab	*Cyp6a8*	680	44.39 (36.10-51.93)	2.57±0.25	30.61	2250	2.18 (1.76-2.53)	3.25 ±0.47	4.11	2500	2.72 (2.19-3.16)	4.22±0.50	0.56
D. simulans													
BG	–	900	0.63 (0.00-1.32)	1.47±0.20	1.00	2500	0.3 (0.09-0.56)	0.43±0.03	1.00	2000	1.79 (0.89-2.62)	1.79±0.14	1.00
OVI	*Cyp6g1*	800	2.46 (0.05-6.83)	0.86±0.10	3.90	2000	2.02	0.74±0.19	6.73	2000	21.42 (18.26-24.23)	3.92±0.30	11.97

[a] Assayed as 2–3 day old adults
[b] Assayed as larvae
[c] P450 gene(s) over-transcribed in each strain, see Table 2
[d] Number of individuals assayed

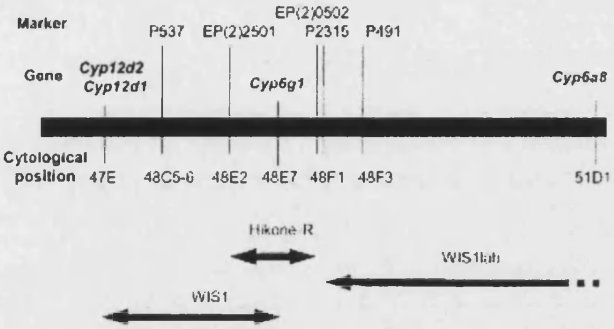

Fig. 1. Mapping of resistance in field and laboratory selected DDT resistant strains of *Drosophila*. The diagram is a linear representation of chromosome II with cytological divisions (47E–51D1) indicated. The relative location of genes and *P*-elements used in mapping are shown. The arrows indicate the region within which resistance in the different strains has been mapped. For simplicity, only cytochrome P450 genes involved in insecticide resistance are shown.

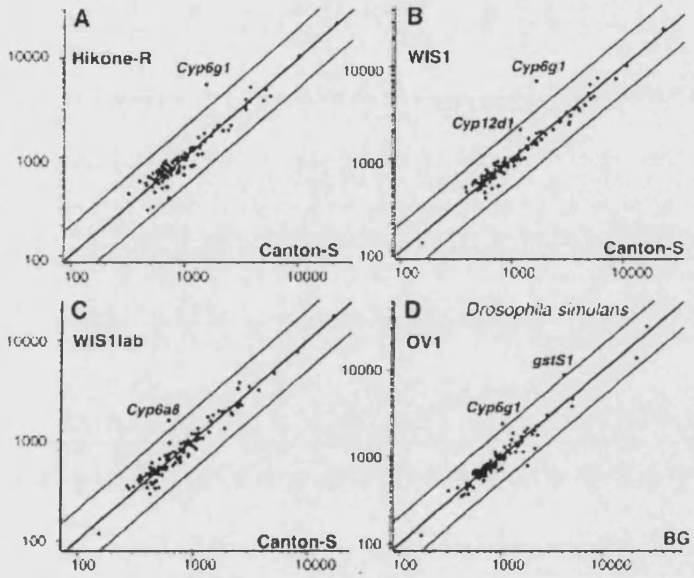

Fig. 2. Microarray analysis of P450 gene transcription in DDT resistant *Drosophila*. (A) The field isolated strain Hikone-R shows over-transcription of *Cyp6g1* alone. (B) The field isolated, but laboratory selected, strain WIS1 shows up-regulation of both *Cyp6g1* and *Cyp12d1*. (C) The sub-strain WIS1lab, from which *Cyp6g1* was genetically removed, shows up-regulation of *Cyp6a8*. (D) A field isolated *D. simulans* strain shows over-transcription of the homologous gene to *Cyp6g1* and also the glutathione-S-transferase gene, *DsGSTS1*. The three parallel lines correspond to three different ratios of resistant versus susceptible signal: 1.0 (center), 2.0 (above) and 0.5 (below).

acetamiprid and nitenpyram, whereas their sibs not expressing the transgenes did not (Fig. 4).

4. Discussion

Here we have shown, using both laboratory selected and field isolated *D. melanogaster* resistant strains, that several different P450 genes can be associated with resistance to DDT. However, resistance in recent field isolates, both of *D. melanogaster* and *D. simulans* is associated with over-transcription of *Cyp6g1*.

The observation that a single gene is selected for in field populations, potentially by numerous insecticides, whereas different genes can be selected for in the laboratory using a single insecticide, is interesting. Selection of a single gene in the field may be related not only to the likelihood of any one gene acquiring a viable resist-

Table 2
Levels of over-transcription of the different P450s in DDT resistant *D. melanogaster* and *D. simulans*

Species/Strain	P450	Fold over-expression (versus susceptible)	P value (T-test)
D. melanogaster[a]			
Hikone-R	*Cyp6g1*	3.1	7.1e-6
Wis1	*Cyp6g1*	4.3	1.6e-8
	Cyp12d1	1.7	5.1e-7
Wis1 Lab	*Cyp6a8*	2.7	1.4e-6
D. simulans[b]			
OVI	*Cyp6g1* homolog	2.1	2.5e-3
	GstS1 homolog	1.8	1.8e-3

[a] Canton-S used as *D. melanogaster* susceptible reference strain
[b] BG used as *D. simulans* susceptible reference strain

ance associated mutation but also to the extent of cross-resistance to different compounds conferred by different P450s, thus genes conferring a wide range of cross-resistance can be selected for by a wider range of compounds. We investigated this question by examining cross-resistance in strains in which different P450s were upregulated by DDT selection in the laboratory and also by examining cross-resistance in transgenic flies only over-transcribing *Cyp6g1*.

Following prolonged laboratory selection with DDT, we were able to co-select over-transcription of *Cyp12d1* in a strain already over-transcribing *Cyp6g1*, as reported previously (Brandt et al., 2002), and also to select a strain only over-expressing *Cyp6a8*. We note however that we are unable to separate between over-transcription of either *Cyp12d1* or *Cyp12d2* on the microarray as these are closely linked genes that differ by only three nucleotide substitutions (http://P450.antibes.inra.fr). Selection of these different genes was confirmed both via genetic mapping of the associated DDT resistance which maps to various locations on chromosome II and corresponds to the known location of these genes (Fig. 1) and also by analysis of a microarray containing all *Drosophila* P450 genes (Fig. 2). The rapidity with which individual candidate resistance associated P450s can be identified using microarray technology stands in marked contrast to the length of time it takes to map resistance genes genetically. Importantly, examination of a complete *Drosophila* microarray, containing all other metabolic enzymes such as esterases and glutathione-S-transferases, also makes no assumptions about the nature of the underlying resistance mechanism beyond over-transcription (Oakeshott et al., 2003). Microarray analysis of insecticide resistance in *Drosophila* and pest insects therefore looks poised to revolutionize the way we analyze metabolic resistance. Further, because microarray

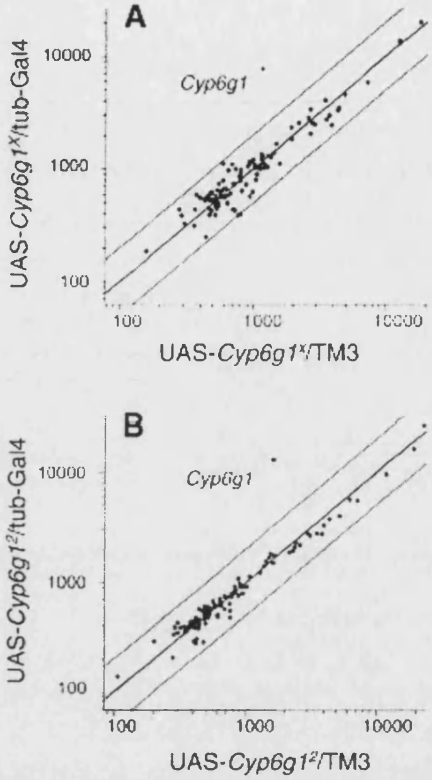

Fig. 3. Scatter plot of microarray analysis showing that only the *Cyp6g1* P450 gene is over-transcribed in transgene expressing progeny. Results for crosses of two different transgenes, one on the X chromosome (A), and one on the second chromosome (B) are shown. Note that in each case, of the 89 other P450 genes samples, no other P450 is over-transcribed in sympathy with the transgenic over-transcription of *Cyp6g1*. For this analysis RNA was isolated from a mixture of males and females, as no X-linked variation in over-transcription was detected. The three parallel lines correspond to three different ratios of resistant versus susceptible signal: 1.0 (center), 2.0 (above) and 0.5 (below).

analysis can recognize genes that are over 80% identical at the nucleotide level (Xu et al., 2001), microarrays constructed for one species, such as *D. melanogaster*, can be used to detect related genes in closely related species, such as *D. simulans* (S. Tares, personal communication).

The extent of cross-resistance afforded by each of the P450 genes, or gene combinations, in each of the strains was examined by looking at the neonicotinoid imidacloprid and the organophosphorus compound malathion. Flies over-transcribing *Cyp6g1*, or both *Cyp6g1* and *Cyp12d1* together, show cross-resistance to both imidacloprid and malathion. Whereas flies only over-transcribing *Cyp6a8* show cross-resistance to only imidacloprid and not malathion by our assay methods. Transgenic

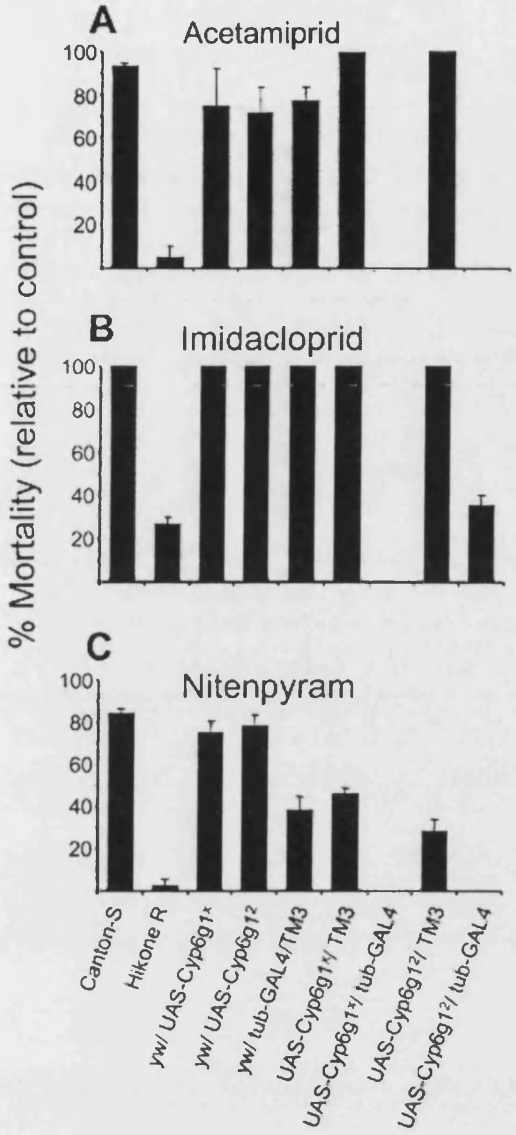

Fig. 4. Discriminating dose larval bioassays to test for cross-resistance to three different neonicotinoids in transgenic flies over-transcribing *Cyp6g1* alone. Percentage mortality at a discriminating dose of (A) acetamiprid (1.2 µg/ml) (B) imidacloprid (0.2 µg/ml) and (C) nitenpyram (2 µg/ml) is shown for the different transgene expressing (*UAS-Cyp6g1*$^{1\text{ or }2nd}$*/Gal4*) and non-expressing (*UAS-Cyp6g1*$^{1\text{ or }2nd}$*/TM3* or *yw x UAS-Cyp6g1*$^{1\text{ or }2nd}$) genotypes. The susceptible Canton-S and resistant Hikone-R were included as negative and positive controls respectively.

flies over-expressing *Cyp6g1* alone also show cross-resistance to two other neonicotinoids, acetamiprid and nitenpyram. Together with earlier data also showing *Cyp6g1* mediated cross-resistance to the growth regulator lufenuron (Daborn et al., 2002), this broad cross-resistance to a wide range of old and new insecticide classes is consistent with the hypothesis that *Cyp6g1* may have been broadly selected due to its ability to metabolise a wide range of compounds. This hypothesis is consistent with the observation that the same gene has been selected for in both *D. melanogaster* and *D. simulans*, presumably by a wide range of different insecticides across the globe. Confirmation of P450 involvement in resistance in vivo using synergists and insecticide metabolism, and metabolism in vitro by CYP6G1, are underway in our laboratory. However, importantly, such broad cross-resistance suggests that old classes of insecticide such as DDT can select for P450 alleles that confer resistance to new classes of chemistry such as the neonicotinoids and lufenuron (Daborn et al., 2002). Given previous observations on the high degree of conservation of different insecticide resistance mechanisms between species it will be interesting to compare the resistance associated mutations found in *Cyp6g1*, putatively an *Accord* transposon, with its resistant homolog in *D. simulans*. Finally, it seems likely that if similar P450s with broad cross-resistance spectra are selected in pest insects they may be hard to control with alternative insecticides in the field.

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References

Amichot, M., Brun, A., Cuany, A., Helvig, C., Salaun, J.P., Durst, F., Bergé, J.B. (1994). Expression study of CYP genes in *Drosophila* strains resistant or sensitive to insecticides. Cytochrome P450. 8th International Conference., Paris, John Libbey Eurotext.

Bergé, J.-B., Feyereisen, R., Amichot, M., 1998. Cytochrome P450 monooxygenases and insecticide resistance in insects. Philosophical Transactions of the Royal Society of London, Series B 353, 1701–1705.

Brandt, A., Scharf, M., Pedra, J.H., Holmes, G., Dean, A., Kreitman, M., Pittendrigh, B.R., 2002. Differential expression and induction of two *Drosophila* cytochrome P450 genes near the *Rst(2)DDT* locus. Insect Mol Biol 11 (4), 337–341.

Chelvanayagam, G., Parker, M.W., Board, P.G., 2001. Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. Chem. Biol. Interactions 133, 256–260.

Chen, B., Chu, T., Harms, E., Gergen, J.P., Strickland, S., 1998. Mapping of *Drosophila* mutations using site-specific male recombination. Genetics 149 (1), 157–163.

Daborn, P., Boundy, S., Yen, J., Pittendrigh, B., ffrench-Constant, R., 2001. DDT resistance in *Drosophila* correlates with *Cyp6g1* overexpression and confers cross-resistance to the neonicotinoid imidacloprid. Mol. Genet. Genomics 266, 556–563.

Daborn, P.J., Yen, J.L., Bogwitz, M., LeGoff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., et al. 2002. A single P450 allele associated with insecticide resistance in global populations of *Drosophila*. Science 297, 2253–2256.

Dunkov, B.C., Mocelin, G., Shotkoski, F., ffrench-Constant, R.H., Feyereisen, R., 1997. The *Drosophila* cytochrome P450 gene *Cyp6a2*: structure, chromosomal localization, heterologous expression and induction by phenobarbital. DNA and Cell Biology, 1345–1356.

Feyereisen, R., 1999. Insect P450 Enzymes. Ann. Rev. Entomol 44, 507–533.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.M., Wides, R., et al. 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298 (5591), 129–149.

Maitra, S., Dombrowski, S.M., Waters, L.C., Ganguly, R., 1996. Three second chromosome-linked clustered *Cyp6* genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*. Gene 180, 165–171.

Maitra, S., Price, C., Ganguly, R., 2002. *Cyp6a8* of *Drosophila melanogaster*: gene structure, and sequence and functional analysis of the upstream DNA. Insect Biochem Mol Biol 32 (8), 859–870.

Misra, S., Crosby, M.A., Mungall, C.J., Matthews, B.B., Campbell, K.S., Hradecky, P., Huang, Y., Kaminker, J.S., Millburn, G.H., Prochnik, S.E., et al. 2002. Annotation of the *Drosophila melanogaster* euchromatic genome: a systematic review. Genome Biol 3 (12), 0083.

Oakeshott, J.G., Horne, I., Sutherland, T.D., Russell, R.J., 2003. The genomics of insecticide resistance. Genome Biol 4 (1), 202.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M.V., Unger, M.F., Collins, F.H., Feyereisen, R., 2002. Evolution of supergene families associated with insecticide resistance. Science 298 (5591), 179–181.

Tijet, N., Helvig, C., Feyereisen, R., 2001. The cytochrome P450 gene superfamily in *Drosophila melanogaster*. Gene 262 (1–2), 189–198.

Xu, W., Bak, S., Decker, A., Paquette, S.M., Feyereisen, R., Galbraith, D.W., 2001. Microarray-based analysis of gene expression in very large gene families: the cytochrome P450 gene superfamily of *Arabidopsis thaliana*. Gene 272 (1–2), 61–74.

Zdobnov, E.M., von Mering, C., Letunic, I., Torrents, D., Suyama, M., Copley, R.R., Christophides, G.K., Thomasova, D., Holt, R.A., Subramanian, G.M., et al. 2002. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. Science 298 (5591), 149–159.