A GENOMIC APPROACH TO UNDERSTANDING METABOLIC INSECTICIDE RESISTANCE TO DIAMIDES IN DIAMONDBACK MOTH (*PLUTELLA XYLOSTELLA*)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Mark Dickson Mallott.

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DECLARATION

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree.

Signed .....(Candidate)

Date .....

Statement 1

This thesis is the result of my own investigation, except where otherwise stated. Other sources are acknowledged and bibliography is appended.

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Date .....

Abstract

A GENOMIC APPROACH TO UNDERSTANDING METABOLIC INSECTICIDE RESISTANCE TO DIAMIDES IN DIAMONDBACK MOTH (*PLUTELLA XYLOSTELLA*)

Mark Mallott

The diamondback moth (*Plutella xylostella*) is a destructive insect pest of cruciferous plants throughout the world. Control of diamondback moth relies heavily on the use of chemical insecticides, resulting in this species developing resistance to most compounds. The diamides are a relatively recently introduced insecticide class used to control *P. xylostella*. Mutations in the diamide target site that confer resistance to diamides have recently been described, however at least one strain exists without these mutations, but remains resistant to diamides.

This study exploited genomic resources including whole genome microarrays to identify candidate detoxification genes. One candidate, a flavin-dependent monooxygenase (*FMO2*) showed approximately a 70-fold increase in expression in microarrays and 14,700-fold overexpression in qPCR when compared to the susceptible ROTH strain. This and other candidate genes were functionally analysed using multiple approaches.

In the absence of diamide selection the resistant Hawaii selected (HS) strain of *P. xylostella* rapidly lost resistance, and this was associated with a drop in the expression of *FMO2*. Transgenic *D. melanogaster* expressing *P. xylostella FMO2* exhibited significant resistance to chlorantraniliprole suggesting it has the capacity to detoxify this compound. In contrast, transgenic *D. melanogaster* expressing other candidate detoxification genes were not resistant. Insecticide bioassays of both *P. xylostella* and transgenic *Drosophila* (over)expressing *FMO*2 revealed significant differences in sensitivity to anthranilic and phthalic diamides, suggesting *FMO*2 can metabolise the former but not the latter. Liquid chromatography-mass spectrometry analysis following diamide exposure showed significant differences in chlorantraniliprole stability in the HS *P. xylostella* and *FMO*2 transgenic *D. melanogaster* strains compared to their susceptible counterparts, implying enhanced metabolism and or excretion may be involved in resistance. The putative promoter region of the *FMO*2 gene in the resistant and susceptible *P. xylostella* strains was found to contain numerous SNPs and indels. These may have in part, resulted from a transposable element insertion in the promoter of the resistant strain, leading to modification in the number and type of predicted transcription factor binding sites. In a dual luciferase reporter assay, the HS promoter variant drove significantly higher levels of expression than the ROTH promoter suggesting a *cis*-acting element is, at least in part, responsible for the overexpression of *FMO*2 in the resistant strain.

In summary, this PhD provides lines of evidence for a novel mechanism of insecticide resistance based on the overexpression of a *FMO*2. The importance of this enzyme family in humans has been well established, the results detailed in this PhD suggest they may also play a role in xenobiotic detoxification in insects. Finally, as diamides are a relatively new and important means of controlling *P. xylostella* the findings presented here hold potential to contribute to the future control of this economically important species.

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Firstly, I would like to thank whole heartedly, Chris Bass for his enduring support throughout this PhD, forever being approachable, pragmatic and focused. I would also like to thank the rest of my academic supervisory team, Lin Field, Mark Paine and Charles Wondji, and my industrial supervisors Jan Elias and Russell Slater, for the many helpful meetings over the last four years. Thank you also to Martin Williamson and Emyr Davies for all of their useful input and advice, and Miriam Daniels for all her help and hosting me at Syngenta and guiding me in the LC-MS work.

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List of Abbreviations

aa	amino acid
Acetyl-CoA	acetyl coenzyme A
AChE	acetylcholine esterase
AHDB	agriculture and horticulture development board
APRD	arthropod pesticide resistance database
BLAST	basic local alignment search tool
bp	base pair(s)
Bt	*Bacillus thuringiensis*
CCE	carboxyl/choline esterase
cDNA	complementary DNA
CTPR	chlorantraniliprole
Da	Dalton(s)
DBM-DB	diamondback moth genome database
DDT	dichlorodiphenyltrichloroethane
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetra-acetic acid
FAO	food and agriculture organization of the united nations
FMO	flavin-containing monoxygenase
FMO2	flavin-containing monoxygenase isoform 2
g	gram
GABA	g-aminobutyric acid
GST	glutathione-S-transferase
HS	Hawaii Selected
IPM	integrated pest management
IRAC	Insecticide Resistance Action Committee
IRM	insecticide resistance management

kb	kilobase pair
KDR	knockdown resistance
L	litre
LB	liquid broth
LC	lethal concentration
LC-MS	liquid chromatography-mass spectrometry
LD	lethal dose
MeCN	acetonitrile
MFO	mixed function monooxygenase
mg	milligram
min	minute
MIRA	mimicking intelligent read assembly
ml	millilitre
mM	millimolar
MOA	mode of action
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	national centre for biotechnology information
ng	nanogram
NGS	next generation sequencing
OP	organophosphate
P450s	cytochrome P450s
PA	pyrrolizidine alkaloid
PBO	piperonyl butoxide
PCR	polymerase chain reaction
QPCR	quantitative polymerase chain reaction
RACE	rapid amplification of cDNA ends
RIDL	release of insects carrying a dominant lethal

RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RyR	ryanodine receptor
SCD	short-chain dehydrogenase
SDW	sterile de-ionised water
sec	second
siRNA	short-interfering RNA
SIT	sterile insect technique
SNO	senecionine N-oxygenase
SNP	single-nucleotide polymorphism
TAE	tris acetate EDTA
Taq polymerase	*Thermus aquaticus* polymerase
TBE	tris/borate/EDTA
TM	melting temperature
tris	tris(hydroxymethyl)aminomethane
UAS	upstream activation sequence
UTR	untranslated region
XDH	xanthine dehydrogenase
µg	microgram
µl	micro litre
µM	micromolar

1 Introduction

1.1 Food Security

Food security is thought of as a 'modern problem' that needs to be addressed as the human population expands at an ever-increasing rate, however, it is an issue that has been officially addressed by civilisations or governments for thousands of years. From the first small scale grain silo or any other method of preservation to the controlled release of food by ancient civilisations during times of famine. It is only in more recent times with a globalised economy that conscious efforts have been made to categorise and define the term 'food security'. The aim is that with definition comes a focusing and (hopefully) coordinated approach to addressing such issues. Food security would likely have different definitions and priorities depending upon the scale at which it is being addressed. At a local scale, consideration would need to be given to 'household' or individuals' behaviour and economic dependencies [1, 2] whereas the impact of this research is at a scale whereby a more global outlook of food security is taken. In consulting for the Food and Agriculture Organization of the United Nations in 2002, Clay [3] highlights that defining a working definition for food security has been a 'flexible concept'. And outlines the changes since the first 'world food conference' took place during November 1974, in Rome (box 1.01).

According to the statistics division of the FAO (FAOSTAT) [4], the number of undernourished people worldwide has fallen from 18.6% in 1991 to 10.8% in 2015. Taken at face value this seems to represent good progress, however, a significant percentage of the global population remains undernourished, for example, in developing countries 12.9% of the populace were undernourished in 2015. While this decrease is apparent despite the world's population increasing, the global population is expected to reach 9.2 billion by 2050 with a stabilisation at around 10 billion people by the year 2200 [5]. The world bank predicts that this continued increase in population, will require a 50% increase in food by 2030 [6]. To date, the world's global aggregate food production has kept pace with the world's population, despite it doubling in the last half a century [5]. However, in the future the amount of land available for cropping and crop yields themselves are likely to be seriously challenged by competition for land, water scarcity, and climate change [5]. Climate change might not immediately have a negative impact [7, 8], as modelling suggests that crop production in developed countries will benefit from low level climate change, compensating

for declines made by developing countries. This will however further increase the disparity in yields between developed and developing countries [8]. Lobell *et al.* make assessments of various climate models, concluding that temperature and individual crops' tolerances to this variation will be of particular importance. They predict that in some instances, for example, maize and wheat in Southern Africa, there is a 95% chance that climate change will harm crop production (in the absence of adaptation) [9]. The authors note though, that their reviews do not consider all variables that may impact crop production as a result of climate change [7, 9] and there are obviously many other factors that may influence crop yields, both biotic and abiotic [10] (fig. 1.01).

- Food security was defined in the Proceedings of the 1974 World Food Summit as: 'availability at all times of adequate world food supplies of basic foodstuffs. . . to sustain a steady expansion of food consumption. . . and to offset fluctuations in production and prices' [11].
- In 1983 FAO expanded its concept to include a third prong: 'Ensuring that all people at all times have both physical and economic access to the basic food that they need.' [12].
- In an influential World Bank report (1986), Poverty and Hunger [13], this concept of food security is further elaborated in terms of: 'access of all people at all times to enough food for an active, healthy life.'
- The 1996 World Food Summit in its Plan of Action adopted a still more complex definition: 'Food security, at the individual, household, national, regional and global levels [is achieved] when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life.' [14].
- This definition is again refined in The State of Food Insecurity 2001: 'Food security [is] a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life.' [15].

Box 1.01. Official concepts of food security as reviewed and detailed by Clay in 2002 [3] highlighting the flexible concept of defining 'food security'.

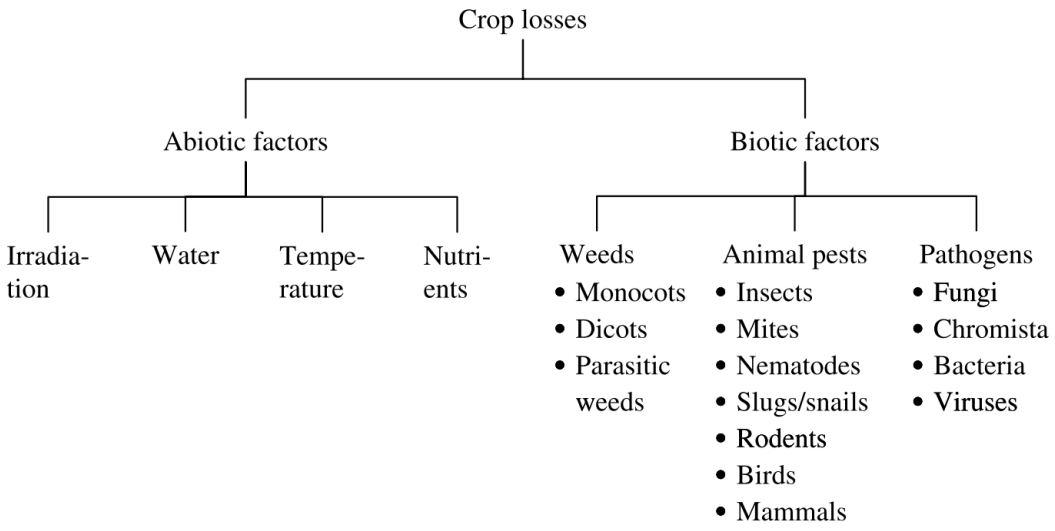


Figure 1.01. Abiotic and biotic factors causing crop losses. Taken from Oerke, 2006 [10].

1.2 Pests of Cruciferae

Cruciferae are an important plant group containing many of the cultivated core vegetable food crops grown as food or for other uses, such as for oil, for example oil seed rape (*Brassica napus* L.) and canola (a modern cultivar of rapeseed). Included in this group is the species *Brassica oleracea,* a particularly important species as it encompasses many common food crops as cultivars. Although not taxonomically distinguished from one another, they are in effect grown as separate crops, with the species as a whole containing cabbage (*Brassica oleracea* L. var. *capitata*), broccoli (*Brassica oleracea* L. var. *italica*), savoy (*Brassica oleracea* L. var. *sabauda*), and cauliflower (*Brassica oleracea* L. var. *botrytis*) amongst others.

It is generally accepted that the principle species of cultivated crucifers originated in Mediterranean and Asian regions [16] and so it is logical that the primary pest species of these crops also originated from these areas. With temperature being a crucial factor in an insect's life cycle and speed of development, particular species will inevitably be more of a problem in some geographic locations than they are in others.

The total number of different pest species present on crucifers has never been quantified *per se*, however, Finch & Thompson estimate that in temperate regions cultivated cruciferae could be attacked by 50-60 insect species, with about 20 species being considered major crop pests [17]. The sixth international workshop on the management of the diamondback moth and other crucifer insect pests note diamondback moth, *Plutella xylostella*, to be the most serious worldwide pest of economically important crucifers. They name the cabbagehead caterpillar (*Crocidolomia binotalis*), web worm (*Hellula undalis*), *Pieris* species of butterfly, flea beetle (*Phyllotreta* spp.) and aphids such as *Brevicoryne brassicae, Liphaphis erysimi,* and *Myzus persicae* as being important secondary pests [18].

Insects can cause damage to crops in a variety of different ways. In the case of lepidopteran species damage is primarily caused through the direct feeding of the moth or butterfly larval stages on various parts of the plant, for example, *Crocidolomia binotalis* or *Pieris* species of butterfly cause damage through the consumption of leaf and/or stem material. Although *Hellula undalis* causes damage through eating crop plant material, rather than living on and consuming from the outside of the plant, the larvae of this species burrows into leaves and mines them from the inside which affords it extra protection from predators. Flea beetle species also cause damage by directly eating the plant, although in this case it is the adult beetles that do the damage, rather than their larvae, which while associated with the plants (root system) do not cause significant damage to the crops. Given ideal conditions and when present in large numbers, aphids are capable of causing significant damage to crops through direct feeding on the plant sap hence weakening the plants. Generally however, greater damage is done to most crop plants through the transmission of plant viruses carried within the insects' saliva. In addition to loss of yield by destroying or killing the crop, even light damage to high value vegetable crops can result in rejection at the point of sale influencing the approaches selected by growers for control.

1.3 Integrated Pest Management (IPM)

Kogan described Integrated Pest Management (IPM) as 'a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interest of and impact on producers, society and the environment' [19]. Put simply it is the integrated

combination of methods that a farmer chooses to employ in order to control potential pests. Optimal IPM will vary with geography, scale, crop, pest(s) being combated, and farming type (e.g. organic) but typically integrates biological, chemical and cultural control methods into an effective and environmentally sensitive approach to control insect pests. Biological control often forms an important component of IPM, however, in certain cases the application of chemical pesticides may be required to augment other control methods.

1.4 Pest Control and Management

There are multiple methods by which crop pests can be controlled. The method or methods adopted will depend upon several factors such as pest species being targeted, geographic location (which would have a large effect given the variation in climatic conditions), cost of control and also the farming practices employed by the farmer. For example, a farm that had adopted organic farming practices would need to employ an entirely different approach of control to that of a conventionally managed farm.

1.4.1 Biological Control

The three main approaches associated with biological control are 'classical' (inoculative), augmentative and conservation [20, 21]. The relationship between biological control and other pest management strategies within the framework of integrated pest management (IPM) is summarized in (fig 1.02).

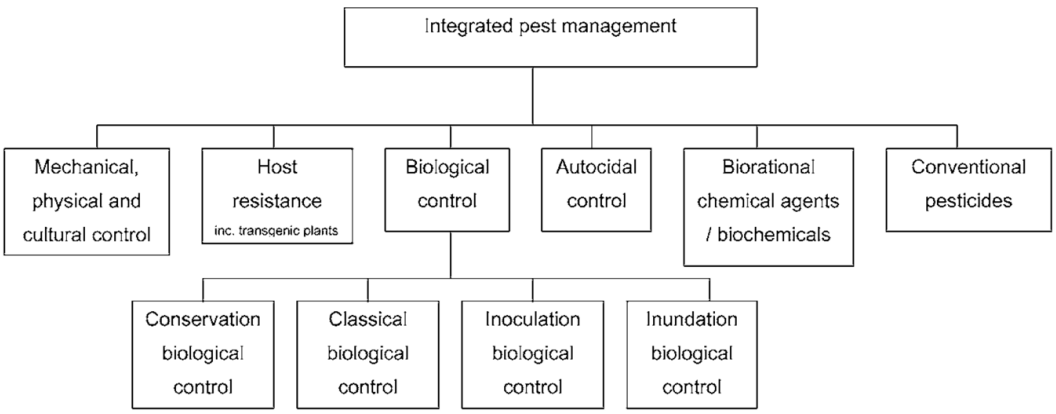


Figure 1.02. Biological control approaches shown alongside other control strategies and within the context of the Integrated Pest Management (IPM). Taken from Eilenberg *et al.* [20].

1.4.1.1 Classical/Inoculation Biological Control

Typically, classical/inoculation biological control is often employed where an alien/invasive pest species requires control due to a lack of control agents occurring naturally in the pest species' new environment. The approach generally involves the release of additional exotic natural enemy species (one or more) upon the pest species infested area with the aim that this 'inoculation' of the biological control species will reduce the target species population to non-pest levels while also establishing itself and more permanently colonising the area providing further protection against future pest outbreaks [22]. Early notable examples of successful classical biological control would be control of the cottony cushion scale, *Icerya purchasi* in California with *Rodolia cardinalis*, an imported predatory coccinellid from Australia in 1888; and use of the tachinid fly *Bessa remota* imported from Malaya to the Fiji Islands in 1925 to control the coconut moth, *Levuana iridescens* [23].

Cowie is critical of previous classical biocontrol experiments and field scale trials suggesting that not enough due diligence or follow-up studies are performed quantifying the impact on non-target species and consequently impacts on the ecology of the inoculated landscape [24]. There are many examples in the literature of unsuccessful attempts at the use of classical biological control, which include the replacement of native species [25], as well as cases of exotic biocontrol species introductions causing extreme unforeseen consequences [26]. One of the most well-known examples of this is the introduction of the cane toad, *Bufo marinus*, from Hawaii into Australia in 1935 as a biocontrol of the greyback beetle, *Dermolepida albohirtum*, a pest species for the economically important sugar cane industry. The introduction of this exotic species as biocontrol has had a devastating impact on native biodiversity (primarily through lethal toxic ingestion by higher trophic levels) [27, 28].

1.4.1.2 Augmentative/Inundative Biological Control

Augmentative biological control refers to techniques whereby natural enemies are periodically introduced to the infested environment and usually requires the commercial production of the released agents [29]. In this instance (unlike that above) it is likely that the introduced control species may exist in the field already at a low density, but too low to affect a significant change and provide protection from the pest species. Therefore the control measure introduction is made with the aim that the density of this predator is increased to

a level whereby they are able to reduce the crop damage inflicted by the pest to below an acceptable economic damage threshold [30]. By supplementing the natural (or depleted) level of biological control species rather than introducing an exotic species as in classical biological control, the hope is that this would negate much of the risk associated with the introduction of an exotic species. The major driver for the development of augmentative biological control approach is to reduce the historic reliance on broad-spectrum pesticides [31]. In releasing green lacewings, *Chrysopa californica* to control the mealybug crop pest of pear orchards in 1949, Doutt and Hagen are likely to have been the first to experimentally apply this technique, with a large number of pest species targeted in this way since [31, 32]. In a review, Collier critically evaluates the augmentative/indundative approach to biological control, concluding that pest populations were suppressed below target densities in only 16% of the cases and by the assessment criteria employed "failed" in 64% of cases. Moreover, the review also concluded that typically, augmentation was less effective than pesticide treatments and can be more expensive or uneconomic. However it has been reported by Trumble *et al.* that where an approach combining both the use of augmentative biological pest control (*Phytseiulus persimilis)* with the use of a pesticide (in this case abamectin) adequate pest control can be both achieved and be economically viable [33].

1.4.1.3 Conservation Biological Control

This form of biological pest control works on a similar principle to the augmentation approach in trying to increase the numbers of naturally occurring control agents in order to control a pest species. The difference is that this is not achieved by directly supplementing the biological control agent in terms of an 'injection of numbers' through release, but where measures are taken to more naturally increase the numbers of natural enemies. Eilenberg *et al.* describe this approach as a combination of protecting biological control agents and providing resources so that they can be more effective, and note that this practice therefore limits pesticide use (although does not rule out its use entirely) [20]. Methodologies employed could take the form of the creation of shelter habitats as refuges for biocontrol agents, or the planting of food sources to aid particular life-stages and increase their fitness and effectiveness [34, 35]. There are of course some negative aspects of added habitat diversity, the primary one being a potential loss of cropping area [31].

1.4.2 Cultural Control

Where a crop is continually farmed over consecutive seasons or years the risk is increased of nutrient depletion as well as a build-up of pathogens and pests, particularly those that are soil born or have part of their life cycle in the soil environment. In addition to 'resting' and 'replenishing' the land, crop rotation can reduce pressure exerted by pest species (in fact on all crops in the rotation) by breaking the pest reproductive cycles. Rotation has been used very effectively in both conventional and organic farming. An early documented example of crop rotation is in the control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, where Forbes concludes that the rotation of corn over the course of at least two years with other crop(s) would provide a complete remedy for damage inflicted upon the crop by *D. virgifera* [36]. However, it has been reported that even this control strategy can prove to be a driver of adaptive change as demonstrated by the evolution of 'behavioural resistance' in the western corn root worm specifically mitigating the impact of crop rotation on its life-cycle [37, 38]. Trap cropping is a form of habitat manipulation involving the use of alternative host plants to attract and retain a pest species thus reducing the negative impact on the crop from that species. Trap crops are often planted as perimeter crops intercepting pest species as they arrive at a crop field [39, 40]. Insects use a number of cues to locate and colonise a target crop whether it be as a direct food source or for oviposition or another stage in their life-cycle such as overwintering. Typically, the cues used to locate host-plants by insects are volatile compounds given off by plants [41]. A trap crop would ideally be more attractive to a pest species than the economically valuable crop as a result of chemical cues that are attractive to the pest. In cases where pests are attracted by a particular volatile compound, for example, isothyanocyanates, which are generalist cues for host location [42] for the cabbage aphid *Brevicoryne brassicae* [43], the cabbage seed weevil, *Ceutorhynchus assimilis* [44], and *Plutella xylostella* [45], plants that have a higher concentration of this compound, or release a volatile ratio composition of this with other semiochemicals, might prove more attractive either for landing or oviposition to the incoming pest [46]. If a trap crop is attractive to a pest insect but a poor host for that pest then it would become a sink for that pest and would be deemed a 'dead-end' trap crop for that pest [47]. For a trap crop to successfully function as a sink for a pest it must be able to 'retain' the pests that arrive [48]. This retention could be as a dead-end, sacrificial (as a preferred food source), or 'hold' the pest to a point whereby it is dealt with through further IPM (for example intervention with pesticide). In a review of approximately 100 cases of trap cropping at a commercial level

it was found that only 10 cases were deemed successful at a commercial level. However, in the cases where it has been proven to work, it can do so very effectively [49].

1.4.3 Biorational Control

The definition of biorational control has been developing for a number of years [50], with more recent literature adopting the term biopesticides. By 2007 the US Environmental Protection Agency (EPA) had over 800 commercial formulations containing 190 registered active ingredients which were categorised into three classifications: 1) microbial pesticides (in which a bacterium, fungus, virus or protozoan is the active ingredient); 2) plant pesticides (in which a pesticidal substance is produced through the genetic modification of the plant); 3) biochemical pesticides (naturally occurring substances that control pests through non-toxic mechanisms) [51]. These biopesticides have in theory a lower general impact both in terms of human health, environmental persistence and in affecting non-target organisms through greater specificity. However, there is overlap between the 'biorational' category and other categories of biocontrol, for example host resistance and conventional pesticides with examples such as genetically modified crop plants incorporating *Bacillus thuringiensis* (*Bt*) and 'naturally occurring' pesticides such as the pyrethrins falling into more than one category (fig 1.02).

1.4.4 Autocidal Control

Autocidal control typically involves the use of the 'sterile insect technique' which is described in more detail in section 1.6.3.5., but involves the release of sterilised male insects of the same species as the target pest. These mate with the pest population, reducing wildtype (fertile) male fertilisation and causing a population crash. In original programs, sterilisation would have been achieved through irradiation [52], but more recently molecular genetic approaches are being employed. These include reduced reproductive performance through RNA interference gene silencing [53] and incorporation of lethal genes through transgenics [54, 55].

1.4.5 Host-plant Resistance

Host plant resistance would originally have exploited a plant's natural ability to resist pest damage through physical or biochemical attributes. In 1961, Ross found evidence for the

development of resistance to tobacco mosaic virus within small areas surrounding lesions that were already present [56]. The reason for this was not completely understood at the time, but demonstrates that resistance does not have to occur systemically. Raffa and Berryman also describe a localised host plant response of *Pinus contorta* var. *latifolia*, a coniferous tree against attack by the bark beetle, *Dendroctonus ponderosae* [57]. The authors note that the relationship between this host and pest is such that a 'stability' is often reached between the two. While the defence itself (essentially a release of sap) can attract further individuals, it is in effect efficient enough to halt the attack before a 'point of no return' is reached, where the plant will die. Dreyer and Campbell detail a chemical basis of host-plant resistance to aphids involving the structure of pectin present within different plants and the ability of different aphid biotypes to enzymatically break down this intercellular barrier [58]. Nottingham *et al.* note the difference in behaviour of aphid species to isothiocyanate compounds by different species of aphid depending upon their host preferences [43]. Host plant resistance now also encompasses the inclusion of genetically modified plants the most famous of which are those expressing *Bt* (described in section 1.6.3.4), but also more recently host plants engineered to release insect pheromones [59].

1.4.6 Use of Synthetic Pesticides

The first use of insecticides (sulphur) was recorded by the Sumerians and the Chinese 2500–1500 BC [10]. Currently there are at least 29 modes of action encompassing nerve and muscle targets, growth and development targets, respiration targets, midgut targets and also those that have as yet unidentified or have non-specific targets [60]. In 2013, in data commissioned from 'Agronova' by Sparks and Nauen, insecticide end-user sales were $17,016 million (excluding fumigants) [61]. At 85% of these sales, the vast majority were for insecticides targeting nerve and muscle action; with growth regulation and energy metabolism occupying 9% and 4% respectively. Insecticides acting on mid-gut membranes and those with unknown mode of action account for the remaining 2% (fig. 1.03). The authors further analysed these data, interrogating it by IRAC mode of action (fig. 1.04). Knowing the number of compounds in each IRAC mode of action group, they note that while there are only 8 neonicotinoids (as opposed to 90 organophosphates), they account for 27% and the largest portion of the market. The diamide class of insecticides affect nerve and muscle action (as detailed in section 1.8.2), falling into the largest category shown by figure

1.04. The market share of 8% shown by the diamides indicates their importance in crop protection, despite their recent release, and low number of compounds [61].

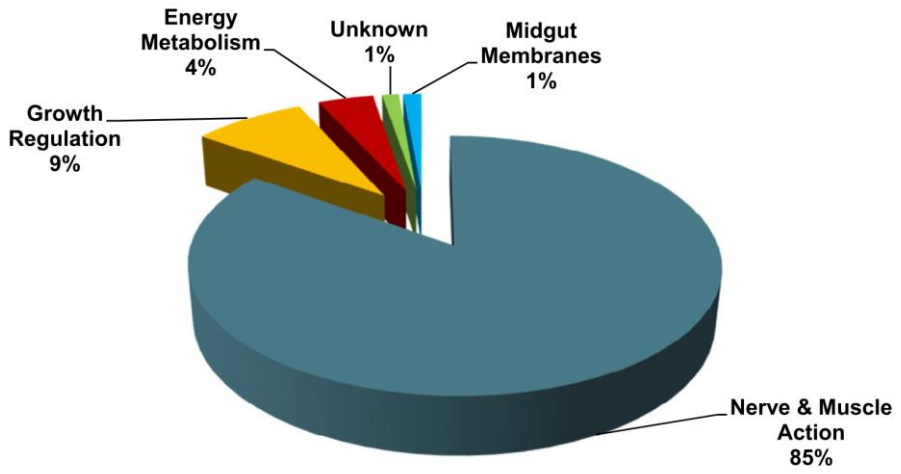


Figure 1.03. Distribution of insecticide sales as percent of total value by broad mode of action. Based on 2013 end-user sales data supplied by Agronova. Sourced form Sparks and Nauen, 2015 [61]

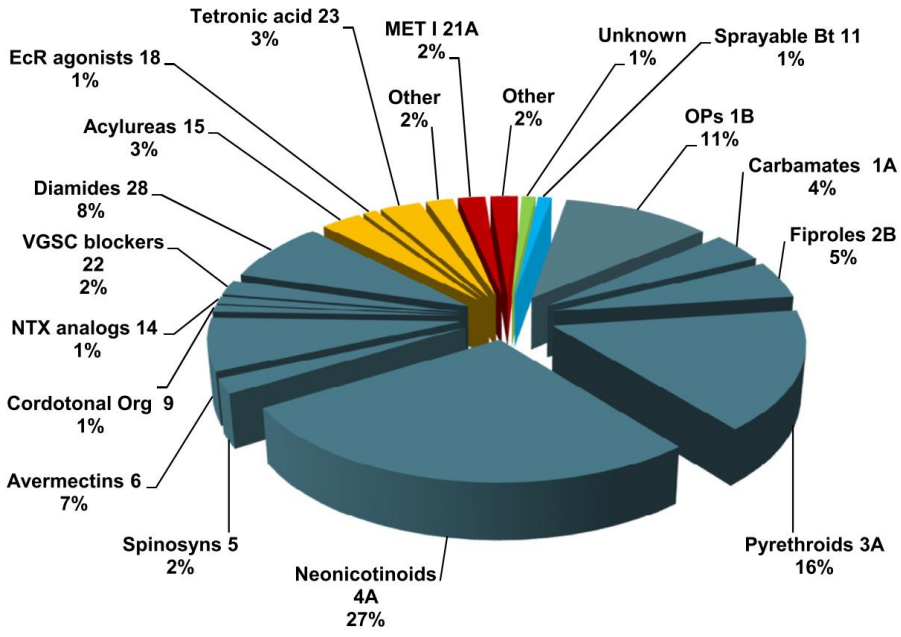


Figure 1.04. Distribution of insecticide sales as percent of total value by IRAC mode of action groups. Based on 2013 end-user sales data supplied by Agronova. Sourced form Sparks and Nauen, 2015 [61]

In addition to an array of modes of action, there are also a number of methodologies by which pesticides can be delivered. The most obvious of which is the direct spraying of crops with a pesticide solution. This method of application can be specifically timed for maximum effect striking a balance between cost and efficacy. For example, a crop may be treated before or after it has become infested, making use of a particular treatment's characteristics, possibly targeting a specific pest life stage [62]. The pesticide could also be delivered to its target by means of a seed treatment whereby the pesticide is absorbed into the plant protecting it systemically. Different delivery methods have positive and negative aspects to their use. Some might enable a greater efficiency of application, longer effective period, or indeed (if desired) have a shorter persistence in the environment which may be beneficial when implemented as part of a wider IPM strategy. Due to their effectiveness, over the years synthetic pesticides have been repeatedly used in such high frequencies that it has resulted in the emergence of pest species resistant to the active ingredient [10]. Depending upon the mode of resistance and how the pesticide is managed, a pest species might become resistant to one, or more, or in a worst-case scenario, all of the available compounds or modes of action available for control.

1.5 Evolution of Resistance

An inevitable outcome of the intensive use of insecticides to control insect pests is the evolution of resistance. This occurs most rapidly when insecticides are misused or overused. Insecticide resistance evolves by natural selection, but the selection pressure through the use of a synthetic chemical agent is extremely high compared to other more 'natural' selection pressures a population will encounter. Given the likely large population sizes and relatively short generation times of insect populations and the high selection pressure, resistance can evolve at a remarkable rate, in some instances, within as little as two years [63, 64].

The process of insecticide resistance evolving within a pest (or for that matter, beneficial) insect population is detailed in figure 1.05 below. The mechanisms by which insect populations develop resistance are varied, involving penetration, behaviour, metabolic detoxification and alteration of insecticide target sites as detailed in section 1.7.

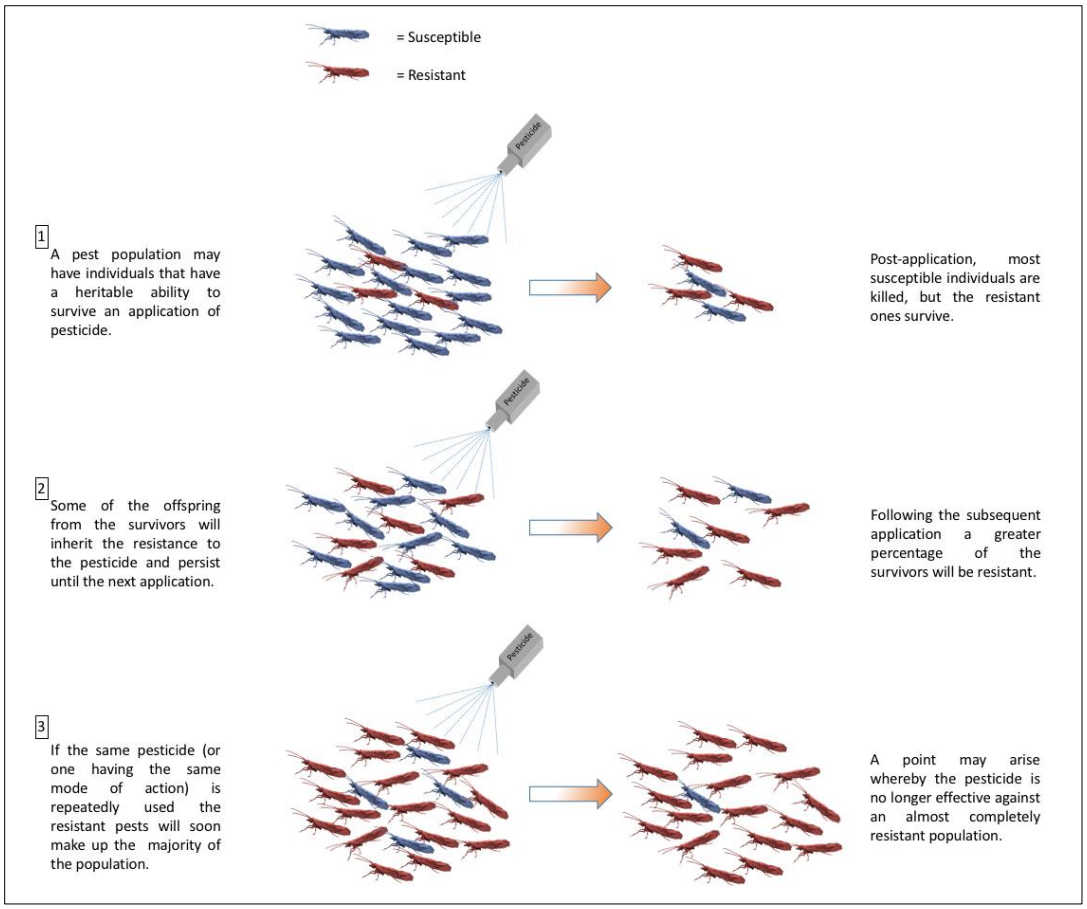


Figure 1.05. The process of the evolution of pesticide resistance. Starting with a predominantly susceptible pest population (1); with selection pressure creating a population primarily composed of resistant pests (3).

1.5.1 Insecticide Resistance Management (IRM)

Insecticide Resistance Management (IRM), sometimes also known as insect resistance management, is usually an important component of IPM. The ultimate aim of IRM is to conserve the susceptibility of pest species to insecticides through strategies aimed at preventing or slowing the development of resistance to insecticides [65]. Resistance to insecticides is essentially a species evolving to withstand a severe environmental stress [66], with the case here being the stress of one or more pesticides, the mechanisms of which are described in section 1.7. There are several strategies that can be implemented, either independently by a grower, or on a larger scale for example, nationally, via a coordinated government approach. As insecticide resistance is fundamentally the selection of individuals that can tolerate a pesticide or group of pesticides that share a particular mode of action, IRM needs to account for the effect of treatment on both the genetic composition of a

population and its total size. The management of resistance entails the prediction of both suppressive and selective effects of potential control strategies [66].

Common strategies of IRM are based on the use of pesticide mixtures, sequences or rotations [67]. The use of a mixture utilises the simplistic theory that if one of the pesticides does not kill the pest, then the other(s) will, a strategy described by Comins as 'redundant killing' [68]. Pesticide mixtures can place pests under high selection pressure for the development of cross-resistance [67]. Therefore, it is important that a proportion of the population should not be exposed to the mixture to ensure that should there be any survivors following exposure (which in theory would be resistant to both pesticides), there will be a cohort of susceptible individuals available to breed with the survivors, diluting the level of resistance in future generations. If not, the risk is that all offspring in future generations will be resistant to both compounds [69]. Of course in a 'real world' environment it is feasible that populations of susceptible pests will remain present in refuges within field boundaries or land in close proximity to the treated crop helping to delay the build-up of resistant genes [70].

Refuges holding susceptible individuals can work alongside the possible cost to fitness an individual may have inherited as a result of the adaptation causing pesticide resistance. In their review of fitness costs associated with insecticide resistance, Kliot and Ghanim define fitness as the ability of an individual of a certain genotype to survive and reproduce relative to other individuals of the same species [71]. Where fitness costs are associated with insecticide resistance, it is likely that the resistance incurs a high energetic cost or other significant disadvantage diminishing the insect's fitness compared to susceptible individuals [71]. This can lead to fitness costs selecting against resistance in refuges [72], and by reducing the frequency of resistance alleles in refuges, fitness costs may then enhance the delaying effect of a refuge on the proliferation of the resistance [73, 74]. As noted by Hackett and Bonsall, the extent by which a fitness cost effects the frequency of resistance alleles depends upon how the cost is realised biologically [73].

Prediction that the fitness of resistant genotypes will increase the longer there is selection for resistance was made by Keiding in 1967 [75]. Kliot concurs, noting that if the evolutionary (pesticide) pressure persists over many generations it is likely that further genetic changes will occur in addition to the original form of resistance to alleviate the fitness cost caused by the initial adaptation [71]. It is possible that these further mutations may entirely remove fitness costs or reduce them to a level whereby the pesticide resistance becomes fixed within a population [71]. Such a case took place in the sheep blowfly, *Lucillia cuprina*, in Australia, where due to a lack of alternative effective insecticides upon its introduction, diazinon was exclusively used with resistance being reported approximately ten years later in 1967 [76]. By comparison with earlier (1969/1970) unpublished data on fitness costs in *L. cuprina* conferred by the diazinon resistance a decade later, McKenzie *et al.* found that individuals carrying the resistance allele were no longer at the disadvantage in a non-diazinon environment than they were previously [77]. The authors hypothesise that a fitness cost will likely be maintained where genetic variability is unavailable or the intensity or the duration of selection is restricted. Also, adding an important factor for IRM, the higher the fitness cost, the increased time is likely to take for a resistant allele to spread within a population [71]. Therefore, preservation of potential fitness costs through alleviation of selective pesticide stress should form part of an IRM strategy.

Rotating pesticides by simple alternation or by using a sequence of compounds of different mode of action provides a way to change the type of selection pressure (provided by using insecticides of different MOA) for a period of time and also works on the assumption that there is a trade-off between resistance and fitness, meaning that individuals that are resistant to one pesticide are in general less fit than susceptible individuals. These will therefore show a greater decline through the application of an alternate pesticide (assuming that they are also not resistant to the alternate pesticide) [67]. The greater the number of unique MOAs involved in a rotation then the greater likelihood there is of delaying the evolution of resistance [78]. Where a pest is resistant to multiple compounds, IPM becomes more complicated as the number of measures for control is reduced. It is exactly this scenario that IRM aims to avoid with IRAC coining the phrase “diversity is the spice of resistance management” [61].

It is widely accepted that the evolution of resistance is fuelled by the inappropriate or ill-advised use of pesticides. This is particularly prevalent in the developing world where farmers are likely to have differing views on the use of pesticides and regulation may be more relaxed [79]. It is likely that a farmer in these circumstances will hold the volume of crop production (and hence value) as by far the most important factor, with this especially viewed in the short-term, rather than holding consideration for the 'long-term' goals of pest management or IRM. In any case, a study by Castle *et al*. assessing and comparing insecticide use strategies on a 'field scale' rather than laboratory experiments found that it was not possible to statistically differentiate between different regimes in the control of *Bemisia tabaci*. The authors demonstrate the difficulties in quantifying a strategy in an external environment in order to make a fully informed strategy decision [80]. This concurs with Sawicki and Denholm who previously reviewed case studies of the management of resistance to pesticides in cotton pests finding no clear winning strategy, unless specific criteria were met [65]. The research all lends weight to the conclusion that the success of an IRM strategy will depend on many factors including the mode of action of any pesticides used, potential mechanisms of resistance to them, previous insecticidal selection pressure or fitness costs to resistant phenotypes [81]. Therefore, any strategy employed should not only incorporate knowledge of the biology and ecology of target pests, but should also make use of any and all other approaches available through IPM.

1.6 Diamondback Moth (*Plutella xylostella*)

1.6.1 Biology and Life Cycle

The life cycle of *P. xylostella* is shown in fig 1.06 and starts with a female moth laying approximately 200 eggs onto the host plant. Eggs hatch into larvae and progress through four larval instars. Following the fourth instar, *P. xylostella* form a pupa surrounded by and held to the underside of a leaf (or other) surface by a silk cocoon where it undergoes maturation into the adult form.

Temperature is the most important factor affecting insect growth, rate of development and survival [82]. With optimal diamondback moth development temperatures between 10^{o}C and 30^{o}C, and a life cycle (fig 1.06) ranging from approximately 13 to 72 days, it is possible

for up to 25 generations to take place in a year [16, 83] with a higher number of generations expected the warmer the region [84]. Therefore, diamondback moth is a particular problem in the tropics and subtropics where year-round brassica cultivation makes this pest a continuous threat to production [83]. In more temperate regions where host crops are not grown continuously, alternative weed hosts are important for sustaining diamondback moth populations in spring before cruciferous crops are planted and become available to *P. xylostella* as its primary host [85].

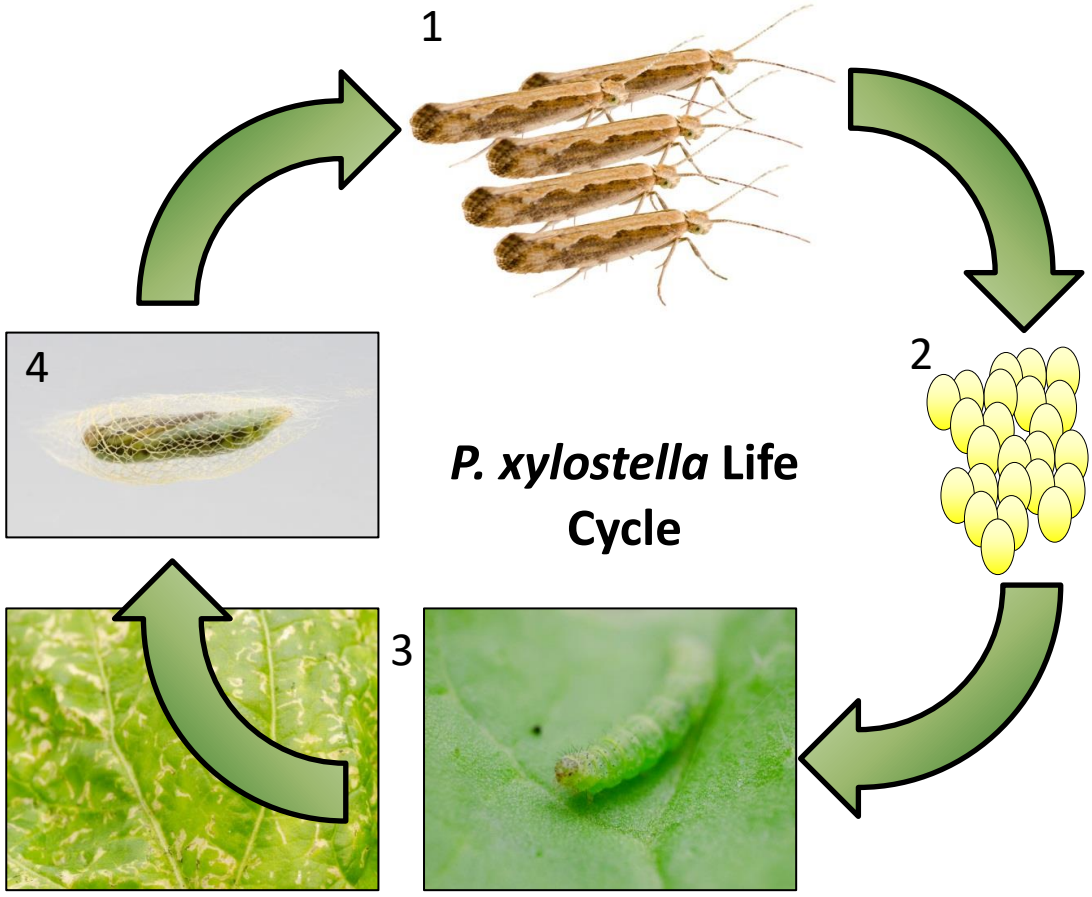


Fig 1.06. Life cycle of *Plutella xylostella*. 1-2. Female adults lay up to 200 eggs; 3. Larvae grow through four larval instars; 4. Larvae form a pupa within a cocoon before emerging as adults.

Crucifers contain high levels of glucosinolates which are absolutely linked with isothiocyanates as a plant defence mechanism through the glucosinolate-myrosinase

system. Glucosinolates and myrosinases are both present, but physically separated within plant tissues; they only come into contact when the plant tissue is damaged. Upon this contact glucosinolates are hydrolysed by the myrosinases into a suite of biologically active compounds including isothiocyanates [86]. To generalist herbivorous pests both of these chemicals act as deterrents and/or are toxic, however, *P. xylostella* (in addition to some other insect species [87]) has evolved to cope with these plant defences by employing glucosinolate sulfatase, an enzyme with a wide glucosinolate substrate range to prevent the formation of toxic hydrolysis products [88]. In fact it has long been documented that *P. xylostella* is a specialist feeder of, and is attracted to, plants containing high levels of the glucosinolates [16, 89], and that isothiocyanate, stimulates oviposition [90] (fig 1.07).

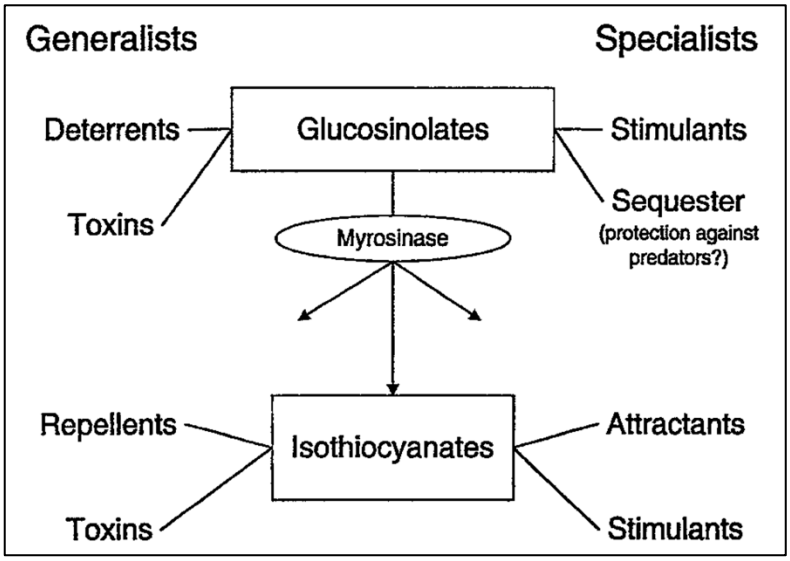


Figure 1.07. Comparative effects of glucosinolates and isothiocyanates on non-adapted (generalist) and adapted (specialist) insects demonstrating the advantages conferred by specialisation [91].

1.6.2 Crop damage and economic Impact

P. xylostella is the most destructive insect pest of cruciferous plants throughout the world and is found in temperate and tropical climates wherever this plant group occurs [85]. It is the larval stage which causes crop damage through the direct feeding of foliage throughout its four larval instars. Starting with the spongy mesophyll leaf tissue and then going on to consume the plant from the lower (abaxial) leaf surface, usually *P. xylostella* larvae generally

leave only the uppermost waxy leaf surface layer which can look like a 'window' between the leaf veins [85]. In the early 1990s Talekar and Shelton stated that the annual cost of managing diamondback moth was U.S. $1 billion [85], however Zalucki *et al.* have updated this estimate and also include yield losses and total cost to the world economy. This revised estimate is U.S. $4 billion – U.S. $5 billion [92]. The primary impact of diamond back moth in relation to food security is the destruction of core food crops. This could affect food availability and increases in cost of control measures could also impact on the price of the affected food commodities.

1.6.3 Control

1.6.3.1 Natural enemies & Refuges

Sarfraz *et al*. review that worldwide, over 135 parasitoid species have been recorded to attack various stages of *P. xylostella*. The most common of these are 6 species of egg parasitoids, 38 larval and 13 pupal parasitoids. According to this review, the principle biocontrol families are shown in table 1.01. While other predators such as lacewings, beetles and spiders and birds will also predate upon *P. xylostella*, these generalist predators would not normally be considered significant *P. xylostella* predators [93].

Life Stage	**Parasitoid**
Egg	*Trichogramma* spp.
	Trichogrammatoidea spp.
Larva	*Cotesia* spp.
	Diagema spp.
	Microplitis spp.
	Oomyzus spp.
Pupa	*Diadromus* spp.
	Pteromalus spp.

Table 1.01. Principle biocontrol parasitoids of *P. xylostella* showing which lifestage each parasitizes [93].

The introduction of a non-endemic enemy can be very successful [94, 95], but this should be employed whilst being mindful of potential consequences such as endemic species displacement [94]. Kirk *et al*. found evidence for the likelihood that multiple strains of the same species of natural enemies exist. Originating from different geographic locations, these are somewhat specialised through behaviour or biology to specific strains of *P. xylostella* coming from the same areas. The authors conclude that this should be a consideration in the selection and release of natural enemies in IPM of *P. xylostella* [96].

Furlong *et al*. made an attempt to quantify the impact of natural enemies on *P. xylostella* in a field environment. Despite relatively low levels of *P. xylostella* during the course of the experiment they found that, at times the impact of natural enemies could be very significant [97]. Following up this study a few years later, the same authors investigated the impact of different IPM strategies on endemic (arthropod) natural enemies [98]. The strategies employed by the farmers ranged from prophylactic application of broad-spectrum pesticides through to sophisticated methods that proactively conserved natural enemies. As would be expected, the study confirmed the effectiveness of IPM in working in unison with natural enemies. The study reports that there was no significant difference in crop yield between sites where there was considerably reduced insecticide input as part of the IPM strategy, compared to those where it was more indiscriminately used. This study supports the potential of natural enemies in the control of *P. xylostella*, but confirms along with Liu *et al.*[99] that this is really only the case where natural enemies are embraced and considered as part of the IMP framework whereby they are not detrimentally affected by the use of pesticides.

1.6.3.2 Synthetic Pesticides

Compounds used for the control of *P. xylostella* will vary from country to country, however, the list of compounds currently registered for use against *P. xylostella* in the United Kingdom are shown in table 1.02 below. At the time of writing, 12 active ingredients are registered for use against *P. xylostella* [100], comprising 6 IRAC modes of action. Fifty percent of the active ingredients are from group 3A, the pyrethroids and pyrethrins, which are sodium channel modulators. *Bacillus thuringiensis* (*Bt*) make up the next largest group, with only one active ingredient approved for each of the spinosyns, benzoylureas, oxadines and diamides. It

should be noted that these are the approved active ingredients and so is not a reflection of market share as many products can make use of the same active ingredient.

Active Ingredient	Example Products	IRAC MoA Group	Example Approvals for outdoor crops
Alpha-cypermethrin	Alert (MAPP 13632)	3A	Lettuce, baby leaf production, herbs, brassicas
Cypermethrin	Cythrin 500 EC	3A	Brassicas (not kale/collards), carrot, red beet
Deltamethrin	Decis	3A	Brassicas, baby leaf production, lettuce, carrot
Esfenvalerate	Sven	3A	Brassicas (not kale/collards)
Lambda-cyhalothrin	Hallmark with Zeon Technology	3A	Lettuce, baby leaf production, herbs, lettuce, red beet, carrot
Pyrethrins	Pyrethrum 5EC	3A	Edible crops
	Cyperkill 10 (MAPP 13157)	3A	Brassicas, (not collard), red beet, baby leaf production, herbs
Spinosad	Tracer	5	Brassicas, lettuce, herbs, baby leaf production
Bacillus thuringiensis var. *aizawai* GC-91	Agree 50 WG	11A	Red beet
Bacillus thuringiensis var. *kurstaki*	Lepinox Plus	11A	Brassicas, baby leaf production, herbs
	Dipel DF (MAPP 14119)	11A	Brassicas, baby leaf production, carrot, herbs, red beet
Diflubenzuron	Dimilin Flo	15	Baby leaf production, herbs, lettuce, brassicas (not kale/collards)
Indoxacarb	Steward	22A	Brassicas, baby leaf production, herbs
Chlorantraniliprole	Coragen	28	Carrot, red beet

Table 1.02. Control options for *Plutella xylostella* in the UK (current as of 22/06/2016). Table shows the IRAC mode of action group and typical crops they are used on. Adapted from compilation made by AHDB and obtained via 'Croprotect' [100].

1.6.3.3 Resistance *in P. xylostella*

Diamondback moth was not regarded as a major pest of crucifers until the introduction of synthetic insecticides in the 1950s [85]. The development of insecticide resistance has been a forte in the evolution of the diamondback moth. *P. xylostella* was the first crop pest to develop resistance to both DDT [101] and *Bt* [102, 103] in 1953 and 1988 respectively, and it continues to quickly develop resistance to modern compounds with different modes of action [64] [104].

The rapid failure of new insecticides for *P. xylostella* control in many countries is driven by the widespread lack of adoption of insect resistance management (IRM) by farmers where *P. xylostella* is a significant problem. The over-use of a new compound as soon as it becomes available, with a lack of regard for IRM, can result in diamondback moth populations developing resistance to a compound in as little as 2 years [105]. This high level of selection pressure through the overuse and non-rotation of synthetic pesticides is compounded by the high fecundity of diamondback moth and results in driving resistance in this species and elevating its pest status [83, 105]. Merely two years after a baseline study reporting relatively low levels of resistance to chlorantraniliprole in 2008-2009 [106], a study was published confirming high levels of resistance in field populations [63]. According to data held in the 'Arthropod Pesticide Resistance Database' *P. xylostella* is resistant to 94 active ingredients spanning all major regions where crucifer crops are grown. As not all geographic populations of *P. xylostella* are resistant to all compounds it is fundamentally important that monitoring studies are used to determine the resistance status of local populations [63, 64, 107, 108]. This information then enables informed decisions regarding IRM and IPM strategies to be made.

1.6.3.4 GM (*Bt*) crops

In contrast to synthetic pesticides, both sprays and genetically modified (GM) crops have been developed to *express Bacillus thuringiensis (Bt)* insecticidal proteins. *Bt* was first discovered through its association with disease in silkworm (*Bombyx mori*) at the turn of last century in 1901 by Shigetane Ishiwata [109], however it was not fully described until 1915 by Ernst Berliner [110]. It was not until the mid 1900's that a renewed interest in this 'bioinsecticide' made a resurgence, whereby details of its structure were starting to be

understood [111, 112] and more comprehensive experiments undertaken investigating its insecticidal potential [113-115]. It is during the sporulation phase of *Bt*'s life cycle that it 'acquires' its insecticidal properties and potential for use as an insecticide. As the bacterium sporulates it produces a spore and parasporal body composed mainly of one or more insecticidal crystal proteins [116] which are selectively toxic to different species of several invertebrate phyla, from Lepidoptera (butterflies and moths) through Diptera (flies and mosquitoes), Coleoptera (beetles and weevils) to Hymenoptera (wasps and bees) [116]. Even the non-insect species *Caenorhabditis elegans*, a nematode has been shown to be susceptible [117]. While *Bt* toxins are known to be very specific, Zhong *et al.* characterised the toxin Cry1Ba as being toxic to three orders [118]. An electron micrograph of a sporulating *Bt* cell can be seen in figure 1.08.

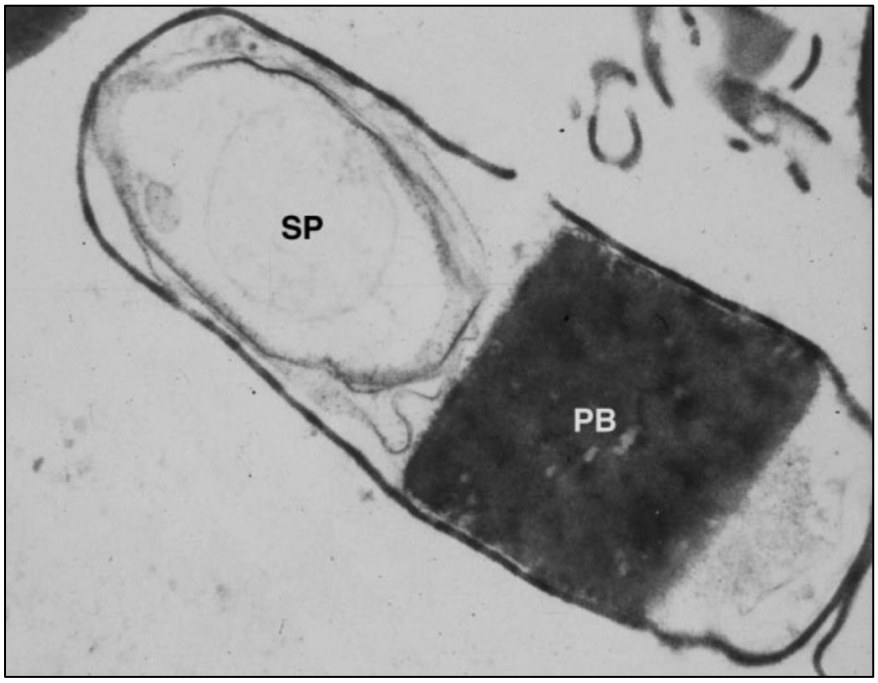


Figure 1.08. Transmission electron micrograph of a sporulating *Bacillus thuringiensis (Bt)* cell. The crystal protein body (PB) and spore (SP) will be released upon rupturing of the vegetative cell wall and become active upon ingestion by target species. Image taken from de Maagd *et al.* [116].

In their 'natural' form, the crystal proteins are relatively harmless, it is only once ingested by their relevant species that they become solubilised and cleaved by proteolytic enzymes that

they become active toxins [119]. It is the very specific nature of these toxins and therefore non-toxicity to non-target organisms, coupled with their low persistence as they degrade rapidly in the environment [120, 121] that has made them popular. It is the lack of persistence that in some cases is likely to have contributed to the overuse of *Bt* as a pesticide, at least in spray form. As with other pesticides, it is likely that it was this overuse that fuelled and enabled *P. xylostella* to evolve resistance to this pesticide, with cases of resistance in the field showing up in the early 1990's [122].

1.6.3.5 GM moths

Genetically modified crops are potentially now not the only GM method of control for *P. xylostella*. The release of genetically modified male-selecting *P. xylostella* [123] is essentially an extension of the previous 'sterile insect technique' (SIT). In a review of this approach, Krafsur describes the fundamental principle of SIT as "Where strong density-dependent phenomena do not occur and high frequencies of sterile matings occur, target populations become progressively smaller, the sterile male to fertile male ratio increases, sterile mating rates increase, and the target population eventually collapses" [124]. In 2003, Benedict *et al*. reviewed the argument for the integration of transgenics in SIT [125], and this is now moving ever closer to reality. In this new approach, rather than insects being sterilised prior to release via irradiation, chemosterilisation, cytoplasmic incompatibility or another approach [126], they are genetically modified to express a male-selecting transgene, resulting in an inherited female-specific lethality [127]. The lethal gene is supressed by feeding on a diet containing tetracycline, in the absence of this antibiotic, females are killed leaving only males to go on to reproduce with a wild pest population. An additional benefit associated with this approach is the dilution of any resistance that is already present within the targeted pest population as released insects are derived from a susceptible strain of *P. xylostella* [123]. This potential *P. xylostella* control approach is built on methods developed for *D. melanogaster* by Thomas *et al*. who named the approach "release of insects carrying a dominant lethal" (RIDL) [128]. To date the work involving *P. xylostella* has taken place in controlled experiments and there has yet to be a published field trial, however, field trials have taken place for the mosquito *Aedes aegypti,* where field populations have been reduced by up to 95% through the sustained release of transgenic male mosquitos clearly demonstrating the potential of this approach [129-131].

1.6.3.6 RNAi

Although not yet in use against *P. xylostella*, RNA interference (RNAi) may be a potential approach for control in the future. Studies have shown RNAi mediated suppression of genes conferring resistance in *P. xylostella* under laboratory conditions through feeding and injection experiments [132-134]; however, the practical hurdles in delivering a technique such as this in the field are significant. In addition to RNA interference concentrating on *P. xylostella* genes, an approach could also be adopted whereby RNAi is used to manipulate expression of key crop host genes, effecting pathways to which *P. xylostella* has become resistant [135].

1.6.3.7 Combinations

Liu *et al.* investigated the use of genetically modified crops expressing insecticidal *Bt* proteins in combination with a natural enemy (*Coleomegilla maculate*) and refuges that were insecticide sprayed or unsprayed [99]. In results that could have implications for the future planning of *P. xylostella* IRM and IPM, the authors found that a combination of the presence of *C. maculate*, and non-B*t* (unsprayed) refuge plants delayed the development of resistance compared to treatments without the predator. In these circumstances, it was found that the inclusion of a refuge was important to delay the evolution of resistance, but that it was also important that this was untreated so that beneficial insects were not killed.

1.6.4 Genetics

1.6.4.1 Genetic resources

During the course of this PhD, two major genomic resources have become available for *P. xlylostella*. The first, draft DBM-DB (diamondback moth database) created by You *et al.* in 2012 [136] was made publicly available as an online genome database (http://iae.fafu.edu.cn/DBM/) in 2013 [137]. The second, is also an online database, 'KONAGAbase' (konaga is the Japanese word for diamondback moth), published by Jouraku *et al.* in 2013; providing transcriptomic and draft genomic sequence and annotation data through a web-based platform (http://dbm.dna.affrc.go.jp/px) [138].

The authors of the DBM-DB used whole-genome shot-gun based Illumina sequencing of individuals from a field population collected from Fuzhou, South-eastern China (Fuzhou-S). Following ten generations of inbreeding, the authors found that the *P. xylostella* genome has a high level of heterozygosity which resulted in an initial poor assembly [136]. Subsequently the authors sequenced 100,800 fosmid clones, comparing them to a set of protein-coding ESTs generated by transcriptome sequencing by He *et al.* [139] and also a bacterial artificial chromosome from an alternative strain. Within the Fuzhou-S strain, the authors found high polymorphism levels including SNPs, indels, and complex segmental duplications, eventually generating a genome of ~343 Mb. In comparison to the genomes of other sequenced insect species, the authors determined that *P. xylostella* has an expanded set of genes (18,071 protein coding genes), with 1,412 being specific to this species. These are associated with information processing, chromosomal replication/repair, transcriptional regulation and carbohydrate and protein metabolism, with the authors suggesting that these additional genes may confer an advantage in adapting to environmental stresses and genetic damage [136]. The study by He *et al.* used the transcriptome they created as a resource to investigate homologs of insecticide targets and detoxification enzymes finding that all of the chemical insecticide and *Bt* resistance related genes from the transcriptome were the same as those from the whitefly, *Bemisia tobaci* and tobacco hornworm, *Manduca sexta* [139]. The authors also identified the expansion of other gene families associated with metabolic detoxification of insecticides, the cytochrome P450s and carboxylesterases. Their analysis found an additional 13,309 unknown unigenes that may represent *P.xylostella* specific transcripts, untranslated regions and/or errors in their assembly. The study by You *et al.* also investigated detoxification pathways and resistance-related gene families finding that in comparison to *Bombyx mori* (which, being relatively domesticated, has been exposed to relatively less insecticide), *P. xylostella* possessed an overall larger set of insecticide resistance-related genes. These included apparent gene duplications of cytochrome P450s, glutathione S-transferases and carboxylesterases [136].

The later KONAGAbase publication made use of combinations of sanger, RNA-seq and Roche 454 GS FLX next generation sequencing of cDNA libraries from midgut, egg and testis and whole body DNA preparations from a *Bt*-susceptible laboratory strain, PXS. In this instance, the authors estimate that the genome size of *P. xylostella* is ~370 Mb, and through clustering of 84,570 unigenes and 34,890 predicted gene-coding sequences, 32,800 sequences were extracted as a putative gene set [138].

A later publication by the same research group that created the DBM-DB, detailed a second version (release 1.2) of the DBM-DB intended to coordinate and integrate data sets from other sources including the putative gene set generated by Jouraku *et al*. from KONAGAbase [137, 138]. As intended by the authors, by becoming a coordinated, 'central' platform, the DBM-DB likely becomes the primary source of *P. xylostella* genome information. Indeed, at the time of writing, KONAGAbase is not available online (although it is not known if this is temporary or permanent).

1.6.4.2 Population genetics

There have been several studies focusing on population genetics of *P. xylostella* as migration and gene flow can have profound implications for IRM within this species. As demonstrated by the publications detailing the development of genomic databases for this species, it seems that *P. xylostella* has a high level of variation [136]. A conclusion confirmed by other more specific studies such as that by Wei *et al.* [140]. In their study, the authors used population genetic approaches to investigate migration patterns of *P. xylostella* within China. By analysing 27 populations for four mitochondrial genes and nine microsatellite markers the authors found a migration of genetic material from southern to northern regions, but rarely in the opposite direction [140]. Endersby *et al.* also used microsatellite loci to investigate population structure and gene flow between populations primarily within Australia, but also investigating populations from New Zealand, Malaysia, Indonesia and Kenya [141]. While populations from Asia and Kenya were statistically different from the Australasian populations, despite distances of over 3600 km between Australian sampling sites and over 5500 km between the Australian and a population sampled from the north island of New Zealand, no differentiation between these populations could be found. The authors put forward that part of the explanation could be the relative recent introduction of this species to the continent, but they also suggest that widespread gene flow through migration may also account for the homology between the populations. Further studies have been carried out investigating genetic variation between populations within the United States [142] and also within Korea, finding no genetic differentiation, but an increased number of haplotypes indicating a high gene flow within a geographic area [143]. Specifically relating to diamide resistance, Steinbach *et al.* collected *P. xylostella* larvae from 11 different countries (Australia, Indonesia, India (Karnataka), Korea (Bongnam-ri), India (Tamil Nadu), Japan, Korea (Sangan-ri), Philippines, Taiwan, Thailand, USA, and Vietnam) between 2012 and 2014, using

pyrosequencing to genotype the populations for the G4946E ryanodine receptor mutation reported as conferring resistance to diamides [144, 145]. Resistant genotypes homozygous for the G4946E mutation were found in 9 of 11 countries, with Australia and Indonesia being the countries where no 4946E homozygotes were found. Having sampled the populations from areas where diamides had proven less effective in controlling the *P.* xylostella (and where diamide control had proven more successful in Australasia), the authors assume that the mutations evolved independently in different geographic locations, going on to use this as support for the mutation conferring diamide resistance [144].

1.7 Resistance Mechanisms

To date IRAC have described 29 different insecticidal modes of action. While there are several ways by which organisms can become resistant to pesticides (and xenobiotics in general), they can be distilled down to two main categories: 1) by modifying the effective dose of the pesticide at the target site; or 2) modification of the target site itself [146]. Of the four main resistance mechanisms, three of them (penetration, behavioural and metabolic) fall into the first category, while target site resistance falls into the later. It is common for resistance to exist through more than one mechanism [147], and where multiple mechanisms do exist, it is possible that higher levels of resistance will be seen comparedto single mechanisms [148-150].

1.7.1 Penetration resistance

Before a pesticide can affect its target it must first penetrate the insect. Penetration of an insecticide into a pest species could be through the cuticle or digestive tract lining [148], and hence any adaptation of an insect that affects the efficiency through which a pesticide can gain access to its target site is likely to confer some level of resistance. Chitin content of an individual tissue can vary depending on the species of insect and tissue function, however it must be maintained at precise levels for the proper growth and development of an organism [151]. Pesticides targeting chitin synthesis have long been developed, primarily focusing on the chitinases and chitin synthase inhibitors [151, 152]. An increase in cuticle thickness or decrease in permeability might result in a level of cross-resistance to pesticides as has been found in a strain of house flies (*Musca domestica*) [153], although the penetration rate of a pesticide is dependent on its chemical structure and hydrophobicity [154].

There are many examples of reduced penetration or absorption conferring insecticide resistance across many different species [155]. Recent examples using modern techniques include the analysis of the cuticle structure and thickness using electron microscopy and radiolabelling, and have shown in resistant fruit flies, *Bactrocera dorsalis*, that modifications to these elements can confer resistance by decreasing the rate of penetration [156]. Other studies have made similar conclusions by examining the sites of the expression of cuticular proteins in the mosquito, *Anopheles gambiae* [157]; and by transcriptome analysis of pyrethroid resistance in the bed bug, *Cimex lectularis* [150]. Very recently, a paper by Balabanidou *et al.* described the cellular locations of two cytochrome P450s, *CYP4G16* and *CYP4G17* within resistant mosquitos, *Anopheles gambiae.* Interestingly, rather than being implicated in metabolism of insecticide, as is often the case, *CYP4G16* is situated in the periphery of oenocyte cells, on the cytoplasmic side of the cell membrane. *In vitro* expression experiments showed *CYP4G16*, which is overexpressed in resistant strains of mosquitoes, is involved in the biosynthesis of hydrocarbons, and may be wholly, or in part, responsible for changes to the hydrocarbon composition of the cuticle of resistant mosquitoes [158].

1.7.2 Behavioural resistance

According to Georghiou [159], the expression of behavioural resistance occurs via two main routes: 1) Stimulus-dependent behaviouristic avoidance; or 2) Stimulus-independent behaviouristic avoidance. The former is the avoidance of an environment or host following sensory stimulation; and the later refers to a natural avoidance by a proportion of the population.

While stimulus-dependent behavioural responses are more readily tested, stimulus-independent studies are less frequently reported. Guedes et al. investigated behaviour in the maize weevil *Sitophilus zeamais,* in response to deltamethrin, finding concentration-independent responses and that within the strains tested, stimulus-independent behavioural resistance was unrelated to physiological resistance [160]. Working on the same species, Haddi noted the co-occurrence of metabolic and behavioural resistance mechanisms which might compromise insecticide efficacy [161]. The tomato leaf miner *Tuta absoluta* has shown avoidance of egg-laying in the presence of the 'biorational' pesticide Azadirachtin in a free-

choice test, however, the same was not true under no-choice conditions. The authors speculate that this pesticide could be used in a push-pull IPM strategy, most likely useful in a small-scale farming scenario [162]. One of the most striking examples of behavioural resistance is that displayed by *Anopheles* species, vectors of malaria. Several studies have noted behavioural adaptations employed by these mosquitoes in response to selection pressure exerted on them through the spraying of insecticides and use of long-lasting insecticide-treated bed-nets (LLINs). These studies have found that while historically these mosquitos have fed on hosts indoors during the night, their behaviour has shifted significantly to feeding outdoors, and earlier in the evening or later in the morning so that their feeding now occurs before or after their hosts (people) are protected by the cover of insecticide impregnated netting or spray protected homes [163-166].

Stimulus-dependent behavioural resistance does not necessarily dictate that a pest will be 'detecting' cues from the presence of the pesticide itself, as demonstrated by a gel bait-resistant German cockroach, *Blattella germanica* [167]. This study found that the 'resistant' strain specifically avoided the combination of phagostimulant ingredients that constituted the bait gel containing the pesticides even in the absence of the pesticides. A similar response was found in *Anopheles atroparvus* that had been selected (using DDT) for an 'irritated' (one of escape) behavioural response [168]. After 32 generations the authors found that either DDT or its solvent greatly enhanced the irritation response [169].

1.7.3 Metabolic resistance

One of the major mechanisms by which insects can evolve resistance to pesticides is through enhanced metabolic detoxification of these compounds. Within pest insect species (and indeed non-pest insects) it is likely that the ability to cope with a toxin rich diet, such as that which comes through feeding on plants, has served as a preadaptation for the acquisition of metabolic insecticide resistance [170]. Many synthetic pesticides resemble plant allelochemicals, or in some cases are directly derived from them, for example the pyrethroids. Therefore, it is likely that enzymes such as cytochrome P450s that can detoxify insecticides [171] evolved from the P450s that originally evolved to detoxify plant allelochemicals [172]. It has been reported by Li *et al.* in *Helicoverpa zea*, that four cytochrome P450s have evolved from common ancestry through gene duplication events

and are inducible (in differing bodily locations) by plant allelochemicals, but also by a drug (phenobarbital) and the insecticide, alpha-cypermethrin [173]. The nature of metabolic resistance, with enzymes often having multiple substrates, can lead to cross-resistance between compounds. This has been demonstrated by Daborn *et al.* in two publications showing a cytochrome P450, *CYP6G1*, confers resistance to both DDT and the neonicotinoid, imidacloprid in *D. melanogaster* [174, 175]. Methodologies by which individual or groups of enzymes are identified and implicated in insecticide resistance varies greatly. Methods have involved the use of synergistic compounds which inhibit specific enzymatic families, rendering resistant populations more susceptible to insecticides in bioassays, such as was employed by Li *et al.* in *Boophilus microplus* [176]. Sun and Ingham both used genetic microarray technologies with qPCR validation to interrogate gene expression variance, with the former studying expression between strains on a whole organism level in *D. melanogaster* [177], and Ingham focusing on expression specificity between organs in *Anopheles gambiae* [178]. Having previously found a candidate gene, *CYP6BG1*, conferring permethrin resistance, Bautista *et al.* used RNA interference mediated knockdown to render *P. xylostella* larvae more susceptible to permethrin [134]. Transgenic approaches such as that carried out by Cui *et al*. and Daborn *et al.*, where *D. melanogaster* were transformed to overexpress candidate detoxification genes have also been employed [179, 180]. The genetic changes that allow metabolic enzymes to confer resistance have been identified as gene duplications/amplifications, *cis*- or *trans*-acting alterations that act as transcriptional enhancements and finally mutations in enzyme coding sequence that result in enhanced metabolism, sequestration and excretion of xenobiotics [172]. The upregulation of detoxification genes is commonly mediated by insertions or deletions in *cis*-acting elements in promoter regions. For example, the presence of an *Accord* long terminal repeat insertion in the 5' end of the P450, *CYP6G1*, causes up regulation of this enzyme, conferring resistance to DDT and imidacloprid [174, 181]. Point mutations can also exist within gene sequences causing amino acid substitutions as demonstrated by Amichot *et al.* [182]. The authors demonstrated, through *Escherichia coli* expression of multiple combinations of three different point mutations in the P450 *CYP6A2*, that these modifications enhanced the metabolism of DDT compared to the wildtype. Three of the main classes of metabolic resistance are cytochrome P450 monooxygenases, glutathione-S-transferases (GSTs) and Choline/Carboxylesterases (CCEs). More details on these are provided below, along with some lesser well known detoxification enzyme families of relevance to this study.

1.7.3.1 Cytochrome P450s (P450s)

P450s are a metabolically important group of enzymes found in virtually all aerobic organisms. P450s are a large superfamily of enzymes with insect genomes reported to often have over 100 P450 genes [183]. Heavily involved in the catabolism and anabolism of xenobiotics such as drugs, plant toxins and pesticides P450s can oxidise a wide array of substrates [171, 184]. In insects, P450s have been shown to metabolise a wide diversity of insecticides including DDT, pyrethroids, neonicotinoids and carbamates [185-188]. In eukaryotes, cytochrome P450s are typically found in the endoplasmic reticulum and mitochondria, where, when they are implicated in insecticide resistance, they are often expressed in the head and midgut and fatbody regions of insects [134, 189]. This makes biological sense considering the common entry-point for insecticides and the requirement to protect the insect brain. Resistance mediated by P450s most commonly occurs as a result of their overexpression. However, in certain cases it is possible that a decrease in P450 metabolism could result in increased resistance where the enzyme is involved in bioactivation of a 'pro-insecticide' [190]. Less commonly P450 resistance has been shown to result from qualitative changes in amino acid sequence rather than quantitative changes in expression [182].

1.7.3.2 Glutathione-S-transferases (GSTs)

Typically, where they are a contributing factor, glutathione-S-transferases (GSTs) confer resistance to xenobiotics by catalysing the conjugation of the xenobiotic with glutathione (GSH), leading to the formation of less reactive product that is more readily excreted [191]. It has been reported that, as with cytochrome P450s, the substrate specificities of GSTs are broad [192] and have been shown to confer resistance to organophosphate insecticide, benzoylureas, DDT and pyrethroids [193-196]. GSTs have also been implicated in resistance to naturally occurring toxins, despite no ecological reason to come into contact them. Mitchell *et al.* describe strains of *D. melanogaster* that were resistant to α-amanitin, a toxin produced by mushrooms, where GSTs (alongside other putative detoxification genes) were implicated in contributing to the resistance [197]. The GST contributions to insecticide resistance have been recently described in lepidopteran, *Plutella xylostella* [194], dipterans, *Anopheles gambiae* [195] and *Aedes aegypti* [198], and coleopteran, *Tenebrio molitor* [196], species demonstrating the commonality of this mechanism across insect groups.

1.7.3.3 Carboxyl/Cholinesterases (CCEs)

Carboxylesterases and cholinesterases catalyse the hydrolysis of carboxylic and choline-based esters respectively with the resulting metabolic products being carboxylates or cholines (along with acid and alcohol by-products). These products pass through further metabolic pathways to be conjugated by other enzymes, following which their solubility is increased and they are able to be more readily excreted. The enzyme, acetylcholinesterase (AChE), a primary cholinesterase, plays a key role as a 'terminator' in synaptic transmission. As such it is the predominant target for organophosphate (OP) and carbamate insecticides. As new enzyme functions and adaptations can be obtained by substitution of only one or very few amino acid changes in these enzymes [199], this group of enzymes are frequently implicated in insecticide resistance, with 12 different target site mutations already discovered conferring resistance to OPs and/or carbamates (number based on compilation of literature made by IRAC). The genetic mutations found within AChE fall into the category of target site resistance (see section 1.7.4), however, carboxylesterases have also been implicated in insecticide (OP) resistance through enhanced metabolic-based mechanisms. The detoxifying carboxylesterases ('A' and 'B'), found on chromosome II of *Culex pipiens* at loci *Est-2* and *Est-3*, confer OP resistance by being overproduced through gene amplification or gene upregulation [200, 201]. OPs and carbamates are often, in effect, inhibitors of esterases because they are poor substrates, but have a high affinity for these enzymes, producing a metabolic bottleneck in the 'natural' metabolic mechanism and overwhelming AChEs. If they are overproduced, esterases can cause insecticide resistance by rapidly sequestering OPs and carbamates, before they reach an insect's AChE [202].

1.7.3.4 Short-chain Dehydrogenases (SCDs)

Modern genomic techniques have confirmed previous thinking that short-chain dehydrogenases/reductases (SCDs) constitute a large family of gene products that are important across all organisms [203, 204]. There is little sequence similarity between SCDs, but there is a large degree of structural similarity [204, 205]. SCDs carry out an enormous number of biochemical roles, with many of them being intermediary metabolic functions. This diversity means that they are involved in many processes including regulation of cellular processes and hormones, and in the utilization and detoxification of ethanol and xenobiotics [204]. Due to the broad spectrum of SCDs and their substrates, Oppermann *et al.* speculate that some SCDs could actually have potential as target sites for new pesticides [206]. Menzel

et al. used DNA microarray techniques to identify significant changes in gene expression in the nematode *Caenorhabditis elegans* in response to polychlorinated biphenyls (PCBs), in this case, PCB52. Following RNAi screening of the primary candidates, the authors confirmed that knockout of SCDs resulted in hypersensitivity to PCB52 and were one of the enzymatic families responsible in the metabolic pathway processing this toxin, confirming an earlier finding by Hulsmeyer *et al*. [207, 208]. While not pesticides, these studies demonstrate the importance of SCDs in the metabolism of synthetic xenobiotic compounds.

1.7.3.5 Flavin-containing Monooxygenases (FMOs)

Flavin-containing monooxygenases have overlapping substrate specificities with P450s [209], and within mammals, have been known to be important in the metabolism of a number of different pesticides for a number of years [210]. Surprisingly, despite the interaction of FMOs with insecticides having been studied in vertebrates, very few studies have focused on insect FMOs and the role of this enzyme class in insects in consequently relatively poorly understood [211].

FMOs oxygenate the nucleophilic oxygen, nitrogen, sulphur and selenium atoms of a wide variety of substrates [209], examples of which, demonstrating their diversity can be seen in table 1.03.

Inorganic
HS^-, I^-, IO^-, I_2, CNS^-
Organic nitrogen compounds
Secondary and tertiary acyclic and cyclic amines
N-alkyl and N,N-dialkylarylamines
Hydrazines
Organic sulphur compounds
Thiols and disulphides
Cyclic and acyclic sulphides
Mercapto-purines, pyrimidines and imidazoles
Dithio acids and dithiocarbamides
Thiocarbamides and thioamides
Others
Phosphines
Boronic acids
Selenides
Selenocarbamides

Table 1.03. Examples of substrates oxidized by porcine liver FMOs. Table adapted from a compilation made by Ziegler, 1990 [212].

FMOs require NADPH and oxygen as co-substrates (in common with most other monooxygenases) in order to oxygenase a third substrate (the xenobiotic compound). However, the FMO catalytic cycle differs from other monooxygenases in that the third substrate (shown by 'S' in figure 1.09) is not needed for either the reduction of flavin by NADPH, or for the re-oxidation by molecular oxygen [212].

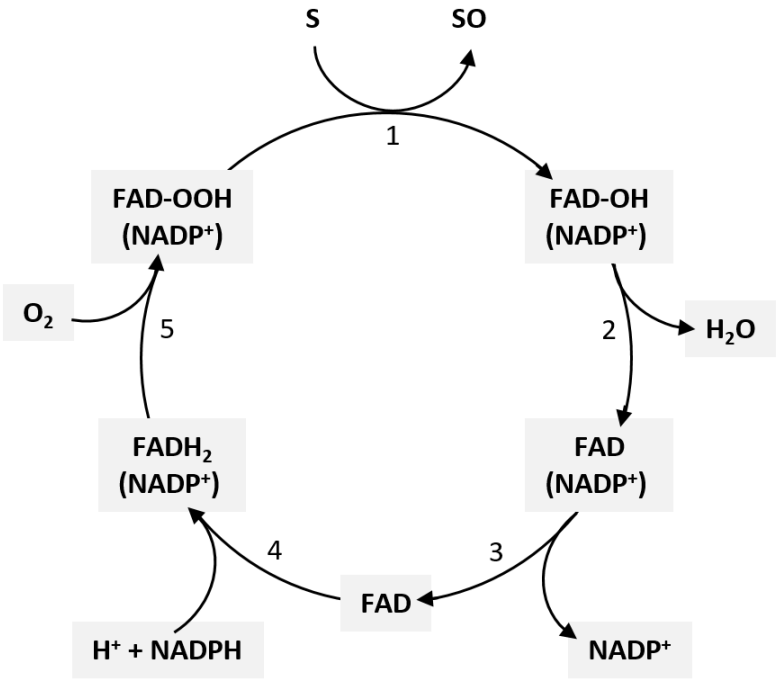


Figure 1.09. The catalytic cycle of FMO. Step 1. Nucleophilic attack of oxygenatable substrate (S) yields oxygenated product (SO). Steps 2-5. Regeneration of the enzyme-bound oxygenating agent from NADPH, O_2 and H^+ [212].

This leaves the FMO cycle 'poised' in a supposedly stabilized hydroperoxyflavin form with the enzyme ready to interact, oxidising a xenobiotic substrate as soon as it becomes available. Ziegler suggests that the FMOs need for only a single point of contact between a xenobiotic substrate and the enzyme-bound oxidant is explanation for FMOs being able to catalyse the oxygenation of structurally dissimilar compounds, in comparison to the more restricted cytochrome P450s, where these might require a more precise fit [212]. Rather than selectively binding xenobiotic compounds, FMOs seem to exclude metabolizing essential physiological nucleophiles. With a few exceptions, evidence suggests that a compound having a charged group may prevent entry to the FMO catalytic site [213]. In separate studies, Guo and Nagata both conclude that FMO substrate specificity seems to be affected by substrate size with differences being found between FMOs isolated from different organs, and which is likely related to different FMO isoforms [214, 215]. In mammals (in which most studies have taken place) five FMO isoforms have been found, the adopted nomenclature being founded by Lawton *et al.* following confusion of duplicate isoforms between both species and organ isolation [216]. While their implication in detoxification is now well

documented, little is still known about pesticide substrate specificity of individual isoforms. In 2002 Ziegler described that while the physiological substrate of mammalian FMO5 is not yet known, it is unlikely to be a significant xenobiotic oxidase [217]. However, in 2004 Hodgson *et al.* implicated mammalian FMO5 in the metabolism of four out of the five tested pesticides, including Fonofos, an organophosphate, methiocarb, a carbamate, and the neonicotinoids, nicotine and imidacloprid [218].

In 1997, Lindigeit *et al.* studied three mixed function monooxygenases (MFOs) and their relationship to the metabolism and sequestration of pyrrolizidine alkaloids (PAs), which insects come into contact through feeding. Due to their original non-descriptive MFO labels, the authors renamed the MFOs as senecionine N-oxgenase (SNO) enzymes derived from their newly ascertained metabolic function. Using radiolabeled substrates and enzymatic assays the authors found that the SNOs isolated from the lepidopteran generalists, *Creatonotos transiens* and *Arctia caja*, showed a broader substrate affinity than the SNO isolated from *Tyria jacobaeae*, an insect specialized to feeding on plants containing high concentrations of PAs [219]. Naumann *et al.* subsequently identified SNOs as part of the FMO gene family and confirmed FMOs as the primary route of PA detoxification in *T. jacobaea* [220]. Following this study, Sehlmeyer *et al.* identified several members of the FMO gene family from the tiger moth family (Arctiidae) and by comparison with FMOs from other insect species, defined three clusters (FMO1, FMO2 and FMO3) based on sequence homology. From this, the authors made predictions that the evolution of FMOs has been through gene duplication events [211]. Wang *et al.* detail that the grasshopper, *Zonocerus variegatus* has independently evolved an almost identical strategy in the sequestration of FMO for the safe accumulation of PAs [221].

A search of the *P. xylostella* genome database, DBM-DB [137] for FMO genes yields four hits, detailed in table 1.04 below. BLAST searching the coding DNA nucleotide sequences through the NCBI database provided information relating to each putative gene. Each hit/accession was an automated annotation, rather than one confirmed through specific publication. The annotations suggest that there may be two variant putative FMOs annotated to date, with NCBI annotations describing the same sequences as, and providing possible links to SNOs.

DBM-DB Gene ID	DBM-DB Annotation/Description	NCBI Accession ID	NCBI Annotation/Description
Px011619	Flavin-containing monooxygenase *FMO GS-OX3*; Similar to GSOX3_ARATH	XM_011565408.1	PREDICTED: *Plutella xylostella* senecionine N-oxygenase-like (LOC105393619), transcript variant X2
Px011620	Flavin-containing monooxygenase *FMO GS-OX3*; Similar to GSOX3_ARATH	XM_011565410.1	PREDICTED: *Plutella xylostella* senecionine N-oxygenase-like (LOC105393620), transcript variant X2
Px011621	Flavin-containing monooxygenase *FMO GS-OX3*; Similar to GSOX3_ARATH	XM_011565412.1	PREDICTED: *Plutella xylostella* senecionine N-oxygenase-like (LOC105393621)
Px015511	Flavin-containing monooxygenase *FMO GS-OX5*; Similar to GSOX5_ARATH	XM_011554543.1	PREDICTED: *Plutella xylostella* senecionine N-oxygenase (LOC105384325), transcript variant X1

Table 1.04. Putative FMO gene annotations from DBM-DB [137] and their corresponding NCBI accessions. All are derived from automated gene predictions with NCBI identifying them as putative SNOs.

A study by Tian *et al.* published in 2014 is the only paper to date making reference to an FMO as a putative gene conferring resistance to an insecticide within an insect [222]. In this study, using synergists, FMO is implicated alongside GST in conferring resistance in the beet armyworm, *Spodoptera exigua* to the insecticide, metaflumizone, a semicarbazone voltage-dependent sodium channel blocker belonging to IRAC group 22. While FMO is described as playing a part in the detoxification, the study stops short of providing conclusive evidence through further expression experiments.

1.7.4 Target site resistance

Different insecticides exert their toxic effect by targeting a variety of important insect proteins, with the majority of targeted receptors part of the insect nervous system. Mutation of one or more amino acid residues at a target site can alter its structure and affect the binding interaction of an insecticide, reducing its sensitivity to the compound (see fig 1.10).

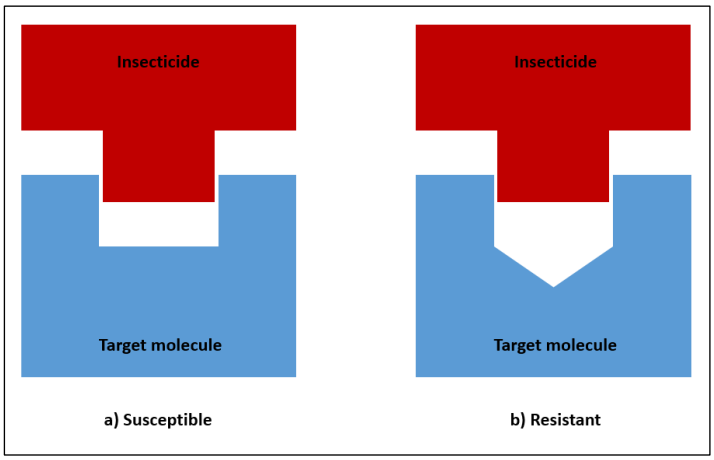


Figure 1.10. Diagrammatic representation of target site resistance. In the susceptible strain (a), the insecticide is able to interact fully with its target molecule. In the resistant strain (b), mutation of amino acids have altered the structure of the target site resulting in reduced binding and efficacy of the insecticide.

Target sites usually carry out vital functions and so most target site proteins are highly conserved, only tolerating a few select amino acid replacements without compromising normal functioning [223]. According to IRAC [224], mutations conferring insecticide resistance have been identified in 12 target sites covering 11 of the 29 modes of action (table 1.05). From the information in table 1.05 it is apparent that the greatest number of documented different mutations is highest for the insecticides targeting the sodium channel. This target site also has the greatest number of different species that have one or more of the mutations. The disparity in numbers for this group of insecticides is likely due to the number of pesticides within this class and length of time that they have been in use, coupled with the broad spectrum of insect classes that they affect.

IRAC MoA Group	Target Site	No. affected species	No. mutations
1A	Acetylcholinesterase (Carbamates)	4	11
1B	Acetylcholinesterase (Organophosphates)	4	8
2	GABA-gated chloride channel	6	3
3	Sodium channel	30	33
4	Nicotinic acetylcholine receptor	3	2
5	Nicotinic acetylcholine receptor	4	3
6	Chloride channel	2	4
10	Chitin synthase	1	1
20	Mitochondrial complex III electron transport	1	4
22	Voltage-dependent sodium channel blockers	1	2
23	Acetyl-CoA carboxylase	1	1
28	Ryanodine receptor	1	4

Table 1.05. Summary of established insecticide target site mutations associated with published cases of insecticide resistance grouped by IRAC mode of action. The 'no. affected species' is the number of species that have one or more of the mutations. Data is based upon review of literature compiled by IRAC [224].

It has been well documented that different species have evolved different mutations conferring resistance to the same target site. *Aphis gossypii* and *Tetranychus urticae* have multiple different mutations within the acetylcholinesterase target site conferring resistance to organophosphates [225-227]. It is also possible for species from different classes to convergently evolve the same resistance conferring mutation, such as the knockdown resistance (KDR) mutation L1014F in the sodium channel conferring resistance to pyrethroids in (amongst other species) *Musca domestica, Tuta absoluta* and *Myzus persicae* [228-230].

1.7.5 *Plutella xylostella* Resistance Mechanisms

P. xylostella is notorious for its ability to rapidly evolve resistance to a range of insecticides and is ranked second in the APRD resistance database of most resistant arthropods [231]. In their 2015 review Sparks and Nauen describe *P. xylostella* as being resistant to 95 different compounds [232]. This is second only to the two-spotted spider mite, *Tetranychus urticae* by number of compounds, and second to the cotton bollworm, *Helicoverpa armigera* in terms of the number of unique reported instances of resistance [61]. This species has developed target site mutations, some of which are in common with other species, but also novel target site resistance mechanisms. *P. xylostella* has developed mutations conferring insecticide resistance at the following target-sites: GABA-gated chloride channel [233], nicotinic acetylcholine receptor [234], glutamate-gated chloride channel [235], voltage-dependent sodium channel blockers [236] and multiple mutations in the sodium channel [237-240] and ryanodine receptors [145, 241].

Metabolic resistance is more complex to quantify than target-site resistance as levels of metabolic resistance are inherently more variable and so less well described in *P. xylostella*. Furthermore, a greater proportion of studies provide indicative evidence for the implication of metabolic resistance as opposed to target-site resistance which tends to be reported in a more conclusive manner. There is also likely to be a greater level of interaction between metabolic mechanisms of resistance which can complicate the picture as more than one enzyme might be interacting in a pathway, rather than acting independently.

In 1993, Chiang *et al.* isolated three glutathione transferase (GST) isozymes from *P. xylostella* (GST-1, GST-2 and GST-3), and using enzyme assays determined their activities against several substrates [193]. The authors found differences in substrate specificities and the presence of different proportions of the same three isozymes in susceptible and organophosphorus resistant strains of *P. xylostella*. The following year, Ku *et al.* isolated a fourth GST isozyme (GST-4) from *P. xylostella* [242]. When compared to the three GSTs already identified it was found to be very similar in terms of its biochemical and toxicological properties to GST-3, but less closely related to GST-1 and GST-2. GST-4 was isolated from a strain resistant to teflubenzuron, belonging to the benzoylurea class of insecticides, with the implication being that one or both isozymes might be involved in conferring resistance.

Further characterisation of GST-3 was made by Huang *et al.* who made comparisons to GSTs from other species, highlighted by other studies. Isolated from a methyl parathion resistant strain the authors concluded that it could be considered identical to the GST-3 characterised by Chiang *et* al. [193] providing further evidence as to its involvement in insecticide resistance [243]. Sonoda and Tsumuki also carried out a study of GST-3 in *P. xylostella*, but in this case in relation to chlorfluazuron resistance, another benzoylurea insecticide [194]. Through sequencing of the gene from resistant and susceptible strains, the authors found no amino acid mutations, suggesting that point mutations are not involved in resistance. The authors also found no evidence of significant differences in the 5'or 3' flanking regions that might explain the differences in gene expression.

The cytochrome P450, *CYP6BG1* was implicated in permethrin resistance in *P. xylostella* by Bautista *et al.* through RNA interference mediated knockdown, with Sonoda *et al.* later noting that in some populations, overexpression of this enzyme and known target-site insensitivity mechanisms both contribute to the resistance [134, 244]. Using synergists and genetic crossings, Pu *et al.* characterized abamectin resistance in *P. xylostella*, finding that while P450 monooxygenase activity in the resistant strain was elevated, it may not have been the major detoxifying mechanism involved [245]. Wang *et al.* found that more than one synergist was capable of increasing the potency of the diamide, chlorantraniliprole but could not account for the total level of resistance [246]. The authors suggested that either metabolic detoxification is not the major mechanism of resistance, or that the synergists used were not specifically addressing the enzymes involved in the chlorantraniliprole metabolism. It is also noted that this study did not genotype the resistant strain for the published target site mutations [145, 241] with the authors admitting that this should probably be clarified. Although the underlying resistance mechanisms to many compounds are known, and include enhanced metabolic detoxification and/or mutation of insecticide target site [234, 244, 247], the molecular mechanism(s) of resistance to compounds such as the avermectins, benzoylureas and diamides have not yet been characterized.

1.8 Diamides

1.8.1 Structure

In 2007 the first diamide, flubendiamide, was introduced to market (fig. 1.11). Flubendiamide was jointly developed by Nihon Nohyaku and Bayer Crop Sciences, and first described by Tohnishi *et al.* in 2005 [248-250]. Very soon after the introduction of flubendiamide, DuPont Crop Protection published work on anthranilic diamides, introducing Chlorantraniliprole [251] which is both structurally (fig. 1.12) and biochemically similar to flubendiamide[252]. Cyantraniliprole [253] (fig. 1.13) is chemically similar to chlorantraniliprole except it has a cyano substituent replacing a chlorine atom on the anthranilic core [249].

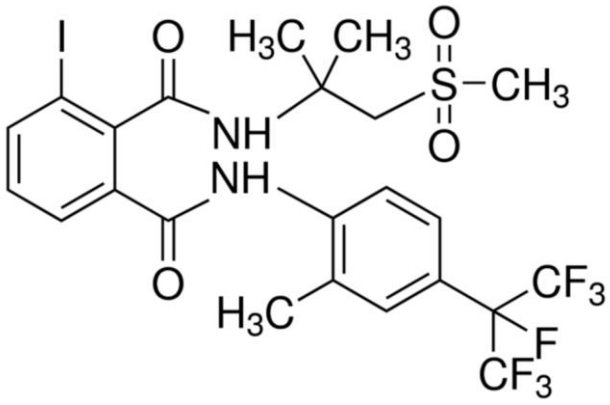


Figure 1.11. Chemical structure of Flubendiamide [254].

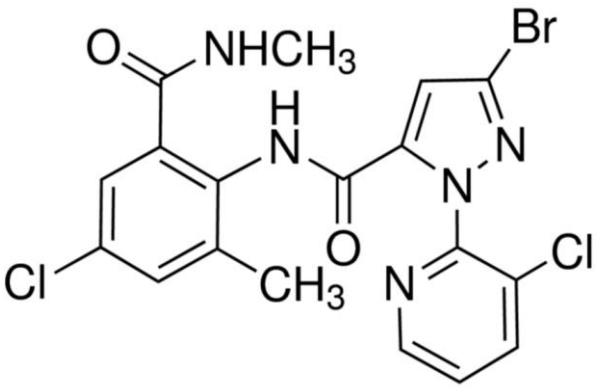


Figure 1.12. Chemical structure of chlorantraniliprole [255].

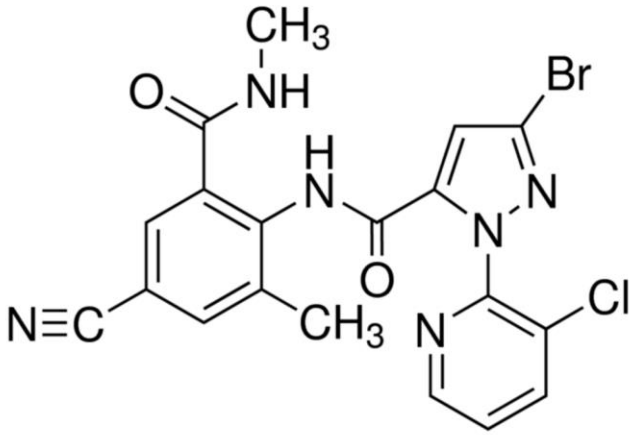


Figure 1.13. Chemical structure of Cyantraniliprole [256]. The chemical structure differs from that of chlorantraniliprole (fig. 1.12) by the substitution of the chlorine with a cyano group.

1.8.2 Mode of Action

Diamides have a unique mode of action (MoA), belonging to IRAC MoA group 28, and acting on insect ryanodine receptors [257]. Calcium homeostasis is important in multiple cell functions especially muscle control [251]. This homeostasis is maintained through the controlled release of calcium (Ca^{2+}) from intracellular stores through calcium channels called ryanodine receptors (RyRs) located in the sarcoplasmic reticulum of muscle, and endoplasmic reticulum of neurons [258]. Diamides 'lock' these RyRs open, depleting the internal stores of Ca^{2+} which interferes in muscle contraction and ultimately results in paralysis and death [248, 252] (fig. 1.14). Both the phthalic acid diamide, flubendiamide, and anthranilic diamides, chlorantraniliprole and cyantraniliprole are potent activators of insect RyRs and show specific target-site selectivity for insects over mammals [258].

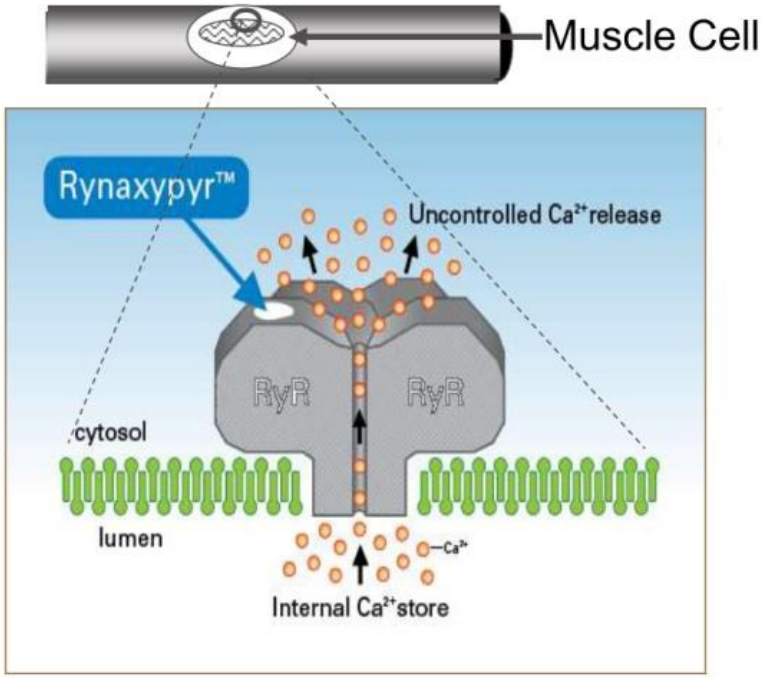


Figure 1.14. Diamide mode of action. Following the application of diamide insecticide, ryanodine receptors are locked open releasing the internal stores of calcium needed for muscle contraction, rendering the muscle paralysed [259].

1.8.3 Resistance to diamides

To date, while extremely efficacious against lepidopteran insects, diamides have been shown to be active against a number of different groups of insects including several significant pest species. Non-exhaustive examples include the oriental fruit fly *Bactrocera dorsalis* [260, 261], Asian citrus psyllid, *Diaphorina citri* [262], Colorado potato beetle, *Leptinotarsa decemlineata* [263], and black vine weevil, *Otiorhynchus sulcatus* [264]. Despite affecting multiple groups, resistance has been mainly described in lepidopteran species. In addition to *Plutella xylostella* diamide resistance have been described in the smaller tea tortrix, *Adoxophyes honmai*, in Japan [265], obliquebanded leafroller, *Choristoneura rosaceana* [266], beet armyworm, *Spodoptera exigua* and related species *Spodoptera litura* [267-269], Asiatic rice borer, *Chilo suppressalis* [270], navel orangeworm, *Chilo suppressalis* [271], and the tomato borer, *Tuta absoluta* [272]. In these other species, while resistance has been reported (in some cases experimentally selected for), the mechanistic basis of resistance has not been elucidated (or not with consensus). This is due, in part, to some of the studies

making use of synergistic bioassays to investigate the possibility of the presence of metabolic detoxification being involved [261, 266, 270, 271].

The mechanistic basis of diamide resistance has been most well investigated in *P. xylostella*. A strain of *P. xylostella* from the Philippines and Thailand which was found to be over 200-fold resistant to chlorantraniliprole and flubendiamide was found to have a mutation (G4946E) in the RyR associated with the resistance [247]. Further evidence was supplied for the involvement of this mutation by the same authors by functionally expressing the cloned mutant gene alongside a susceptible wildtype in *Spodoptera frugiperda* Sf9 cells [273]. This demonstrated that receptors with the mutation are significantly less sensitive to diamides. Furthermore, studies made independently by Yan *et al.* and Steinbach *et al.* described a significant association between the frequency of the G4946E mutation with phenotypic resistance in field populations [144, 274]. Since the discovery of the G4946E variant, three additional novel mutations have been found in a strain of *P. xylostella* from China with 2128-fold resistant to chlorantraniliprole [275]. These consist of a glutamic acid (GAA) to valine (GAT) substitution (E1338D); a glutamine (CAG) to leucine (CTG) substitution (Q4594L); and an isoleucine (ATA) to methionine (ATG) substitution (I4790M). In addition to their identification, the authors examined combinations of the mutations and their impact on the resistance profiles. The authors reported that the RyR containing multiple mutations showed significantly lower affinity for chlorantraniliprole and that the co-existence of different combinations of the four mutations conferred resistance. Steinbach *et al.* reported finding the G4946E mutation in 9 populations of preserved *P. xylostella* larvae from 11 countries, all from areas where diamide control of *P. xylostella* had failed [144].

Possible metabolic resistance has already been documented in *P. xylostella* to non-diamide insecticide compounds through the employment of cytochrome P450s, esterases, and glutathione S-transferase [134, 194, 276]. As with other lepidopteran species, it is possible that metabolic mechanisms may also play a role in diamide resistance in *P. xylostella*. Indeed, several detoxifying enzymes have been implicated in the metabolism of diamides; through the use of synergists that inhibit P450s (microsomal oxidases), esterases and GSTs [246, 277]. Not all of the studies have concurred in their findings with several contradictions in the implication of certain enzyme families [134, 278]. It is feasible that different populations

have evolved different or a combination of different metabolic mechanisms (which may or may not also involve target site resistance) [277, 279]. The use of synergists to identify metabolic mechanisms, while well proven in confirming metabolic interactions involving specific families of enzymes, is self-limiting when applied to the discovery of new enzymatic mechanisms or pathways. Because for only those enzymes for which synergists are being tested, (or indeed known) will information be yielded, this method by definition will not result in the discovery of novel metabolic interactions. Typically, only a handful of synergists are ever used, with diethyl maleate (DEM), piperonyl butoxide (PBO) and S,S,S-tributylphosphorotrithioate (DEF) being by far the most common.

1.9 Aims of this project

The overall aim of this project was to characterise the molecular basis of diamide resistance in *P. xylostella* with a particular focus on metabolic resistance as this has been much less well studied.

Specific objectives were to:

1) Identify candidate detoxification genes through the comparison of whole genome microarrays performed on multiple resistant strains in comparison with laboratory susceptible strains and validate their overexpression using qPCR.

2) Obtain full length cDNA sequences of candidate genes and carry out a range of *in vivo* and *in vitro* functional approaches to examine the strength of association between the overexpression of detoxification genes and resistance.

- Compare phenotypic data obtained through bioassay experiments of live strains alongside molecular expression data to provide further corroborative evidence of candidate gene(s) participation in resistance.
- Create transgenic *Drosophila melanogaster* expressing *P. xylostella* candidate resistance genes and examine the effect on phenotypic resistance
- For both *P. xylostella* and transgenic *D. melanogaster*, use liquid chromatography-mass spectrometry (LC-MS) to look for differences in parent compound uptake and presence temporally post exposure, and where possible identify any secondary metabolites.

2 Materials and Methods

2.1 *Plutella xylostella* material and live strains

2.1.1 Strains only available as frozen (whole larvae) material

Material was provided by Troczka *et al.*, Rothamsted Research.

CM (Chiang Mai) – A field collected susceptible strain from Chiang Mai,Thailand [247].

UNT (Untreated) – A field collected diamide resistant strain collected from Bang Bua Thong, Thailand [247]. Not selected with insecticide, it was the parent strain of CHL and FLU lines.

CHL (Chlorantraniliprole) – A line with UNT strain parentage, selected with Chlorantraniliprole approximately every two weeks.

FLU (Flubendiamide) – A line with UNT strain parentage, selected with Flubendiamide approximately every two weeks.

2.1.2 Live strains

HS (Hawaii Selected) – Originally from Hawaii, this strain was supplied by Cornell University, USA. The strain exhibits resistance to diamides and is regularly selected with Chlorantraniliprole to maintain this resistance.

ROTH = Untreated laboratory strain maintained under laboratory conditions at Rothamsted Research for more than 30 years without insecticide selection.

2.1.3 *P. xylostella* Rearing

Four trays (12.5cm wide x 17cm length x 5.5cm deep) each containing between 8-12 Chinese cabbage (*Brassica rapa* spp.) (5-6 weeks old) plants were placed in a 44.5cm wide x 44.5cm deep x 69.5cm high clear perspex rearing cage. Each cage was equipped with an extractor fan and mesh covered holes to aid ventilation and to reduce the build-up of condensation and fungal pathogens (plate 2.01).

Plate 2.01. *P. xylostella* cages. Extractor fans are visible on the back panels and ventilation grills on the front. The plants are kept watered through capillary matting in the bottom of the trays.

Approximately 500-800 eggs per cage were infested by cutting aluminium egg sheets into pieces of about 250 eggs each, and placing the pieces on leaves of different plants within the cage. Cages were kept in a quarantine facility at 25°C ± 1°C with 16h light / 8h dark photoperiod, 70% relative humidity. Providing the number of eggs was not excessively exceeded the number of plants were able to sustain the larvae to adulthood. If larvae ran short of host plant material new Chinese cabbage plants were added to the cage. Once the larvae had pupated adults were collected and transferred to an egg laying chamber to mate and lay eggs on a fresh egg sheet to become the next generation.

2.1.3.1 Egg-laying chamber

The design and construction of the egg laying chamber was custom built by myself specifically for this purpose, but inspired by that used by Collins [280]. It consists of a 13cm tall x 11cm diameter clear plastic beaker with lid. The pot was upturned with the bottom removed and replaced with a fine mesh capable of retaining *P. xylostella* adults, whilst allowing a large amount of ventilation. A hole just bigger than the size of the insect pooter collection vial used to collect the adults was cut in the lid (now the bottom of the chamber) and was given a latex 'collar' creating a seal to contain the moths when the vial is pushed

through to transfer them to the chamber. An additional mesh cover was held in place using sticking tape to cover the orifice when not in use.

Eggs were collected on cabbage-treated aluminium foil sheets; foil strips 6cm wide x 13cm long folded in half lengthways to give a strip 3cm wide x 18cm long. As *P. xylostella* would normally lay their eggs in the undulations and crevices on host plants, the aluminium sheets were gently crumpled before being formed into a 'U' shape. This mimicked a host plant and experience showed that it increased the oviposition of eggs onto the egg sheets (figure 2.01).

Each sheet was dipped into cabbage juice (65g cabbage blended in 500 ml distilled H_20, then strained), and stood on its long side to dry (for up to 24 h). Once dry, an egg strip was added to the egg laying chamber and held in place in a 'u' shape by trapping each end in the join between the body of the chamber and the lid.

Adults were added to the egg laying chamber and left under the same conditions as the rearing cage for 24 hours. As many adults as possible were collected for breeding in order to maintain the genetic diversity of the population and avoid inbreeding.

By rearing *P. xylostella* using this methodology each rearing cage only contained one generation and so all individuals in the cage were the same age +/-1day. Additionally, the cage only needed to be opened for collecting adults at the end of a generation reducing the risk of individuals escaping.

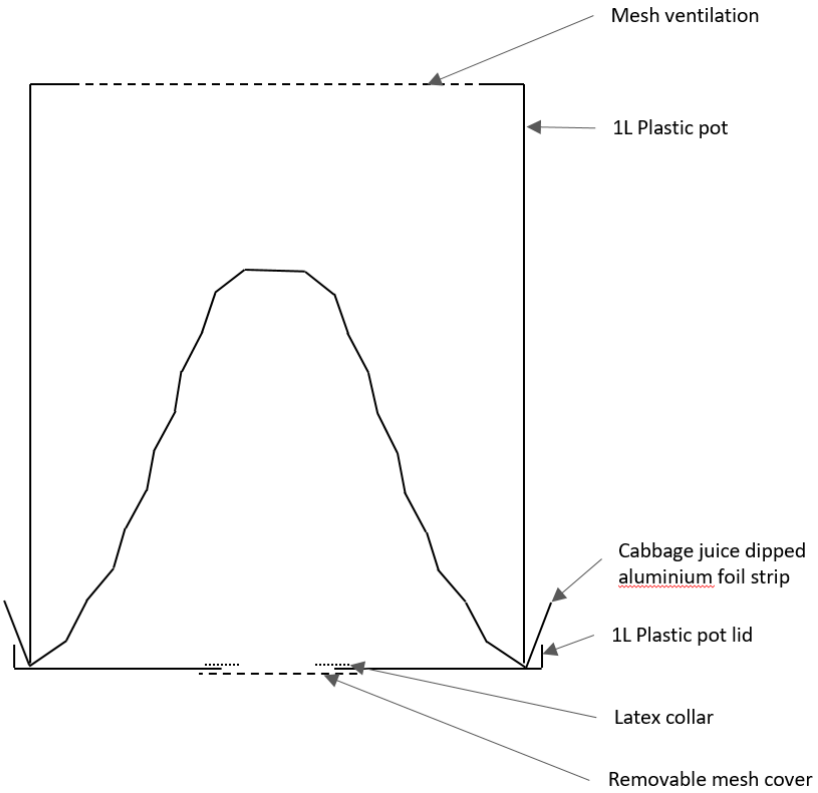


Figure 2.01. *P. xylostella* egg laying chamber containing egg laying sheet. Insects were added to the chamber through the hole in the bottom of the chamber, which was covered with a removable mesh cover (when not in use this was held in place using sticky tape).

2.1.4 *P. xylostella* Selection with Chlorantraniliprole (Hawaii Selected strain)

Following the dilution of chlorantraniliprole using agral (6-10ppm), 4-5 week old Chinese cabbage leaves were dipped in insecticide for 10 seconds (with gentle agitation) before being placed in water saturated oasis (22x11x2.5cm). Holes had previously been made in the oasis ready to receive the cabbage leaves, as this seemed to aid leaf survival for the course of the selection. The oasis was contained in a 35cm x 21.5cm watertight tray with the water depth up to just below the surface of the oasis. A custom made 'lid' consisting of an upturned 24x15.5x20cm clear plastic box with the bottom removed and replaced with a fine net mesh was used to cover the treated leaves once 2nd instar larvae had been added. Insects were kept on the leaves for 72 hours (at rearing conditions) before being transferred to a new rearing cage containing fresh cabbage leaves as described earlier. The second-generation post selections (G2) were used for experiments or procedures.

2.2 Insecticide/Synergist Dilutions

All dilutions for leaf dip bioassays and selections were made using 0.01% Agral (wetting agent) in 1l containers that were large enough to accommodate large leaves or leaf disks without damaging them while dipping. The exception to this was the initial dilution of Piperonyl Butoxide (PBO) from 900,000 ppm to 10,000 ppm which was made using acetone, after which further dilutions were made using 0.01% Agral. The compounds used and their source can be found in the relevant experimental chapters.

2.3 Liquid broth for growing of cell cultures

Liquid broth was made by combining 2.5-5g NaCl, 5g Tryptone, 2.5g Yeast Extract, made up to 500ml with deionised H_2O. The pH was then adjusted 7 using 5M NaOH (approx. 100µl). The solution was autoclaved and left to cool until needed.

2.4 Luria-Bertani agar plates for cloning procedures

LB Agar was made by in the same manner as for liquid broth, with the addition of 7.5g Bacto-agar before being made up to 500ml with deionised H_2O. Once autoclaved, the solution was left to cool to 50-55°C then antibiotic added. E.g. Kanamycin (50ug/ml) or Ampicillin (100ug/ml).

2.5 RNA extraction

Approximately 30mg insect material (*P. xylostella* larvae) was ground in a 1.5ml eppendorf tube cooled using liquid nitrogen using a micro-pestle. RNA was then extracted using the Bioline 'Isolate RNA Mini Kit' according to manufacturer's instructions. The quality and quantity of the RNA obtained was assessed by spectrophotometry (Nanodrop Technologies) and by running an aliquot on a 1.5% agarose gel. For the latter, RNA was mixed with 1x loading buffer (95% formamide; 0.025% xylene cyanol; 0.025% bromophenol blue; 18 mM EDTA; 0.025% SDS), heated for 5 minutes at 65 °C to denature, and briefly chilled on ice prior to loading.

2.6 First-strand cDNA synthesis

cDNA was synthesised from RNA using SuperScript III Reverse Transcriptase according to the manufacturer's protocol (Invitrogen) using 50-250ng of random primers and 2µl total RNA. Samples were incubated at 25°C for 5 minutes, 50°C for 45 minutes and 70°C for 15 minutes. cDNA stocks were stored at -80°C.

2.7 Standard Primer Design

Primers were manually designed within 'Geneious' (v6) and tested for self-complementarity and melting temperature (TM) (aiming for 58-60°C) using 'Oligo Calc' [281].

2.8 Standard PCR Protocol

Typically, a standard PCR reaction consisted of 10µl PCR Mix (DreamTaq Green), 1µl of each forward and reverse primer (10µM), 1µl cDNA or PCR product made up to 20µl total with sterile distilled water (SDW). The standard thermal cycling program can be seen in table 2.01 below. Where longer fragments were amplified, the extension time was increased proportionally (1 min for each 1 kb of amplicon size).

Stage of Program	Temperature (°C)	Duration	Number of cycles
Hold	95	2 mins	-
Cycling	95	10 secs	40
	57	15 secs	
	72	20 secs	

Table 2.01. Standard PCR program.

2.9 Agarose gel electrophoresis

Agarose gels were made by dissolving 1-2g agarose in 150ml TBE buffer (x1). 4µl Ethidium Bromide (0.01mg/µl) was added and mixed after cooling prior to pouring. TBE buffer (x1) was used in the gel tank and gels were typically run at 90-100v for approximately an hour or until products were differentiated using UV visualisation. When greater definition was required stronger percentage gels were made and run at a lower voltage (65-85v). Where a product was to be used for cloning TAE (x1) buffer was used in place of the TBE buffer above.

2.10 Purification of PCR products

Purification of PCR products (both directly and through gel extraction) were performed using the 'Wizard SV Gel and PCR Clean-up System' (Promega) according to the manufacturer's protocol.

2.11 Ligation of vector and insert DNA

DNA fragments were ligated into plasmids using T4 DNA ligase (New England BioLabs) following the manufacturer's protocol.

2.12 Single colony PCR

Single colony PCR was used to check that an insert of the correct size had successfully ligated into the vector in transformant colonies. PCR tubes were prepared each containing a 20µl aliquot of SDW. Using a sterile pipette tip a colony was picked and a small streak made onto a numbered LB-Agar (+ antibiotic) plate and the tip then placed in the corresponding PCR tube (stirring as this was done). This was repeated for the desired number of colonies. The plated colonies were incubated at 37^{o}C for several hours or overnight.

The water in which tips had been placed was used as template in PCR reactions to test that the desired inserts (gene/gene fragment) had been successfully isolated and inserted into the vectors. Standard PCR reaction volumes were used as described above, with the exception that 2µl of template was used (with the volume of SDW adjusted appropriately). The PCR cycling regime used is shown in table 2.02 below.

Stage of Program	Temperature (^{o}C)	Duration	Number of cycles
Hold	95	5 mins	-
Cycling	94	20 secs	30
	50	30 secs	
	72	45 secs	
Hold	72	5 mins	-

Table 2.02. Single colony PCR program

2.13 Cloning

Unless, otherwise stated, cloning of PCR product was performed using the 'StrataClone PCR Cloning Kit' (Agilent Technologies) following the manufacturer's protocol. Standard cultures were set up in 5ml LB (+ antibiotic) in 14ml BD Falcon tubes by picking colonies using sterile pipette tips and inoculating the LB (by dropping the tip in). The cultures were incubated overnight at 37^{o}C with shaking (225-250rpm). Following the incubation they were spun down at 4000rpm for 4 mins after which the liquid was poured off and pipette tip removed.

2.14 Plasmid harvesting and purification

Following cloning, plasmids were harvested and purified using a 'GeneJET plasmid miniprep kit' (ThermoFisher Scientific) according to the manufacturer's instructions. If the purified plasmids were to be used directly for expression work the final elution step was carried out using water, for other purposes elution buffer was used.

2.15 Sequencing

Other than the next generation sequencing detailed in chapter 6, all sequencing was performed by Eurofins Genomics using their 'value read' service. Premixed samples (DNA + primer) were submitted according to the service criteria defined by the company.

3 Phenotypic resistance in *Plutella xylostella* strains

3.1 Introduction

The Hawaii (selected with chlorantraniliprole) (HS) strain of *P. xylostella* was received with the prior information that it was resistant to the diamide chlorantraniliprole (private correspondence). However, it was important to confirm the resistance status of this strain. In addition to chlorantraniliprole, the resistance status of HS to the other diamide compounds, flubendiamide and cyantraniliprole was also determined to identify if this strain was cross-resistant across all of the (current) diamide compounds.

Resistance to chlorantraniliprole in *P. xylostella* has been widely reported both in field populations and developed through laboratory selection. Cross-resistance across diamide compounds has already been reported, with Troczka *et al.* reporting cross-resistance between chlorantraniliprole and flubendiamide in a field strain from the Phillipines [145]. Wang *et al.* also reported cross-resistance between chlorantraniliprole and flubendiamide in Chinese collected field samples, despite the populations not having encountered flubendiamide in the field (as at the time of collection flubendiamide had not been used for the control of *P. xylostella*) [246]. Liu *et al.*, alongside compounds from other insecticide classes, made a comparison of resistance to all three diamide compounds (i.e. including cyantraniliprole) [282]. In this case, the authors selected a strain for 50 generations in the laboratory with chlorantraniliprole, and found it to be cross-resistant to all three diamides (and also abamectin).

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique used to separate chemicals through chromatography, and then, following vaporisation of a sample, detect ionised, charged molecules, and measure the mass-to-charge ratio of these particles. It can produce both quantitative and qualitative information on the component parts of a sample and has been used in many studies investigating insecticidal fate and metabolism. Much of the LC-MS work carried out in this field is in the study and detection of pesticide residues in plants and products such as those performed by Klein and Alder, developing methodologies for the simultaneous mass screening for multiple pesticide residues [283], or Ferrer *et al.,* who investigated improved strategies for the determination of persisting pesticide residues in olives and olive oil [284]. Investigations integrating the use of LC-MS in the study of insecticide metabolism have also been performed such as the identification of metabolites involved in detoxification pathways. Approaches to the use of

this technique differ between studies, with some using radio-labelled parent compounds, whereby the metabolic route and/or sequestration of a compound and its by-products can be followed through radioactive traces. Such an approach was used by Wang *et al.* to investigate the recruitment of a flavin-dependent monooxygenase (FMO) by the grasshopper, *Zonocerus variegatus* [221]. Other studies, such as that by Alptekin *et al.*, have used *in vitro* techniques in combination with LC-MS, such as expression of candidate P450 genes from honey bee in *E. coli* cell lines to study the metabolism of thiacloprid and imidacloprid [285].

A study by Bass *et al.* used LC-MS to analyse washes and extracts on whole insect bulk preparations of the aphid, *Myzus persicae* to investigate neonicotinoid (imidacloprid) metabolism [286]. Following an initial topical application of imidacloprid, over a 24-hour time period the authors showed that the recovery of this insecticide decreased in a time-dependent manner in the resistant clone, in contrast to a susceptible clone, which accumulated the insecticide. This chapter employs a similar methodology using chlorantraniliprole as a parent insecticide compound. Using washes of the external insect surfaces and whole insect bulk extractions using acetonitrile the aim was to explore potential differences between the uptake and metabolism of this insecticide between susceptible and resistant *P. xylostella* strains.

3.2 Methods

3.2.1 Insecticide/Synergist Dilutions

All dilutions for leaf dip bioassays were made using 0.01% Agral (Syngenta) (wetting agent) in 1l containers that were large enough to accommodate large leaves or leaf disks without damaging them while dipping. The sources of the compounds used can be seen in table 3.01 below.

Active Ingredient	Grade	Commercial name	Source
Chlorantraniliprole	Formulated	Coragen	DuPont (via Syngenta)
Chlorantraniliprole	Technical	-	Sigma-Aldrich
Flubendiamide	Technical	-	Sigma-Aldrich
Cyantraniliprole	Formulated	Cyazypyr	DuPont

Table 3.01. Grade and sourcing of insecticide compounds.

3.2.2 Standard *P. xylostella* Bioassays

Bioassays were carried out by placing 10 second instar *P. xylostella* larvae on a 65mm Chinese cabbage leaf disk. Leaf disks were dipped in a pesticide or control solution for 10 seconds (with gentle agitation) and dried for 30 mins – 1 hour on paper towel before being laid abaxial side uppermost on two sheets of 90mm filter paper that had been moistened with approximately 2.5ml distilled water contained in a 90mm petri dish. Two sheets of filter paper and the correct volume of water were required to sustain the leaf disk, but not leave enough unabsorbed water so that the insects could drown. During the course of the experiment additional water was added (~1ml) to keep filter paper moist and maintain the leaf disk in good condition. It was ensured that larvae were from at least the second generation (G2) post the most recent selection. Once larvae had been added, the lids were placed back on the petri dishes and they were then stored at 18-19^{o}C.

Typically, 30 larvae (3 replicates of 10 larvae) were tested at each concentration and assessed for mortality after 72 hours. An individual was assessed as being alive if it showed signs of movement following agitation with a paintbrush. The larvae were assessed for mortality at an endpoint of 72 hours.

3.2.2.1 Analysis of Bioassay Data

Raw bioassay data was corrected for any control mortality using Abbott's formula [287] (see fig. 3.01), following which the data was analysed using probit regression analysis using GenStat (18th Edition). To enable comparison of strains tested during the same bioassay, the probit analyses used employ a generalised linear model fitting parallel regression lines to determine LD_{50} values (the dose expected to kill 50% of individuals) and confidence limits. Where 95% confidence intervals did not overlap, populations were considered to be significantly different in sensitivity to the compound in question. LD_{50} fold changes (resistance ratios) were calculated using the formula shown in figure 3.02.

$$\frac{x - y}{x}$$

Figure 3.01. Abbott's formula used for correction of raw bioassay data to account for natural mortality. X = percent living in control; y = percent living in the treated sample.

$$LD50\ Fold\ Change = \frac{LD50\ Resistant\ or\ selected\ population}{LD50\ Susceptible\ or\ unselected\ population}$$

Figure 3.02. Formula used for calculation of LD_{50} fold change (resistance ratio).

3.2.3 Stability and metabolism of chlorantraniliprole in susceptible (ROTH) and resistant (HS) strains of *P. xylostella* using liquid chromatography-mass spectrometry (LC-MS)

Two LC-MS assays were carried out, each using a different method of exposing the insect to the chlorantraniliprole insecticide. Firstly, *P. xylostella* larvae were fed chlorantraniliprole (see section 3.2.3.1.1 below), in the second method larvae were exposed by topical application (see section 3.2.3.1.2 below). Chlorantraniliprole was used for the assays over the other diamide compounds as it had been used to select the resistant HS population (in order to maintain its resistance level) and because the HS strain showed the greatest resistance ratio to this compound (see section 3.3.1).

3.2.3.1 Assay procedures

3.2.3.1.1 Feeding assay

Chinese cabbage (4-5 weeks old) leaf discs (θ 65 mm) (4 replicates for each strain at each timepoint) were dipped in 0.1 ppm chlorantraniliprole (Coragen) solution and gently agitated for 10 s. The leaf discs were then dried for 30-60 min resting on absorbent paper, abaxial side uppermost. The discs were placed (abaxial side upwards) in Petri dishes (θ 90 mm) containing 2 sheets of 90 mm filter paper pre-moistened with ~2.5 mL dH_2O, after which they were infested with 10 *P. xylostella* larvae (2nd instar) per disc. An aliquot of the treatment solution was stored at -20°C. At each timepoint (0, 1, 5, 24 and 72 h after infestation), larvae were harvested by brushing into pre-weighed microcentrifuge tubes and the larval weight recorded. Larvae were then snap-frozen using liquid nitrogen and stored at -20°C. In addition, 10 cohorts of untreated larvae (10 individuals per cohort) per strain were sampled by snap-freezing and stored at -20°C to be used for standard matrices.

3.2.3.1.2 Topical dosing assay

Cohorts of 5 second instar *P. xylostella* larvae were placed in 35mm diameter petri dishes and cooled on an ice block to temporarily reduce larval movement. Using a Burkard micro-applicator and syringe, each larva was dosed with 0.25µL of 10ppm chlorantraniliprole (in acetone). Lids were placed on the petri dishes and stored until the allotted timepoint was reached. Each cohort was transferred to a pre-weighed microcentrifuge tube and snap frozen using liquid nitrogen at the appropriate sampling time before being stored at -20°C.

3.2.3.2 Sample preparation for LC-MS

Two extract samples from each assayed cohort were analysed through LC-MS with preparative steps for each. For topically dosed larvae, an additional wash/extraction was made from the petri dishes used for dosing.

3.2.3.2.1 Insect Wash: to extract any residual compound from the external surface of the insects.

250µl MeCN (Acetonitrile) was added to a cohort of insects in a 1.5ml eppendorf tube. The tube was inverted 4 times and then the supernatant removed (~250µl) to a LC-MS vial and capped.

3.2.3.2.2 Extraction from Insects: to extract any compound consumed/absorbed by the insects.

A washed cohort of insects was added to 500µl MeCN:H_2O (50:50) in an MP lysing matrix tube with the ceramic sphere removed (the lysing matrix alone was adequate for cell disruption). Tubes contained lysing matrix 'A' containing garnet matrix. Using a FastPrep 24 5G sample preparation system the sample was macerated at 10m/s for 30 seconds after which 125mg DisQuE (4 parts magnesium sulphate, 1 part sodium chloride, 1 part trisodium citrate dehydrate, 0.5 part disodium hydrogencitrate sesquihydrate) was added and mixed by shaking. Tubes were then centrifuged (15 mins @ 13000rpm), following which the MeCN supernatant was removed to LC-MS vial and capped.

3.2.3.2.3 Petri Dish Wash: to collect any compound transferred to the dishes during dosing.

1mL MeCN was added to the petri dish, swirled and removed. The MeCN was re-applied to the dish, swirled and removed again. This was repeated one further time, with the MeCN removed to an LC-MS vial and capped.

3.2.3.3 LC-MS analysis

Samples were analysed using an Agilent 1290 liquid chromatography system with Thermo Q-Exactive mass spectrometer. The separation was achieved using a Waters Acquity 1.7 µm BEH C18 column (50 x 2.1 mm) with a 6 minutes gradient elution at 0.7 mL/min of A (Water + 0.1% Formic acid) and B (Acetonitrile): T=0 min, A=95%; T=0.5 min, A=95%; T=4.5 min, A=5%; T=4.9 min, A = 5%; T=5 min, A=95%; T=6, A=95%. Mass spec analysis was carried out using positive electrospray in full scan, AIF (all-ion fragmentation) and tSIM (targeted selected ion monitor) modes. Examples of the output are shown in figures 3.03a and 3.03b along with explanations of what they represent. Data output from the LC-MS was analysed through MS-Excel 2016.

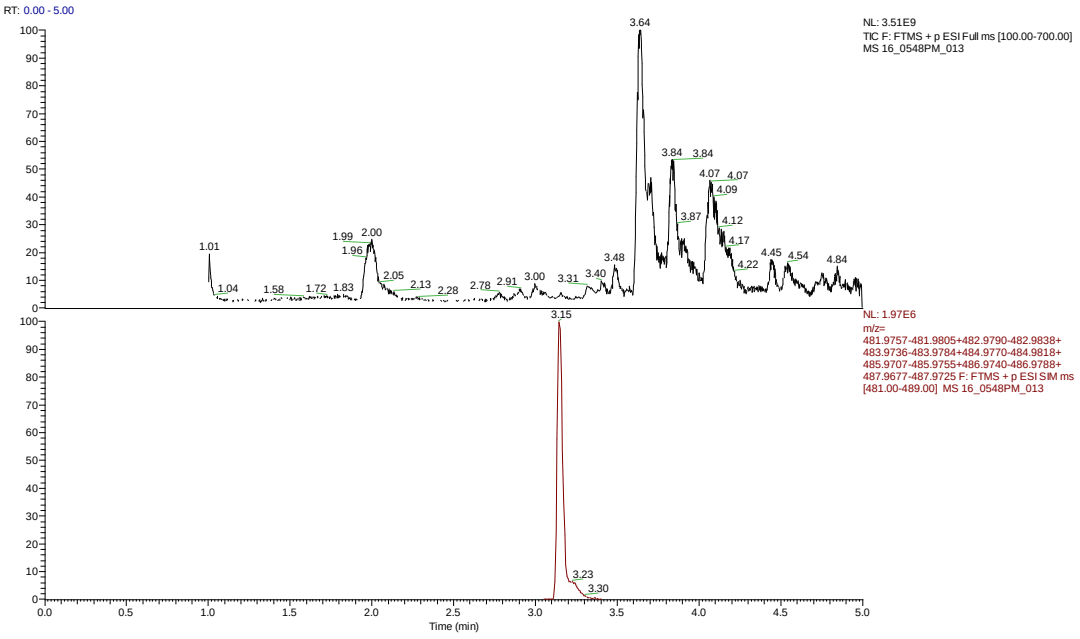


Figure 3.03a. Representative LC-MS output traces taken from standard curve (0.025ppm chlorantraniliprole) from *P. xylostella*. **Top trace**: Trace of all peaks in the sample from 100-700Da (Daltons). **Bottom trace**: Chlorantraniliprole contains chlorine and bromine, these have several isotopes and therefore several masses on the mass spectrometry. The signal peak shown is for chlorantraniliprole that is cumulative of these several isotopes.

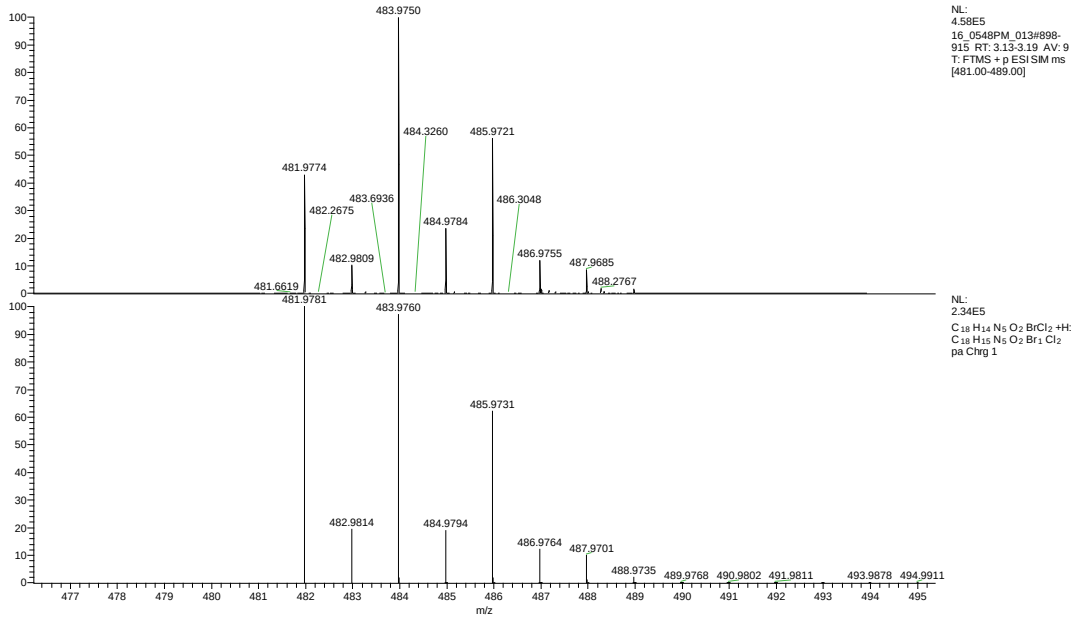


Figure 3.03b. Representative LC-MS output traces taken from standard curve (0.025ppm chlorantraniliprole) from *P. xylostella*. **Top trace**: Shows the component parts actually making up the cumulative signal peak shown in trace 3.03a (bottom trace). **Bottom trace**: A simulation of computer predicted isotopes for chlorantraniliprole. This prediction is made by the computer software through inputting the chemical formula of chlorantraniliprole.

3.3 Results

3.3.1 Phenotypic resistance of Hawaii strain

A bioassay of the Hawaii selected (HS) strain alongside the ROTH strain indicated that it was approximately 125 times more resistant at the LD_{50} than the susceptible strain confirming its significant resistance status to chlorantraniliprole. Follow up bioassays confirmed that the HS strain was 9- and 89- fold more resistant (at the LD_{50}) to flubendiamide and cyantraniliprole respectively. The results of which can be seen in table 3.02 and figures 3.04-3.07 below.

Treatment	Strain	LD	Estimated Dose (ppm)	Lower 95%	Upper 95%	LD_{50} Fold Change	Slope	Slope Standard Error
Chlorantraniliprole	Hawaii	50	12.50	4.70	34.00	125.00	0.917	0.118
		95	773.40	224.35	4914.00			
	ROTH	50	0.10	0.04	0.00			
		95	7.00	2.06	42.00			
Flubendiamide	Hawaii	50	2.45	1.31	4.56	9.42	1.0167	0.094
		95	101.59	45.26	296.13			
	ROTH	50	0.26	0.14	0.50			
		95	10.96	4.96	30.95			
Cyantraniliprole	Hawaii	50	8.90	4.52	17.60	89.00	0.9704	0.0911
		95	440.50	181.55	1432.00			
	ROTH	50	0.10	0.03	0.10			
		95	3.10	1.29	9.60			

Table 3.02. Dose-response probit analysis results for diamide bioassays. The compared to the susceptible ROTH strain, the Hawaii selected strain shows 125-fold, 9-fold and 89-fold greater resistance to chlorantraniliprole, flubendiamide and cyantraniliprole respectively.

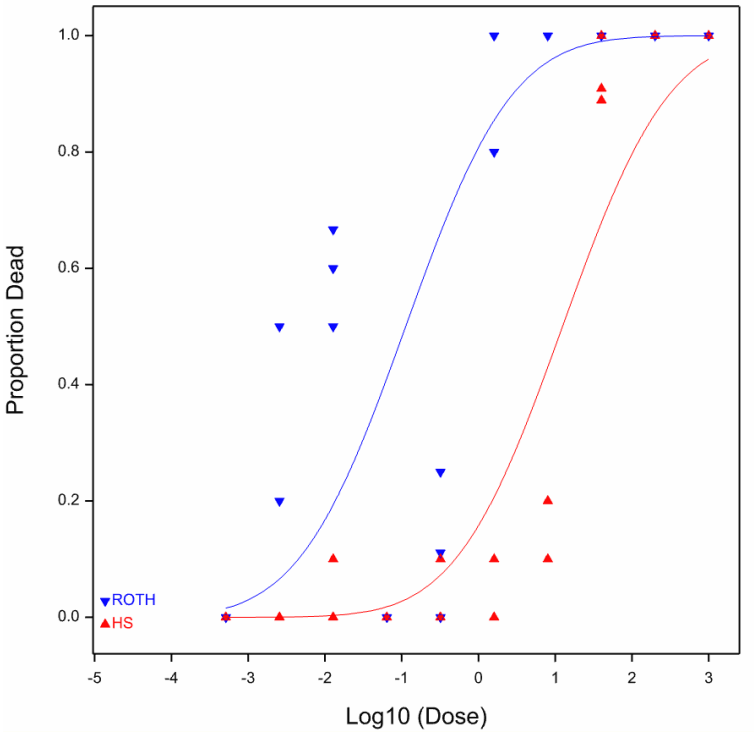


Figure 3.04. Fitted and observed relationship of dose-response for chlorantraniliprole bioassay for resistant Hawaii strain vs susceptible ROTH strain.

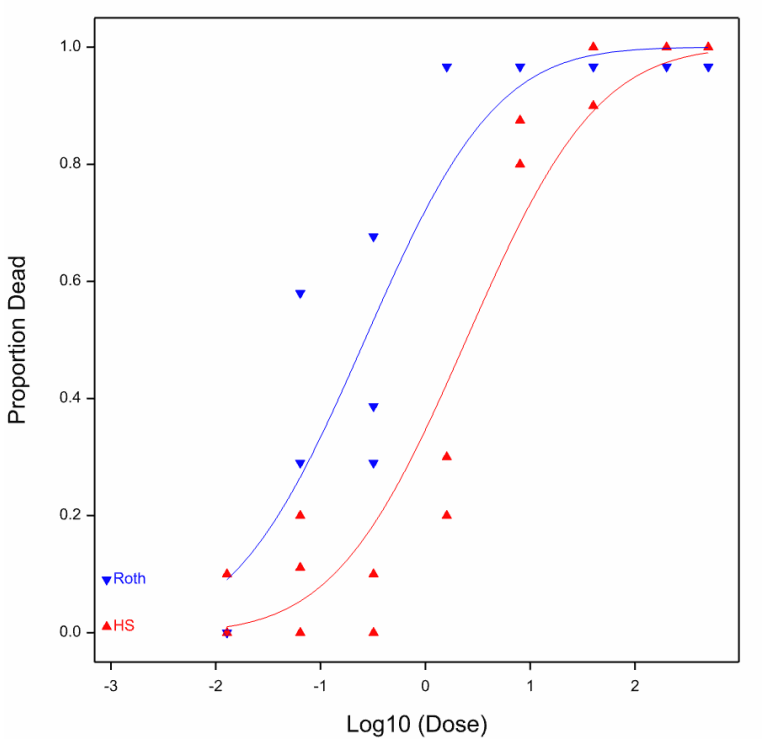


Figure 3.05. Fitted and observed relationship of dose-response for flubendiamide bioassay for resistant Hawaii strain vs susceptible ROTH strain.

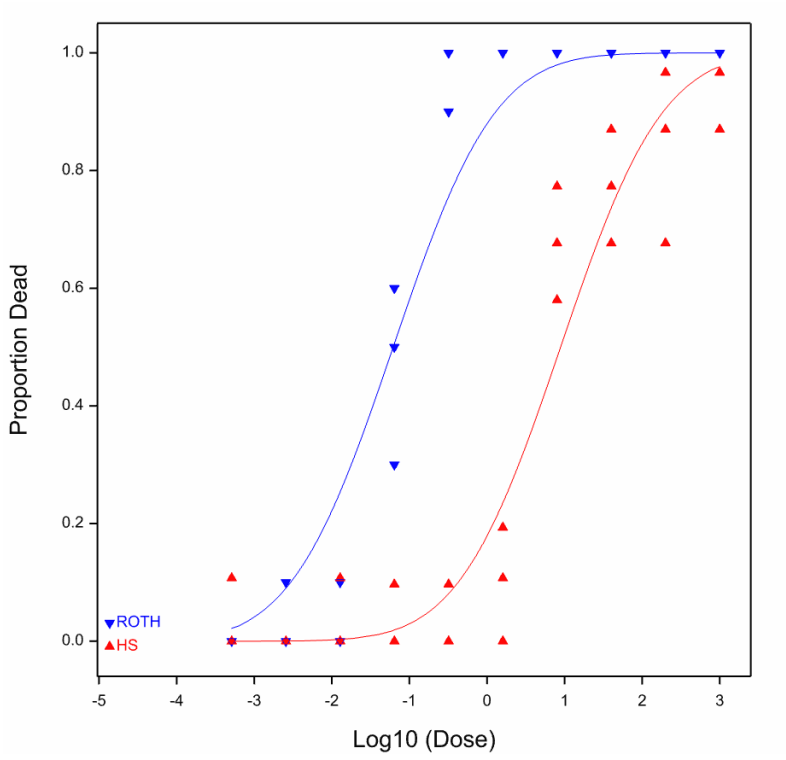


Figure 3.06. Fitted and observed relationship of dose-response for cyantraniliprole bioassay for resistant Hawaii strain vs susceptible ROTH strain.

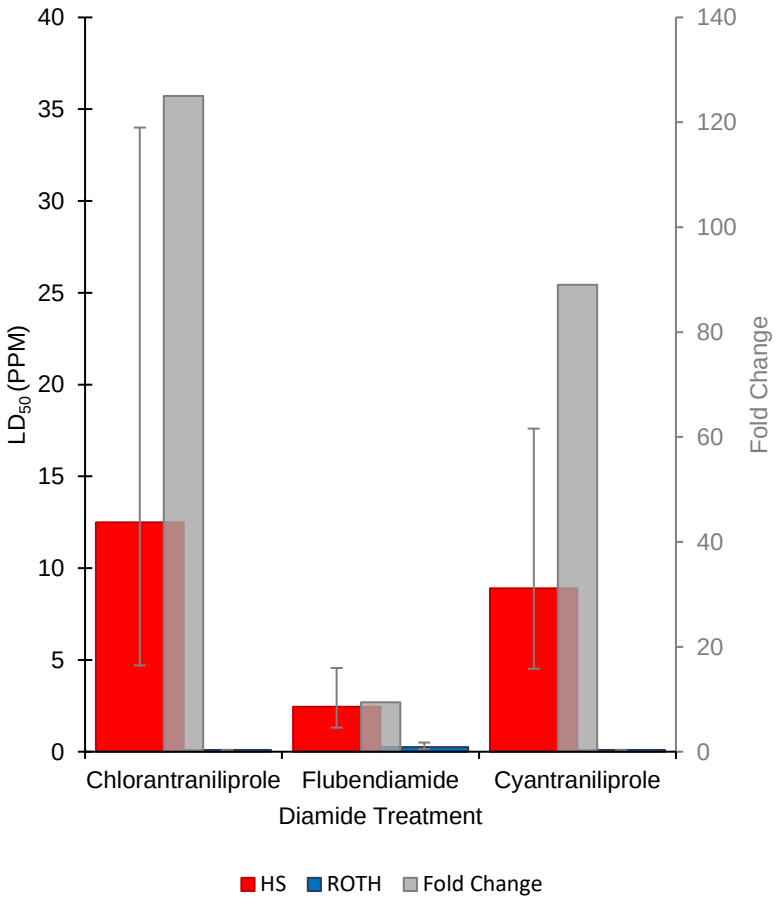


Figure 3.07. LD_{50} for resistant, Hawaii Selected (HS) and susceptible, ROTH strains for diamide compounds 72 hours post treatment. Error bars indicate 95% confidence limits. Secondary axis (grey bar) indicates fold change between HS and ROTH for each treatment. The HS strain shows significant resistance to all compounds.

3.3.2 Stability and metabolism of chlorantraniliprole (CTPR) in susceptible (ROTH) and resistant (HS) strains of *P. xylostella* using liquid chromatography-mass spectrometry (LC-MS)

3.3.2.1 Chlorantraniliprole recovery following insecticide consumption

Following initial peaks of chlorantraniliprole recovery one-hour post exposure, the trends of the experiment reveal very little change in the amount of chlorantraniliprole recovered from the ROTH strain at the different time points, while the HS strain shows a trend of a decline in this compound over time. However, there was no statistically significant difference (p-values were: T1=0.37, T5=0.82, T24=0.12, T72=0.52) in chlorantraniliprole recovery between

the resistant HS strain and susceptible ROTH strain over the time course of 72 hours (see figure 3.08).

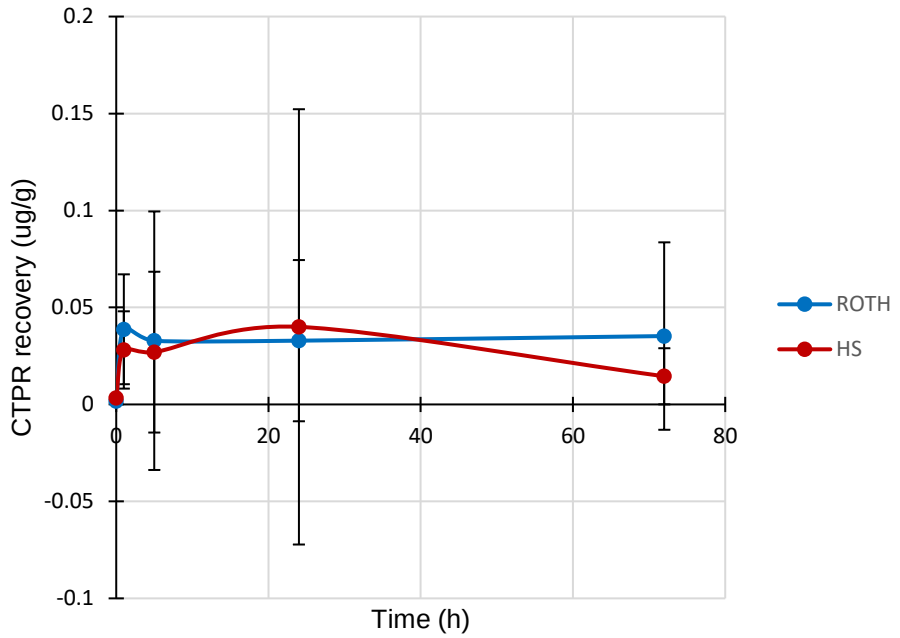


Figure 3.08. Average Chlorantraniliprole (CTPR) recovery in *P. xylostella* extracts from feeding assay (µg/g) determined through LC-MS over 72 hours. Error bars indicate 95% confidence limits. There was no significant difference in the concentration of chlorantraniliprole recovered between the susceptible (ROTH) and resistant (HS) strains over the time.

3.3.2.2 Chlorantraniliprole recovery following topical application

Analysis of the LC-MS data following topical dosing of *P. xylostella* with chlorantraniliprole (Figure 3.09) showed that while the 95% confidence limits overlap there is a significant difference between the HS and ROTH strains at 5 hour and 18 hour timepoints (p-values were: T1=0.13, T5=0.03, T18=0.03, T24=0.09). It appears that there is a difference between the changes in chlorantraniliprole recovery from insect washes, implying the HS strain is showing comparatively lower penetration of the insecticide.

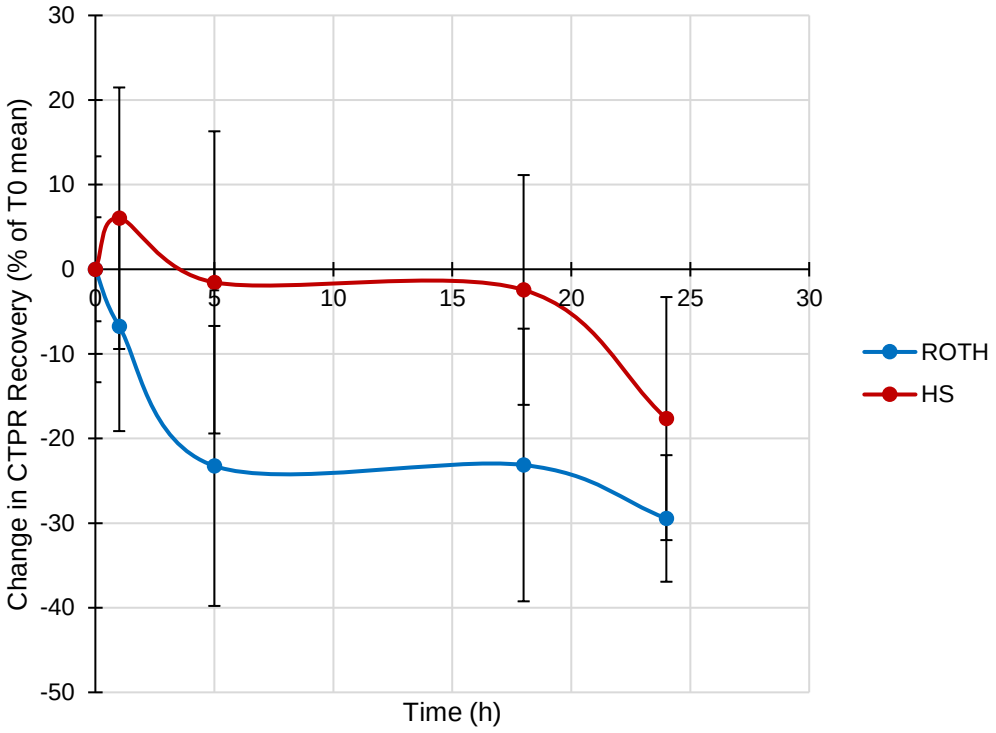


Figure 3.09. Percent change in chlorantraniliprole (CTPR) recovery by LC-MS in *P. xylostella* washes over 24 hours following topical application. Error bars indicate 95% confidence limits. There is a significant difference between the changes in chlorantraniliprole recovery at 5 hour (p=0.03) and 18 hour (p=0.03) time points.

There were significant differences for all timepoints (p-values were: T1=0.003, T5=0.02, T18=3.3×10^{-6}, T24=0.001) between the two strains in the percentage change of chlorantraniliprole recovery in the insect extracts compared with the average at time of application (T0). This is shown in figure 3.10, where, 24 hours post application, the resistant HS strain has 40% less chlorantraniliprole in the extract than at T0, whereas the susceptible ROTH strain has accumulated 238% more chlorantraniliprole than at T0. The significant decrease in percentage of recovered chlorantraniliprole from the HS strain in comparison to the accumulation in the ROTH strain implies that the chlorantraniliprole is being metabolised in HS.

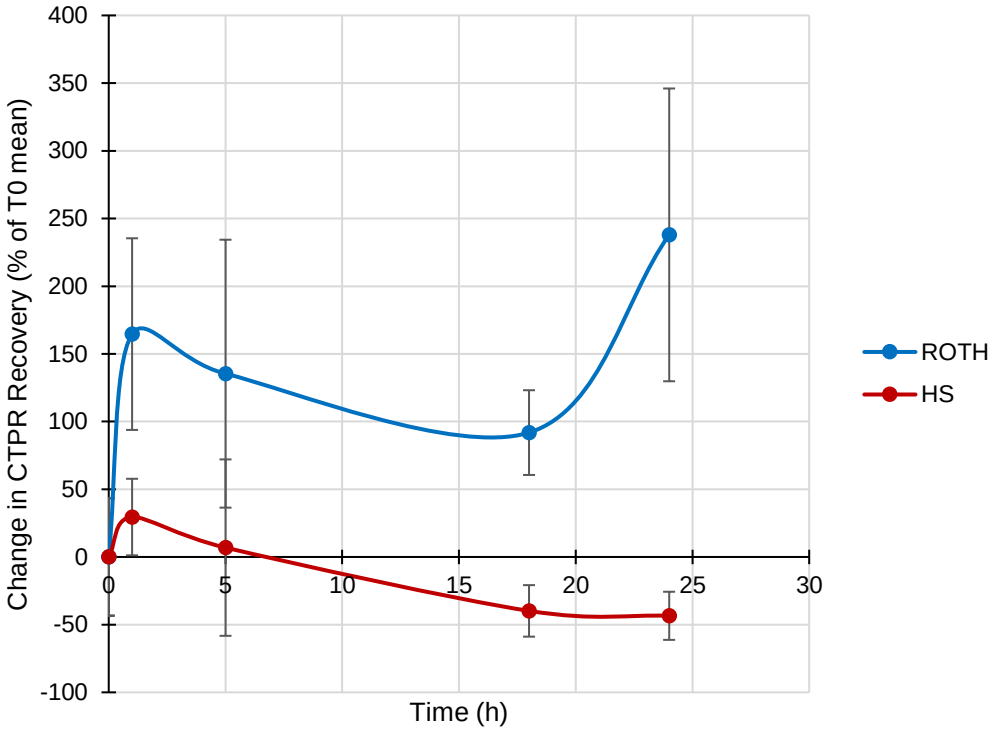


Figure 3.10. Percent change in chlorantraniliprole (CTPR) recovery by LC-MS in *P. xylostella* extracts over 24 hours following topical application. Error bars indicate 95% confidence limits. There are significant differences in recovery between the two strains at all time points (p-values were: T1=0.003, T5=0.02, T18=3.3x10^{-6}, T24=0.001).

3.4 Discussion

3.4.1 Phenotype

The Hawaii selected strain was found to be significantly resistant to all three (current) diamide compounds but exhibited different levels of resistance to each. The minority of studies investigating cross-resistance include all of the insecticides within the mode of action, with most relying upon one or two insecticides to be representative of the class. It is of value to test for cross-resistance as previous work has clearly shown examples where the substrate specificity of detoxifying enzymes may result in one compound being more readily metabolised than another, even between compounds within the same mode of action group [288]. Possibly due to the lack of use in China (full registration was only completed in 2013), many of the investigations screening *P. xylostella* to assess susceptibility of field populations have not tested flubendiamide. A recent such study by Zhang *et al.* looked at 11 different

insecticides, including chlorantraniliprole and cyantraniliprole, but did not include flubendiamide [289]. Conversely studies such as those by Troczka *et al*. [145] and Wang *et al.* [246] concentrated on chlorantraniliprole and flubendiamide at the exclusion of cyantraniliprole. There are however some studies, such as two published in 2015 by Liu *et al.* that have looked at all three diamides and found significant, but differing levels of resistance to each compound. Following the laboratory selection of a *P. xylostella* strain with cyantraniliprole by Liu *et al.*, the authors found that resistance to cyantraniliprole had increased between 30.6- and 326-fold in comparison to a susceptible population, but alongside this, the strain was also found to be resistant to flubendiamide and chlorantraniliprole by 14.1- and 24.3-fold respectively [279]. In a separate publication, through selecting *P. xylostella* with chlorantraniliprole, the same authors demonstrate 48.2-fold resistance to chlorantraniliprole with cross-resistance ratios of 7.3-fold to flubendiamide and 3.3-fold to cyantraniliprole [282]. In contrast to laboratory selected populations, five field populations of *P.xylostella* from China were monitored over two years by Zhang *et al.* [289]. It was found that the resistance ratio for chlorantraniliprole on average ranged from 1.19 to 14.26, and from 0.4 to 2.15 for cyantraniliprole. Another study by Wang *et al.* found field strains with resistance ratios of 18 and 30 for chlorantraniliprole and 15 and 17 for flubendiamide. This study also detailed a resistant field population with a resistance ratio of 1150 for chlorantraniliprole and 800 for flubendiamide [246]. The HS strain phenotyped in this PhD showed significant cross resistance to all three diamide compounds compared to the susceptible laboratory strain with LD_{50} resistance ratios of 125.0, 89.0 and 9.42, for chlorantraniliprole, cyantraniliprole and flubendiamide respectively. While lower than the very high instances that have been found as detailed above, these resistance ratios are significant within this study and within the ranges found in other resistant strains.

Comparison of fold changes between different studies should be performed tentatively due to any differences in susceptibility between the control strains or experimental methodologies used. For example, Troczka *et al.* reported a resistant strain with a resistant ratio of >4100 [145] and Wang *et al.* have reported a resistance ratio of 2000 fold in a different strain [63]. However, the two studies used different susceptible strains for calculation of the resistance ratios and the strains used had marked differences in sensitivity to chlorantraniliprole, with an LC_{50} of 0.048 gm/L for the former and 0.132 mg/L for the latter. In fact, all other variables being equal, if the study by Troczka *et al*. had used a susceptible

strain with the same LC50 as that used by Wang *et al.*, the resistance ratios calculated by the two studies would have been the same (both ~2000).

The label rate for control of *P. xylostella* in brassicas using chlorantraniliprole (foliar application) is 108.4mg/L. While the LD_{50} for the HS strain is well below this rate at 12.5 mg/L, the LD95 is over 7 times the label rate at 773.4mg/L. With the susceptible strain LD95 well below the label rate at 7 mg/L this is a strong indication that the HS strain would partially survive exposure to the field rate of this insecticide. A similar resistance profile can be observed for flubendiamide compared with the manufacturer's label rate of 48 mg/L where HS has an LD_{50} of 2.45 mg/L and LD_{95} of 101.56 mg/L. However, while the HS strain is significantly more resistant than the susceptible ROTH strain (by 89-fold) to cyantraniliprole, in comparison to the manufacturer's label rate (880 mg/L), the LD95 for this strain falls at about half the label rate at 440.5 mg/L. Although the label rate would fall within the confidence limits of the HS LD_{50}, this would still imply that an application of cyantraniliprole in a field environment would result in control of this strain, however, the phenotypic difference compared to the ROTH strain means that it is still a valid diagnostic compound. Phenotyping cross-resistance across the current diamides highlighted a large difference in the resistance ratios of HS between the diamide compounds. This is particularly obvious between the results for chlorantraniliprole and flubendiamide, where the resistance ratio of HS is 13.3 times higher for chlorantraniliprole than it is for flubendiamide. This suggests that the underlying basis of resistance is much more effective against chlorantraniliprole than it is against flubendiamide, with cyantraniliprole placed somewhere between these two. Variation in levels of cross-resistance between compounds with the same mode of action has been documented previously where Roditakis *et al.* found that the cytochrome P450, *CYP6CM1vQ* in *Bemisia tabaci* conferred resistance to the neonicotinoids imidacloprid, clothianidin and thiacloprid, but not acetamiprid or thiamethoxam [288]. This finding is almost certainly related to the chemical structure of the three compounds, as shown in figure 3.11. Chlorantraniliprole and cyantraniliprole are both anthranilic diamides and differ only in the side group on the benzene ring with chlorantraniliprole having a Cl at this position and cyantraniliprole a CN. In contrast, flubendiamide is categorised as a phthalic diamide and is structurally more different to chlorantraniliprole and cyantraniliprole.

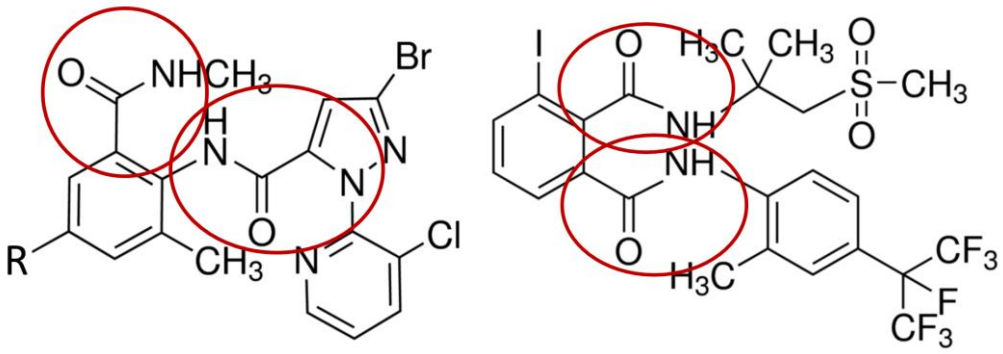


Figure 3.11. Chemical structures of anthranilic (left) and phthalic (right) diamide molecules. Circles highlight the common 'diamide' structures between the two forms. The anthranilic diamides chlorantraniliprole and cyantraniliprole differ only in the 'R' group, where for chlorantraniliprole R = Cl and for cyantraniliprole R = CN. Flubendiamide shows a significantly different structure.

3.4.2 LC-MS

3.4.2.1 Chlorantraniliprole recovery following oral dosing

Comparison of the levels of chlorantraniliprole recovery in the resistant HS and susceptible ROTH strains revealed no significant differences after exposure by feeding. When performing LC-MS to quantify the recovery of chlorantraniliprole from the strains, the levels of chlorantraniliprole detected were at the threshold of detection by the machine. This was because the concentration used (0.1 ppm) was based upon the LD_{50} for the susceptible ROTH strain. This dose was selected because while the LD_{50}, for the resistant HS strain is 125-fold this concentration, it was important that an adequate number of individuals of both strains were able to survive through the course of the assay. Compounding the relatively low dose used, because the assay was based on a leaf-dip methodology it is not easy to know exactly how much compound was consumed by the larvae. Even running treated leaf material through washes and extraction followed by LC-MS would not give an adequate estimation of quantity actually consumed by the larvae because the precise amount of compound on each leaf in the first place would not be exactly known as this would likely have been affected by variation in each individual leaf. Leaf dip assays have been used successfully by Nauen *et al.* with *Spodoptera frugiperda* prior to LC-MS analysis, however, in this study the leaf disks were only 4mm in diameter [290]. This is a stark contrast to the 65mm diameter disks used in this PhD study. Nauen *et al.* only went on to use individual larvae that had consumed the entire disk within 20 minutes. Other methods of orally administering treatments have been used.

When observing the metabolism and sequestration of ^{14}C-labelled senecione and its *N*-oxide in the lepidopterans *Creatonotos transiens* and *Spodoptera littoralis,* the larvae were fed small pieces of artificial diet which incorporated the radioactive tracer [219]. The same study also included the orthopteran *Zonocerus variegatus* whereby the tracer was administered via a droplet on a glass-fibre disk. Studies investigating gene knockdown through RNA interference in lepidopterans, such as those by Bautista *et al*. [134] and Yang *et al*. [291] have directly orally fed *P. xylostella* and *Diatraea saccharalis* larvae droplets containing dsRNA via pipette. The majority of these studies enforced a starvation period of 12 to 24 hours on the insects prior to them being offered the 'treated' food sources. Even though these other studies have different focuses, in hindsight, it may have been more effective to have adopted a different methodology for a feeding assay, concentrating on one that is more quantifiable.

3.4.2.2 Chlorantraniliprole recovery following topical dosing

LC-MS following the topical application of *P. xylostella* with chlorantraniliprole yielded more robust results with a significant reduction in the recovery over time of chlorantraniliprole from the resistant HS strain compared to the susceptible ROTH strain.

Triplicate start and end doses were made into Eppendorf tubes and chlorantraniliprole extractions made from these for each of the strains. The recovered chlorantraniliprole from the insect extracts and washes for each strain from each time-point was compared to the average of these 3 replicates for the respective strain. This effectively normalised the results, allowing a comparison of the washed or extracted chlorantraniliprole with what the insects were actually dosed with. In terms of the percentage of chlorantraniliprole actually applied, there was no significant difference between the chlorantraniliprole recovery from the insect washes between the two strains, but the insect extracts indicated greater disparity (significant for 18 and 24-hour time points). Based on this further interrogation of the data was performed, normalising the two sets to show chlorantraniliprole recovery as a percentage change from at time (T) =0. This enables the data-sets for the two strains to be fairly compared, resulting in clear significant differences between the two strains to be visualised. Working from percentage rather than from actual amounts accounts for any differences in chlorantraniliprole recovery efficiencies between the two data sets.

Significantly less chlorantraniliprole was recovered from the HS extract samples as a percentage of what was present at T=0 compared to the ROTH strain. The trend of higher percentage recovery of chlorantraniliprole in the HS strain washes, significant at 5 and 18 hour timepoints, is suggestive that this strain might have some form of resistance to the penetration of chlorantraniliprole. The significant depletion of chlorantraniliprole in the HS strain extracts suggests that the rate of chlorantraniliprole metabolism and excretion is greater than the rate of penetration and/or accumulation. In contrast chlorantraniliprole accumulated in the ROTH strain over time suggesting it is not as rapidly metabolised/excreted.

As detailed in sections 1.6.1 and 1.6.3, an increase in cuticle thickness (or decrease in permeability) or metabolic detoxification might result in insecticide resistance. Experiments on *P. xylostella* by Doichuanngam and Thornhill used similar methods to study the penetration and metabolism of the organophosphate insecticide, malathion, labelled with ^{14}C [292]. The authors found differences in cuticular penetration, metabolism and excretion of the insecticide between a resistant and a susceptible strain. Counter-intuitively, it was found that the insecticide penetrated the cuticle of the resistant strain significantly faster than for the susceptible strain, but this was offset by the increase in metabolism and excretion. Given the results produced in this chapter, it is likely that metabolic detoxification of the diamide compounds (particularly chlorantraniliprole) is a greater contributor to the resistance status of the HS strain than penetration resistance. However, it is possible that the two mechanisms are linked, where detoxification could be taking place as the insecticide penetrates the insect tissues rather than 'passing through' the digestive tract. An early publication in 1952 authored by Sternburg and Kearns reported that the insecticide DDT can be metabolised during penetration through the cuticle in the grasshopper, *Melanoplus differentialis* [293]. Ahmed and Gardiner have reported activation of the insecticide malathion through its conversion to malaoxon by the locust (*Schistocerca gregaria*) body-wall [294]. Additionally, while cytochrome P450 enzymes are frequently associated with metabolic insecticide detoxification [185-188], a recent study by Balabanidou *et al.* describes the activity of cytochrome P450 enzymes active in the cuticular layers of *Anopheles gambiae* [158].

3.4.3 Summary

In summary, through insecticide bioassays, the HS strain was confirmed as being phenotypically resistant to all three of the current diamide compounds compared to a laboratory susceptible strain. LC-MS analysis of the resistant HS strain revealed enhanced metabolism and/or excretion may be involved in the resistance shown by HS. The distinct differences between the resistance ratios of HS to the different diamide compounds and the initial conclusion through the LC-MS results that metabolic resistance may be involved in detoxification imply that there may be structure activity relationships involved with any enzyme(s) that are responsible for metabolic resistance. Further chapters will make use of comparative transcriptome profiling between the resistant HS and susceptible ROTH strains to identify differentially expressed genes that might underlie the profile of resistance shown by this strain.

4 Molecular Characterisation of Resistance Mechanisms

4.1 Introduction

As detailed in section 1.6, a number of mechanisms exist which can confer resistance to various pesticide modes of action. These range from physical barriers inhibiting penetration (such as cuticle thickness), behavioural responses, target-site mutations affecting insecticide binding sites and enhanced metabolic detoxification. Target site resistance to diamides has been previously associated with a mutation in the membrane-spanning domain of the ryanodine receptor in *P. xylostella*. This consists of a point mutation in the region of the gene encoding the proposed binding site of the diamide insecticides. The polymorphism within the triplet GGG/GAG at coding position 4946 causes an amino acid substitution from Glycine (G) in the susceptible wild type to Glutamic acid (E) in resistant strains [145, 241, 273]. Previous unpublished work by Syngenta using inhibitors of detoxification enzymes in insecticide bioassays suggested both target-site and metabolic resistance may be involved in the resistance of the CHL, FLU and UNT *P. xylostella* strains to diamide compounds (Jan Elias, personal communication). In contrast, the molecular and/or metabolic basis of resistance in the resistant Hawaii strain was unknown. The aim of the work described in this chapter was to investigate the basis of resistance in these strains using molecular and transcriptomic approaches to identify candidate resistance genes. Because target-site resistance to diamides has been well characterised in *P. xylostella* previously, greater effort was invested to characterise any metabolic resistance.

Some studies specifically focus on investigating the role of one or a handful of metabolising enzymes that have been previously implicated in insecticide resistance in a range of insect pests, with cytochrome P450s one common example [295]. While identifying a detoxification enzyme as upregulated in a resistant strain might infer it as the primary metabolic resistance mechanism, it is possible that further genes or other less well known metabolic processes are also involved in detoxification. The use of microarray technology, as employed in this chapter, allows gene regulation to be assessed on a 'global' scale, with a holistic overview of a multitude of genes and possible detoxification pathways. Other studies have taken similar approaches, with Zhu and Luttrell identifying five cytochrome P450 and nine esterase genes (amongst others) significantly upregulated from 369 annotated candidates from a imidacloprid resistant strain of *Lygus lineolaris*, the tarnished plant bug [296]. The authors

note the need for expression studies (possibly involving RNAi techniques) to provide further evidence as to individual genes' contributions to resistance. Fifteen genes, including P450s and GSTs from phenobarbital treated *Drosophila melanogaster* were validated using qPCR following microarray identification by Sun *et al.* [177]. Several of the genes were found to have been previously associated with insecticide resistance, including DDT. Ingham *et al.* took the microarray approach a step further by comparing results from not only resistant and susceptible strains, but also between the microarray output from different dissected parts of the insects [178]. While several new candidates were identified through comparisons of transcriptomes between abdomen integuments, as most genes associated with metabolic insecticide were also identified through the whole-body transcriptomes the authors did not view the comparison of dissected body parts to be advantageous. The use of transcriptome profiling by microarrays or RNAseq opens up the possibility of identifying gene associations in resistance that were not necessarily expected by the researchers. Using microarrays to investigate α-Amanitin resistance in *D. melanogaster,* Mitchell *et al.* found that a suite of genes and metabolic pathways were likely responsible for resistance. This was in contrast to the previous studies implicating two genes (a multidrug resistance gene, Mdr65, and a protein kinase, Pkc98E) accounting for the resistance [197]. For this study, because no assumptions were made regarding the nature of any metabolic resistance present, an approach that would consider all possibilities was undertaken.

As shown in table 4.01 , the number of gene accessions represented on the microarray for each of the gene classes described in section 1.7.3 is higher than the number that have been annotated in the DBM-DB [137]. The NCBI database typically reports higher numbers of gene annotations for each of the classes, but given the more generic nature, and larger pool from which to draw predictions, this is to be expected. It is possible that the majority of, if not all of the putative genes for each of the classes were encompassed within the microarray. However, it should be borne in mind that many of the annotations are automated predictions, and therefore may contain inaccuracies that may later be corrected through scientific research and/or improved algorithms. This could result in gene annotations changing with and between all gene classes. Accession numbers for those putative genes targeted on the microarray can be seen in the appendix (section 8.1).

Gene Class	Number of probes on microarray	Number of accessions represented on microarray	Number of annotated accessions in DBM-DB	Number of annotated accessions in NCBI
Cytochrome P450s	232	116	85	102
Gutathione-S-transferases	60	30	24	47
Carboxyl/Cholinesterases	160	80	21	70
Short-chain dehydrogenases	86	43	3	59
Flavin containing Monooxygenases	20	10	4	0

Table 4.01. Gene content of microarray for gene classes associated with metabolic resistance. Numbers of annotated genes resulting from online searches for given gene classes for *P. xylostella* from DBM-DB and NCBI databases are also shown.

Because microarrays can generate false positives it was important to validate the association of candidate genes with diamide resistance using a second method. Quantitative polymerase chain reaction (qPCR) was used to corroborate the candidate genes expression profiles either confirming their selection, or ruling them out, further narrowing the list of candidates.

Before investigating possible metabolic mechanisms of detoxification it was important to first establish the presence or absence of the known ryanodine receptor target site resistance mutations, G4946E [145], I1338D, Q4594L and I4790M [275]. Confirmation of the absence of the mutations would lend greater weight to any findings by ruling out previously described sources of resistance.

4.2 Methods

4.2.1 Genotyping strains for target site resistance to diamides

All target site mutations were genotyped through the amplification of DNA fragments spanning the polymorphic sites and subsequent sequencing of the resulting PCR products. Twelve larvae of each of the CHL, FLU, UNT and Hawaii strains were genotyped for the G4946E [145] mutation using primer combinations MUT1 and MUT2 (see table 4.02 for primer sequences) for both amplification and sequencing (supplied by Troczka *et al.*) using standard PCR reactions (chapter 2) with the minor change of 12.5µl PCR mix being used and then made up to 25µl totals. Genotyping the mutations described by Guo *et al.* [275] was completed separately using two biological replicate cDNA bulks for each of CM, CHL, FLU, HS

and ROTH strains. The PCR program used for all amplifications used is shown below (table 4.03).

Primer name	Primer sequence	Mutation (Forward or Reverse primer)
Mut1_F	CTATACTGATCGGGTACTACCATTTG	G4946E (F)
Mut1_R	CCTCCTGCACGTAGAACTTGCGG	G4946E (R)
Mut2_F	TACTGATCGGGTACTACCATTTGAAG	G4946E (F)
Mut2_R	GCACGTAGAACTTGCGGAAG	G4946E (R) and I4790M (R)
Px.N-5Fb	CTCCGGCAAGTGGTACTTCGAG	I1338D (F)
Px.Nterm1-R	TCCTCTTGACCGTCATCATAGTCGCGG	I1338D (R)
Px.qPCR-cterm-F1	CTATATTGGATACGGAGCTTTGG	Q4594L (F)
Px.2Kb-R3	CCCGATCAGTATAGCCAGCGATAC	Q4594L (R)
Px.cterm-seq2F	GACGGAGGCAGCGGTGAAGAAGACG	I4790M (F)

Table 4.02. Primer sequences used for genotyping published target site mutations. The primer 'MUT2_R' is shared for genotyping both G4946E and I4790M. All primers were supplied by Troczka *et al.* Target sites were amplified using primers, with PCR product then sequenced to assess genotype.

Stage of Program	Temperature (°C)	Duration	Number of cycles
Hold	95	2 mins	-
Cycling	95	10 secs	40
	50	20 secs	
	72	1.5 mins	
	72	5 mins	-

Table 4.03. PCR Program & primer sequences used for G4946E genotyping.

4.2.2 Microarrays

Dr Heiko Vogel's (Max Planck Institute) *P. xylostella* array (unpublished) was used for all microarray experiments, this was an array developed from 46,280 expressed sequence tag (EST) contigs. Two different probes were designed for all contigs to give around 90k probes with the remainder of the array filled with replica probes. The final slide layout consisted of four arrays of 180k 60-mer probes produced by Agilent by *in situ* oligonucleotide synthesis. Total RNA was extracted from four pools of 5 fourth instar larvae as described in chapter 2. 200ng of each total RNA was used to generate labelled cRNA, which was hybridized to arrays and washed as described in Agilent's Quick Amp Labelling Protocol (Version 6.5). The microarray experiment consisted of four biological replicates and incorporated a dye swap design whereby the Cy3 and Cy5 labels were swapped between resistant and susceptible strains. Microarrays were scanned with an Agilent G2505C US10020348 scanner, and fluorescent intensities of individual spots were obtained using the Agilent Feature Extraction software with default Agilent parameters. Data normalization, filtering, dye flipping and statistical analysis were performed using the GeneSpring GX 11 suite (Agilent). For statistical analysis, a t-test against zero using the Benjamini-Hochberg false discovery rate (FDR) method for multiple testing corrections was used to detect significantly differentially expressed genes. Genes meeting a p value cut-off of 0.05 and showing a transcription ratio >2-fold in either direction were considered to be differentially transcribed between the two strains.

4.2.3 Comparison and filtering of multiple microarray data

Spreadsheets were designed using Microsoft Excel to compare the resulting lists from the CHL and FLU strains to identify common genes that were differentially expressed in both microarray experiments. By comparing the microarray results in this way the list of candidate genes was narrowed to those that might be involved in resistance to both of the compounds. As they have the same mode of action this works as a filter to focus on relevant genes. By comparing microarray data across the strains it should be possible to identify components of the biological response whether it be a 'specific' detoxifying gene or maybe a 'core' set of genes that might constitute part or all of a resistance pathway and that these candidates might be void of irrelevant artefacts of the individual microarrays. This refining meta-analysis technique is comparable to other studies looking at both intra, and cross-species gene expression data sets [297-300]. It does not simply take into consideration just the most highly

expressed genes, but those that are consistent in being over or under expressed across all samples.

Specifically, once the data tables had been sorted and arranged appropriately, the spreadsheets were designed (in a formulaic sense) making a focused use of the 'VLOOKUP' function in a nested formula approach that made direct comparisons between tables of microarray data output by 'GeneSpring' (described in section 4.2.2). The resulting tables collated information for those genes that showed up or down regulation in all tables for each comparison. Conditional formatting was then used to highlight up or down regulated genes to make visualisation easier (table 4.10).

4.2.4 qPCR validation of candidate genes

4.2.4.1 Primer Design for Quantitative PCR

Using the coding sequence of the selected genes of interest from the microarray data, the correct reading frame was identified and primers designed using 'Primer3' webpage/application (http://frodo.wi.mit.edu). Typically primers were designed with a TM of 60^{o}C, avoided lengths of sequence that contained stop codons, and also avoided the first and last 20 bases pairs of an EST sequence which tend to be more error prone. For longer sequences, additional primers were designed to cover the length of the sequence, but care was taken that the primer pairs would amplify non-overlapping products. Primers were ordered from 'Sigma-Aldrich', re-suspended as a stock 100µM and an aliquot diluted to 10µM as a working concentration.

4.2.4.2 Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reactions (qPCR) were performed using each of the primer pairs with cDNA from susceptible and resistant strains and SDW (as a control) to verify primer amplification and specificity using the melt curve. All qPCRs were performed on a Corbett Research Rotor-Gene RG6000 and analyses performed within Rotor-Gene 600 software (version1.7). See tables 4.04 & 4.05 for PCR volumes and qPCR program respectively.

PCR Volumes	µl
PCR Mix (SYBR Green)	7.5
Forward Primer (10µM)	0.5
Reverse Primer (10µM)	0.5
SDW	5
cDNA (10ng/µl)	1.5
Total	15

Table 4.04. Reaction volumes used for primer testing

Stage of Program	Temperature (°C)	Duration	Number of cycles
Hold	95	2 mins	-
Cycling	95	10 secs	40
	57	15 secs	
	72	20 secs	
Melt	95		-

Table 4.05. Primer testing qPCR program

Identifier	Primer Name	Forward Primer Sequence	Reverse Primer Sequence	Efficiency
1528_Xanthine dehydrogenase	1528_2	AGGGCTGCATAC AAAAATGG	GAAAGCAGCATTG ACCCAGT	1.04
39731_Solute family carrier family 2	39731_1	GGTTCCTCTCTTG ACTCTGTGC	CGAACGCTAGCAGC CAAT	1.00
42163_Short-chain dehydrogenase	42163_2	GAGGAACTAGGA CGCCCTTC	GATCGCTGGCCAGA TAAACA	1.02
45938_ Cytochrome p450	45938_1	AGGAAGAAAATC GTGCCGTA	CACGCGGTAACTCT TGAACA	1.15
42842_Dimethylanilin e monooxygenase	42842_2	GGAAGACATGAT GGCGAAGT	TCTTGTACGTGCCT GACTCG	1.00
AB372008.1_ CYP6BG1	CYP6BG1_2	ATG TTG TTA TTG GTA GCT CTG GT	TGT GGC GGA AAC ATA GAG AGT	0.93
35855_ Cytochrome p450	35855_2	GCATGGCATATTG AAGTCCA	CCTGGTAGGTCAGA CGCAGT	1.01
33985_ Carboxylesterase	33985_1	CCGTAGAGATGC GGCTAAAG	AGGTGAACGACGG ATAGACG	1.02
Actin	PxActin_2	TCGGTATGGGAC AGAAGGAC	AGGTGTGGTGCCA GATCTTC	0.93
GAPDH	PxGAPDH_1	CCCTTCATCGGTC TGGACTA	CAGCGATCTTGTTT CCGTT	0.87
Tubulin	PxTubulin_1	ATCCCGAACAAC GTCAAAAC	AGCTCTTGGATTGC TGTCGT	0.92

Table 4.06. qPCR Primer sequences and calculated efficiencies used for genes of interest (highlighted in pink) and housekeeping genes (highlighted in blue).

Based on the initial testing, primers were selected for efficiency testing and to develop a standard curve to be used in further analyses. One primer pair for each candidate gene was selected based on the efficiency of amplification (table 4.06). Efficiency testing is a qualitative test to check if the primers are usable for comparison. In each case, series dilutions from 0.01ng to 100ng of the cDNA (per reaction) were made of cDNA prepared from the susceptible strain which was used for the efficiency testing/standard curve qPCRs with 3 replicates for each dilution. The reaction volumes can be seen below (table 4.07). The PCR program was the same as that used for the primer testing (table 4.05) minus the 95°C melt step at the end.

PCR Volumes	**µl**
PCR Mix (SYBR Green)	7.5
Forward Primer (10µM)	0.5
Reverse Primer (10µM)	0.5
SDW	2.5
cDNA	4
Total	15

Table 4.07. PCR volumes used for efficiency testing

In order to produce a standard curve for each primer any obvious outlying replicates were removed from the dataset to give the highest possible efficiency.

Having developed a standard curve, candidate genes were tested alongside reference housekeeping genes that exhibited stable expression between the strains. Having tested three possible housekeeping genes, Actin, Tubulin, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); In most cases Actin and GAPDH were selected to be used as the controls. Expression testing qPCR used the same PCR program and reaction volumes as that for the standard curves with 20ng cDNA in each reaction. Calculation of relative gene expression was made using the $2^{-\Delta\Delta}$*C*t method described by Livak and Schmittgen [301] using spreadsheets specifically designed for this purpose within Microsoft excel.

4.2.4.3 qPCR optimisation for *FMO2* gene

The *FMO2* gene (42842) displayed such low levels of expression in the susceptible ROTH strain that further optimisation of the qPCR was required. The same qPCR program was used as for previous qPCRs, however it was necessary to increase the amount of cDNA to 100ng per reaction and extend the number of cycles in order to get a viable result (table 4.08). Extra biological replicates were also included to gain the most accurate results for this GOI.

PCR Volumes	µl
PCR Mix (SYBR Green)	7.5
Forward Primer (10µM)	0.5
Reverse Primer (10µM)	0.5
SDW	5.5
cDNA (100ng/ul)	1
Total	15

Table 4.08. PCR volumes used for *FMO2* qPCR expression

4.3 Results

4.3.1 Genotyping the strains for target site resistance to diamides

Genotyping of CM, CHL, FLU and UNT for the previously reported target-site mutation, G4946E, had previously been carried out by our research group prior to this PhD studentship commencing. Alignment of the sequences of cDNA fragments from 12 individual larvae from the Hawaii strain encompassing the G4946E mutation region with the other strains, revealed only silent single nucleotide polymorphisms apart from at the position encoding G4946. While larvae of the CHL, FLU and UNT strains had the GAG triplet encoding glutamic acid (conferring diamide resistance) at this position, in contrast, all of the individuals tested from the HS strain had the GGG triplet encoding glycine (susceptible allele) at this locus (figure 4.01). The susceptible CM strain was sequenced previously, and from 20 larvae sequenced, 18 individuals were homozygous for the wild type allele (G4946) and two heterozygous for G4946E (overall frequency of 5% for E4946) [145]. All of the strains (HS, ROTH, CM, CHL and FLU) genotyped for the mutations presented by Guo *et al.* [275] showed wildtype nucleotides for each of the mutations with no mutations being present in any of the strains.

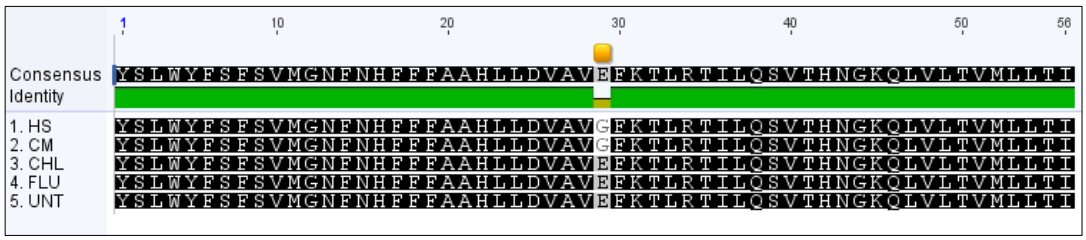


Figure 4.01. Genotyping results showing amino acid substitution at G4946E in resistant strains, CHL, FLU and UNT and wildtype genotypes for the susceptible CM strain and resistant HS strain. Amino acid position is indicated by yellow band above consensus. The HS and CM strains are of the G4946 genotype; CHL, FLU and UNT are of the E4946 genotype.

4.3.2 Microarray analysis and qPCR of strains of *P. xylostella* for candidate gene selection

As detailed above, despite CHL, FLU and UNT carrying the G4964E mutation conferring diamide resistance, enzyme inhibitor bioassays carried out by Syngenta have suggested that additional metabolic mechanisms may underlie diamide resistance in these strains. The Hawaii strain lacked this mutation, yet is resistant to diamides, suggesting an alternative mechanism of resistance in this strain. To investigate the role of metabolic resistance in these strains whole transcriptome microarrays were used to examine global gene expression levels and identify genes that were significantly differentially expressed in resistant compared to susceptible *P. xylostella* strains.

Initially, the following microarray comparisons were carried out:

- CM x CHL
- CM x FLU
- CM x UNT

These microarrays produced lists of 1597, 3331 and 2726 probes significantly differentially expressed between the CM and CHL, FLU and UNT strains respectively (p value of 0.05 (with Benjamin Hochberg correction [302]) and fold change greater than 2). Because probes on the array were duplicated, the total number of contigs (on which probes were designed) differentially expressed was 1192 contigs for CHL, 2641 contigs for FLU and 2117 contigs for UNT. When combined this was a total of 5864 separate probes representing 4551 contigs differentially expressed in one or more of the three (CHL, FLU, UNT) microarrays (fig. 4.02).

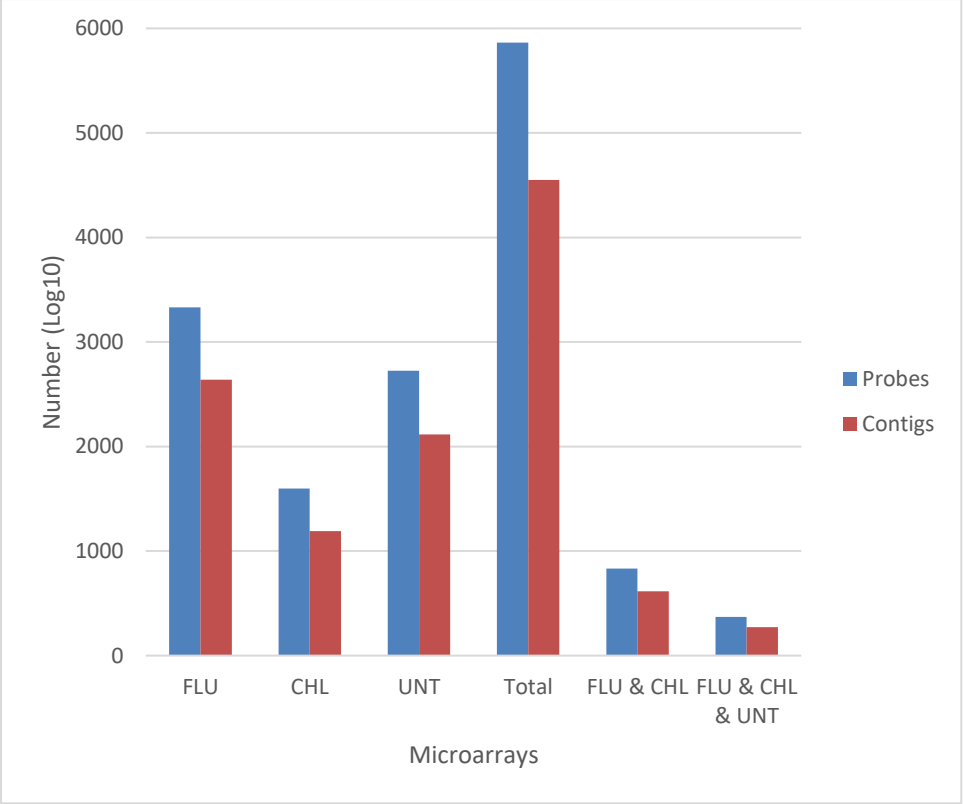


Figure 4.02. Number of probes and corresponding contigs differentially expressed in one or more microarrays.

In this study, a significant expression result was accepted as such if it was presented by at least one duplicate probe. In the majority of cases duplicate probes corroborated gene expression profiles within any one microarray, however, expression levels estimated from probe pairs was not always equivalent, therefore in these cases an average of the ratios was taken as has been done previously [303].

Comparison of the lists of differentially expressed genes produced in different array comparisons was used to identify genes that were commonly differentially expressed in resistant strains. This comparative approach produced a reduced list of 621 contigs (once duplicates had been removed) that were common in both the FLU and CHL diamide selected strains from the original list of 8234. Both microarrays have analogous hypotheses that have been tested in each dataset which is that genes are differentially expressed between the normal ('wildtype') susceptible and resistant strains. To further filter the list of common genes, microarray data for the UNT strain (resistant but not selected strain) was included in the comparison resulting in a further reduction in the number of common genes down to

286 (fig. 4.03). Further analysis of the data was employed (again through spreadsheet design) to consider whether expression of a contig was positive or negative, generating gene counts of 140 and 146 respectively.

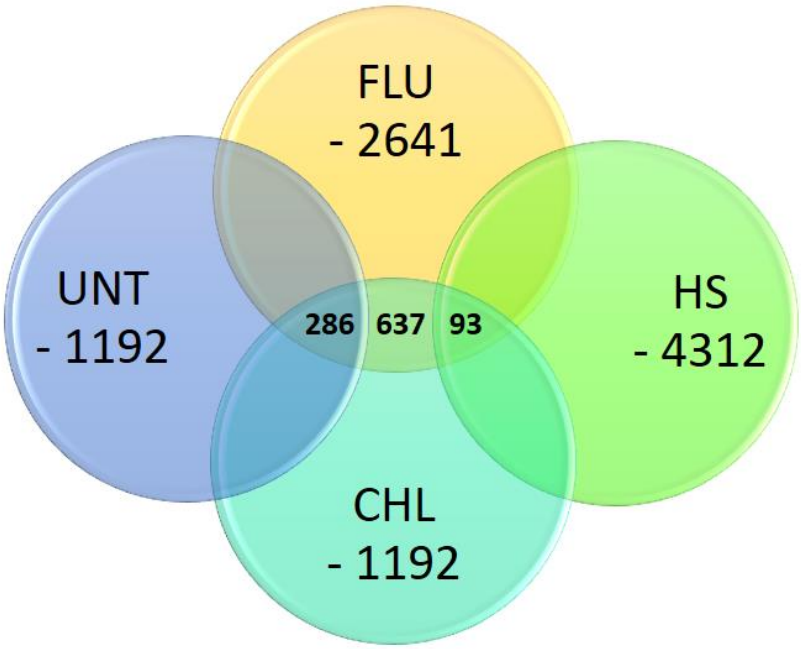


Figure 4.03. Using data from multiple microarrays reduces the number of candidate genes. Intersections indicate the number of differentially expressed genes in common between microarrays.

An initial subset of these genes was in the process of being selected and validated through qPCR (fig. 4.04) before the diamide resistant Hawaii strain became available. A candidate gene was selected to continue through to further expression experiments if the qPCR validation confirmed overexpression in both of the selected strains with at least one of the strains showing significant overexpression in comparison to the susceptible strain. This validation stage effectively rejected 2 of the 8 candidate genes leaving 6 candidates to be studied further (table 4.09).

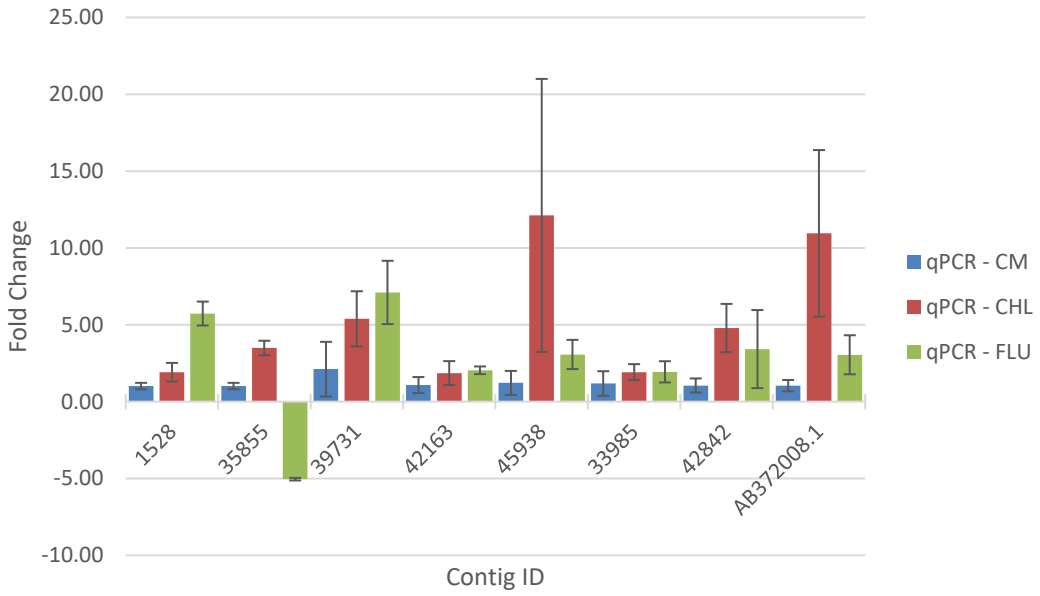


Figure 4.04. Fold change through qPCR validation of early candidate genes (identified through CHL & FLU microarray meta-analysis). Error bars indicate 95% confidence limits. Genes showing consistent expression (up or down) between CHL and FLU strains, and where one of these was significantly different to the CM strain (1528, 39731, 42163, 45938, 42842, AB372008) were selected to continue through to further expression studies.

Selected	**Rejected**
1528_Xanthine dehydrogenase	35855_Cytochrome p450
39731_Solute family carrier family 2	33985_Carboxylesterase
42163_Short-chain dehydrogenase	
45938_Cytochrome p450	
42842_Dimethylaniline monooxygenase	
AB372008.1_CYP6BG1	

Table 4.09. Candidate genes selected or rejected through qPCR validation. A candidate gene was selected to continue through to further expression experiments if the qPCR validation confirmed overexpression in both of the selected strains with at least one of the strains showing significant overexpression in comparison to the susceptible strain. Candidate genes 35855 and 33985 did not meet these criteria and were rejected.

As it had become available, an identical array was carried out using the diamide resistant Hawaii strain in a comparison with a susceptible laboratory strain (ROTH). The resulting microarray data was analysed as before producing a list of 5119 differentially expressed genes with a fold change of more than 2. This list was reduced to 4312 candidate genes once the duplicates (based on contig name) were removed. Microsoft Excel was again used to compare the lists of candidate genes produced by each of the microarrays (all with the same correction and p values and fold change of +/- 2) to highlight common genes of interest. The microarrays used to filter the total list in two different comparisons were: CMxCHL, CMxFLU, CMxUNT and HSxROTH. One comparison used all four of the microarrays and another used three of the four microarrays (CMxCHL, CMxFLU, HSxROTH). The comparison using all four microarrays yielded 43 common contigs, 26 of which had sequence descriptions. Nine of these are up-regulated in all four microarrays and 1 is down-regulated in all four arrays. The rationale for comparison with and without the CMxUNT array data was that the UNT strain is the untreated parental source of the CHL (chlorantraniliprole) and FLU (flubendiamide) selected strains. While it does have some resistance to diamides, it is not as resistant as the other strains due to lack of selection pressure. Indeed, Syngenta were unable to provide an accurate assessment of its resistance level (as assessed by diamide bioassays). Consequently, it is possible that the microarray data from this strain could mask possible candidate genes that would be highlighted through the other array data, caused by lower levels of expression through a lack of exposure to insecticides. Had a live strain been available it would have been possible to compensate and/or test for this fact through bioassay and selection work as it has been reported that where resistance is a product of metabolic detoxification, this can decline through a lack of exposure to xenobiotics and fitness costs [276, 304].

Comparison of the data from the 3 microarrays involving diamide selected strains (CMxCHL, CMxFLU and HSxROTH) yielded a list of 93 individual contigs (fig. 4.03). Of these, 62 had sequence descriptions. The list is further narrowed to 27 described genes of interest that are consistently up-regulated across all 3 microarrays and 2 that are down-regulated in all 3 (table 4.10).

Contig	FC in CMxCHL	FC in CMxFLU	FC in ROTHxHS	Sequence description
Contig_1924	30.58	11.36	-10.21	n-acetylneuraminate lyase
Contig_3261	19.34	11.69	56.34	myo-inositol oxygenase
Contig_46169	14.24	12.23	-6.59	gloverin-like protein
Contig_2963	12.17	6.43	31.45	n-acyl-l-amino-acid amidohydrolase
Contig_42842	12.10	6.42	20.82	dimethylaniline monooxygenase
Contig_3379	11.92	4.49	-5.11	short-chain dehydrogenase
Contig_42837	11.02	3.30	13.19	cytochrome p450
Contig_1694	10.40	9.97	2.10	3-hydroxyacyl-coa dehyrogenase
Contig_2416	8.44	2.89	9.29	sugar transporter
Contig_19228	8.15	5.15	42.89	n-acyl-l-amino-acid amidohydrolase
Contig_39731	6.88	3.70	7.07	solute carrier family 2 (facilitated glucose transporter) member 8
Contig_42163	6.70	3.93	3.50	short-chain dehydrogenase
Contig_42182	6.66	4.30	114.76	2-oxoisovalerate dehydrogenase subunit mitochondrial
Contig_25748	6.42	4.52	66.52	dimethylaniline monooxygenase
Contig_33985	6.14	5.91	43.69	carboxylesterase
Contig_1528	5.54	2.70	19.67	xanthine dehydrogenase
Contig_4020	5.35	3.64	-38.29	hypothetical protein VOLCADRAFT_107620 [Volvox carteri f. nagariensis]
Contig_2566	4.56	5.13	4.29	beta- -glucan recognition protein 2
Contig_40035	4.32	3.50	92.38	amp dependent coa ligase
Contig_1685	3.93	2.40	3.99	selenium-binding protein
Contig_29807	3.66	4.28	-2.06	protease inhibitor epi11
Contig_3596	3.45	2.46	6.09	glutamine-oxaloacetic transaminase
Contig_34882	3.42	3.51	-75.07	isoform b
Contig_4158	3.42	2.38	9.37	15 protein
Contig_2518	3.40	5.16	15.48	helicase conserved c-terminal domain containing protein
Contig_3271	3.28	3.30	3.54	serine protease inhibitor
Contig_2648	3.22	2.46	3.59	selenium-binding protein
Contig_2356	3.20	2.12	30.82	phosphogluconate mutase
Contig_4134	3.02	2.52	17.80	phosphogluconate mutase
Contig_2422	3.01	2.00	18.18	glucosyl glucuronosyl transferases
Contig_19431	2.80	4.49	12.50	protein
Contig_24697	2.71	4.66	-25.88	transposable element p transposase
Contig_42715	2.65	2.32	5.84	zinc finger protein
Contig_2909	2.26	2.95	132.32	low density lipoprotein
Contig_23886	2.11	4.60	-3.06	bel12_ag transposon polyprotein
Contig_1673	2.06	3.72	-2.60	suppressor of profilin 2
Contig_36192	-2.00	-2.43	31.42	protein
Contig_36107	-2.03	-4.17	4.05	cg12608
Contig_3436	-2.08	-2.18	11.48	ecto-nox disulfide-thiol exchanger 1
Contig_45256	-2.10	-2.19	-2.30	nucleolar protein 10
Contig_40671	-2.12	-2.52	6.81	something about silencing protein 10
Contig_1217	-2.14	-2.14	10.62	ecto-nox disulfide-thiol exchanger 1

Contig_5837	-2.14	-2.69	3.58	polycomb protein l
Contig_36865	-2.30	-2.78	-4.28	thioredoxin family trp26
Contig_38726	-2.30	-2.31	93.88	tp53-regulating kinase
Contig_22487	-2.31	-2.11	6.53	endonuclease-reverse transcriptase
Contig_44988	-2.51	-3.71	6.89	uncoordinated family member (unc-89)
Contig_44529	-2.54	-4.45	10.44	isoform b
Contig_35465	-2.57	-6.30	10.18	heat repeat-containing protein 3
Contig_3387	-2.58	-3.27	4.25	h aca ribonucleoprotein complex subunit 2-like protein
Contig_41133	-2.62	-3.10	2.28	ccaat enhancer binding protein zeta
Contig_43719	-2.67	-3.67	4.70	upf0399 protein v1g245966
Contig_36551	-3.73	-2.09	13.35	retrovirus-related pol polyprotein from transposon tnt 1-94
Contig_2368	-3.78	-5.28	11.38	mki67 fha domain-interacting nucleolar phospho
Contig_37812	-3.95	-4.62	10.71	nucleolar protein 10
Contig_38673	-4.18	-3.31	7.20	tyrosine recombinase
Contig_34113	-4.27	-2.87	6.19	tyrosine recombinase
Contig_3514	-7.77	-4.79	8.92	salivary gland secretion 3
Contig_1198	-10.29	-10.87	74.96	cytochrome p450
Contig_41661	-15.17	-12.70	58.96	cytochrome p450
Contig_45299	-26.18	-28.52	57.02	cytochrome p450

Table 4.10. Comparison of microarray results. Sequence descriptions highlighted in blue indicate up-regulation in all 3 microarrays. Sequence descriptions highlighted in pink indicate down-regulation in all microarrays. Contigs for which no sequence description was available have been removed. Order is by decreasing fold change shown by CMxCHL.

'Average linkage' cluster analysis was performed using the open source 'Cluster 3' program [305], the results of which were visualised using 'Java TreeView' [306] as both a dendrogram and heatmap (figure 4.05). The aim of this was to help visualise commonalities in expression of candidate genes and provide insight into any hierarchal groupings to aid selection of genes for further functional characterisation [307, 308]. Microarray data included in the cluster analysis were those up or down regulated in all 3 resistant strains (CHL, FLU and HS) for which sequence descriptions were available. Because sequence descriptions are not contig specific, sequence descriptions for the clustering were modified to include the contig ID so that the actual sequences could be identified. This gave a list of 62 candidates to be included for cluster analysis.

As would be expected, an initial study of the dendrogram shows the CMxCHL and CMxFLU arrays are more related than the HSxROTH confirming their relatedness through their shared parental strain (at least in comparison to HS). Figure 4.05 also highlights a node clustering 27 genes with a correlation value of 0.76. This node contains all of the overexpressed contigs that were highlighted by the spreadsheet analysis.

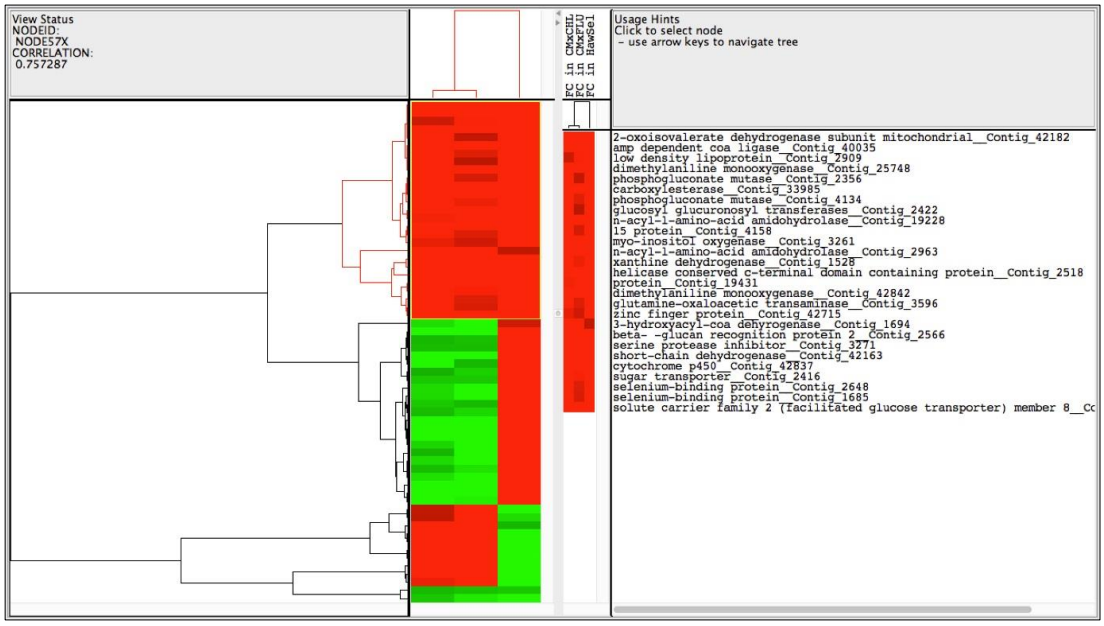


Figure 4.05. Dendrogram showing clustering of microarray results. Highlighted node has correlation value of 0.76 and includes all of those GOIs highlighted through spreadsheet analysis.

The clustering analysis supports the spreadsheet analyses in highlighting the same subset of contigs. While the spreadsheet analyses serve to perform an indicative meta-analysis on the microarray data, cluster analysis provides a more quantitative data breakdown with dendrograms providing a visually simple of way of viewing associations between various contigs/genes.

Literature searches were conducted on all of the 27 candidate genes with a known function to further assess their potential role in resistance. Based on this, cluster analysis and preliminary qPCR results, 6 genes of interest were selected to be further investigated (table 4.11). This analysis involving the HS microarray alongside the others altered the list of candidates from those shown in table 4.09 to that shown in table 4.11.

1528_Xanthine dehydrogenase
39731_Solute family carrier family 2
42163_Short-chain dehydrogenase
2416_Sugar transporter
42842_Dimethylaniline monooxygenase
AB372008.1_CYP6BG1

Table 4.11. Genes of interest selected for further investigation

Additional qPCR validation of these genes was carried out this time including the Hawaii and Roth strains (see figure 4.06). This revealed particularly profound differences in the expression of two candidate genes. Dimethylaniline monooxygenase, otherwise known as flavin-dependent monooxygenase (*FMO2*) revealed a 14,700-fold over expression in the Hawaii-selected versus the Roth strains and *CYP6BG1*, a cytochrome P450 that has previously been implicated in permethrin resistance in *P. xylostella* [134, 309], was overexpressed in excess of 33,000-fold. However, the 95% confidence limits were much greater for the *CYP6BG1* gene suggesting this result should be treated with more caution. Due to these results and further literature searching, the following candidates were prioritised for further functional characterisation: the flavin-dependent monooxygenase (*FMO2*), short-chain dehydrogenase (*SCD*), xanthine dehydrogenase (*XDH*) and *CYP6BG1*.

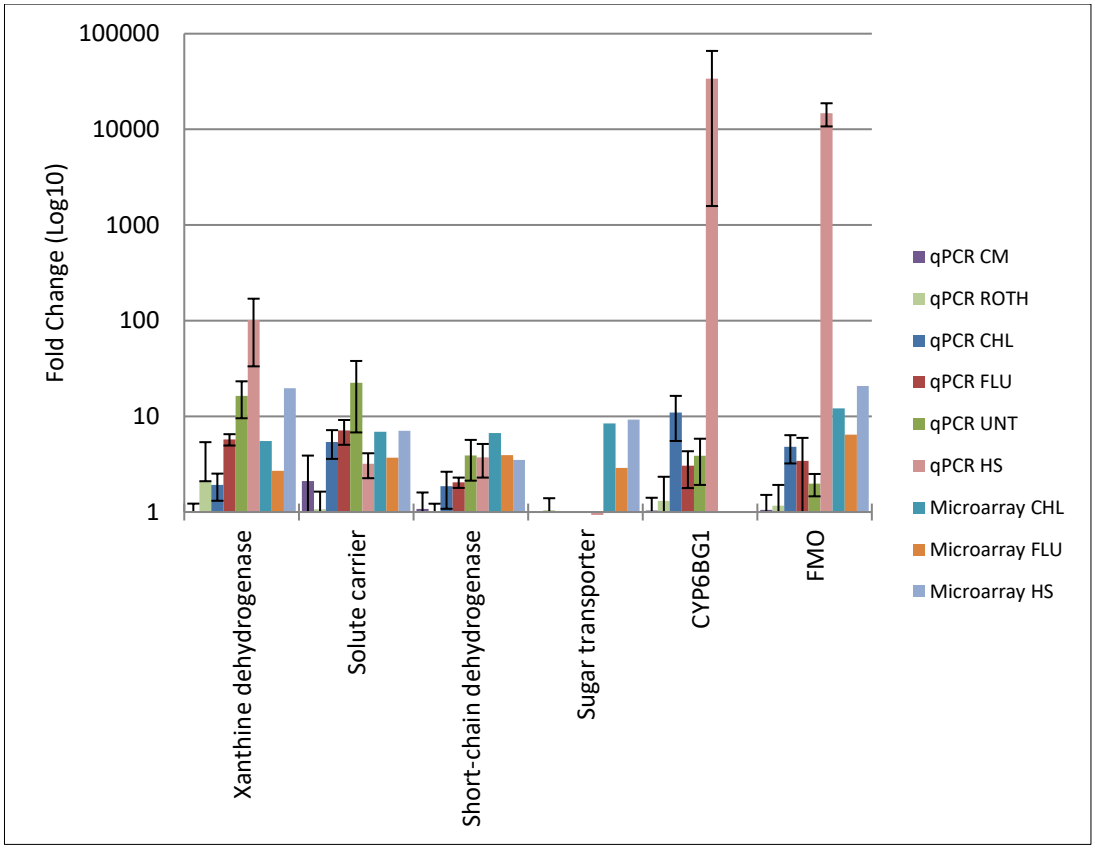


Figure 4.06. QPCR and microarray expression (fold change) of candidate genes. Error bars for qPCR data indicate 95% confidence limits. *FMO2* and *CYP6BG1* candidate genes showed particular overexpression through qPCR of 14,700-fold and 33,000-fold respectively in the HS strain.

4.4 Discussion

Prior to investigating metabolic resistance to diamides in *P. xylostella* it was important to investigate the frequency of the previously reported target-site mutations in these strains. It was particularly necessary to rule out this mutation as the cause of diamide resistance in the recently acquired Hawaii population as doing so would provide a strain with non-target site resistance alone. Data obtained confirmed that this strain lacks the G4946E substitution in contrast to the FLU, CHL and UNT strains. Based on these results it is likely that the resistance of the HS strain is metabolic in nature, however, it is possible that other mutations within the ryanodine receptor may be responsible for or contribute to diamide resistance. In 2014 Guo *et al.* published a paper identifying three new point mutations (E1338D, Q4594L, and

I4790M) [275] in the ryanodine receptor, which genotyping confirmed also are not present in the Hawaii strain.

It has been suggested that metabolic detoxification may be involved in chlorantraniliprole resistance in *Plutella xylostella* [106, 304]. In other insects, (*Spodoptera exigua* and *Choristoneura rosaceana*) several synergistic inhibitors have been tested to see how they affected the toxicity of chlorantraniliprole [310]. The authors concluded that mixed function oxidases, esterases, glutathione S-transferases and carboxylesterases might not be the major drivers of chlorantraniliprole resistance. Wang *et al.* [304] found that the synergistic effects of metabolic enzyme inhibitors were limited in *P. xylostella* against chlorantraniliprole which, while it was suggested that this might indicate that metabolic detoxification is not the major mechanism, it was also suggested that the chosen synergists were not specifically addressing those enzymes involved in chlorantraniliprole metabolism.

To explore metabolic resistance in diamide resistant *P. xylostella* strains, transcriptome profiling was carried out using microarrays. The microarray design used is based on extensive sequencing of the *P. xylostella* transcriptome (H. Vogel, personal communication) and so should represent the majority of expressed genes in this species.

Genes with a potential role in diamide resistance were initially selected through a meta-analysis of multiple microarrays used to generate expression profiles for *P. xylostella* strains selected with different diamide compounds and different levels of resistance. Further cluster analysis, qPCR and literature searching was used to select a small number of genes for further functional validation. There is an inherent risk in such an approach in disregarding potential genes as candidates on the basis that different genes may play a role in resistance in the different strains, or a role in resistance is not supported due to the current lack of data in the literature. It is possible that enzymes can be recruited into new metabolic pathways if they have broad substrate specificity [311]. However, because of cost and time constraints, prioritisation of genes for functional characterisation must be carried out and multiple lines of evidence were used to inform this process.

Like Mitchell *et al.*, it was found that the microarray analysis seemed to report an underestimation of the fold changes compared to the qPCR validation [197]. A check of the qPCR primer binding sites for the genes of interest with alignments of the microarray sequences, resistant HS and susceptible ROTH strain sequences for the candidate genes confirmed that there are no discrepancies in their targets. It is possible that differences in the location of the qPCR primers relative to the microarray probes could account for this [312], however, as the threshold for fold-change for individual genes was set at 2, it is likely that despite discrepancies between the actual fold changes, there was good correlation between the microarray and qPCR results. In a study specifically addressing correlations between microarray and qPCR results, Morey *et al*. concluded that genes exhibiting at least 1.4-fold change had significantly higher correlations than those demonstrating less change by both microarray and qPCR [313]. Taking this into consideration, the possibility must be considered that candidate genes were disregarded due to the 2-fold change threshold imposed in this PhD study.

Figure 4.05 demonstrates cluster analysis confirmation of the group of candidate genes with a correlation of 0.76. Following validation of the candidate genes, further interrogation of the clustering and dendrogram produced reveals correlation between them. The original node containing all of the genes of interest separates into two further nodes, one of which contains (figure 4.07) the solute carrier, short-chain dehydrogenase (*SCD*) and sugar transporter, all of which were discounted during the course of validation. The other node (figure 4.08) contains the *FMO2* (dimethylaniline monooxygenase) and xanthine dehydrogenase (*XDH*), both of which have been implicated in resistance (particularly the *FMO2*) throughout the validation processes.

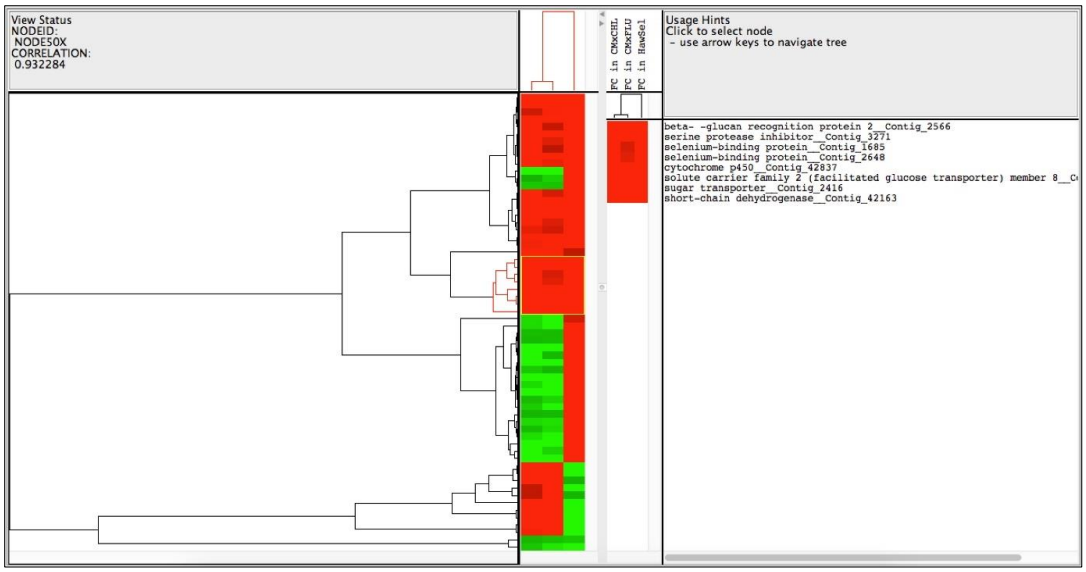


Figure 4.07. Dendrogram showing clustering of microarray results. Highlighted node has a correlation of 0.93 and includes the GOIs rejected through the validation process.

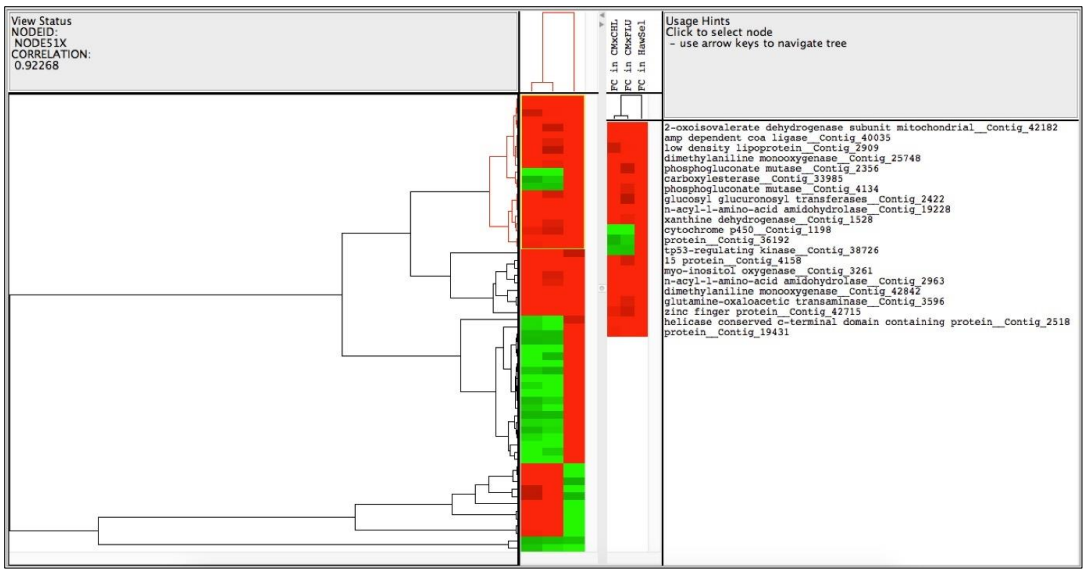


Figure 4.08. Dendrogram showing clustering of microarray results. Highlighted node has a correlation of 0.92 and includes the primary (*FMO2*) and secondary (*XDH*) genes of interest confirmed through the gene validation in chapter 5.

At the early stages of the project it would not have been possible to make a prediction based on the cluster analysis on which node of candidate genes to focus resources. However, this result does demonstrate the value of adopting more than one approach to candidate gene selection, and the potential of cluster analysis to interrogate data sets.

One of the strongest candidate genes to emerge from the microarray and qPCR analyses was an *FMO2*. There is very little literature on the role of FMOs in insects, and the studies that do exist focus on the detoxification and sequestration of pyrrolizidine alkaloids [211, 220, 221, 314-316]. To date, there is only one study suggesting a relationship between FMOs and the metabolic detoxification of insecticides [222]. In contrast, the relationship between FMOs and xenobiotics in vertebrates is well studied where P450 enzymes are commonly supplemented by FMOs in xenobiotic detoxification [317]. Even though mammalian FMOs show a monophyletic origin and have evolved well separated from polyphyletic insect FMOs [318], they do share common functional pathways in their oxygenation of nitrogen or sulphur nucleophilic substrates [217]. The ability of FMOs to oxidise these so called soft-nucleophiles means that they can interact with substrates such as amines, amides, sulphides and thiols amongst others giving a very broad spectrum of specificity [217, 319]. The spectrum of these substrates suggests that FMOs could be involved in the metabolism of diamides, with chlorantraniliprole containing numerous nitrogens and flubendiamide containing a sulphur dioxide moiety (see figs. 1.11 and 1.12).

Short chain dehydrogenases/reductases are one of the oldest and largest families of enzymes [203] and contain about 25% of all dehydrogenases [320]. Having investigated this family from an evolutionary perspective, Jornvall *et al.* note that even though it is functionally possible for enzymes such as cytochrome P450s to have replaced short chain dehydrogenases this is not known to have occurred, implying that they must have functions seemingly more important than might be initially assumed by their category [203]. Originally classed as alcohol dehydrogenases, it is now known that SCDs have an enormous variety of biochemical roles and comprise many intermediary metabolic functions [204] including enhanced insecticide catabolism [321].

As with FMOs, Xanthine dehydrogenase (XDH) has not been previously associated with pesticide resistance. XDH plays a primary role in the excretion of uric acid [322]. Much of the literature describes the metabolic action and importance of xanthine oxidase, a key enzyme involved in the metabolism of nitrogenous compounds. Although XDH is the common *in vivo* form of this enzyme in insects [323-325], the two catalytic forms are so closely related in the excretion pathway that it is worth considering information about one form to be informative

of the other. Xanthine dehydrogenase oxidises hypoxanthine to xanthine and then xanthine to uric acid, playing an indispensable role in nitrogen metabolism, especially in uricotelic animals, e.g. lepidoptera [326, 327]. Xanthine oxidase has been highlighted as an important non-P450 in drug metabolism [328] and Slansky has suggested that although XDH is generally not considered within the context of xenobiotic detoxification, it may be associated with phytochemical detoxification [329]. It is possible that even if *XDH* is not directly metabolising diamide insecticides, it may be playing a part in the more rapid excretion of these xenobiotics or their metabolic wastes and therefore warrants further investigation.

CYP6BG1 is a cytochrome P450 which as described earlier is a class of enzymes that have long been associated with metabolism and detoxification of insecticides [184, 244]. In fact *CYP6BG1* itself has been implicated in permethrin resistance in *P. xylostella* [134, 309] providing strong justification for its inclusion as a gene of interest.

The major blood (Haemolymph) sugar in insects is often the disaccharide trehalose [330]. It is synthesised from glucose phosphates in fat body tissue and serves as a source of carbohydrates for various tissues throughout the body [331]. While being identified as separate candidate genes through the microarray analyses, within the insect, the sugar transporter and solute carrier family 2 perform essentially the same role in transporting solutes (sugars) across cell membranes so making them available as energy sources for any number of biological processes and pathways. They are not specifically associated with xenobiotic detoxification; however, it is possible that increased expression in a detoxification pathway necessitates an increase in sugar availability to ensure the efficient functioning of said pathway/mechanism and so as is the case with *XDH*, while these genes may not directly metabolise xenobiotics, it is possible that their upregulation is a factor in a diamide metabolising pathway.

4.5 Summary

In summary, transcriptome profiling of diamide resistant *P. xylostella*, including a strain with no known target-site mechanism, identified a number of potential candidate genes that may be involved in metabolic detoxification. In the following chapters (chapters 5 and 6) the strength of the association of these genes with resistance to diamides was examined to determine which, if any, have the capacity to metabolise this insecticide class.

5 Validation of candidate genes in diamide resistance

5.1 Introduction

Having identified *FMO2*, *CYP6BG1*, *SCD*, sugar transporter and *XDH* as candidate genes it was important to examine the association of these genes with diamide resistance in the HS strain in order to identify which, if any, have a causal role. A combination of different approaches were used to achieve this and are introduced briefly below.

5.1.1 Stability of chlorantraniliprole resistance in resistant strain

During routine culturing, selection and bioassay of the resistant Hawaii strain it became apparent that diamide resistance may be unstable. This observation correlates with work by Wang *et al*. [304] who found that resistance to chlorantraniliprole was not stable in the absence of selection pressure with resistance declining slowly for 2 generations post selection, followed by a rapid decrease in resistance from 2040-fold to only 25-fold in just seven generations. The instability of resistance in the HS strain suggests a significant fitness cost in the absence of selection and also provides an opportunity to explore the association of the candidate genes listed above with resistance. I designed an experiment to monitor the stability of resistance to chlorantraniliprole of the HS strain in the absence of diamide selection by insecticide bioassays. I hypothesised that the resistance status of the unselected line (UnSel) would decline as generations progressed compared to the selected line (HS). Comparison of the level of phenotypic resistance during this process with the expression of candidate genes (as assessed by QPCR) would reveal the strength of the association of these genes with chlorantraniliprole resistance in the HS strain.

5.1.2 Synergists

Synergists are chemicals that inhibit specific genes/enzymes, increasing the potency of an insecticide. Synergists have frequently been used to implicate specific families of metabolic enzymes in insecticide resistance. In this regard the most widely used synergist is piperonyl butoxide (PBO) which inhibits monooxygenase genes (typically, cytochrome P450s), and to a lesser extent certain insect esterases which are often implicated in metabolic resistance. For example, Bautista *et al.* realised a 4-fold reduction in the resistance ratio of a resistant strain of *P. xylostella* to permethrin through the use of PBO [309]. Wang *et al.* also tested a field

population of *P. xylostella* with PBO and chlorantraniliprole, finding a synergistic ratio of 2.2 [246]. In the same study, the authors also tested the glutathione-S-transferase depleter, diethyl maleate (DEM) and S,S,S-tributylphosphorothioate (DEF), an esterase inhibitor, observing synergism ratios of 2.3 and 2.9 respectively. Often synergist assays are used in studies as a precursor to, or to provide corroborative, supporting evidence to further more complicated (or expensive) methodologies validating putative metabolic detoxification genes. Synergists can have such a significant impact on the efficacy of an insecticide that in addition to their use in laboratory diagnostics, they are also used in field applications to aid pesticide efficacy [66]. As discussed in previous chapters, FMOs have been primarily studied in other (non-insect) taxa. Methimazole (MEM) has been the primary inhibitor of FMOs employed by these studies, being used by Tugnait *et al.* and Ripp *et al.* as a competitive inhibitor in studies investigating rabbit, pig and human FMO isoforms [332, 333]. Methimazole has, at the time of writing, only been used as an FMO inhibitor once in insect *in-vivo* studies of insecticide resistance, with the study by Tian *et al.* also being the only currently published work linking insecticide (metaflumizone) resistance with FMO detoxification in insects [222].

Following selection of the candidate genes, synergist bioassays were performed for the *CYP6BG1* and *FMO2* candidate genes as they were the most over-expressed in HS shown through the qPCR validation, and exhibited the greatest decline in expression over time in the resistance stability experiment (figure 5.04). PBO was used as a likely inhibitor of *CYP6BG1*, while MEM was used as a possible synergist for *FMO2* to investigate if the resistance phenotype of HS could be affected by either of these compounds.

5.1.3 Sequence characterisation of candidate genes

As a prerequisite for the further functional analyses detailed in this chapter, it was essential to obtain the full-length sequences of the candidate genes from the resistant HS strain. Several resources were available to be mined to obtain the sequences needed, and in some instances, multiple approaches needed to be combined in order to obtain the necessary information.

The starting point for each of the candidate genes was the transcriptome sequence on which microarrays were designed (chapter 4). Web searchable databases such as NCBI (also integrated into sequence analysis programs such as Geneious) enable efficient searching and comparison of genes from many organisms. In addition to generic databases, two diamondback moth genome databases have been published by Jouraku *et al.* and Tang *et al.* in 2013 and 2014 respectively [137, 138]. By definition, these genomes provide genomic and hence sequence data specific to the species studied here and so are a powerful resource in identifying sequences. They were however, not available from the start of this project and so sequences from and comparisons between other insect species were utilised, specifically using other lepidopteran model insects (such as the silk moth, *Bombyx mori*).

5.1.4 Transgenic *Drosophila*

D. melanogaster is a model insect species, in part because it is easily cultured in the laboratory, has a relatively short life-cycle, and robust methodologies have been developed for the creation of transgenic lines. The genetic modification of *Drosophila melanogaster* provides a powerful tool for understanding the function of genes of interest [334]. In contrast, transgenic approaches are yet to be developed for non-model insects such as *P. xylostella*. For this reason, the GAL4/UAS system was employed in *Drosophila* to better understand if any of the candidate genes, *FMO2*, *CYP6BG1*, *SCD* or *XDH* are involved in the detoxification of diamide compounds. In the GAL4/UAS system, a gene of interest is designed to be expressed under the control of an upstream activating sequence (UAS), which is activated by the binding of the GAL4 transcription factor. It is only upon crossing the UAS/transgene line with a GAL4 driver line that the transgene will be expressed. GAL4 can itself be placed under the control of a diverse range of promoters. Driver lines containing different and, if required, very specific promoters are commercially available and can be used to express the transgene in a host of different tissues.

In the case of this study, all of the transgenes were expressed under the control of an actin promoter, which expresses the GAL4 ubiquitously throughout the insect. Actin was selected as the aim of the experiments was to ascertain if the genes were implicated in detoxification; expressing the genes in a specific tissue would, at this stage, complicate any results that were

obtained (or result in experimental failure) as it was not known in what tissues the genes would normally be expressed.

This approach was used to create individual transgenic *D. melanogaster* lines expressing each of the candidate genes. In bioassays, these flies were compared with flies of the same genetic background but minus transgenes as a control. The same experimental methodology was used by Daborn *et al.* in a GAL4/UAS study of the insecticide resistance potential of eight different cytochrome P450 genes by transgenic overexpression [180]. In their study the authors were able to differentiate between the selection of P450 genes, enabling associations to be made between some genes and resistance to specific insecticides, while discounting others.

5.1.5 RNA Interference

RNA interference (RNAi) is a reverse genetics approach that has been previously used to investigate the association of a candidate gene with insecticide resistance. Since the first indicative study and hint at the potential of RNAi discovered by Napoli *et al*. in 1990 when studying violet colouration in petunias [335], it took until the publication of a paper in 2006 by Bernstein *et al*. for the process to be elucidated with the authors identifying involvment of the enzyme, 'dicer' [336]. In a short review of the history of RNAi, Sen and Blau note the widespread use of RNAi to assess the effect of loss of gene function in organisms and the relative ease with which the technique can be applied [337]. This is reiterated by Kim *et al.* in a later review of applications and advances in RNAi, detailing the technique's use to identify and validate genes encoding major target enzymes or proteins; reveal the role of the genes encoding detoxification enzymes and transporters; elucidate the mechanism of insecticide-induced up-regulation of detoxification genes; and develop RNAi-based transgenic plants as proof of concept for insect pest management [338]. In relation to diamide resistance Wan *et al.* and Yang *et al.* used RNAi mediated knockdown of the ryanodine receptor gene in the Colorado potato beetle, *Leptinotarsa decemlineata* and whitebacked plant-hopper, *Sogatella furcifera*, and demonstrated that this reduced the sensitivity of these species to chlorantraniliprole [339, 340]. In addition, RNAi has been successfully applied in the characterisation of multiple detoxification genes confering insecticide resistance within *P. xylostella.* For example, Bautista *et al.* used RNAi to silence

the cytochrome P450, *CYP6BG1* and reduce the resistance of *P. xylostella* to the pyrethroid, permethrin [134]; Zhang *et al.* detail the RNAi knockdown, through injection of dsRNA, of the ubiquitin gene UBL40 in this species resulting in a 1.8 – 1.9-fold decrease in resistance to deltamethrin [341]. Because the *FMO2* gene became the primary candidate gene prior, based on other functional tools, attempts were made to use RNAi to silence this gene and investigate if individuals dosed with double stranded or short interfering RNA become more sensitive to diamide pesticides than control individuals injected with control RNAs. As a first step it was necessary to confirm that the RNAi dosing reduced the expression of the *FMO2* gene before exploring the effect of this on the resistance phenotype.

5.1.6 Syngenta LC-MS (*Drosophila melanogaster*)

Following the liquid chromatography-mass spectrometry performed on *P. xylostella* in chapter 3, the availability of transgenic *D. melanogaster* expressing the *FMO2* gene provided the opportunity to investigate the stability and metabolism of chlorantraniliprole in this model system. Unlike the native situation in *P. xylostella*, where several genes might contribute to resistance, the GAL4/UAS transgenic *D. melanogaster* line enabled the quantitative measurement of chlorantraniliprole metabolism compared to controls whereby the *FMO2* gene is the only gene differing between the experimental and control lines.

One study to have made use of the GAL4/UAS system linked with biochemical studies incorporating LC-MS, is that by Mukherjee *et al.* where the transgenic GAL4/UAS system has been used incorporating the reporter gene, yellow fluorescent protein (YFP) to aid in organ tissue dissection. LC-MS was then subsequently used to identify proteins in these tissues [342]. As discussed in chapter 3, the majority of pesticide-based studies published focus on the use of LC-MS in the detection of pesticide residues in crops, and those that do focus on insecticide metabolism in insects tend to use *in-vitro* platforms on which to perform LC-MS. An example is that by Zimmer *et al.* investigating P450 mediated resistance to pyrethroids in pollen beetles who demonstrated a correlation between *in-vitro* formation of the 4-OH-deltamethrin metabolite and *in-vivo* synergist experiments [343]. As for *P. xylostella*, both feeding and topical dosing assays with chlorantraniliprole were conducted for the transgenic *FMO2* expressing *D. melanogaster*.

5.2 Methods

5.2.1 Stability of chlorantraniliprole resistance in resistant HS strain

Starting from a selected population (8ppm chlorantraniliprole), 2nd generation post selection (G0) offspring were separated into two populations. One of these populations was subject to 6ppm chlorantraniliprole selection every 2nd or 3rd generation, while the other was not. Where possible, every other generation was subjected to a bioassay as well as a subsample of 40 individuals (4 cohorts of 10 individuals) snap frozen using liquid nitrogen. Upon conclusion of the experiment, RNA was extracted and cDNA synthesised from each of the frozen cohorts (see general methods sections 2.5 and 2.6 respectively). The cDNA was used as template in qPCR to identify any change in the expression status of each of the candidate genes between the selected and unselected strains. The protocols for qPCR and analyses were the same as reported in section 4.2.4.2. Chlorantraniliprole series dilutions, bioassay protocols and analyses were the same as reported in section 3.2.

5.2.2 Synergist *P. xylostella* Bioassays

Synergist bioassays using either methimazole (MEM) or piperonyl butoxide (PBO) were conducted following the standard bioassay protocol but insects were exposed to the relevant synergist for 24 hours prior to insecticide exposure (section 3.2.2). After 24 hours on the synergist (or agral control solution) treated leaf disks larvae were moved to fresh insecticide treated leaf disks and standard bioassay methods followed.

5.2.2.1 Insecticide/Synergist Dilutions

Insecticide and synergist dilutions were made using the methods detailed in section 3.2.1. The exception to this was the initial dilution of Piperonyl Butoxide (PBO) from 900,000 ppm to 10,000 ppm, which was made using acetone, after which further dilutions were made using 0.01% Agral. Technical grade synergist compounds were sourced from Sigma-Aldrich. Following preliminary assays, PBO and MEM were used at concentrations of 100ppm and 200ppm respectively.

5.2.3 Identifying candidate gene sequences

Of the candidate genes identified, *SCD* and *CYP6BG1* had their sequences identified through the repeated sequential design of primers, PCR and sequencing according to the general methods laid out in chapter 2. Primers were initially designed based upon the transcriptome sequences used for the microarray design. Following successful amplification of target product from the actual strains being investigated (initially CM, CHL and FLU, with a later shift to the live strains of ROTH and HS), this known sequence data from the biological material was aligned with sequences obtained from both *P. xylostella* and other species. Typically, the other species sequences used were from other lepidopterans such as the cotton bollworm, *Helicoverpa armigera,* silk moth, *Bombyx mori,* and monarch butterfly, *Danaus plexippus*. Primer pairs were designed with one of the two primers based on known sequence from the *P. xylostella* strains with the other primer designed in sequence regions that were conserved between the different species. More often than not, multiple primer pairs were designed for any one target region, allowing, if needed, a nested PCR approach to be used (see general methods chapter). All the sequences were identified from pools of larvae in order that a true consensus sequence would be identified for each of the candidate genes.

The *FMO2* and *XDH* genes required additional approaches to be used to identify key regions of their sequences. For the *FMO2* gene, random amplification of cDNA ends (RACE) was employed to identify the 5' end, and next generation sequencing was used to identify the 5' end of the *XDH* candidate. The need for both of these approaches was required because of heterogeneity between the reference sequences and repeated failure to amplify these regions using conventional PCR.

RACE was performed using a 'FirstChoice RLM-RACE Kit' (Ambion) according to the manufacturer's protocol with some minor changes, notably smaller PCR reaction volumes (25µl total volumes), a lower temperature during the annealing stage of PCR (58^{o}C), and an increase of the PCR extension time (1min 30secs). The primers used and the process by which the technique was employed are detailed in section 5.3.3.1.

Initially, next generation sequencing (NGS) took place for chapter 6 and so the NGS platform and methodology used for genome sequencing is detailed in that chapter. The NGS data produced was utilised in this chapter by mining the 'MIRA' (Mimicking Intelligent Read Assembly) assembled scaffold database using Geneious. The process used has been detailed alongside the results in section 5.3.3.2.

5.2.4 Transgenic *Drosophila melanogaster*

5.2.4.1 Preparation of *SCD*, *CYP6BG1* and *FMO2* genes for expression

Having identified the sequences for the *SCD*, *CYP6BG1* and *FMO2* candidate genes isolated from the resistant HS strain, the sequences were appended by the addition of restriction site sequences to the 5' (*Bgl*II) and 3' (*Xba*I) ends to enable subsequent cloning into plasmids. Genes were then synthesised by Geneart (Invitrogen). The synthesised genes were provided by the manufacturer in their inhouse plasmid vectors and were subsequently sub-cloned into the vector, pUAST using the restriction sites engineered into the constructs. 'XL1-Blue Competent Cells' (Stratagene) were transformed with the pUAST plasmids according to the manufacturer's protocol, grown overnight and plated. Following this, colonies were selected, and colony PCRs and product sequencing (via Eurofins) performed. Standard PCR conditions were used with the primers shown in table 5.01. Sequencing confirmed that the sequence in each plasmid was correct.

Primer Name	Primer Sequence
CYP6BG1 fly seq R	CGA GGT GTC AAT AAT AAG AGG CT
CYP6BG1 fly seq F	TTG TTC ACG CTG TCG CCA G
CYP6BG1 fly SCP	ATG CGC GCG GGA CTT CGA
FMO fly seq F	GTT GGT AGT TGG ACG TGG AC
FMO fly seq R	AGT GAA CCT CGC CAC ATC AG
FMO fly SCP	CCG GAG AAT ATG TTA TGG ATG AC
SCD fly seq F	CCG TGT ACT TAC TGA CCA GC
SCD fly seq R	TCC AGG GCG GCT TTA GAA AC
SCD fly SCP	TAG ACT CGG ACG CAG ACG T

Table 5.01. Primers used for colony PCR and sequencing of pUAST plasmids containing candidate genes. 'SCP' and 'seq R' primers were used for colony PCR, with 'seq F' and 'seq R primer pairs used for sequencing.

The plasmids were then sent to the University of Cambridge fly facility for injection into *Drosophila melanogaster* embryos and the creation of balanced lines. The transgenic *D. melanogaster* lines containing candidate transgenes downstream of the UAS promoter were crossed with driver lines expressing GAL4 under the control of a *Drosophila* actin promoter. F1 progeny of these crosses expressing the genes were then subjected to insecticide bioassays and compared to flies of the same genetic background but without the transgene.

All the genes initially synthesised and used to create transgenic *D. melanogaster* lines were the original lepidopteran sequences identified directly from *P. xylostella*, however, upon obtaining bioassay results from these lines, a second version of the *FMO2* gene was synthesised that was codon optimised for *Drosophila* expression. Codon optimisation was conducted through the online 'gene optimisation' portal provided by ThermoFisher Scientific as part of their gene synthesis service (https://www.thermofisher.com/uk/en/home/life-science/cloning/gene-synthesis/geneart-gene-synthesis/geneoptimizer.html). The gene was synthesised and transformation of the *D. melanogaster* line carried out as above. Figure 5.01 shows a side-by-side comparison of the 5' and 3' ends of the *FMO2* gene, demonstrating the silent nucleotide substitutions that took place.

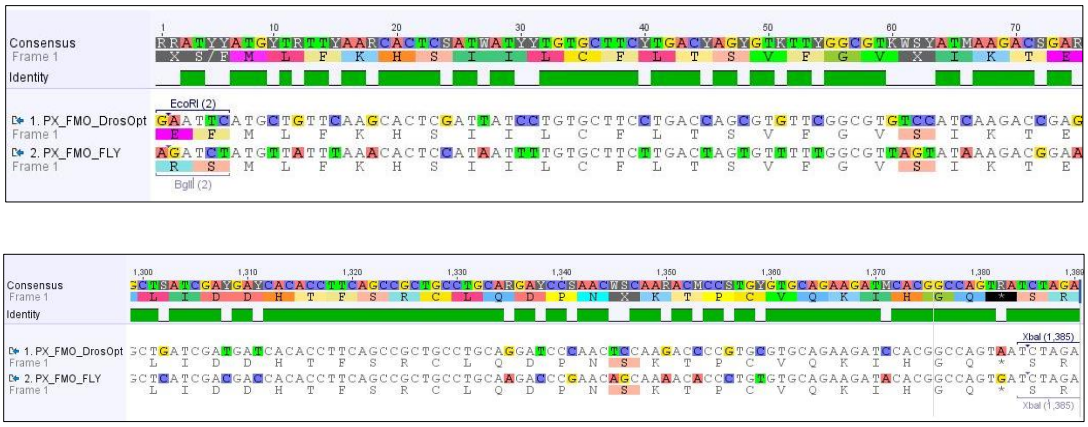


Figure 5.01. Sequence comparisons of original lepidopteran *FMO2* and codon optimised *FMO2* variants. Top shows the 5' end extract. Bottom shows 3' extract. Engineered restriction sites used for cloning have been labelled up and down-stream of the 5' and 3' ends respectively. Highlighted nucleotides indicate silent base pair substitutions that have been made.

5.2.4.2 Creation of transgenic *D. melanogaster* lines

While the *FMO2*, *SCD* and *CYP6BG1* transgenic *D. melanogaster* lines were commercially created through a third party, by the time the *XDH* sequence was fully resolved a transgenic micro-injection facility had been set up at my host institute. This provided the opportunity to create a transgenic line, rather than purchase it.

Unlike the other candidate genes which were synthesised, the *XDH* gene was amplified from the HS strain using 'Q5' high fidelity DNA polymerase (New England BioLabs) and the primers in table 5.02 which incorporated the restriction sites *Xho*I (CTCGAG) on the forward primer, and *Xba*I (TCTAGA) on the reverse primer. Then, as for the previous genes, it was ligated into pUAST and cloned.

Primer Name	Primer Sequence
HS_XDH_XHoI_5'_F	aattctcgagATGTCCATGTACACTTTACTGC
XDH_XbaI_3'_R	aatttctagaCTAAGGCACAATGTTCCATGG

Table 5.02. Primers used for PCR amplification of *XDH* gene from HS strain. Lower-case letters denote additional base pairs incorporated into the primer design. At the start of each primer, 'aatt' is a leader sequence to assist with restriction enzyme digestion. The leader sequence is followed by restriction site sequence (6-bases), after which capital letters denote *XDH* hybridisation sequence.

Purified plasmids were injected into *D. melanogaster* embryos (Y w M(eGFP, vas-int, dmRFP)ZH-2A; P[344]attp40) using a method detailed by Gompel and Schröder [345]. A motorised micromanipulator, TransferMan NK2 (Eppendorf) and a Femto Jetexpress microinjector (Eppendorf) in conjunction with an inverted microscope, an eclipse Ti-U (Nikon) were used for the injections.

5.2.4.3 *D. melanogaster* crossing for bioassays

Virgin female *D. melanogaster* (this line, 'y[1]w[*]; P{Act5C-GAL4-w)E1/CyO' was obtained directly from Bloomington *Drosophila* stock center; stock number 25374; FlyBase ID FBst0025374) heterozygous for GAL4 (under the control of actin) and CyO (curly wings) and having white eyes, were crossed with males homozygous for the gene of interest (GOI) (also containing the upstream activation sequence (UAS)) and having red eyes from the injected

line. Resulting F1 female progeny displaying the phenotypes (reporter genes) of pink eyes, and straight wings were used for bioassays (figure 5.02).

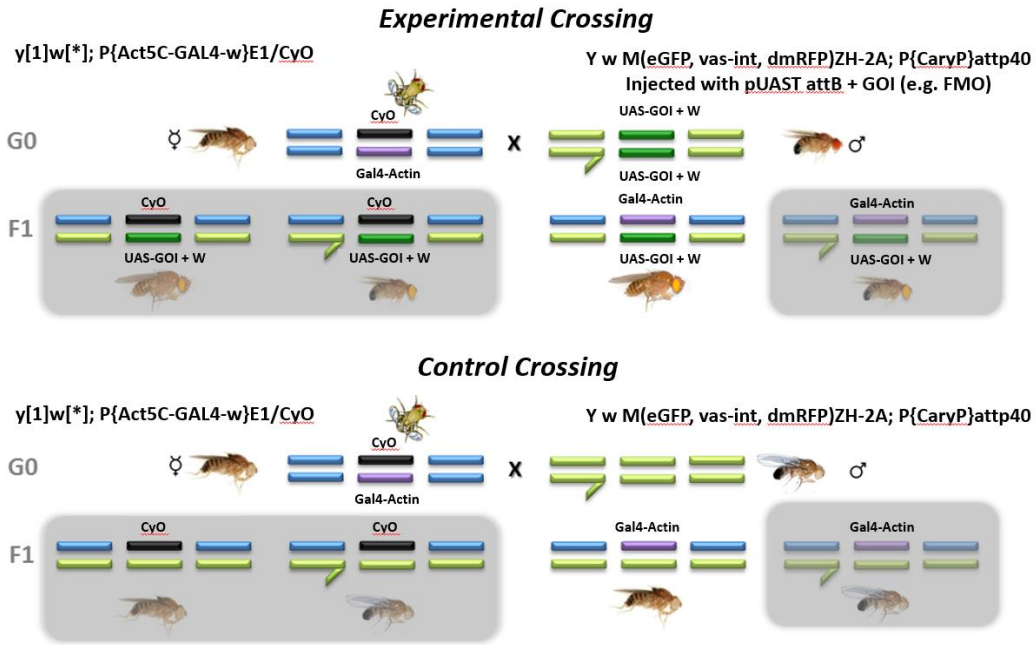


Figure 5.02. Diagrammatic representation of crossing of *D. melanogaster* lines to achieve experimental lines expressing genes of interest and individuals suitable for bioassay experimentation.

5.2.4.4 *D. melanogaster* bioassays

1.5g agar and 3g sugar were added to 100ml of tap water and boiled in a microwave until the agar was completely dissolved. 2ml of the sugar-agar solution was added to 95mm tall x 25mm diameter vials and allowed to become solid. The vials were placed in a drying incubator (~50°C) for 2-3 hours after which 100 µl of insecticide solution was added. It was ensured that the whole agar surface was in contact with the insecticide solution by gently shaking the vial. The insecticide solution was allowed to soak into the agar and dry-off in a fume hood for ~5 hours before the addition of 10 female *D. melanogaster* adults for experimentation. Flies were assessed for mortality at 48 hours and 72 hours and probit analyses performed in the same manner as for *P. xylostella* (section 3.2.2.1).

5.2.5 RNAi

5.2.5.1 RNAi template isolation

The *FMO2* gene was amplified using a plasmid (pUAST) containing the HS *FMO2* gene as template to aid efficient amplification. The plasmid had been produced for the creation of the transgenic *D. melanogaster* as detailed in sections 5.2.4 and 5.3.3. The primers 'PX_FMO_dsRNA_F1' and 'PX_FMO_dsRNA_R2' were used, each having had a T7 promoter sequence (TAATACGACTCACTATAGGG) attached to the 5' end (table 5.03). Reaction volumes can be seen in table 5.04, and PCR conditions in table 5.05.

Primer name	**Primer sequence**
px_fmo_dsR_f1	taatacgactcactatagggAATCACCTTGTCGAGTCCGT
px_fmo_dsR_r2	taatacgactcactatagggGAAGTTATCCGGGAACGGTG

Table 5.03. Primers used for amplification of ds RNA template. Both sequences have had the addition of T7 promoter sequences to the 5' end, denoted by lower-case letters.

	x1 (µl)
DreamTaq	12.5
FP (10µM)	0.5
RP (10µM)	0.5
Diluted Plasmid	(1)
SDW	10.5
Total	25

Table 5.04. RNAi template PCR volumes.

Stage of Program	**Temperature (°C)**	**Duration**	**Number of cycles**
Hold	95	5 mins	-
Cycling	94	30 secs	35
	59	30 secs	
	72	45 secs	
Hold	72	5 mins	-

Table 5.05. RNAi template PCR program.

PCR amplified target fragments were purified using the Promega 'Wizard SV Gel and PCR Clean-Up System', eluted in SDW and quantified using a Nanodrop spectrophotometer.

5.2.5.2 Double stranded RNA synthesis using MEGAscript RNAi Kit (Life Technologies)

The manufacturer's protocol was followed with the following points of note: The transcription reaction was incubated for 4 hours and for purification using a filter cartridge, liquid was drawn through using centrifugation.

5.2.5.3 RNAi experimental delivery and sampling

Petri dishes containing Chinese cabbage leaf disks were set up using the method described for standard bioassays but were not dipped in insecticide (section 3.2.2). 100 second generation post chlorantraniliprole selection individuals were injected with 41.4nl *FMO2* double stranded RNA (dsRNA) equating to 20.3ng dsRNA using an nanoliter2010 (world precision systems). A further 100 individuals were injected with the buffer used to elute the dsRNA, and a further 100 were not injected as an additional control. Only 80 individuals were required for each treatment for the experiment (20 for each time point) but additional larvae were injected to allow for any mortality caused by the injection itself. Each larva was pinned down using a soft paintbrush and injected between abdominal segments approximately midway along the abdomen. Treated larvae were placed on the leaf disks (10 individuals per disk) and stored under bioassay conditions. 20 individuals for each treatment were snap frozen using liquid nitrogen (4 cohorts of 5 individuals) at each of the following time points post injection: 18 hours, 24 hours, 42 hours and 48 hours. These time points were selected based on previous reports of RNAi in insects. Following snap freezing, RNA extractions and cDNA synthesis was carried out as per the methods already described in chapter 2 and qPCR analyses performed using the primers Px_FMO_F3 (ACC ATG GTG CTG CTG GGA TT) and Px_FMO_R3 (GAG CGC CAC GGC GTA TCT) using the standard conditions.

5.2.6 Stability and metabolism of chlorantraniliprole in transgenic *D. melanogaster* expressing *FMO2* gene using liquid chromatography-mass spectrometry (LC-MS)

As for the *P. xylostella* LC-MS assays (chapter 3), two dosing methods were used; feeding and topical exposure to chlorantraniliprole. While there were variations in the methodologies between the two species for feeding and dosing (detailed below), following the assays, sample preparation for LC-MS, the LC-MS itself, and subsequent analyses were identical.

5.2.6.1 Feeding assay

The feeding assay was performed exactly as that for the phenotyping bioassays detailed in section 5.2.4.4, where each vial was dosed with 100µl of 10ppm chlorantraniliprole diluted in equal parts of water and acetone. Four replicates of 10 *D. melanogaster* per vial for each line (control, and transgenic flies carrying the codon optimised *FMO2* gene) for each sampling time-point (0, 1, 5, 24, 72 hours) were assayed.

As for the *P. xylostella* assays, flies were snap-frozen at each of the time-points using liquid nitrogen, although the flies were anaesthetised using CO_2 first to enable transfer from dosing vials to 1.5ml Eppendorf tubes.

5.2.6.2 Topical assay

D. melanogaster were anaesthetised using CO_2 and each individual dosed with 0.25µl of 10ppm chlorantraniliprole in acetone. The flies were dosed and kept in 35mm diameter petri dishes. For each time-point (0, 1, 5, 18, 24 hours), 6 replicates of 5 individual flies per petri dish were sampled by anaesthetising and snap freezing using liquid nitrogen.

5.3 Results

5.3.1 Stability of chlorantraniliprole resistance

Following selection with 8ppm chlorantraniliprole, over the course of 17 generations an initial statistical comparison of the bioassay data indicated that there was no statistical difference between the slopes of the lines for the selected (HS) and unselected (UnSel) populations. However, this is a result of the variation observed in the results as depicted by the standard error in figure 5.03. Taking this into consideration, further (weighted regression) analysis showed that the selected (HS) strain LD_{50} showed no significant difference in the slope or intercept compared with zero (the null hypothesis). However, the slope and intercept for the unselected (UnSel) strain was significantly different from zero ($p=0.016$), rejecting the null hypothesis, and resulting from the marked decrease in resistance over time (fig. 5.04).

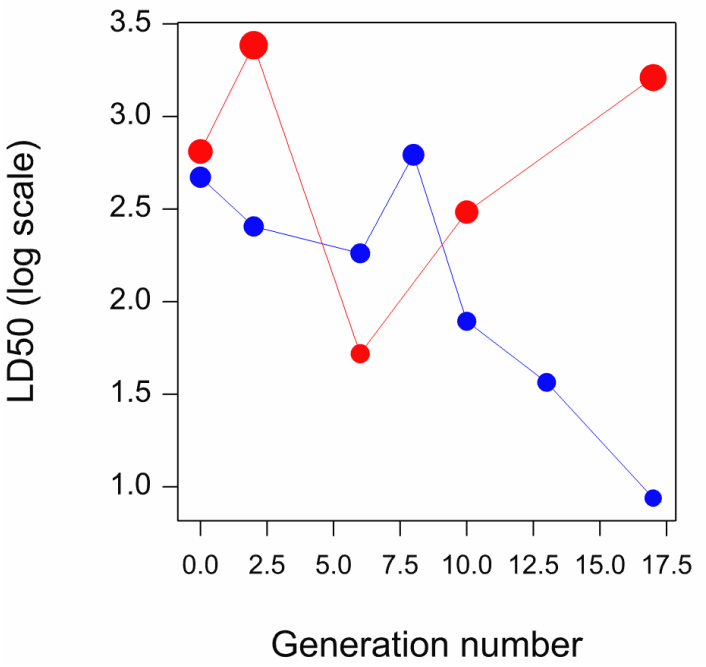


Figure 5.03. LD_{50}s of HS populations selected with chlorantraniliprole (Red) and unselected (Blue) over the course of 17 generations post initial selection. The size of each point is proportional to standard error. The LD_{50} of the unselected population showed a 52-fold decrease from 469ppm to 9ppm over 17 generations.

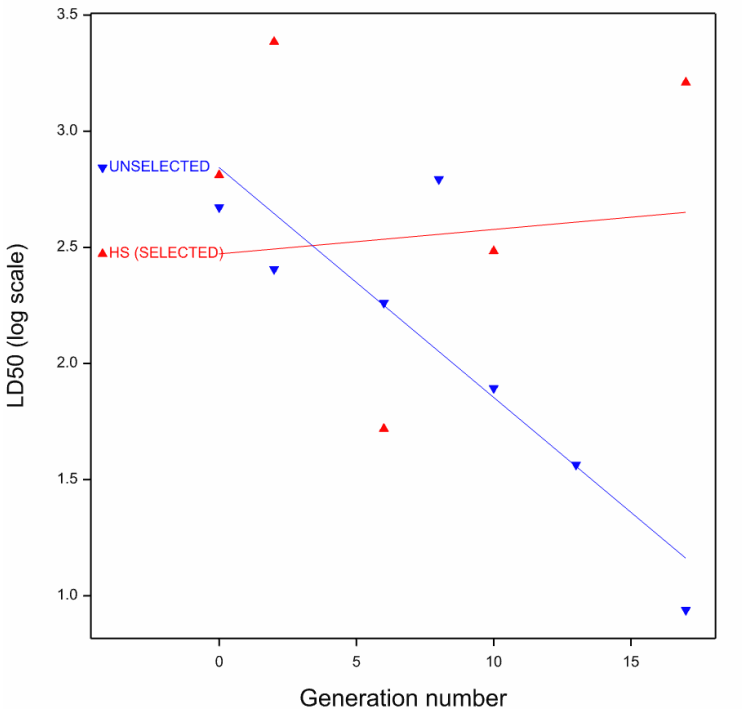


Figure 5.04. Weighted regression of the LD_{50}s for HS populations selected with chlorantraniliprole (Red) and unselected (Blue) over the course of 17 generations post initial

selection. While the selected population has no significant difference to zero, the unselected population slope and intercept shows a significant difference from zero (p=0.016).

Analysis of qPCR data for the earlier identified candidate genes of interest from cDNA isolated from the snap frozen larval samples at times G0 and G17 indicates that there is a significant reduction in expression of all but one of the genes of interest between the two time points. *XDH*, *SCD* and *CYP6BG1* genes showed 3.6-, 1.2- and 5.6-fold change reductions respectively between G0 and G17. The sugar transporter was the only gene to show no significant difference between the two generations, and the *FMO2* showed by far the largest decrease with a 111.1-fold reduction between the two generations (fig. 5.05).

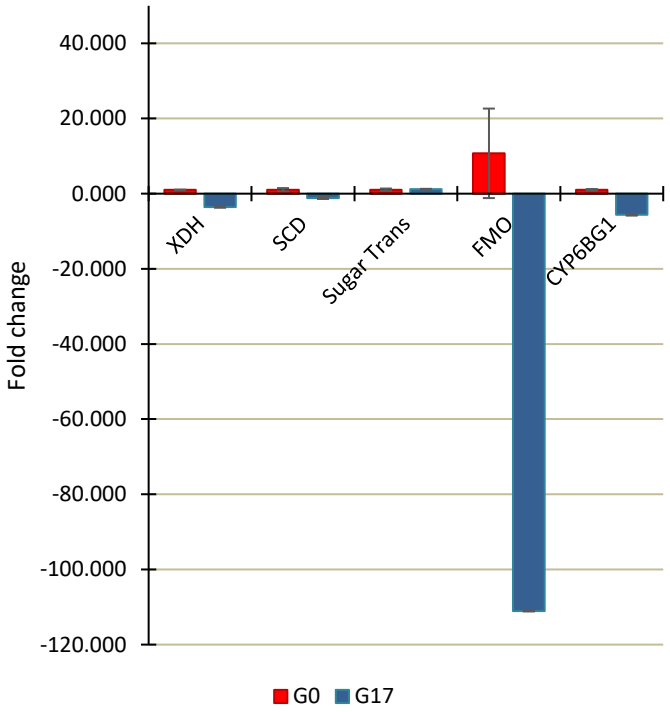


Figure 5.05. Differential expression of genes of interest for UnSel strain between (G0) and (G17) post selection. Error bars indicate 95% confidence limits. *FMO2* is showing a decrease in expression of 111-fold over the 17 generations.

While the *FMO2* candidate displayed 111-fold reduction in expression over 17 generations, there is obviously natural variation of *FMO2* expression within the HS population with one of the G0 cohorts expressing *FMO2* less than the other two (expression of the the control

housekeeping genes did not show the same variation). On an experimental level, this population is showing a valid natural variation and has been included within the analyses, however, if these suspected outliers are removed from the qPCR data, the level of expression between G0 and G17 would be a reduction as high as 1800 fold (fig. 5.06).

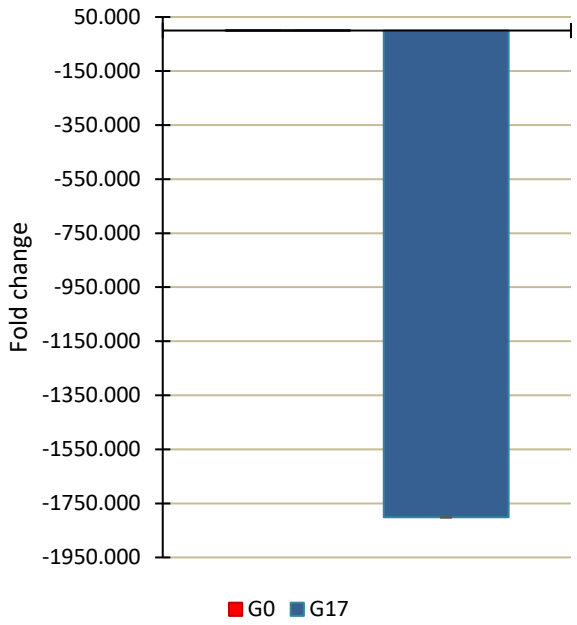


Figure 5.06. QPCR differential expression of *FMO2* gene for UnSel strain two generations post selection with 8ppm chlorantraniliprole (G0) compared with 17 generations (G17) post selection having removed the suspected G0 outliers. Error bars indicate 95% confidence limits. The gene shows 1800-fold decrease in expression after 17 generations and G0 shows a normalised value of 1.051.

5.3.2 Synergists

Following treatment with the synergist PBO, the LD_{50} of the resistant strain showed an overall decrease of 1.56-fold from 3.9ppm to 2.5ppm (Figure 5.07). There was no significant difference between the two treatments indicating no significant change in susceptibility of this strain to chlorantraniliprole when compared to the same strain not exposed to the synergist. Surprisingly, when HS was treated with MEM prior to being exposed to chlorantraniliprole, rather than becoming more sensitive, the LD_{50} resistance ratio increased 4-fold from 4.2ppm to 16.8ppm.

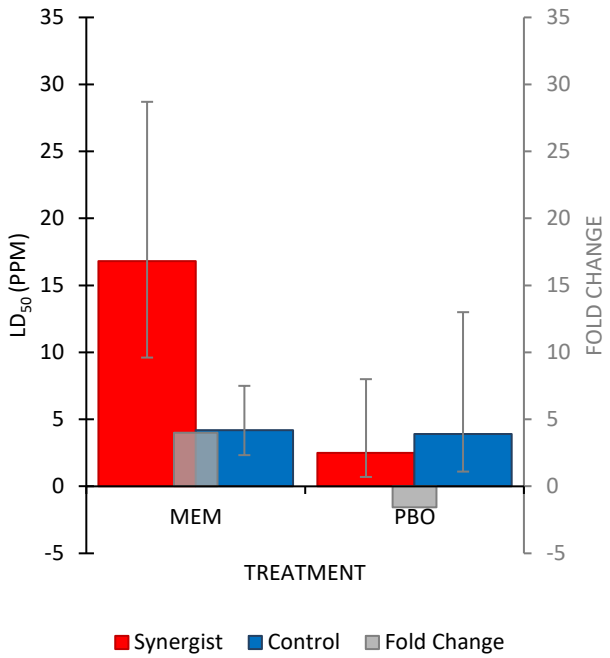


Figure 5.07. LD50 for resistant, HS strain 72 hours post treatment with the synergists methimazole (MEM) or piperonyl butoxide (PBO) followed by chlorantraniliprole. Controls were exposed to chlorantraniliprole only. Error bars indicate 95% confidence limits. Secondary axis (grey bar) indicates fold change between synergist treated and control for each synergist compound. PBO had no significant effect on the susceptibility of HS to chlorantraniliprole. In contrast, MEM significantly increased the resistance of HS by 4-fold.

5.3.3 Obtaining full length sequence of candidate genes

NCBI accession numbers for the *FMO2*, *SCD*, *CYP6BG1* and *XDH* genes from the resistant HS strain can be seen in the appendix (section 8.1). The results from the additional processes needed for sequence identification of the *FMO2* and *XDH* genes can be seen in the following sections of this chapter.

5.3.3.1 Identification of *FMO2* sequence using RACE technique

In the microarrays (table 4.3), the overexpressed *FMO2* was represented by two EST contigs (Contig_25748 and 42842). Upon alignment of these sequences, the 3' end of the cDNA sequence was complete (as indicated by the presence of a poly-A tail), but the 5' end was incomplete. To obtain the sequence for the 5' end, Random Amplification of cDNA Ends (RACE) was employed with primers designed from conserved regions of the gene. Figure 5.08

shows an extract of the two contigs aligned showing the 5’ end of the alignment and the position of the primers used.

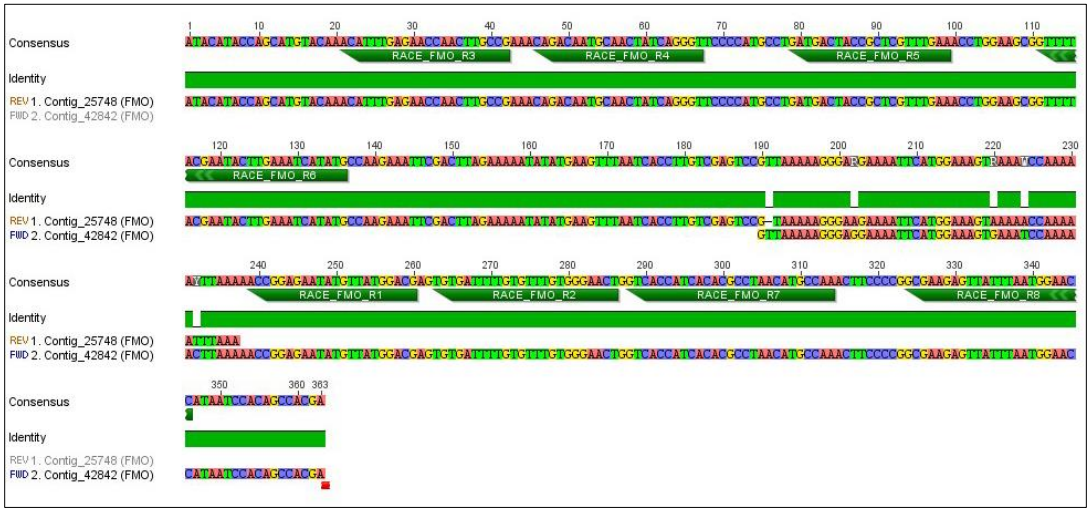


Figure 5.08. Extract of *FMO2* contig alignment of known 5’ end showing positions and sequences of RACE primers.

Primers RACE_FMO_R4 and RACE_FMO_R3 failed to amplify a product, and while a nested PCR approach using primers RACE_FMO_R2 (first round PCR) and RACE_FMO_R1 (nested, second round PCR), yielded a fragment, this sequence did not align to the rest of the *FMO2* gene or return any relevant BLAST hits. Therefore, further primers were designed (RACE_FMO_R5 to R8) and tested in the same manner. Plate 5.01 shows that the two new nested primer combinations (‘R5’ was nested using ‘R6’ PCR product, with ‘R7’ using R8 product) both amplified fragments in the CM3 strain.

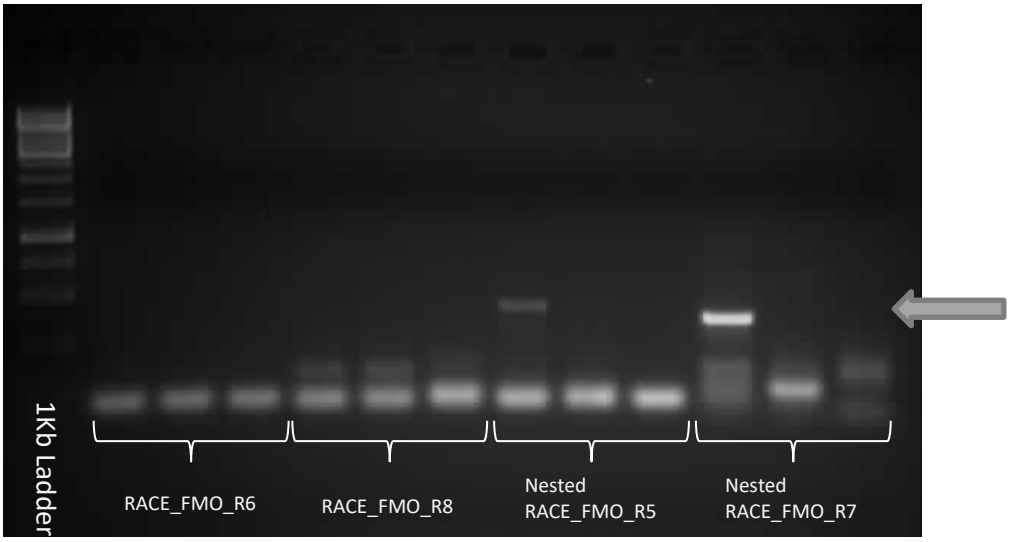


Plate 5.01. RACE PCR products visualised on agarose gel. Sample order for each primer: CM3, CHL5, SDW. Arrow indicates the target bands successfully amplified using a nested PCR approach. 'RACE_FMO_R5' was nested using 'RACE_FMO_R6' PCR product, with 'RACE_FMO_R7' using 'RACE_FMO_R8' product.

When purified and sequenced, the RACE PCR product amplified from CM3 aligned perfectly with the reference contigs with a 51bp overlap. Having translated the sequence in the correct reading frame, the 5' end of the *FMO2* gene was identified and showed that RACE had amplified 237bp of coding sequence that were missing from the original *FMO2* gene sequence, plus 55bp of the 5' untranslated region (UTR). Primers were then designed (Fig. 5.09) to amplify the whole gene in one PCR which was necessary for the expression studies that followed.

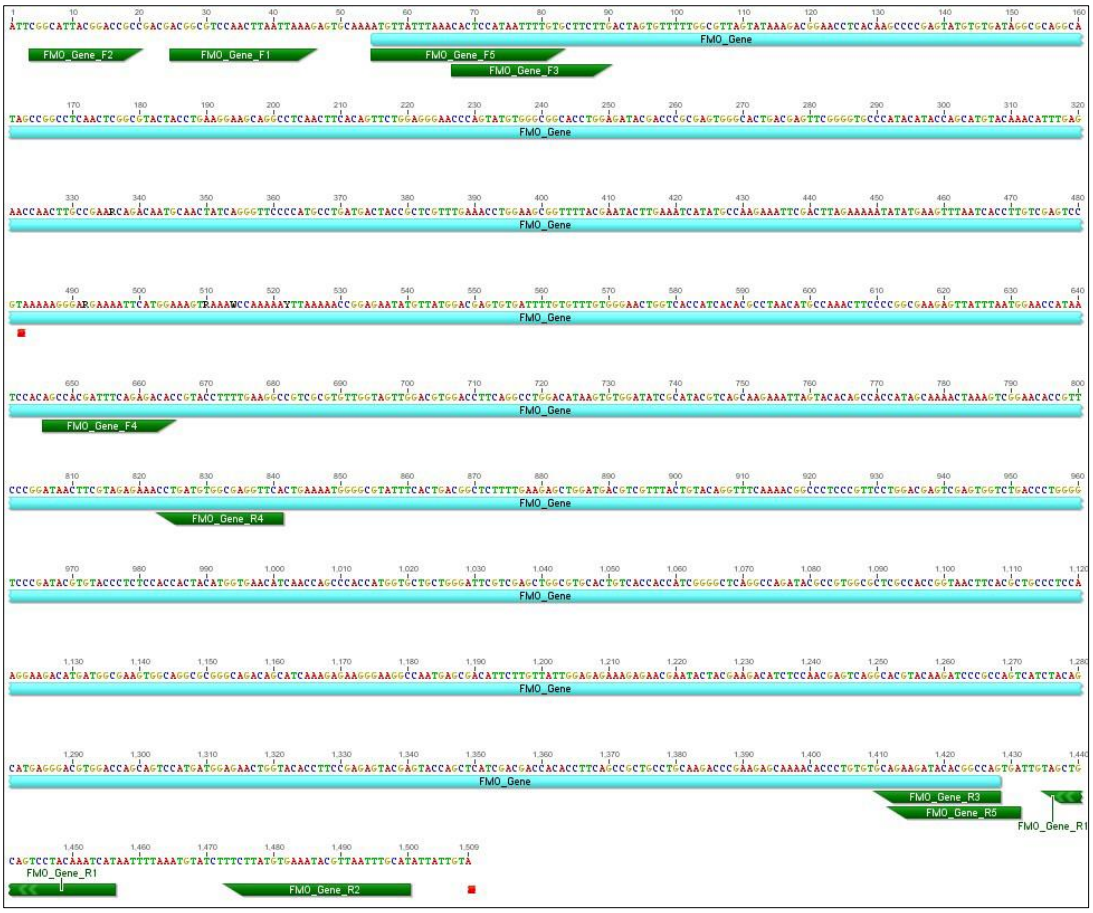


Figure 5.09. *FMO2* gene sequence showing primers used for gene amplification.

Following optimisation of PCR conditions (annealing temperature was decreased to 56°C, and extension time increased to 2 mins 10 secs, and the number of cycles set to 35) and a nested PCR approach, the whole gene was successfully amplified in susceptible (CM) and resistant (HS, CHL and FLU) strains. Sequencing of these products confirmed the gene sequence was that of *FMO2.*

Sequences of the *FMO2* gene for the resistant HS, CHL and FLU strains were aligned with the susceptible CM strain showing there were no indels in the coding sequence of the gene associated with the resistance phenotype. The only nonsynonymous single nucleotide polymorphism (SNP) present was in the HS strain at position 1337 (G to C) causing an amino acid change from lysine (K) to asparagine (N) (Fig. 5.10). However, this is not present in the other resistant CHL or FLU strains indicating it is unlikely to confer resistance by enhancing the activity of the enzyme in relation to chlorantraniliprole metabolism.

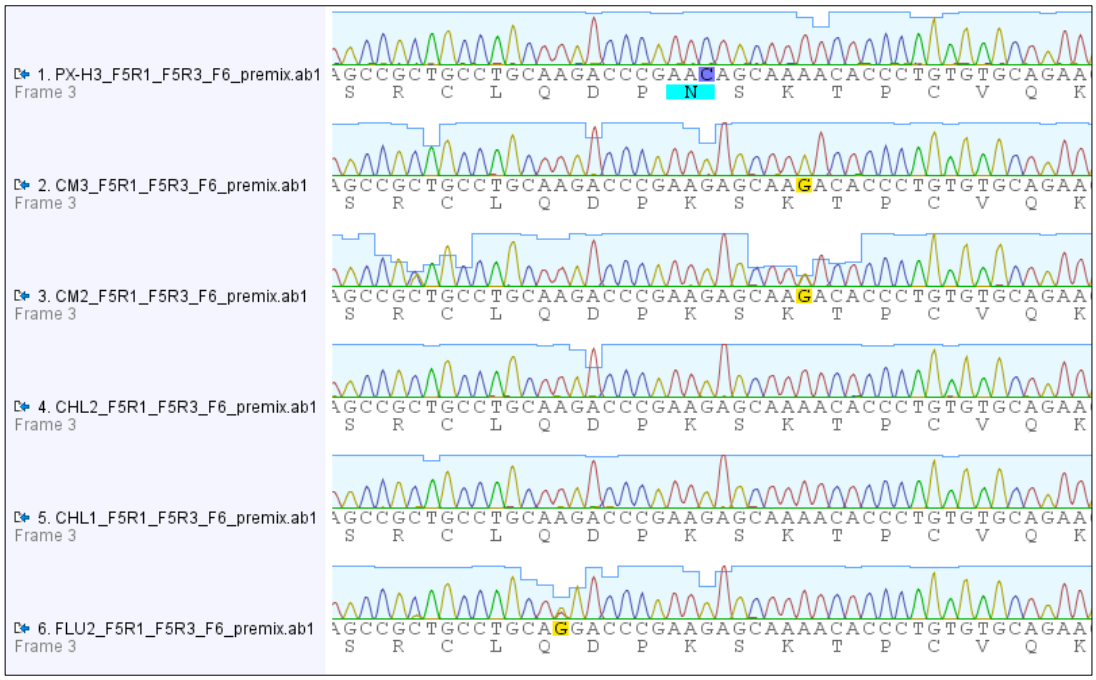


Figure 5.10. Amino acid change from Lysine (K) to Asparagine (N) in resistant HS strain caused by single nucleotide polymorphism G to C at position 1337. The amino acid substitution can be seen highlighted.

Accession details (NCBI) for the *FMO2* gene can be seen in the appendix (section 8.1).

The complete *FMO2* cDNA sequence was BLAST searched, with the top results all originally being from other lepidopteran species (*Bombyx mori*, *Danaus plexippus* and *Helicoverpa armigera*). Analysis of the phylogenetic relationship with these other lepidopteran isoforms of FMO indicated it to likely be an *FMO2* (figure 5.11). However, a more recent BLAST search returns different results, with the candidate *FMO2* showing high similarity to the senecionine N-oxygenase-like gene (from *P. xylostella*), accession XM_011565412.1. This is the same accession detailed earlier in table 1.04, annotated in the DBM-DB as an FMO (accession Px011621). Therefore, a second phylogenetic alignment was made including the putative FMOs annotated within the DBM-DB (figure 5.12). Within this alignment, HS_FMO and Px011621 share a 99.5% amino acid pairwise identity resulting from a 96.8% nucleotide pairwise identity. While figure 5.28 shows the HS_FMO and Px011621 separate from the other FMO clusters, given that a BLAST search of the protein sequence of Px011621 returns a mixture of other senecionine N-oxygenase-like and *FMO2* hits from other lepidopteran species, it is feasible that this putative SNO is an FMO2.

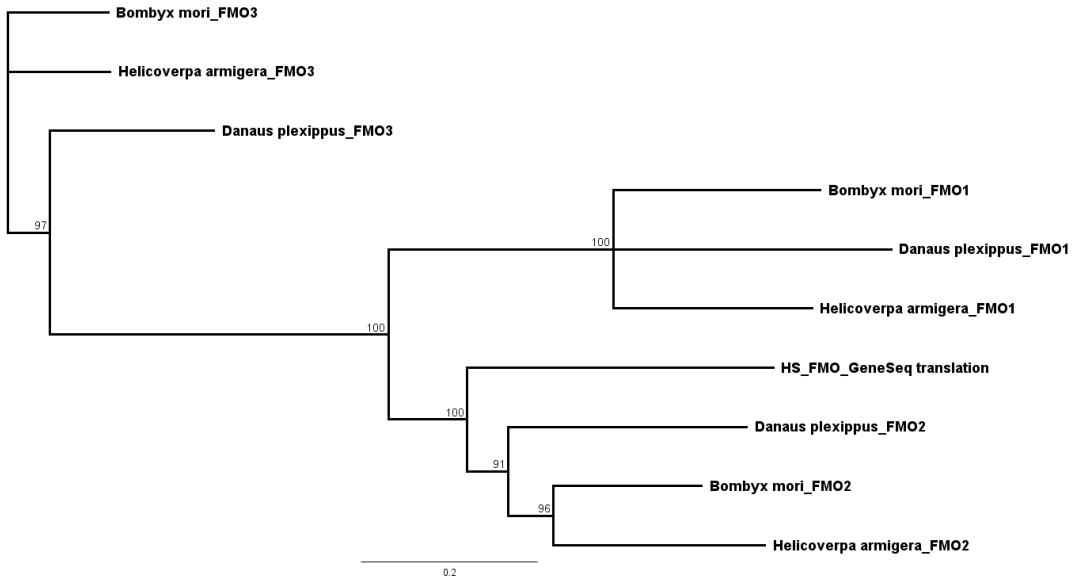


Figure 5.11. Relationship of protein sequence of putative *FMO2* from *P. xylostella* HS strain with FMO isoforms in other species of lepidoptera. Bootstrap value was set at 75. Branch labels indicate percentage similarity. The *FMO2* clusters with *FMO2*s from other species.

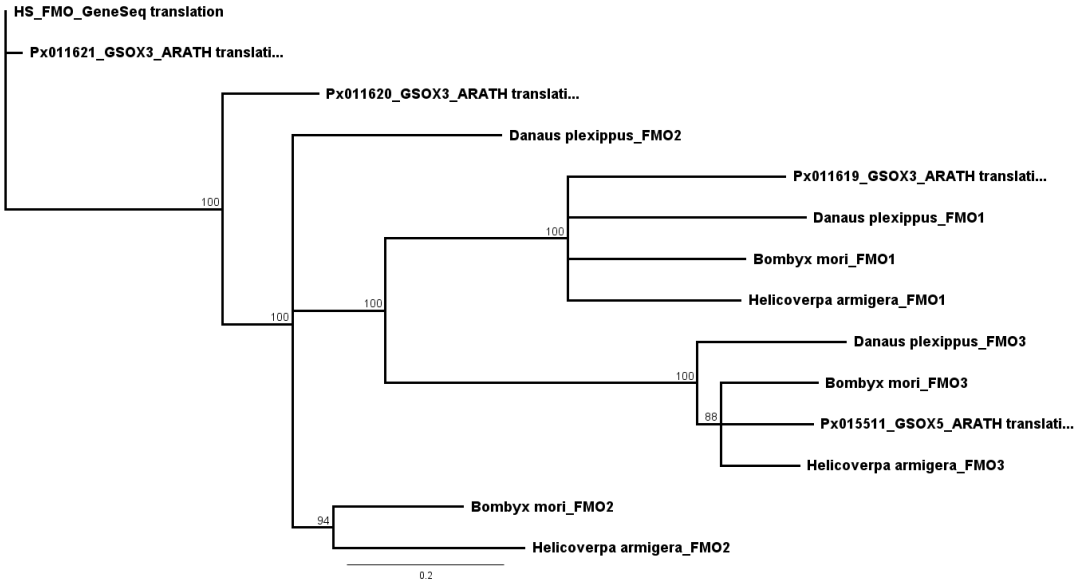


Figure 5.12. Relationship of protein sequence of putative *FMO2* from *P. xylostella* HS strain with FMO isoforms in other species of lepidoptera and FMO accessions from DBM-DB. Bootstrap value was set at 75. Branch labels indicate percentage similarity. The candidate *FMO2* shares greatest homology with accession Px011621, an FMO from the DMB-DB.

5.3.3.2 Identification of the sequence of xanthine dehydrogenase using next generation sequencing

It was not possible to obtain the full-length sequence of the xanthine dehydrogenase gene (*XDH*) from assembled contigs, and PCR amplification proved problematic due to high levels of sequence variation between reference species used for primer design.

The ROTH consensus protein sequence (reading frame 2) was aligned with protein sequences for xanthine dehydrogenase genes from *Danaus plexippus, Culex quinquefasciatus, Bombyx mori and Acromyrmex echinatior*. It can be seen in figure 5.13 that both the 5' and 3' ends were still missing. Based upon the other species' sequences, between 722 and 738 5' residues and 87 3' residues were predicted to be missing.

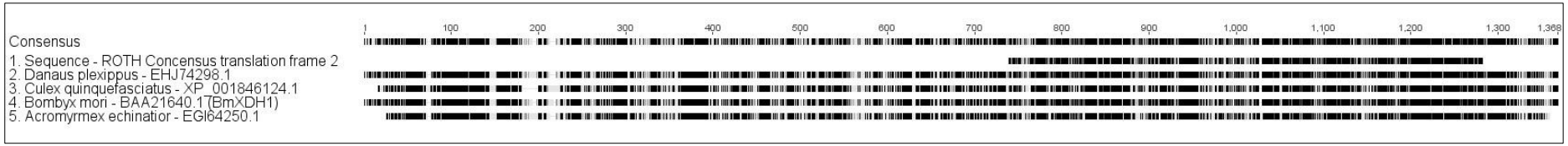


Figure 5.13. Xanthine dehydrogenase ROTH consensus protein alignment with other species showing likely missing 5' and 3' ends. Degree of similarity of each amino acid with others in the same position indicated by tone (black = 100% similarity; grey = 60-99% similarity; white = <60% similarity).

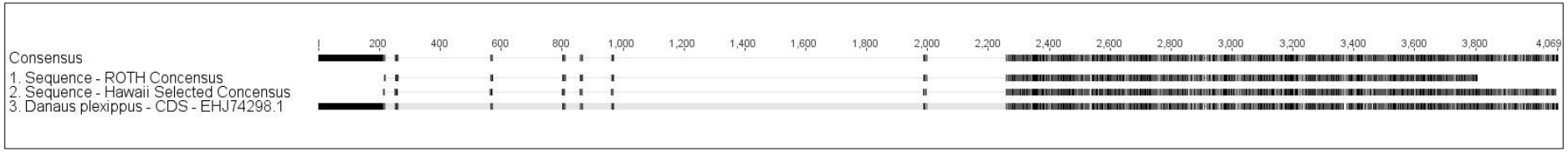


Figure 5.14. Alignment of known ROTH & HS *XDH* nucleotide sequences with that of *D. plexippus.* Degree of similarity of each nucleotide with others in the same position indicated by tone (black = 100% similarity; grey = 60-99% similarity; white = <60% similarity).

Considering the homology between sequences of the other species, the *XDH* gene sequence of *Danaus plexippus*, the monarch butterfly, was used as a representative reference and the *XDH* consensus nucleotide sequences for HS and ROTH strains that have been identified so far were aligned with this (figure 5.14). While the 3' end was incomplete for the ROTH strain, it was complete for HS.

The known ROTH 3' sequence was relatively conserved with that derived from HS (fig. 5.15) and the last 18bp were selected and BLAST searched against the database of NGS MIRA contigs (chapter 6) using Geneious, returning a single hit with contig 'MIRA_c12172'. When assembled with the ROTH consensus, this contig provided an overlap of 402bp and extension of 1002bp. There were two silent SNPs and 1bp indel between the two sequences in the overlap section (figure 5.16), but these could be due to sequencing errors (within either sequence) or the natural variation within the strain as the MIRA sequence represents only one individual as opposed to the bulk used for the other sequence.

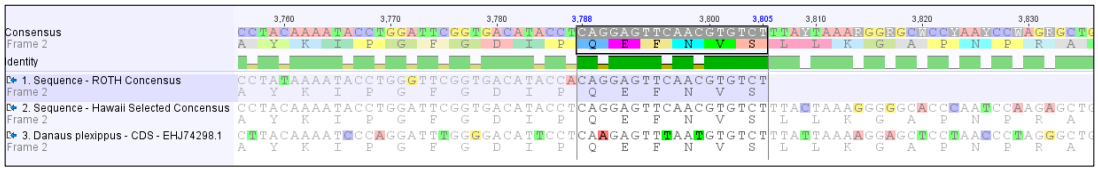


Figure 5.15. Extract of sequence alignment of known ROTH & HS *XDH* nucleotide sequences with that of *D.plexippus* highlighting region conserved between the ROTH and HS.

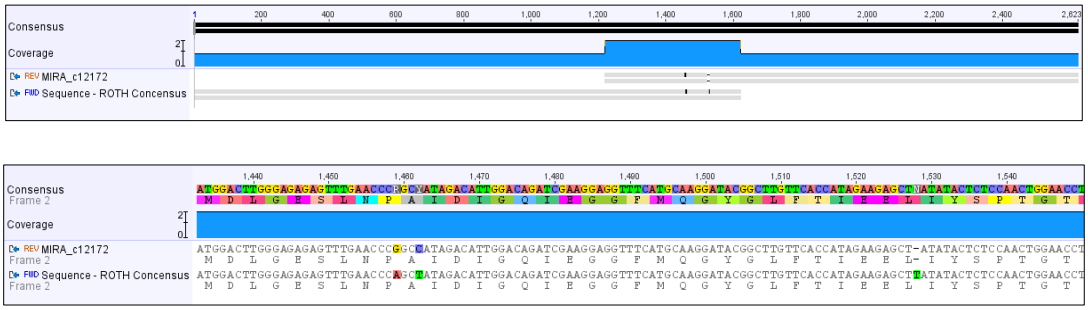


Figure 5.16. Extract of ROTH consensus sequences assembled with NGS MIRA_c12172 highlighting two silent SNPs and 1bp indel between the two sequences.

In the same manner as for the 3' end, a BLAST search of the MIRA contig database was also performed for a 20bp sequence taken from a conserved region towards the 5' end of the known sequence for the ROTH and HS strains. This again resulted in a single hit with contig MIRA_c2493. The ROTH consensus was aligned to the MIRA_c2493 contig showing 3 indels and 3 SNPs between the sequences over a 920bp overlap (figure 5.17). An accession/annotation of *Plutella xylostella* xanthine dehydrogenase-like (LOC105382860) mRNA (NCBI Reference Sequence: XM_011552829.1) added to the DBM-DB (13/03/2015) confirmed that two of the indel discrepancies between the sequences were introns present in the NGS MIRA-c2493 data.

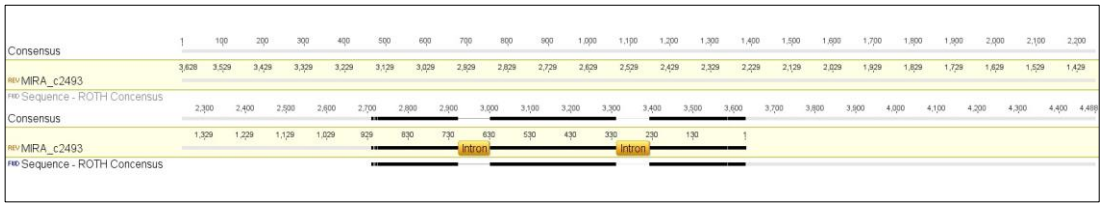


Figure 5.17. Alignment of known ROTH consensus cDNA sequence with NGS MIRA_c2493 genomic DNA sequence. Degree of similarity of each nucleotide with others in the same position indicated by tone (black = 100% similarity; grey = 60-99% similarity; white = <60% similarity). The two highlighted gaps in the ROTH consensus sequence are the product of introns present in the genomic sequence of MIRA_c2493.

Following the discovery of the *P. xylostella* xanthine dehydrogenase XM_011552829.1 accession, it was BLAST searched against the NGS MIRA database, retrieving MIRA contigs c2493, c12172 and an additional contig, c39930. These contigs were 'de-novo' assembled giving a consensus sequence of 5,614bp in length (figure 5.18).

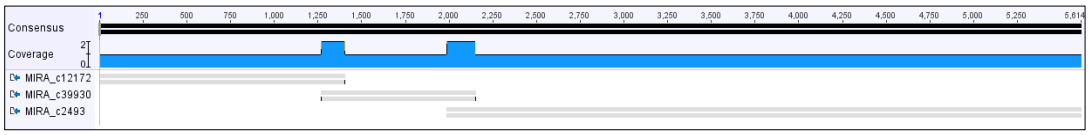


Figure 5.18. Assembly of NGS MIRA contigs covering the *P. xylostella XDH* gene.

The ROTH consensus and *P. xylostella* XM_011552829.1 sequences were assembled to the MIRA consensus sequence and aligned with a high degree of agreement except for the first 24bp of the *P. xylostella XDH* XM_011552829.1 accession gene sequence (figure 5.19). A BLAST search of the MIRA database with this fragment yielded no further contigs and alignment/mapping of an extraction of the start of this gene also yielded no contigs. A BLAST search of the MIRA database of 30 nucleotides from the *Danaus plexippus* gene from a region conserved amongst this and other insect species (approx. 150bp downstream of the start codon) yielded no hits.

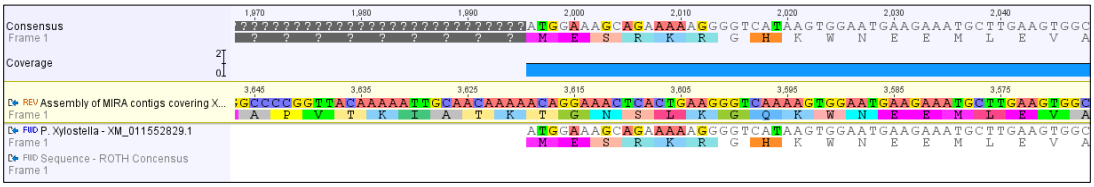


Figure 5.19. Extract of alignment of *P. xylostella XDH* XM_011552829.1 accession with MIRA consensus covering *XDH* gene. The two sequences showed a high level of homology except for the 5' 24 nucleotides of XM_011552829.1.

Not achieving any BLAST hits from a search of the MIRA database indicated that it was likely that the start sequence of the *XDH* gene was very different from that of the published gene and there were many possible start codons within the MIRA consensus. As it was possible that the start codon may be preceded by a 'Kozak' sequence [346], the first 2050 bases of the MIRA consensus sequence upstream of the so far confirmed 5' end of the ROTH consensus were run through the online 'atgpr' program which has been designed to identifying the initiation codons in cDNA sequences [347, 348]. The query using the atgpr program was set to return the top 20 results, the top 5 of which can be seen in table 5.06.

No.of ATG from 5' end	Reliability	Frame	Identity to Kozak rule A/GXXATGG	Start (bp)	Finish (bp)	ORF Length (aa)	Stop codon found?	Protein Sequence
17	0.37	1	tXXATGc	787	2050	421	No	MHGLAVTTVEGIGSTKSKLHPVQERIAKAHGSQCGFCTPGIVMSMYTLLRSSDHIKFEDMEVAFQG NLCRCTGYRAIIEGYKTFIEDWENRRVSNDSNVQNGKGVCAMGKDCCKNKKNEETDDSETQYIFD KSSFLPYDASQEPIFPPELKLSPVYDEQELIYRGKQVTWYRPTELKTLLKLKQQHPDAKIVVGNTEVG VEVKFKHCVYPVIIMPNSIPELNSIMETKNGFTVGASVTLSEMDNVFRKHIDILPKYKTRTLKTIADML NWFAGKQIRNVAGIGGNIMTGSPISDLNPILMSLKVKLNLLSEKGGHRSVQMDETFFTGYRKNVVK PDEILLSIEIPFTTKFQYVKAFKQAKRREDDISIVTAAINVEFEDNSNVIKNINIAYGGMAPVTKIATKT GNSLKGQKWNEEMLEVA
18	0.25	1	GXXATGt	913	2050	379	No	MSMYTLLRSSDHIKFEDMEVAFQGNLCRCTGYRAIIEGYKTFIEDWENRRVSNDSNVQNGKGVCA MGKDCCKNKKNEETDDSETQYIFDKSSFLPYDASQEPIFPPELKLSPVYDEQELIYRGKQVTWYRPTE LKTLLKLKQQHPDAKIVVGNTEVGVEVKFKHCVYPVIIMPNSIPELNSIMETKNGFTVGASVTLSEM DNVFRKHIDILPKYKTRTLKTIADMLNWFAGKQIRNVAGIGGNIMTGSPISDLNPILMSLKVKLNLLS EKGGHRSVQMDETFFTGYRKNVVKPDEILLSIEIPFTTKFQYVKAFKQAKRREDDISIVTAAINVEFED NSNVIKNINIAYGGMAPVTKIATKTGNSLKGQKWNEEMLEVA
31	0.16	1	AXXATGG	1459	2050	197	No	METKNGFTVGASVTLSEMDNVFRKHIDILPKYKTRTLKTIADMLNWFAGKQIRNVAGIGGNIMTGS PISDLNPILMSLKVKLNLLSEKGGHRSVQMDETFFTGYRKNVVKPDEILLSIEIPFTTKFQYVKAFKQA KRREDDISIVTAAINVEFEDNSNVIKNINIAYGGMAPVTKIATKTGNSLKGQKWNEEMLEVA
39	0.15	1	cXXATGG	1744	2050	102	No	MDETFFTGYRKNVVKPDEILLSIEIPFTTKFQYVKAFKQAKRREDDISIVTAAINVEFEDNSNVIKNINI AYGGMAPVTKIATKTGNSLKGQKWNEEMLEVA
30	0.14	1	AXXATGc	1426	2050	208	No	MPNSIPELNSIMETKNGFTVGASVTLSEMDNVFRKHIDILPKYKTRTLKTIADMLNWFAGKQIRNVA GIGGNIMTGSPISDLNPILMSLKVKLNLLSEKGGHRSVQMDETFFTGYRKNVVKPDEILLSIEIPFTTK FQYVKAFKQAKRREDDISIVTAAINVEFEDNSNVIKNINIAYGGMAPVTKIATKTGNSLKGQKWNEE MLEVA

Table 5.06. Results produced by 'atgpr' program identifying possible start codons for the *XDH* gene. The top two results returned, having 'reliability scores' of 37 and 25 were investigated further as potentially being the correct start codon.

The atgpr results table shows a score for 'reliability' derived from the number of times predictions are normally correct when a particular linear discriminant function (LDF) score has been achieved. Specifically, the estimate of reliability is derived from tests using a non-redundant set of 660 sequences. The reliability scores of the top two candidate start codons predicted by atgpr were 0.37 and 0.25 meaning that in tests, the reliability of the LDF score was only 37% and 25% respectively. The low reliability scores meant that neither start codon candidate could be assumed to be correct from this result alone. The sequence analysis software, Geneious, was used to predict start codon open reading frames (ORFs) and the two start codons predicted by atgpr selected from these. The ORFs extend 961bp into the *P. xylostella* - XM_011552829.1 gene and 247bp into the known ROTH consensus. Start codon candidates 1 and 2 are 1209bp and 1083bp upstream of the supposed start of the *P. xylostella* - XM_011552829.1 gene (figure 5.20).

Using the assembly of MIRA contigs covering the whole *XDH* gene, primers were designed around the two start codon candidates from the next generation sequence and used to amplify a ~2050bp region from the 5' end covering both predicted start codons and providing overlap with both *XDH* reference sequences. Primers used for fragment amplification were ROTH_XDH_CODON_F4 (GCGTGTCTGGCCCCAGT) and ROTH_XDH_CODON_R4 (ATTCGACATTCGCCTTCAACAAT), under standard PCR conditions with a 2min 15sec extension time. Alignment of the 5'end fragment with the known ROTH *XDH* sequence and ORF analysis revealed which of the two candidates was the correct start codon, giving an ORF length of 3681bp for the *XDH* gene. Alignment of the susceptible ROTH, and resistant HS strains revealed many SNPs, resulting in 18 amino acid substitutions between the two. Having obtained the full length *XDH* gene from the ROTH strain, it was amplified from the resistant HS strain using Q5 high fidelity taq polymerase (New England BioLabs) and primers (HS_XDH_XHoI_5'_F and XDH_XbaI_3'_R) that added the required restriction sites needed for ligation into pUAST as preparation for embryo injection. Details of the NCBI *XDH* sequence accession from the resistant HS strain can be seen in the appendix (section 8.1).

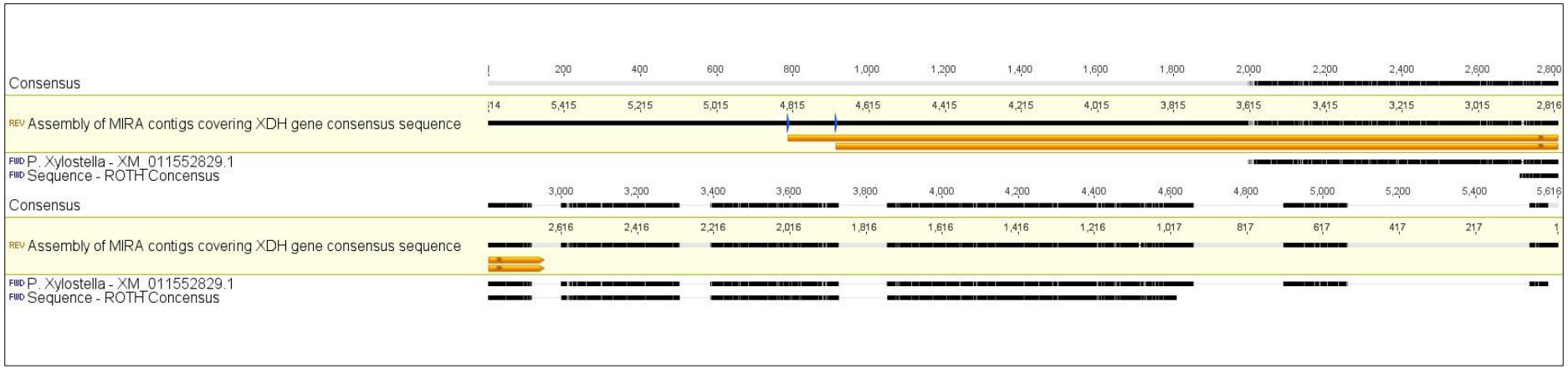


Figure 5.20. Alignment of NGS sequence, *P. xylostella* XM_011552829.1 gene and known ROTH sequence. Blue arrows indicate predicted start codons, with the ORFs designated by orange arrows. Degree of similarity of each nucleotide with others in the same position indicated by tone (black = 100% similarity; grey = 60-99% similarity; white = <60% similarity).

5.3.4 Phenotyping transgenic *D. melanogaster* to diamide insecticides

Bioassays of transgenic *D. melanogaster* with chlorantraniliprole showed that after 48 hours those lines expressing either *CYP6BG1* or *SCD* were significantly more sensitive to the insecticide than the control strain (figure 5.21). Conversely the line carrying the lepidopteran *FMO2* gene was significantly more resistant. The LD_{50} for the *FMO2* line was 1.9-fold higher at 36.2ppm than for the control line which had an LD_{50} of 19.26ppm (table 5.07).

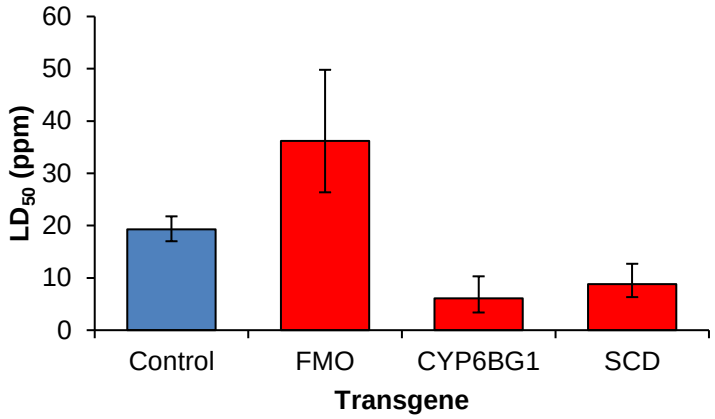


Figure 5.21. LD_{50} of transgenic *D. melanogaster* carrying the *FMO2*, *CYP6BG1* or *SCD* transgene compared to control flies of the same genetic background, but not carrying a transgene bioassayed with chlorantraniliprole at 48 hours. Error bars show 95% confidence limits. Transgenic flies carrying the *FMO2* gene were significantly more resistant than the control line. Those expressing the *CYP6BG1* or *SCD* genes were rendered significantly more sensitive to the insecticide.

Strain	LD_{50} (ppm)	Lower 95%	Upper 95%	LD_{50} Fold Change compared to control	Slope	Slope Standard Error
Control	19.26	17.01	21.77	-	3.632	0.253
FMO2	36.20	26.37	49.80	1.88	2.684	0.236
CYP6BG1	6.09	3.38	10.30	0.32	2.187	0.2858
SCD	8.81	6.32	12.70	0.46	0.745	0.166

Table 5.07. Dose-response probit analysis results for chlorantraniliprole at 48 hour endpoint for transgenic *D. melanogaster* expressing *P. xylostella* genes of interest. The *FMO2* line showed 1.88-fold resistance over the control line, *CYP6BG1* and *SCD* both displayed a reduction in resistance to chlorantraniliprole.

Following codon optimisation of the primary candidate gene, *FMO2*, a further bioassay took place to compare the level of resistance between *D. melanogaster* expressing the original lepidopteran gene, and those with a variant codon optimised for *D. melanogaster*. After 48 hours, the *D. melanogaster* carrying the codon optimised *FMO2* gene variant had an LD_{50} of 21.0ppm, slightly higher than for the original lepidopteran *FMO2* which had an LD_{50} of 14.2ppm for chlorantraniliprole (figure 5.22 and table 5.08). However, there was no significant difference between the two lines based on overlapping 95% CLs. The two *FMO2* lines also showed overlapping 95% CLs with the control strain, However, when the same assay was scored at a later time point (72hours) the results for the codon optimised *FMO2* line were significant from the control based on this criteria although still no difference was observed between the two *FMO2* variants (see Table 5.08).

Two separate chlorantraniliprole assays of transgenic *D. melanogaster* were performed in this chapter. The first assessed transgenic *D. melanogaster* expressing the *P. xylostella FMO2*, and the second, assessed the same gene alongside the variant codon optimised for *D. melanogaster*. A comparison of the results for the two experiments revealed differences between the two experiments in the LD_{50} values calculated for both the control and *P.* xylostella *FMO2* lines (see tables 5.08 and 5.09). As the same lines and experimental protocols were used for each experiment, it would be expected that these values should be the same, however, the results show different values for the two experiments. A criticism could be made of the GLM approach employed through the dose-response probit analysis in calculating the LD_{50}s as this statistical analysis requires each of the regression lines to have the same slope. However, this is unlikely to have produced the differences observed between the experiments. It is likely that the variation is caused by the range of insecticide concentrations used within the experiments being broad and introducing a level of error. Both experiments used 5-fold series dilutions ranging from 1.6 ppm to 1000ppm providing a range of five concentrations plus a control. In hindsight, more accurate and consistent data would have been obtained by increasing the number of concentrations used and/or focussing this range around the discriminating doses.

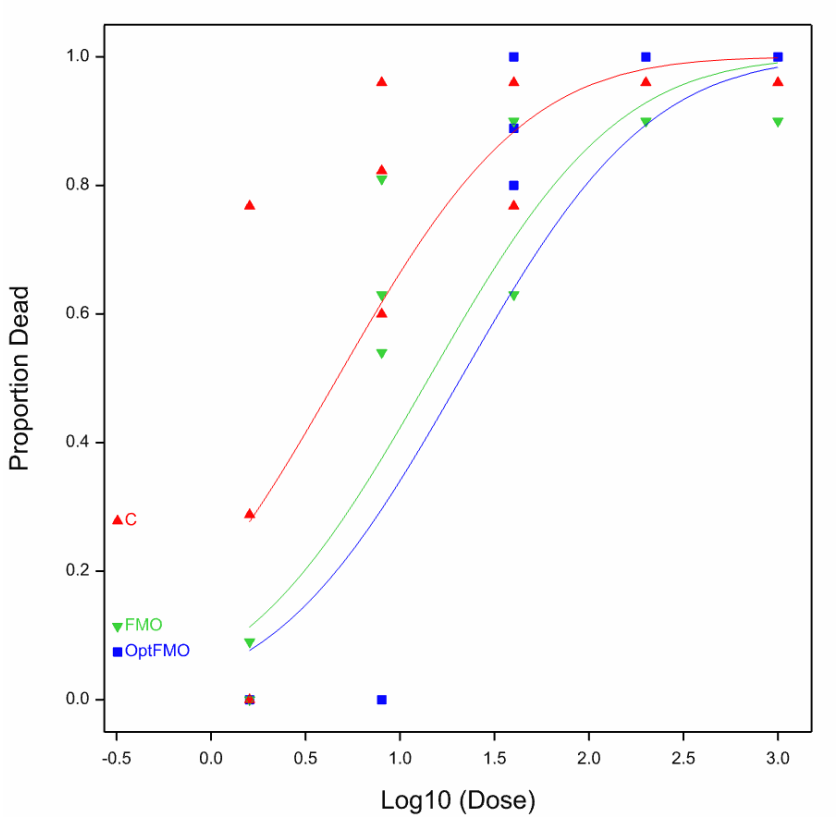


Figure 5.22. Fitted and observed relationship of dose-response for chlorantraniliprole bioassay after 48 hours for transgenic *D. melanogaster* expressing either original lepidopteran *FMO2* gene, or *FMO2* gene codon optimised for *D. melanogaster* (OptFMO). There was no significant difference between the two.

Time point (hours)	Strain	Estimated Dose (ppm)	Lower 95%	Upper 95%	LD_{50} Fold Change compared to control	Slope	Slope Standard Error
48	Control	4.70	1.70	11.60	-	1.277	0.169
	FMO2	14.20	6.36	30.50	3.02		
	OptFMO2	21.00	9.53	45.20	4.47		
72	Control	3.70	1.36	9.20	-	1.293	0.169
	FMO2	19.70	9.08	41.60	5.32		
	OptFMO2	21.80	10.14	46.00	5.89		

Table 5.08. Dose-response probit analysis results at 48 and 72 hours for transgenic *D. melanogaster* expressing original lepidopteran *FMO2* gene and *FMO2* gene codon optimised for *D. melanogaster* assayed with chlorantraniliprole. There is no significant difference between the two *FMO2* gene variants at either time point.

Further bioassays of the *FMO2* gene were carried out with the phthalic diamide, flubendiamide. As can be seen in figure 5.23, there was no difference in the dose-response between the control, original *FMO2* or codon optimised *FMO2* lines at 48 hours. Given the LD_{50}s for both the *FMO2* variant lines and the control line, and lack of differences between them, it appears that *D. melanogaster* is inherently more resistant to the phthalic diamide, flubendiamide than it is the anthranilic diamides chlorantraniliprole and cyantraniliprole (table 5.09).

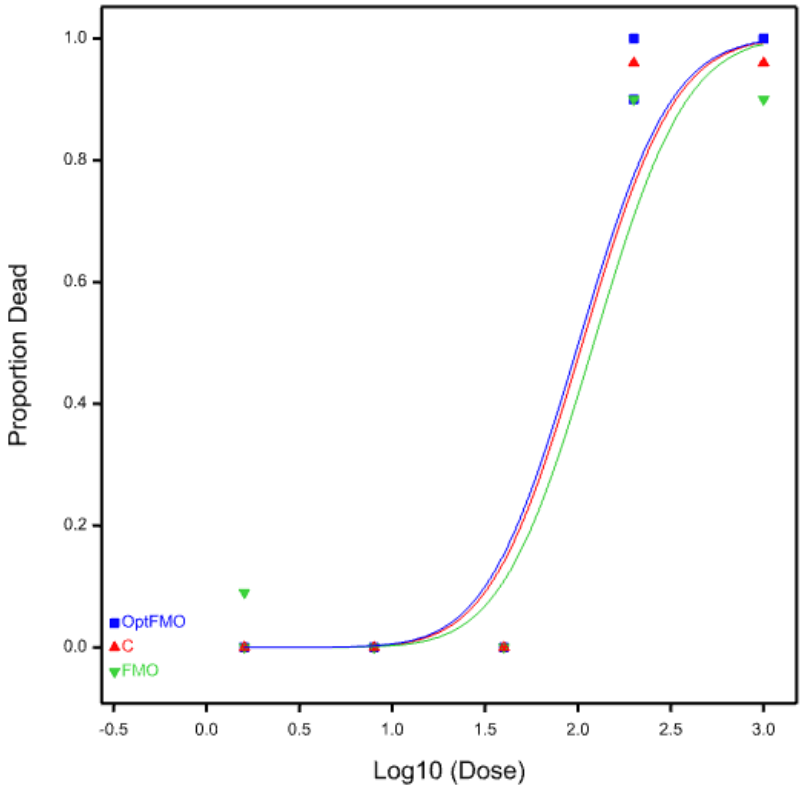


Figure 5.23. Fitted and observed relationship of dose-response for flubendiamide bioassay after 48 hours for transgenic *D. melanogaster* expressing either original lepidopteran *FMO2* gene, or *FMO2* gene codon optimised for *D. melanogaster* (OptFMO). There was no significant difference in the susceptibility between any of the three lines.

Strain	Estimated Dose (ppm)	Lower 95%	Upper 95%	LD_{50} Fold Change compared to control	Slope	Slope Standard Error
Control	105.9	62.9	179.3	-	2.546	0.349
FMO2	121.8	75.5	197.4	1.15		
OptFMO2	100.8	62.7	162.3	0.95		

Table 5.09. Dose-response probit analysis results at 48 hours for transgenic *D. melanogaster* expressing original lepidopteran *FMO2* gene and *FMO2* gene codon optimised for *D. melanogaster* assayed with flubendiamide. There was no significant difference in the susceptibility between any of the three lines.

Transgenic *D. melanogaster* carrying the lepidopteran *XDH* gene were bioassayed separately from the lines carrying the other genes of interest because it took longer to isolate the gene sequence. When compared, the *XDH* line was not significantly different to the control line, although for chlorantraniliprole the confidence limits only very marginally overlap (figure 5.24 and table 5.10).

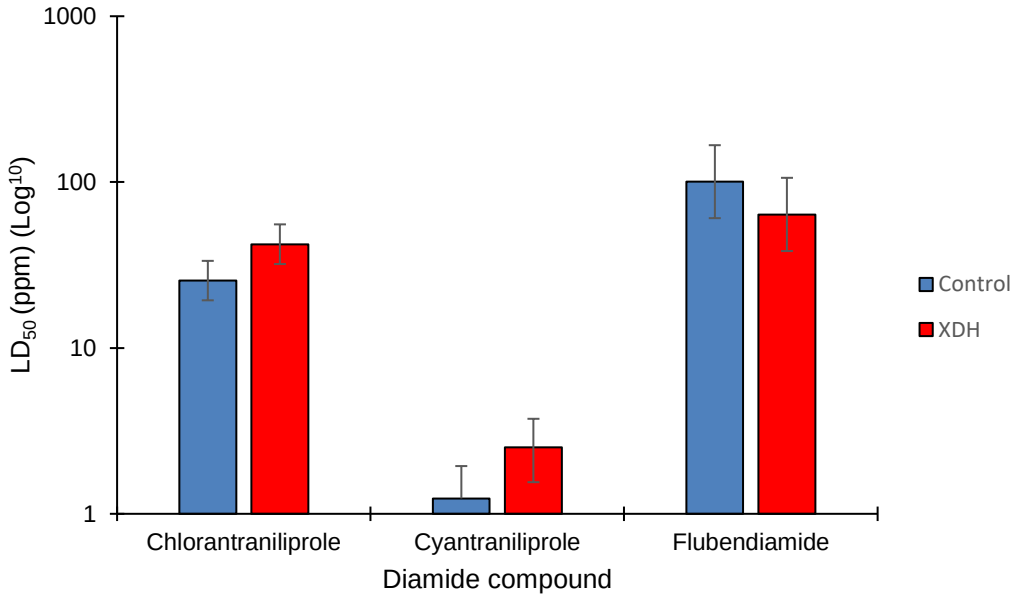


Figure 5.24. LD_{50} of *D. melanogaster* carrying lepidopteran *XDH* gene for current diamide compounds at 48 hours compared to control line. Error bars indicate 95% confidence limits. The *XDH* line is not significantly different to the control line in its response to any of the insecticides.

Diamide Compound	Strain	Estimated Dose (ppm)	Lower 95%	Upper 95%	LD_{50} Fold Change compared to control	Slope	Slope Standard Error
Chlorantraniliprole	Control	25.50	19.37	33.50	1.65	2.302	0.194
	XDH	42.20	32.06	55.60			
Cyantraniliprole	Control	1.24	0.66	1.94	2.03	2.097	0.35
	XDH	2.52	1.55	3.74			
Flubendiamide	Control	100.60	60.60	166.90	0.63	2.727	0.477
	XDH	63.60	38.40	106.10			

Table 5.10. Dose-response probit analysis results for current diamide compounds at 48 hour endpoint for transgenic *D. melanogaster* expressing *P. xylostella XDH* gene. The *XDH* line is not significantly different to the control line in its response to any of the insecticides.

5.3.5 RNAi

For all treatments, post injection survival was at least 80% allowing all replicates (cohorts) to be fairly compared. Analysis of the qPCR data (figure 5.25) showed that there was no significant difference between the *FMO2* dsRNA or buffer injected treatments. It also shows that there was no significant difference between either of the injected treatments and the non-injected control.

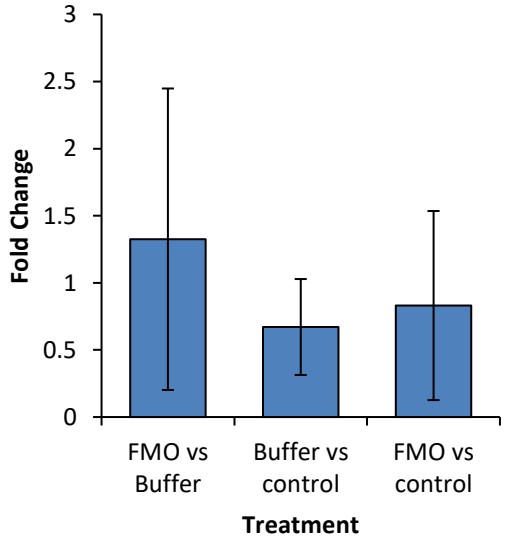


Figure 5.25. Comparison of qPCR fold change in the expression of *FMO2* in the HS strain following different injection treatments. FMO: dsRNA injected; Buffer: buffer injected; Control: not injected. Results shown for 48 hour timepoint. There was no significant difference between any of the treatments.

5.3.6 Stability and metabolism of chlorantraniliprole in transgenic *D. melanogaster* expressing *FMO2* using liquid chromatography-mass spectrometry (LC-MS)

5.3.6.1 Feeding assay

By the 1 and 5 hour time points, there was no significant difference between the control *D. melanogaster* line and that carrying the *FMO2* gene, however figure 5.26 demonstrates that the *FMO2* transgenic line continues to accumulate chlorantraniliprole to the 24 hour timepoint, while the control line plateaus after the 5 hour timepoint. The difference between the two lines at the 24 hour timepoint is significant (p=0.024) with *FMO2* line extracts containing 1.8-times more chlorantraniliprole per gram of insect tissue than the controls. Between the 24 hour timepoint and the endpoint at 72 hours, the concentration of chlorantraniliprole recovered decreases in the experimental *FMO2* line to the same level as that of the controls with no significant difference between the two at the 72 hour endpoint. The p-values for all the time points were: T0=0.68, T1=0.46, T5=0.84, T24=0.02, T72=0.98.

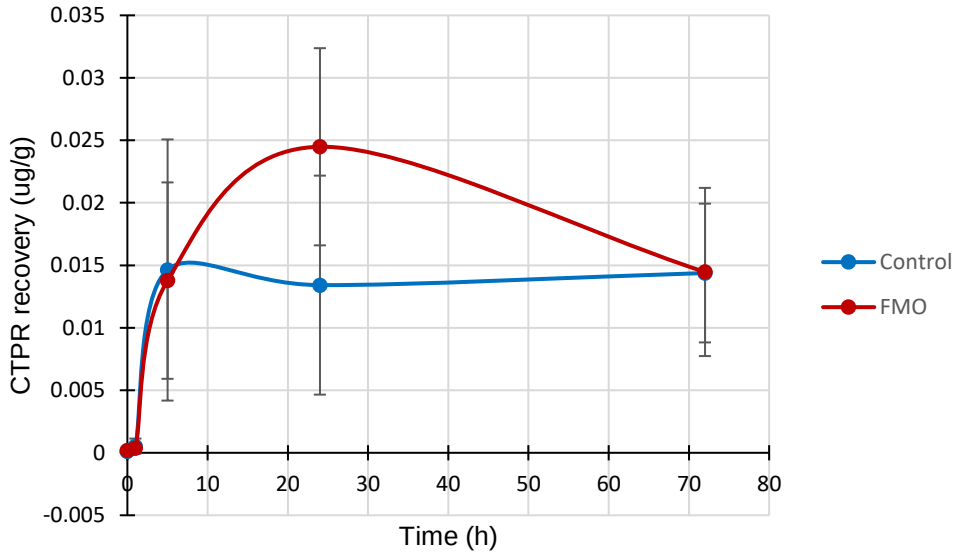


Figure 5.26. Average Chlorantraniliprole (CTPR) recovery in *D. melanogaster* extracts from feeding assay (µg/g) determined through LC-MS over 72 hours. Error bars indicate 95% confidence limits. There was a significant difference in the concentration of chlorantraniliprole recovered between the control and transgenic (*FMO2*) strains at the 24 hour timepoint (p = 0.02).

5.3.6.2 Topical assay

As can be seen in figure 5.27, there was no difference in the percent change in chlorantraniliprole recovery from insect washes between the *FMO2* and control lines following topical application of chlorantraniliprole. Statistical analysis of the results confirms no significant differences at any of the timepoints (p-values were T1=0.40, T5=0.71, T18=0.20, T24=0.51). Both the experimental and control lines show distinct decreases in chlorantraniliprole recovery over time, indicating that both lines had a similar level of insecticide penetration.

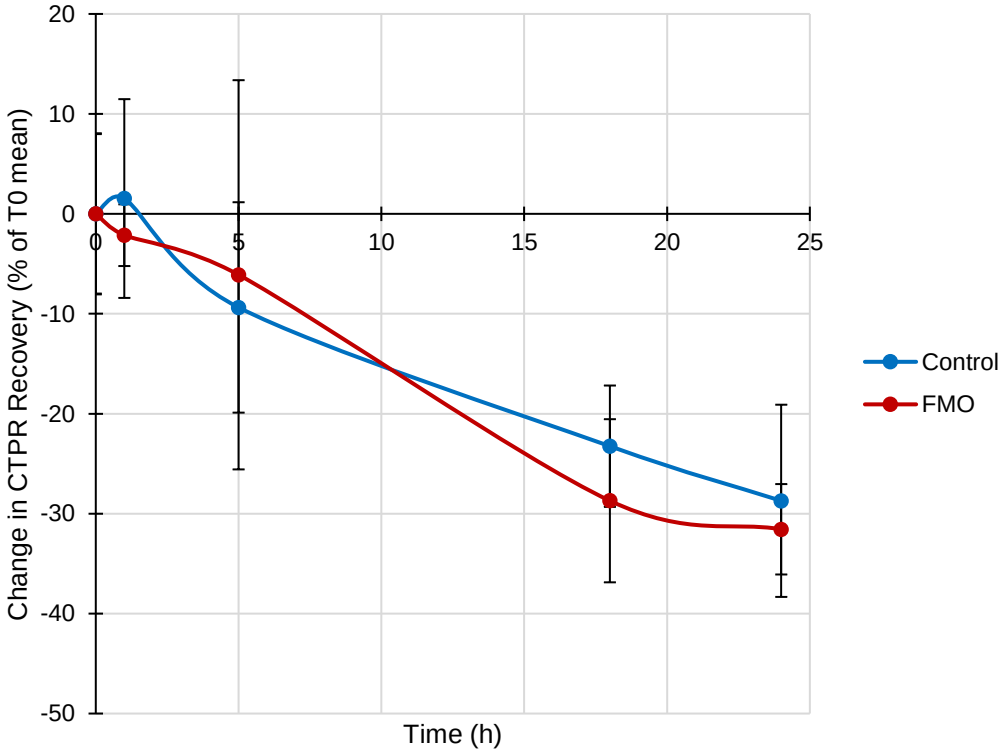


Figure 5.27. Percent change in chlorantraniliprole (CTPR) recovery by LC-MS in transgenic *D. melanogaster* expressing *FMO2* gene washes over 24 hours following topical application. Error bars indicate 95% confidence limits. There is no significant difference between the two *D. melanogaster* lines.

As with the washes, analysis shows that there was also no significant differences in chlorantraniliprole recovery from insect extracts of the two *D. melanogaster* lines at any of the timepoints (extract p-values were T1=0.30, T5=0.94, T18=0.41, T24=0.55) (figure 5.28). Both lines show the same accumulation of chlorantraniliprole at the 72 hour time course.

The control strain consistently shows a slightly higher accumulation of chlorantraniliprole than the experimental *FMO2* line, but even by 72 hours it is marginal (1.2 times more) and not significant.

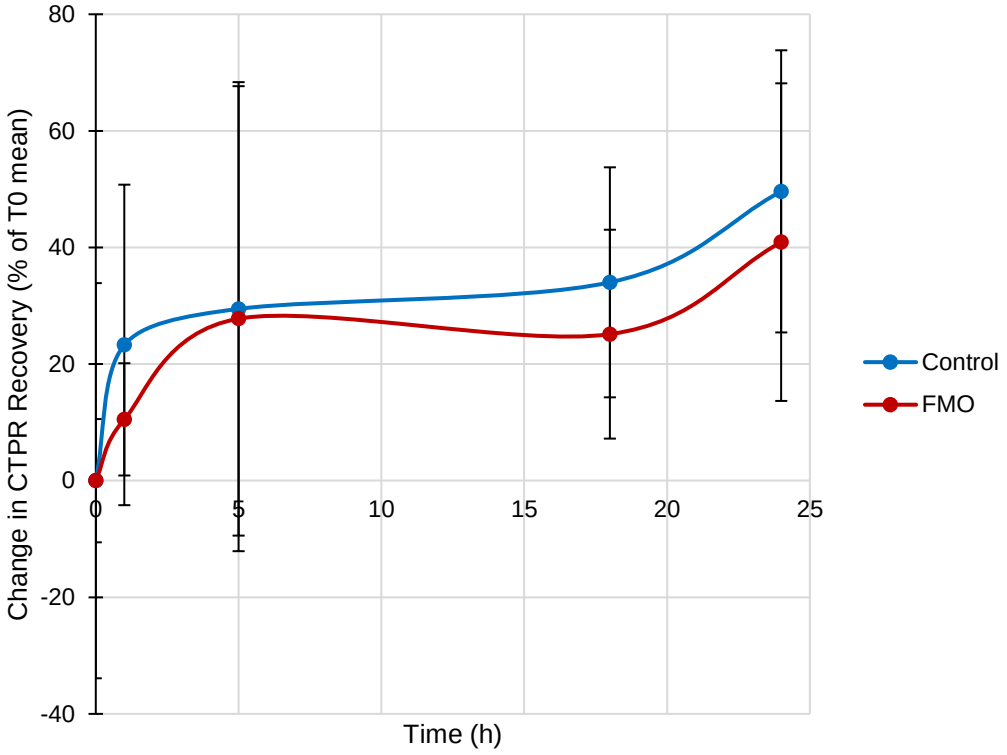


Figure 5.28. Percent change in chlorantraniliprole (CTPR) recovery by LC-MS in transgenic *D. melanogaster* expressing *FMO2* gene extracts over 24 hours following topical application. Error bars indicate 95% confidence limits. There is no significant difference in recovery between the two *D. melanogaster* lines.

5.4 Discussion

5.4.1 Stability of chlorantraniliprole resistance

It was accepted through the course of this experiment that it would not be possible to adhere to a strict sampling timetable for bioassays and the snap freezing of larvae between the selected, HS and unselected, UnSel populations to produce truly paired datasets. This was due to asynchronisation of the populations caused through the need to select one population with insecticide, but not the other. Han *et al.* reports sublethal effects of chlorantraniliprole resulting in the mean generation time of chlorantraniliprole treated groups increasing as

dose increased [349]. A further paper by Guo *et al.* also reported sublethal effects of chlorantraniliprole treatment of *P.* xylostella resulting in longer generation times, but also found negative transgenerational effects in F1 generation individuals that were never directly exposed to the insecticide [350]. It was important that these factors were taken into consideration when sampling and bioassaying the two populations and the reason why the 2nd generation post selection (F2) were subject to insecticide bioassays.

The results of the stability experiment clearly demonstrates that the level of resistance to chlorantraniliprole is unstable in the HS population and declines over time in the absence of selection. This is strong evidence that the mechanism(s) underlying resistance confer a strong fitness cost when insecticides are not used. Resistance-associated fitness costs have been well documented in the literature with recent publications by Ribeiro *et al.* and Liu *et al.* specifically focusing on the diamides, chlorantraniliprole and cyantraniliprole in *P. xylostella* respectively [279, 351]. Ribeiro *et al.* found an increase in the larval cycle, and weight reduction in resistant insects even in the absence of chlorantraniliprole exposure and Liu *et al.* reported that their cyantraniliprole selected strain, Cya-Sel, had lower reproductive ability and relative fitness than other populations.

The significant reduction in expression of several of the genes of interest implies that it is possible that more than one gene is involved in the diamide resistance shown by the HS strain. However, the overiding dominant overexpression shown by the *FMO2* candidate in the initial microarrays and qPCR (chapter 4), and the subsequent significant reduction in expression alongside an increase in susceptibility in the resistant strain following a lack of exposure to chlorantraniliprole (shown in this chapter) cements this gene's status as the primary candidate involved in diamide detoxification.

5.4.2 Synergists

A baseline bioassay had previously been carried out to investigate the toxicity of PBO and MEM in the HS strain to generate an LD_{50} and guide the concentration at which the synergists could be used in the actual experiments (data not shown). It was found that there was no significant toxic affect resulting in larval death from either of the inhibitors up to concentrations of 500ppm. At the higher doses, there was some evidence that the synergists

did have some effect on the larvae, but not enough to cause mortality within the 48-hour test period. Previous studies on *P .xylostella such* as that by Wang *et al.* [246] have used 100mg/L (100ppm) concentrations of PBO and this concentration seemed to be supported by the baseline bioassay performed here.

PBO is an inhibitor of cytochrome P450's and did not have a synergistic affect on diamide efficacy against the HS strain, casting some doubt on the contribution of P450s to the resistance shown by this strain. Results using the methimazole inhibitor bioassay were surprising, with the MEM treated population showing a significant decrease in sensitivity to chlorantraniliprole. The only chemical inhibitors of FMOs are competitive substrates [352]. Methimazole has been used as an FMO inhibitor in many (non-insect) studies, for example Usamni *et al.* used this compound as one of several approaches (and alongside inhibitors of P450s) in the identification of putative human FMO and cytochrome P450 enzymes involved in the metabolism of Lorcaserin [353]. Yeniceli *et al*. also used MEM as a competitive inhibitor of FMOs investigating the metabolism of tamoxifen [354], although these other studies used *in vitro* cell based techniques rather than an *in vivo* whole organism approach. MEM has also been extensively used as a substrate for FMO sulfoxidation as used by Wyatt *et al.*, Lattard *et al.* and Scharf *et al.* in their respective studies in the measurement of FMO activity [355-357]. Scharf *et al.* noted from *D. melanogaster* differences in sulfoxidation between the two FMO isoforms present in that species, with weak sulfoxidation observed from *FMO1* compared to *FMO2*. Differences in the substrate specificity between the different isoforms of FMO is well documented and reviewed by Ziegler [358]. As so little information is known regarding FMOs in insects, it is possible that alternative inhibitors (for example imipramine) should be investigated. It has been shown that a modulator can activate or inhibit FMO's catabolism depending on substrate concentration. For example, in the publication by Wyatt *et al.*, imipramine activates methimazole metabolism catalysed by rabbit *FMO1* or *FMO2* at higher methimazole concentrations, and inhibits at lower methimazole concentrations. The extent of the activation increases as the substrate concentration increases, and inhibition increases as the substrate concentration decreases [357]. In the experiments carried out in this chapter, the putative substrate of *FMO2* is chlorantraniliprole, with methimazole used as an inhibitor/modulator, but it is possible that a similar mechanism is at work here. Wyatt *et al.* states that with either inhibition or activation, the magnitude of the effect is dependent on the concentration of the inhibitor, therefore it may be that the synergist bioassay would

benefit from further optimisation, specifically in regards to the concentration of methimazole being used. Of course, the possibility must be acknowledged that the *FMO2* is not responsible for metabolising chlorantraniliprole. However, given the complexities that are involved in the successful synergistic inhibition of this insect *FMO2*, about which very little detail is known, a great deal of optimization needs to be performed before conclusions should be drawn solely from synergist bioassays. In fact even in the more closely studied class of mammalian FMOs, Cashman and Zhang state that FMOs are not easily induced nor readily inhibited [359]. Further synergist bioassays could be performed possibly incorporating other possible inhibitors of FMO's and investigating other genes of interest such as Xanthine dehydrogenase with the synergist allopurinol [329].

5.4.3 Characterisation of GOIs

Identification of the full-length coding sequences of the candidate resistance genes from the resistant HS strain, enabled functional studies to be conducted. Comparison of the primary candidate (*FMO2*) gene sequence with the other strains, revealed that there were not any obvious mutations (either SNPs or indels) present that would be predicted to significantly impact the activity of the encoded enzymes.

Most insects have three isoforms of FMO [211], and differences in isoform substrate specificities and tissue specific expression patterns have been reported in other organisms [360]. Different isoforms can also differ in other characteristics, for example, Hodgson reports that heat inactivation of FMO activity is more effective for some isoforms than for FMO2 [361]. Therefore, the identification of which FMO isoform is likely being investigated may be informative for future studies and experiments.

5.4.4 Transgenic *D. melanogaster* bioassays

Drosophila melanogaster represents a powerful tool to explore gene function using the array or transgenic approaches that have been developed for this model insect species. More recently *Drosophila* has also been used to examine the role of the genes of other insects in resistance [362, 363]. The advantage of this approach is it allows the effect of a gene on resistance to be explored in a common genetic background. The potential disadvantage of

this approach is that *Drosophila* may not accurately reflect the genetic or physiological environment of the native insect.

The bioassay results showed that the transgenic *D. melanogaster* expressing the *SCD* or *CYP6BG1* lepidopteran genes were detrimentally affected by the over-expression of these genes. The inclusion of these genes resulted in significant increased sensitivity to chlorantraniliprole compared to the control line. The fact that the line expressing the *FMO2* conversely resulted in a significant increase in the resistance of the flies, led to the lowered prioritisation of these other candidates and cemented focus on the *FMO2* as the primary candidate gene. The same can be said of the *XDH* gene, although, while not a significant result, the addition of *XDH* gene does seem to confer a marginal level of resistance in *D. melanogaster* to both the anthranilic diamides, chlorantraniliprole and cyantraniliprole (despite the extreme sensitivity to the later). This was not true for the phthalic diamide, flubendiamide where the *XDH* line was more susceptible.

Previous studies expressing pest insect genes in *Drosophila* have used the native gene sequences and, to date, comparison with gene sequences codon optimised for expression in this model species have not been made. My results revealed that no significant phenotypic difference was detected between transgenic flies expressing the original lepidopteran *FMO2* gene and those with the gene variant codon optimised for *Drosophila*. QPCR confirmed increased expression levels for the original lepidopteran and codon optimised variants of the *FMO2* gene in the transgenic drosophila of 152 and 277-fold respectively over the parental control lines carrying one or other of the genes, but without the driver (figure 5.29). QPCR also showed that expression of the codon optimised *FMO2* variant is higher (but not significantly so) than the native sequence isolated from the *P. xylostella* HS strain, mirroring the dose-response findings for the two lines assayed with Chlorantraniliprole.

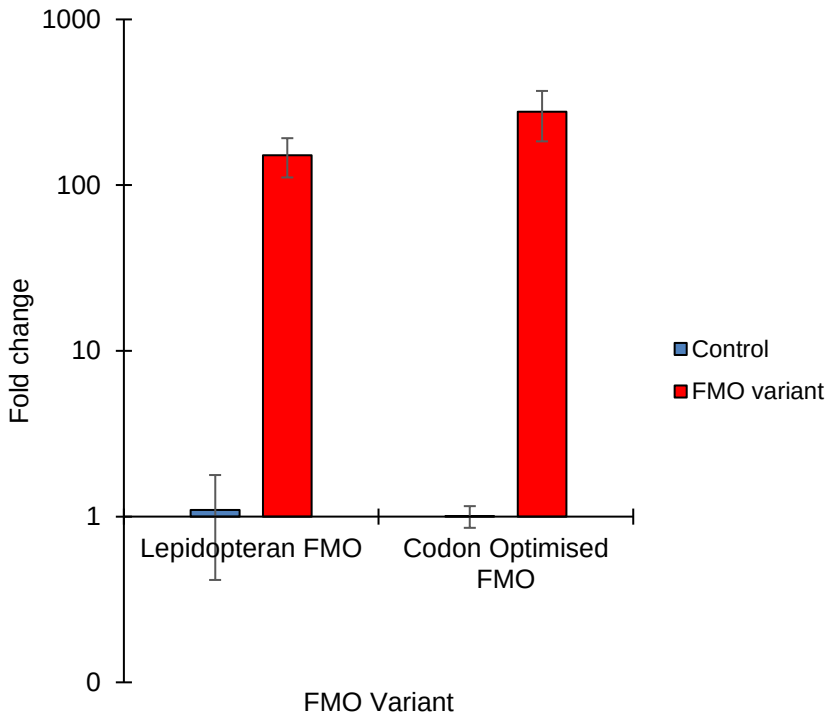


Figure 5.29. Transgenic *D. melanogaster* qPCR expression for original lepidopteran *FMO2* gene and *FMO2* gene codon optimised for *D. melanogaster*. Error bars indicate 95% confidence limits. While both show overexpression compared to the control, there is no significant difference between the two forms.

The hypothesis was that codon optimisation of the *FMO2* gene would increase expression of the gene. Differential expression of genes sharing amino acid sequences, but differing nucleotide sequence results from different organisms using specific subsets of codons to encode the same protein, known as codon bias [364, 365]. There are 61 different codons to encode 20 amino acids, and three codons to terminate translation. Kane details that an excess of organism specific 'rare' codons (as might be introduced in a transgenic system such as the GAL4/UAS used here) could create translational problems, either in quality of quantity of the produced protein [364, 366]. While there was a marginal increase in *FMO2* expression, and resistance, in the codon optimised *FMO2* line, the lack of a greater difference might be due to the similarity between the two nucleotide sequences on the basis that they are both insects. Had the study organism been from another taxonomic order, it is possible that the codon optimisation would have elicited a greater differential response. Burgess-Brown *et al.* demonstrate the significant effect that codon optimisation can have in the expression of human genes in *Escherichia coli* [367]. The authors found that 18 of the 30 human genes

tested exhibited increased expression compared to their native counterparts, although as SDS-PAGE protein visualisation was used, actual differences in expression are not given.

It has been found that the ubiquitous overexpression of the cytochrome P450, *CYP12A4* in a *D. melanogaster* GAL4/UAS system under the control of tubulin results in late embryonic lethality [368]. In the same study, the authors reported that in the same experimental system, overexpression of the same P450 gene conferred resistance to lufenuron when it was expressed in specific tissues, namely the larval midgut and malpighian tubules. It is possible that more focused tissue expression patterns for the candidate genes could result in a greater resistant phenotype, or in the case of *SCD* and *CYP6BG1* carrying *D. melanogaster* a phenotype that is at least less sensitive. Unfortunately, there were not the resources or time available to investigate this further during this PhD.

5.4.5 RNAi

Silencing of the *FMO2* gene through RNA interference knockdown was unsuccessful. Therefore phenotyping of the resistant HS strain using RNAi treated individuals was not pursued. This aspect would only be investigated upon confirmation of successful gene silencing, and so while, this work was not uninformative, it should be deemed a preliminary experiment.

The primers designed to isolate template DNA for the double stranded RNA (dsRNA) synthesis were designed from the CM strain sequence as this was carried out before sequencing of the *FMO2* gene from the HS strain had been completed. Once sequencing of the whole of the HS *FMO2* sequence was complete, it was compared to the *FMO2* target template from the CM strain. The two were identical within the sequences of the forward primers, however, a SNP was present within the reverse primer. Ideally (and with hindsight) the primers would have been designed from and therfore exactly match the HS strain. In practice it is unlikely that this made a difference because the actual template amplified was from the HS strain. As the process of RNAi involves the template double stranded RNA (approx 400bp) being 'cut' into smaller 'short interfering RNAs (~22bp long) by the enzyme, dicer, the fact that one of these siRNA's might have the wrong sequence is unlikely to effect whether the double stranded RNAi works.

Several different approaches for the application of RNAi have been used in *P. xylostella*. Zhang *et al.* and Dong *et al.* both used injection into *P. xylostella* larvae in their respective studies [341, 369], whereas Lee *et al.* made injections into pupae [370]. Several studies also have taken the approach of feeding dsRNA or short-interfering RNA (siRNA) to pest species. Gong *et al.* investigated feeding siRNA to larvae which acted as a pesticide itself, resulting in larval death [371]. Conversely, Yang *et al.* achieved a decrease in susceptibility to the *Bacillus thuringiensis* produced insecticidal protein, Cry1Ab through the silencing of aminopeptidase N proteins via the oral feeding of dsRNA to the sugarcane borer, *Diatraea saccharalis* [291]. Bautista *et al.* fed dsRNA to *P. xylostella* achieving the silencing of the cytochrome P450, *CYP6BG1*, resulting in a decrease in larval resistance to permethrin [134].

Preliminary injections of water into 3rd to 4th instar larvae were trialled to test the equipment and technique. Between 2.3nl and 69.0nl of liquid was injected and mortality assessed (results not shown) resulting in a volume of 41.4nl being chosen to be injected for the RNAi experiment. Despite extra individuals undergoing injection to account for any injection mortality, survival was excellent with neither the volume of liquid nor the injection itself effecting the result. It is possible that knockdown of the *FMO2* gene would have been observed if a greater quantity of dsRNA was injected, although Rinkevich *et al.* propose that other factors such as injection site may influence effectiveness of RNAi [372]. In this experiment 20.3ng of *FMO2* dsRNA was injected which is less than has been typically injected in other studies with 100-500ng [341, 369] injected into larvae and injection of 100ng in 100nl [65] into pupae. Experiments performed by Bautista *et al.* directly fed 250ng per larvae in 0.3µl elution solution, whereas Yang *et al.* fed 50ng per individual within 0.1µl totals [134, 291]. Gong *et al.* also fed short interfering RNA (siRNA) at up to 200µg/ml to larvae via coated leaves [371].

Techniques involving feeding double stranded or short interfering RNA obviously have advantages over injections as they are non-intrusive and so control mortality is likely to be kept to a minimum. However, with feeding, it is difficult to control the precise amount of RNA consumed per insect. In view of the low mortality rate of the above experiment the primary focus will be to optimise RNAi experiments through injection, primarily regarding

the concentration of dsRNA being utilised. Additionally, experiments could be made using short interfering RNA.

As mentioned, even if RNAi knockdown of the target gene is successful in the future (confirmed through qPCR), this will not prove that this gene is responsible for the resistance seen in this strain. To test the latter, it would be necessary to perform a standard bioassay using chlorantraniliprole and/or other diamides on RNAi treated larvae to confirm a reduction in their resistance factor against a non-treated control.

5.4.6 Stability and metabolism of chlorantraniliprole in transgenic *D. melanogaster* expressing *FMO2* using liquid chromatography-mass spectrometry (LC-MS)

The experimental and control line washes showed the same decreases in chlorantraniliprole recovery over time, indicating that both lines had a similar level of insecticide penetration and suggesting the *FMO2* gene does not result in any changes to the insect cuticle. The fact that chlorantraniliprole recovery from the insect extracts increased over time while the washes show a decrease indicates that the pesticide was penetrating the cuticle of the insects as expected. For these topically dosed *D. melanogaster*, the level of chlorantraniliprole found in the extracts did not differ significantly between the transgenic and control lines at any of the time points with the same level of accumulation over time. At face value this implies that chlorantraniliprole is not metabolised or excreted at a greater level by the flies expressing the *FMO2* gene than controls lacking this transgene.

Results from the feeding assay show a different picture, where the transgenic *D. melanogaster FMO2* strain accumulated a significantly higher level of chlorantraniliprole by the 24-hour sampling point, but by the 72hour experimental endpoint, the amount of chlorantraniliprole had decreased to the same level as for the control strain. It is possible that the plateaux in the amount of chlorantraniliprole present in the control strain from 5 hours onwards is symptomatic of a reduction or cessation in feeding, likely brought about by the insecticide. The fact the experimental strain initially accumulated a significantly higher level of chlorantraniliprole, which then decreased over time might indicate the metabolism of this insecticide in this transgenic strain. This is similar to the early accumulation of the primary intermediary imidacloprid compound, olefin-imidacloprid in the termite, *Reticulitermes flavipes*, prior to its further metabolism into secondary products shown by

Tomalski *et al*. The authors reason that this may be because the metabolism of imidacloprid to olefin-imidacloprid equalled the metabolism of olefin-imidacloprid to other products [373]. A similar scenario might have been at play here, where the chlorantraniliprole accumulated as the influx of insecticide matched or exceeded that of its metabolism. Beyond 24 hours though, the accumulation of chlorantraniliprole was depleted, possibly as a result of the rate of metabolism exceeding intake.

The differences seen in the amount of chlorantraniliprole in the *D. melanogaster* extracts over time between the oral and topical deliveries is likely related to the point of entry of the insecticide into the insect. The resulting locations of parent compound in the insect tissues was not assessed, although it would seem logical that topically applied chlorantraniliprole penetrating through the cuticle of the insect would take longer to reach a physiological environment such as the fat body, midgut or Malpighian tubules, whereby it can be more readily metabolised and/or excreted than orally fed compound. Because the topical insecticide application assay only took place over 24 hours, as opposed to the longer (72-hour) duration of the feeding assay, it is impossible to say what differences (if any) the two lines would have demonstrated beyond this time-point in the topical assay. It would be interesting to explore this in future studies.

While informative, if this approach were to be repeated, it would be very interesting to be able to track both the parent compound and metabolites as Tomalski *et al.* demonstrated by radio-labelling imidacloprid [373]. Radio-labelling chlorantraniliprole would allow its progression both in terms of parent compound depletion and the identification of and changes in the levels of metabolites carrying the radiolabel to be followed. Bull *et al.* were also able to assess the metabolism of the pyrethroid, permethrin, in a resistant strain of German cockroach, *Blattella germanica*. Again, the insecticide was radio—labelled with C^{14}, with the authors measuring radioactivity present in insect washes, bodily extractions and frass extractions to assess cuticular penetration, internal accumulation and excretion [374].

5.5 Summary

In summary, multiple techniques and experiments detailed in this chapter have been conducted in order to provide the best assessment possible of the involvement of the genes

identified in chapter 3 in diamide resistance in *P. xylostella*. No single approach was used to draw conclusions, which might have biased one gene over another. While the experiments involving synergistic compounds and RNAi failed to provide definitive conclusions, they did provide valuable information which would serve to take the research further in future. Other experiments detailed in this chapter proved informative, providing corroborative evidence from which conclusions may be drawn.

The stability of chlorantraniliprole resistance was proven to be unstable over time given a lack of selection pressure with this insecticide with the decline in resistance reflected in reduced expression levels of several of the candidate genes. One of the genes, the *FMO2* showed a particularly significant reduction in expression over the time-course compared to the other genes. Bioassays involving compounds that show synergistic relationships with the two primary candidates, *FMO2* and *CYP6BG1* in the literature showed no synergistic effect for *CYP6BG1*, and actually significantly increased the resistance ratio for *P. xylostella* treated with methimazole. The reasons for this are unclear and will take further work to resolve.

Sequences of the top four candidate genes, *FMO2*, *CPY6BG1*, *SCD* and *XDH* were identified from the resistant HS strain allowing the creation of transgenic *D. melanogaster* lines each carrying one of the candidate genes. Phenotyping of these lines with diamide compounds resulted in *CPY6BG1* and *SCD* both indicating significantly more sensitive phenotypes. The transgenic *XDH* line showed an increase in resistance to the anthranilic diamides, but this was not significant with 95% confidence limits. The *D. melanogaster* line expressing the *FMO2* gene showed a significant increase in resistance to chlorantraniliprole, but not to the phthalic diamide, flubendiamide. Codon optimisation of the lepidopteran *FMO2* gene for *D. melanogaster* resulted in a marginal, but not significant increase in expression of this gene and resistance phenotype.

Given the concurring results for the *FMO2* being putatively involved in the *P. xylostella* resistance shown, an attempt at RNA interference was attempted to silence expression of this gene. This was performed through the injection of dsRNA into resistant HS larvae, but did not result in reduced expression of this gene compared to control injections. It is likely

that this is a result of the experimental procedure not being optimal, but the resources were not available to pursue this line further.

Liquid chromatography-mass spectrometry assessment of parent compound present in washes and whole body extractions from transgenic *D. melanogaster* following topical application of chlorantraniliprole over time showed that there was no reduction in insecticide penetration of the cuticle conferred by the *FMO2* gene, nor was there any difference in chlorantraniliprole levels within the insect compared to the control line. The transgenic line orally fed chlorantraniliprole displayed an ability to tolerate and accumulate this diamide above that of the control line, which after 24 hours appears to have then been metabolised to the same level as the control line by the end of the experiment.

The transgenic *D. melanogaster* allowed the assessment of each of the candidate genes in isolation, again, providing evidence for the contribution of the *FMO2* gene to the resistance seen in the *P. xylostella* HS strain. It is possible however, that the co-expression of more than one candidate gene in a *D. melanogaster* line may produce a line with a more complete metabolic pathway. For example, while the *FMO2* gene does seem to confer a level resistance to chlorantraniliprole, the *XDH* gene did not, although only marginally. It is possible that a transgenic *D. melanogaster* line co-expressing both the *FMO2* and *XDH* genes could result in a phenotype that is more resistant than either of the lines expressing individual transgenes. Speculatively, as *XDH* is involved in the excretion pathways, it might be biologically more efficient in a functional relationship either directly or indirectly involving an *FMO2* mediated metabolite of chlorantraniliprole than it is of chlorantraniliprole itself. This could be true for any, or none of the combinations of candidate transgenes.

Strong evidence has been detailed here implicating *FMO2* as a major contributor to metabolic detoxification of diamides, particularly in that of the anthranilic diamides. Studies of FMOs in insects have primarily focused on their function in the safe sequestration and accumulation of pyrrolizidine alkaloids from host plants as a form of defence [220, 221, 375] (also see section 1.7.4). Regarding pesticide resistance in insects, only a single publication by Tian *et al*. implicates FMO in detoxification of insecticide (metaflumizone) in beet armyworm, *Spodoptera exigua.* However, there is evidence of pesticide detoxification from other taxa

(mammals) with Hodgson *et al.* reporting differences in the metabolism of organophosphates (phorate and fonofos), neonicotinoids (nicotine and imidacloprid) and a carbamate (methiocarb) from a selection of species (mouse, rat and human) [218]. All of these pesticide classes were metabolised by FMOs with few significant differences between the species, although there were significant differences between the isoforms regardless of species.

The reason for *FMO2* overexpression is not investigated in this chapter, although in a sequence comparison with the susceptible strain there was no difference in the sequence of this gene that would likely cause a qualitative effect on enzyme function. The following chapter (chapter 6) details a possible reason for overexpression of this gene.

6 *FMO2* promoter region and *cis*-acting regulation

6.1 Introduction

Work in chapter 5 identified a primary candidate gene, *FMO2*, that above all the other candidate genes, appears to confer resistance to the diamide chlorantraniliprole, however the reasons for the overexpression of this gene in the HS strain of *P. xylostella* were unknown. For this reason, and given the challenges encountered with PCR amplification of target regions for the *XDH* gene (despite the publication of the DBM-DB genome [137] to aid with primer design and alignments), next generation sequencing was employed to identify the genomic sequence from the susceptible ROTH strain. The ROTH strain was selected over the resistant HS strain primarily because it was this strain with which problems were being encountered in PCR. Secondly, the ROTH strain is commonly used as a susceptible standard in *P. xylostella* studies worldwide [63, 376, 377], therefore once adequately assembled and annotated, it could potentially provide a valuable resource for the wider scientific community.

It is known that a range of genomic elements and processes in regions upstream of a gene can affect gene expression. Indeed, it is within the proximal promoter region that transcription of a gene is initiated. A publication by Morra *et al.* compares the induction of three P450 promoter regions by caffeine and phenobarbital using a dual luciferase reporter assay, finding differences between the promoter regions. The authors then went on to employ a bioinformatic approach to identify upstream DNA sequence motifs that might be involved in transcriptional regulation [378]. Dual luciferase reporter assays have also proved a useful tool to investigate the differences in promoter region activity and the implications for overexpression of putative resistance genes in other studies. Having identified numerous predicted transcription factor motifs and indel polymorphisms in the promoter of the P450 *CYP6AY1* between resistant and susceptible strains of the brown planthopper, *Nilaparvata lugens*, Pang *et al.* used a dual luciferase reporter assay to assess their effect on expression. Testing numerous combinations of putative elements, the authors found 12 variations that discriminated between the resistant and susceptible strains [379].

Fireflies (*Photinus pyralis*) use a biochemical reaction allowing them to bioluminescent signals as part of their courtship behaviours [380]. Due to the highly efficient conversion of luciferin to oxyluciferin and the emission of light as a by-product of the reaction by the enzyme, luciferase, assays involving this reaction are extremely sensitive, allowing small changes in bioluminescence to be quantified. In a dual luciferase reporter assay this reaction is employed by the cloning of the regulatory region of the gene of interest (in this case *FMO2*) upstream of the luciferase gene in an expression vector. This expression vector is introduced into cells, which are then grown on to increase the amount of proteins available. Once the cells are harvested, their lysates are used to perform the chemical reaction with cofactors and the luciferin enzyme with the enzymatic activity quantified using a luminometer. In a dual luciferase reporter assay a second reporter luciferase (from *Renilla reniformis*) is also quantified from within the same reaction mixture and used as an internal baseline control against which the firefly luciferase measurement can be normalised. It is the measurement of the light output by both reactions that allow for assessment and quantification of the reaction. By cloning the regulatory region from the *FMO2* from the resistant HS and susceptible ROTH strains upstream of the luciferase gene, the transcription activity of each region can be compared through comparison of the relative amount of light produced in each case.

This chapter details the next generation sequencing that took place, subsequent completion of remaining sequencing, and experimental comparison of the transcription of the putative promoter regions using a dual luciferase reporter assay. Following a significant result produced through the reporter assay, a computational investigation was made into sequence and structural differences present between the promoter regions of the resistant and susceptible strains that might explain the difference in expression levels between the promoter regions of the two strains.

6.2 Methods

6.2.1 Next generation sequencing

6.2.1.1 Extraction of genomic DNA

DNA was extracted from a single 3rd-4th instar larvae using a EZNA insect DNA kit (Omega Bio-Tek) according to the manufacturer's protocol, with a minor modification. An additional RNAse incubation (2µl - incubated for 10 mins @ 60°C) step was performed after the proteinase K step and the proteinase K step was incubated for 2 hours. The additional RNAse step was made because from previous experience, extractions using the original protocol (where an incubation with RNAse took place before the addition of proteinase K) had resulted in RNA contamination of the final extraction, which in turn affected accurate DNA quantification when using a nanodrop spectrophotometer. All other steps were carried out as per the manufacturer's protocol. The final elution was made in 50µl elution buffer.

6.2.1.2 DNA quantification

DNA quantification was performed using a 'Qubit' fluorometer. The method involved vortexing 199µl of buffer and 1µl reagent with 2µl DNA sample.

6.2.1.3 Next generation sequencing

For whole genome sequencing, fragment libraries were generated from 1ug genomic DNA using the Ion Xpress Plus Fragment Library Kit (ThermoFisher Scientific) with the Ion PI Template OT2 200 Kit v3 (ThermoFisher Scientific) used for template preparation and the Ion PI 200 Sequencing Kit v3 (ThermoFisher Scientific) used for sequencing on an Ion Proton semi-conductor sequencer, all according to the Manufacturer's protocols.

The sequence produced was uploaded and analysed in the open-sourced, web-based platform, Galaxy (https://usegalaxy.org). Data quality was assessed using galaxy 'FastQC', following which the reads were aligned to the DBM-DB reference genome using the 'Bowtie2' tool.

In addition to this mapping, a standalone MIRA (Mimicking Intelligent Read Assembly) assembly of the reads was performed, with the resulting contigs imported into Geneious as a BLAST-searchable database.

6.2.2 Dual luciferase reporter assay

It should be noted that while all other work is my own, the practical experimental work detailed specifically in this section (6.2.2) was completed by a laboratory technician late during this PhD, during the period when I was writing up.

Following identification of the proximal promoter regions from the resistant HS and susceptible ROTH strains, a nested PCR primer approach was adopted to amplify the regions from extracted DNA. In order to amplify the largest region possible, but remain as consistent as possible between the strains, given the large number of discrepancies between the 5'UTR sequences, forward primers ('1) Px_FMOProm_F1' and '3) Px_FMOProm_F2') were designed from conserved sequence at the furthest 5' end of the ROTH strain with the same primers being used for each strain. The first round reverse PCR primer ('2) Px_FMOProm_R1') was also the same for each strain, being designed from conserved sequence. The reverse primers ('4) Px_R_FMOPromNcoI_R2' and '5) Px_HS_FMOPromNcoI_R2') for the nested PCR were located in the same relative position for the two strains, straddling the 3' end of the UTR and the 5' start of the *FMO2* gene. Primer positions can be seen in figures 6.01 (a)-(c), with primer sequences shown in table 6.01. The HS strain PCR product (used for the dual luciferase reporter assay) is 1530 ungapped base pairs in length, and the ROTH PCR product is 1312 ungapped base pairs (both include the first 7 bp of the *FMO2* gene).

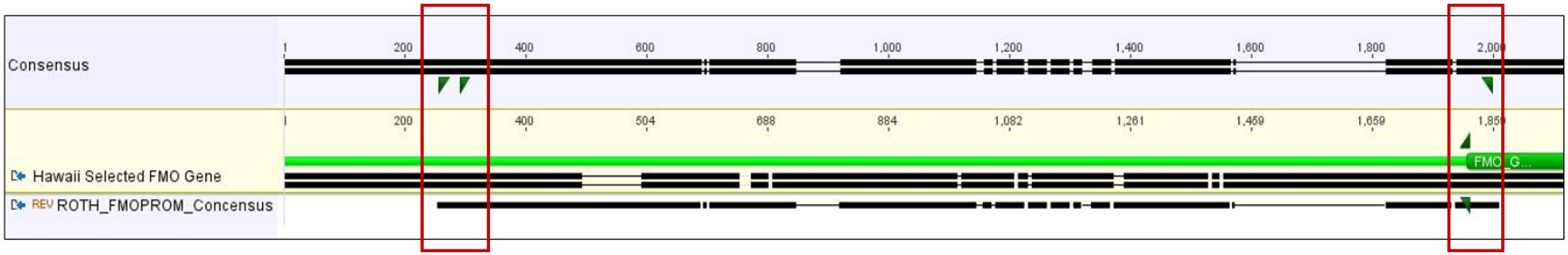


Figure 6.01 (a). Alignment of HS and ROTH 5' proximal promoter sequences showing positions of primers used for PCR amplification of this region. Magnifications of red boxes can be seen in figures 6.01(b) and 6.01(c).

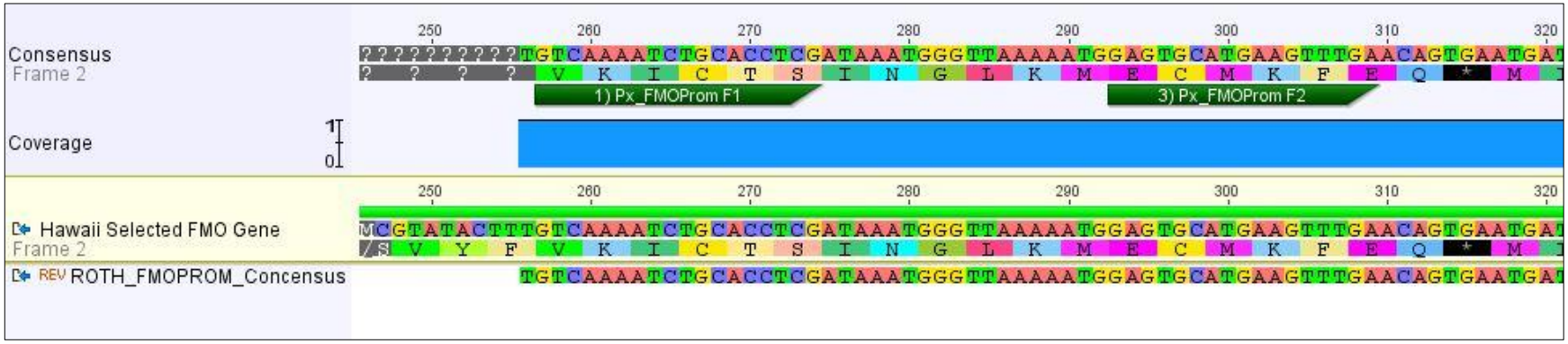


Figure 6.01 (b). Extract of figure 6.01(a) showing primer sequences and positions for forward primers. The same primers were used for PCR amplification in both HS and ROTH strains as they are from 100% conserved sequence.

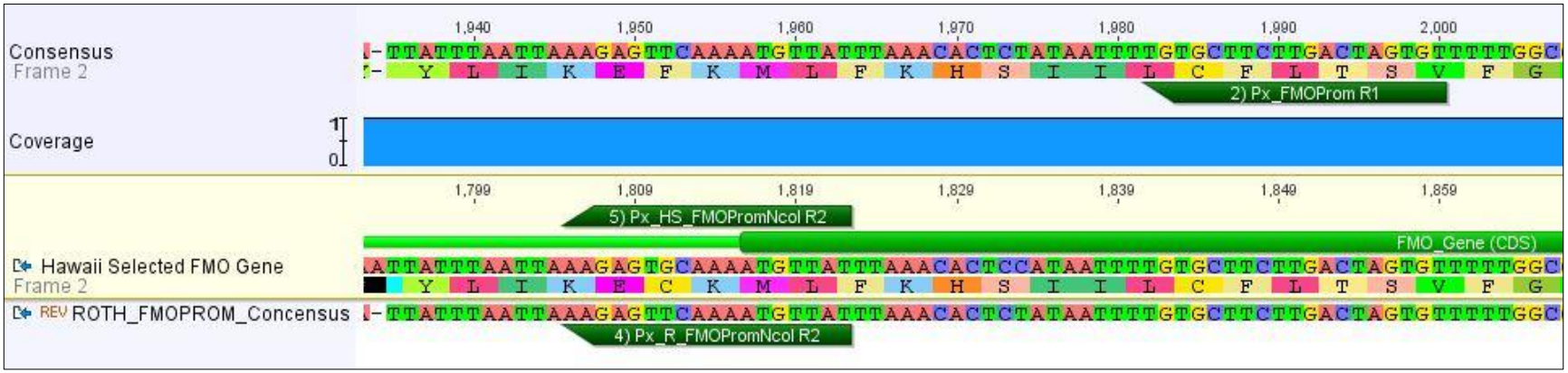


Figure 6.01 (c). Extract of figure 6.01(a) showing primer positions for reverse primers. The first-round PCR primer, '2) PxFMOProm_R1' was used for amplification from both strains. Primers '4) Px_R_FMOPromNcoI_R2' and '5) Px_HS_FMOPromNcoI_R2', used for nested PCR were strain specific, accounting for a SNP between the two strains.

The HS and ROTH promoter fragments were PCR amplified using 'Phusion high fidelity DNA polymerase' (New England BioLabs) using a nested PCR approach. The first round of PCR used primers '1) Px_FMOProm_F1' and '2) Px_FMOProm_R1'. A second (nested) round of PCR took place using 2µL (1:50 dilution) of first-round PCR product using primers '3) Px_FMOProm_F2' and '4) Px_R_FMOPromNcoI_R2' for amplification from the ROTH strain, and primers '3) Px_FMOProm_F2' and '5) Px_HS_FMOPromNcoI_R2' for amplification from the HS strain. The nested forward primer, '3) Px_FMOProm_F2' incorporated the *Sac*I restriction site, with reverse primers '4) Px_R_FMOPromNcoI_R2'and '5) Px_HS_FMOPromNcoI_R2' incorporating the *Nco*I restriction site, each of which was required for ligation into the pGL3-basic luciferase reporter vector (Promega). The PCR conditions can be seen in tables 6.02 and 6.03. PCR product was purified using Wizard SV gel and PCR clean up kit (Promega), before being subject (alongside pGL3 plasmids) to enzymatic digestion with *Sac*I and *Nco*I restriction enzymes (CutSmart from New England BioLabs) according to the manufacturer's protocol.

Primer name	Primer sequence	Restriction Site
1) Px_FMOProm_F1	GTCAAAATCTGCACCTCG	-
2) Px_FMOProm_R1	ACACTAGTCAAGAAGCACA	-
3) Px_FMOProm_F2	aattgagctcGAGTGCATGAAGTTTGA	*Sac*I
4) Px_R_FMOPromNcoI_R2	aattccatggATAACATTTTGAACTCTT	*Nco*I
5) Px_HS_FMOPromNcoI_R2	aattccatggATAACATTTTGCACTCTT	*Nco*I

Table 6.01. Primers used for PCR amplification of the proximal promoter of the HS and ROTH strains. Lower-case letters denote additional base pairs incorporated into the primer design to incorporate restriction sites where needed. Where found, 'aatt' is a leader sequence added to assist with restriction enzyme digestion. The leader sequence is followed by restriction site sequence (6-bases), after which capital letters denote primer hybridisation sequence.

Stage of Program	Temperature (°C)	Duration	Number of cycles
Hold	95	2 mins	-
Cycling	95	30 secs	35
	51	30 secs	
	72	1 min 30 secs	
Hold	72	5 mins	-

Table 6.02. PCR conditions for first-round PCR of promoter sequence from HS and ROTH *P. xylostella* strains.

Stage of Program	Temperature (°C)	Duration	Number of cycles
Hold	95	2 mins	-
Cycling	95	30 secs	40
	45/50	30 secs (ROTH/HS)	
	72	1 min 30 secs	
Hold	72	5 mins	-

Table 6.03. PCR conditions for second-round (nested) PCR of the promoter sequence from HS and ROTH *P. xylostella* strains. Different primer annealing temperatures were used for each of the strains as indicated in the table.

Digested products were run on a TAE gel with target fragments then excised and purified using a Monarch gel purification kit (New England BioLabs). Ligation of the PCR fragments into the pGL3 vectors was made at 3:1 ratio of insert:vector using T4 DNA ligase (New England BioLabs). The resulting pGL3-HS (containing the HS promoter) and pGL3-R (containing the ROTH promoter) recombinant plasmids alongside pGL3-basic and pRL-CMV (Renilla luciferase, used as an internal control) were cloned into library efficiency DH5α competent cells (ThermoFisher Scientific) according to the manufacturer's instructions, and following purification using a GeneJET plasmid miniprep kit (ThermoFisher Scientific) the plasmids were sequence verified (Eurofins).

In preparation for the reporter assay, Sf9 cells were maintained at 37°C in Gibco serum free medium (ThermoFisher Scientific). For three replicates for each transfection, 2.5 mL of Sf9 cells at a density of ~$4x10^5$ cells/ml were plated into 6-well plates one hour prior to simultaneous transfection with 2µg recombinant pGL3 plasmid and 3ng Renilla luciferase pRL-CMV plasmid using Insect GeneJuice transfection reagent (Novagen) according to the manufacturers' guidelines (user protocol TB359). Controls were simultaneously transfected with pGL3-basic and pRL-CMV. After four hours, the transfection medium was replaced with fresh serum free medium and the cells incubated at 27°C for 48h. Cells were harvested according to the dual-luciferase assay II (DLRII) kit protocol (Promega). Luminescence readings from both firefly and Renilla luciferase activities were taken for each of the replicates of the pGL3-HS, pGL3-R and pGL3-basic control lysates in white opaque 96-well plates using a BioTek Synergy 2 microplate reader. Luminescence readings were also taken of 3 wells containing cell lysate from non-transfected cells plus the matching amounts of DLRII reagents as a measure of background luminescence which through the analyses was

effectively subtracted from the experimental readings. Data were normalised to Renilla luciferase activity as a control for transfection.

6.3 Results

6.3.1 Next generation sequencing

Next generation sequencing produced a total of 33,334,336 total reads with an average read length of over 200bp (Figure 6.02). When aligned to the DBM-DB [137] scaffolds using 'Bowtie2' 79 % of the bases aligned (Figure 6.03).

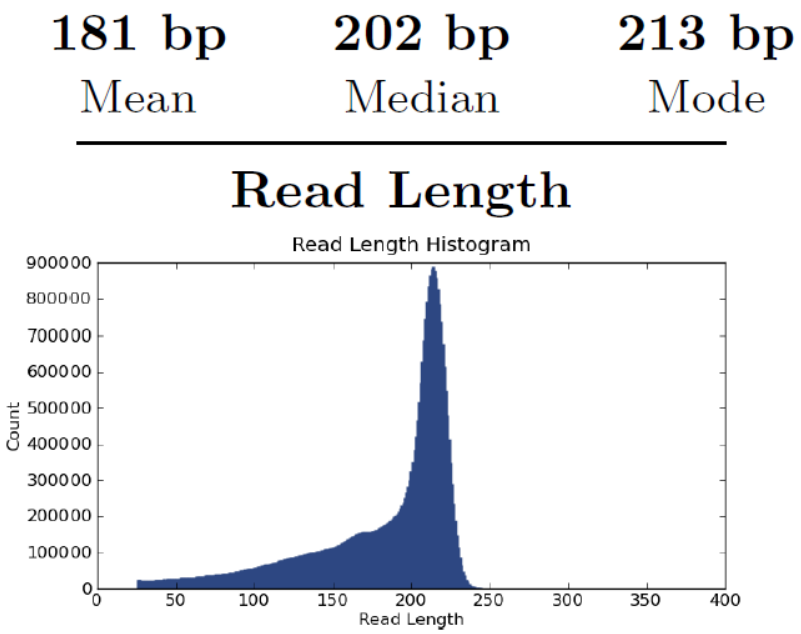


Figure 6.02. Read length metrics for IonTorrent genome sequencing of ROTH *P. xylostella* strain.

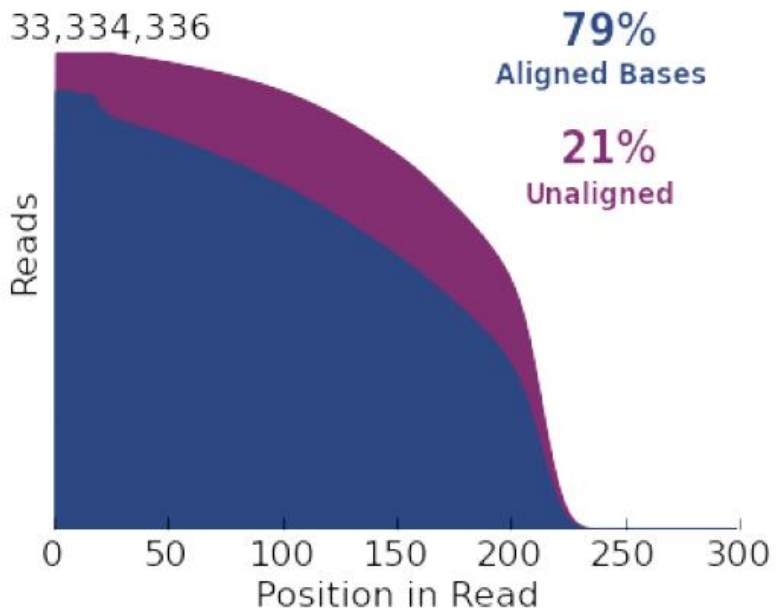


Figure 6.03. Alignment metrics for IonTorrent genome sequencing of ROTH *P. xylostella* strain against DBM-DB scaffolds. 79% of 33,334,336 bases aligned with DBM-DB scaffolds.

The NGS reads were mapped to DBM-DB scaffold_443 along with (for reference) the HS strain *FMO2* coverage and the DBM-DB putative FMO accession/annotation, Px011621 (figure 6.04). The mapping showed a relatively uneven distribution of reads with a number of gaps in the sequence, although as can be seen in figure 6.04, all of the putative CDS regions had good coverage. The alignment showed that the annotations for the Px011621 gene are incomplete, missing an intron and first exon of the gene.

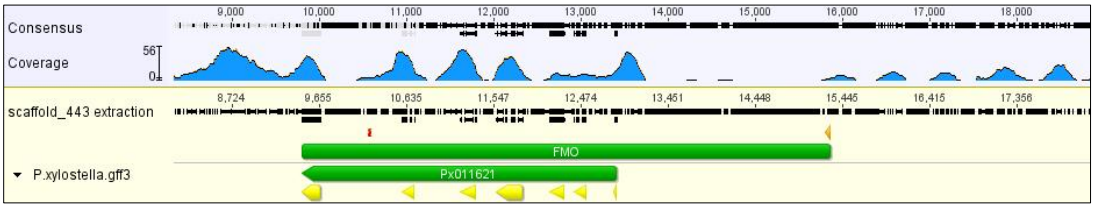


Figure 6.04. Extract of DBM-DB 'scaffold_443' showing coverage of NGS read alignments (blue cumulative peaks). Note that figure reads 3' to 5', left to right. Exons of the DBM-DB *FMO2* (Px011621) annotation are indicated with yellow arrows. The 5', orange arrow indicates the location of the start codon and first exon for the HS FMO2 gene. This alignment shows the first exon and intron missing from the Px011621 annotation.

The independent MIRA assembly (named 'ROTH_MIRA') produced a consensus of 273,397,079bp in length, composed from 219,629 contigs. The mean contig length was 1447bp, with the maximum contig length of 13,143bp.

6.3.2 Identification of promoter regions using MIRA assembly

The sequence was built up starting with identification of the gene sequence itself. The *FMO2* region of scaffold_443 (DBM-DB) was BLASTn searched against the 'ROTH_MIRA' database, with the resulting top 3 hits (MIRA_c125155, MIRA_rep_c177480 and MIRA_c59818) then assembled to produce a larger consensus contig (figure 6.05).

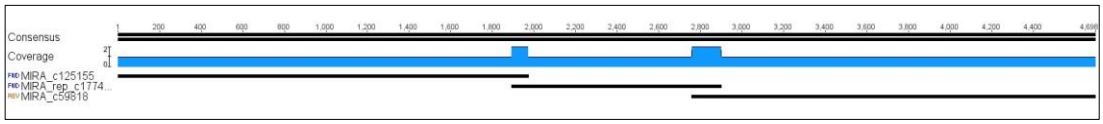


Figure 6.05. Assembly of top 3 hits resulting from *FMO2* BLAST search of ROTH_MIRA database. Nucleotides matching the consensus are highlighted in black and show the overlapping sequences are the same.

As can be seen from figure 6.06, the consensus of the 3 assembled contigs ('FMO MIRA Relevant cont…') represent 3927 bases of the 3' end of the gene plus 886 further downstream. These figures are approximate as they are based on an alignment with the scaffold 443 sequence and so contain gaps (in the alignment sequence). Further BLAST searches were made of the ROTH_MIRA database using sequence extracts from DBM-DB scaffold 443 *FMO2* region and the region directly upstream in the putative 5' UTR. These identified one additional contig, MIRA_rep_c256489 from the *FMO2* putative region (figure 6.06) and two matching contigs, MIRA_c80686 and MIRA_c102638 traversing the 5' end of the *FMO* gene and upstream into the 5'UTR. These are shown in figures 6.06 and 6.07.

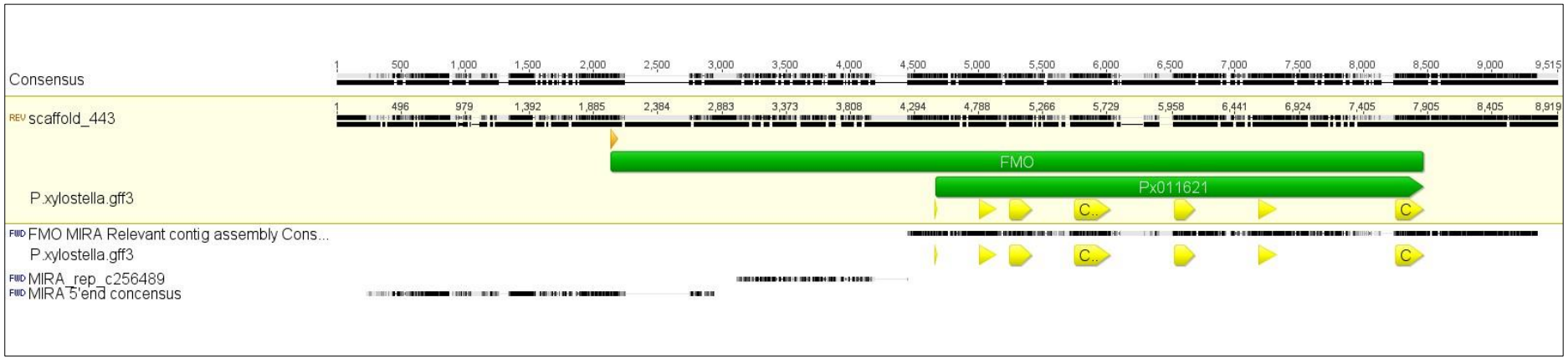


Figure 6.06. MIRA NGS contigs aligned to extract of DBM-DB 'scaffold_443'. Exons of the DBM-DB FMO (Px011621) are indicated with yellow arrows. Nucleotides matching scaffold_443 are highlighted in black. The 5', orange arrow indicates the location of the start codon and first exon for the HS *FMO2* gene. 'FMO MIRA Relevant cont…' is the consensus contig comprised of those shown in figure 6.05. 'MIRA 5' end concensus' is comprised of those contigs shown in figure 6.07.

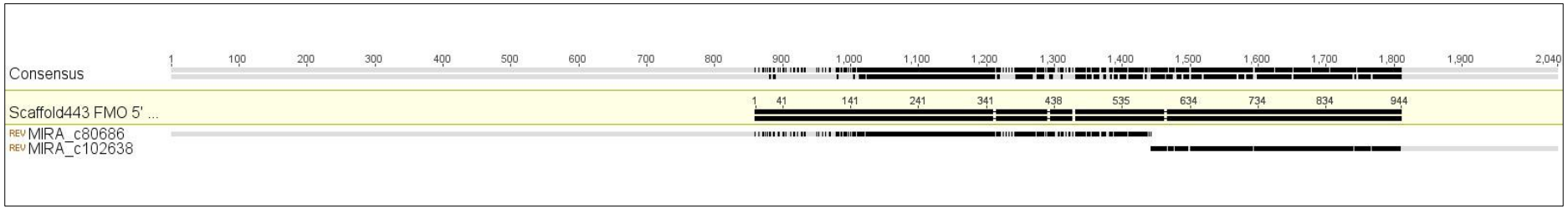


Figure 6.07. Named 'MIRA 5'end concensus', this figure shows the alignment of MIRA contigs to an extract of DBM-DB scaffold_443. Nucleotides matching scaffold_443 are highlighted in black.

A series of primers were designed from the NGS sequencing alignments, enabling the amplification and sequencing of the *FMO2* gene plus the proximal promoter from DNA bulks. PCR of the promoter regions from each of the strains resulted in additional products being amplified. Upon gel extracting and purifying the product of the correct size, the resulting concentrations were not efficient for multiple sequencing runs, therefore a nested PCR approach was used to further isolate the desired product. A single product was amplified for HS and this PCR product was subsequently purified and sequenced. However, more than one product was again amplified from the ROTH strain and gel extractions yielded low concentrations, therefore the purified products were cloned using a 'StrataClone PCR cloning kit' (Agilent Technologies) according to the manufacturer's instructions, with the purified plasmids directly sequenced. Alignments of the sequences resulted in promoter regions of 1824bp and 1395bp for HS and ROTH respectively. Details of each promoter sequence NCBI accession can be seen in the appendix (section 8.1).

6.3.3 Dual luciferase reporter assay

The raw data output from the microplate reader for the HS and ROTH experimental putative promoter regions, measured as relative light units (RLU) was normalised for variation in the expression of each construct using the *Renilla* measure. Data was then normalised to the pGL3 control and reported as relative luciferase activity. Figure 6.08 shows that the putative promoter region from the resistant HS strain caused significantly more relative luciferase reporter activity than the ROTH putative promoter region ($p = 5.8x10^{-6}$).

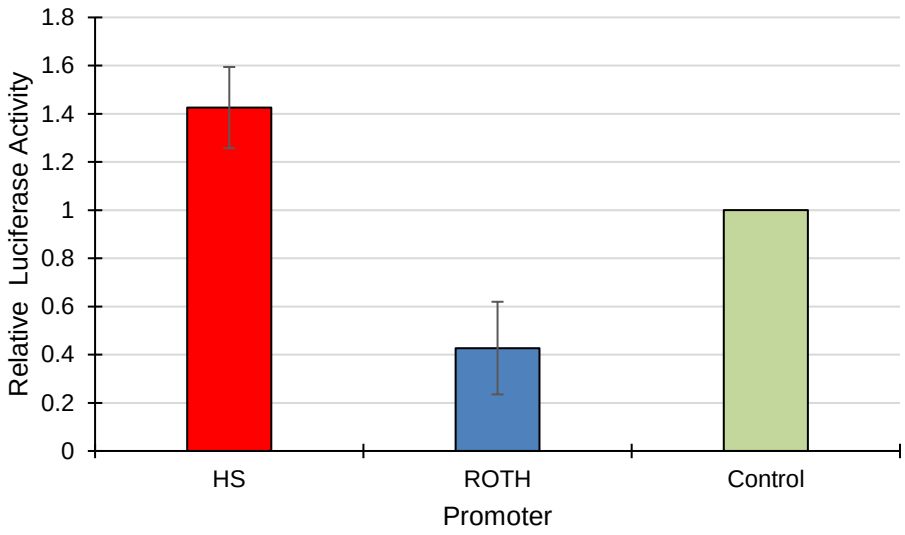


Figure 6.08. Relative luciferase activity for resistant HS and susceptible ROTH putative promoter regions from isolated 5' UTRs. Error bars indicate 95% confidence limits. The data has been normalised to that of the control (pGL3) with the HS promoter showing significantly greater activity than that of the ROTH strain.

6.3.4 Bioinformatic analysis of proximal promoter regions

Alignment of the HS and ROTH promoter sequences (figure 6.09) revealed low levels of sequence conservation with only 63.8% pairwise identity. Numerous 'minor' polymorphisms are present between the two strains with 7 indels of 10 bp or larger present. The largest of these indels are 96 bp, 71 bp and 248 bp in length respectively.

Using the 'EMBOSS' (v6.5.7) plugin for the Geneious analysis software, searches were made of the HS and ROTH promoter sequences to identify the presence of any putative transcription factor binding sites. The default search criteria were used which included a minimum 7 bp length for returned hits. Within the region analysed, the resistant HS strain has 6 predicted transcription factor binding sites compared to 5 for the susceptible ROTH strain (table 6.04 and figure 6.10). Both strains also share an additional predicted transcription factor binding site (TBP) positioned just inside the *FMO2* CDS downstream of the start codon.

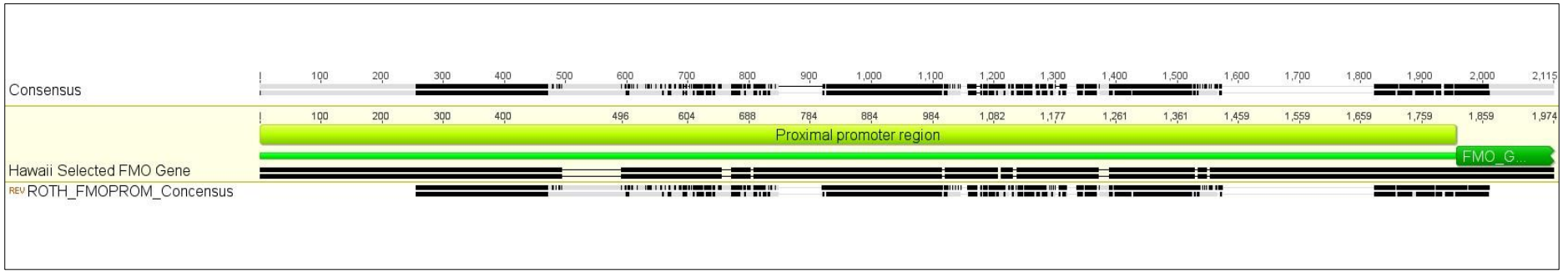


Figure 6.09. Alignment of proximal promoter sequences for resistant HS and susceptible ROTH strains. The 5' *FMO2* gene CDS was included on the HS sequence as a point of refence showing the ROTH fragment overlaps with the start of the gene. Nucleotides matching between the two sequences are highlighted in black.

Strain (sequence name in figure 6.09)	Name	Minimum	Maximum	Length	Species (transcription factor from)
Hawaii Selected (Hawaii selected FMO…)	AP-1	463	469	7	*Drosophila melanogaster*
	TBP	473	479	7	*Drosophila melanogaster*
	CF2-I	598	606	9	*Drosophila melanogaster*
	Bcd	624	633	10	*Drosophila melanogaster*
	TBP	1,777	1,783	7	*Drosophila melanogaster*
	TBP	1,795	1,801	7	*Drosophila melanogaster*
	TBP	1,820	1,826	7	*Drosophila melanogaster*
ROTH (ROTH_FMOPROM…)	AP-1	208	214	7	*Drosophila melanogaster*
	DEP2	524	531	8	*Drosophila melanogaster*
	CF2-I	957	965	9	*Drosophila melanogaster*
	TFIID	1,307	1,314	8	*Homo sapiens*
	TBP	1,322	1,328	7	*Drosophila melanogaster*
	TBP	1,347	1,353	7	*Drosophila melanogaster*

Table 6.04. Predicted transcription factor binding sites within HS and ROTH strain promoter sequences and immediate 5' *FMO2* CDS region. The resistant HS strain has 6 predicted transcription factor binding sites and the susceptible ROTH strain, has 5. They both share one transcription factor binding site within the 5' *FMO2* gene CDS.

Figure 6.10. Alignment of predicted transcription factor binding sites to HS and ROTH strain promoter regions (including the 5' CDS region of *FMO2* gene). Transcription element descriptions can be seen in table 6.04.

Comparing the sets and positions of the predicted transcription factor binding sites in the promoter (working from 5' to 3'), both strains share the same AP-1 transcription factor binding site, with a further 7 unmatched transcription factor binding sites between the two strains (4 present in the HS promoter and 3 in the ROTH promoter) before a final shared 'TBP' transcription factor binding site.

In addition to the predicted transcription factor binding sites it was noted that the first 935 bp of the HS promoter form a long inverted repeat, potentially creating a stem-loop structure. The stem is potentially comprised of 247 bp separated by 441bp (which form the loop). It is possible that the 5' complementary 'stem' is longer which would create a longer stem towards the *FMO2* CDS, but this is the limit of what was sequenced. The two corresponding complementary regions that comprise the possible formation of a stem are highlighted in the linear sequence depicted in figure 6.11. Pairwise assembly of the two LIR region sequences results in 99.6% pairwise identity. It would be a 100% match except for heterozygosity shown for two nucleotides (figure 6.12). Figure 6.13 shows a graphical representation of the potential stem-loop structure formed by folding of the DNA as predicted by the Geneious software.

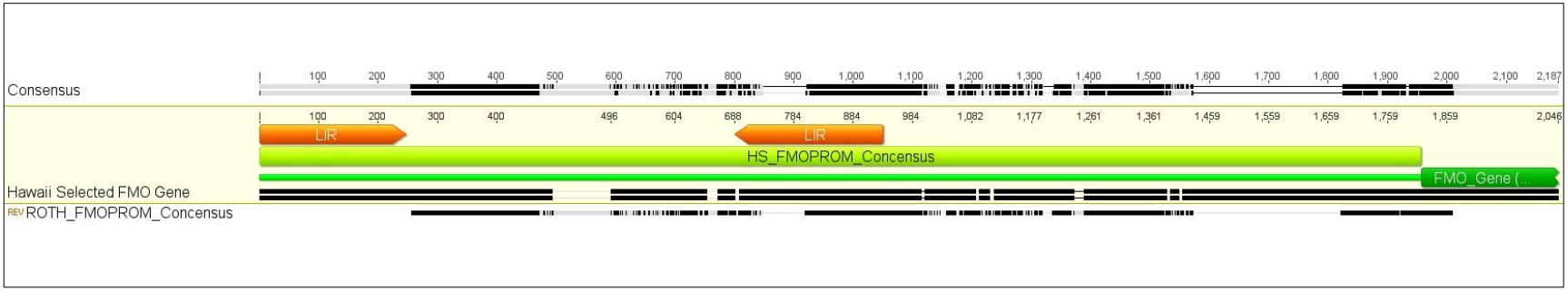


Figure 6.11. Locations of complementary sequence regions found within HS strain 5' UTR. Orange arrows indicate the regions of sequence that form the basis for the putative long inverted repeat (LIR) possible formation of stem-loop structure. The LIR regions are 247 bp in length, separated by 441 bp.

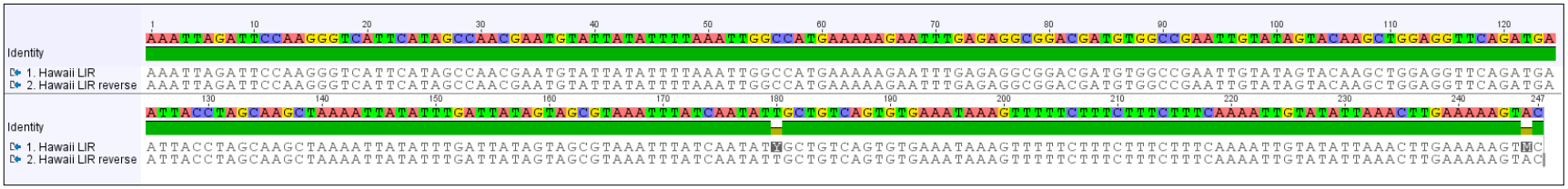


Figure 6.12. Pairwise assembly of the two 5' UTR LIR region sequences from the resistant HS strain. The complementary regions share a 99.6% pairwise identity with heterozygosity shown by just two nucleotides.

Figure 6.13. Graphical representation of putative stem-loop structure produced by folding of DNA caused by long inverted repeat in the resistant HS strain.

As long inverted repeats are a signature of transposons, particularly retrotransposons, the HS promoter region was subject to analysis using the online tool, Repbase [381] to predict the presence of any transposable elements in the sequence. The sequence returned a single hit, designated 'Kolobok-N6_LMi'. This Koloblok transposable element of 72 bp in length was located at position 664-735 in the HS sequence, found on the transition between the loop and stem of one side of the LIR.

6.4 Discussion

Constitutive overexpression of genes associated with resistance appears to most commonly result from mutations in *cis*-acting promoter sequences and/or *trans*-acting regulatory loci. However, evidence for trans-acting factors regulating resistance associated genes, such as P450s, comes from just a handful of studies. In *M. domestica*, overexpression of *CYP6A1* (which maps to chromosome 5) confers diazinon resistance and there is evidence that this is controlled by one or more loci located on chromosome 2 [382], however, the location and nature of the mutations involved has not been identified. Examples of *cis*-acting mutations that result in altered resistance gene expression are both more common and well defined [383]. A widely cited example of adaptation mediated by *cis*-regulatory change is the overexpression of *CYP6G1* in DDT resistant *Drosophila*, discussed further below [174].

Because sequence differences in the proximal promoter region of the *FMO2* gene were observed between the diamide resistant and susceptible strains of *P. xylostella* it was predicted that these may increase the expression in the HS strain. To test this a reporter gene assay was used to examine the level of expression driven by the two promoter variants. This provided evidence that elements within the 5' proximal promoter of the *FMO2* gene in the HS strain result in increased expression compared to the susceptible Roth strain.

In fact, the dual luciferase reporter assay showed that the proximal promoter from the ROTH strain drove expression even less than the control. The results of the assay are relative, so it is difficult to say whether the ROTH promoter is actually inactive, but it is clear that it is much less active. It is acknowledged that given the reasonably low shared pairwise identity between the proximal promoter regions of the two strains of 63.8%, they may not be orthologous regions. However, alignment of the proximal promoter regions from the two

strains identifies four regions of 213 bp, 190 bp, 136 bp and 140 bp that share high sequence identity between the two strains. The former two sharing 100% sequence identity and the latter, 98.5% and 95% respectively. These have been highlighted in figure 6.14. Additionally, a BLAST search of the ROTH proximal promoter region nucleotide sequence returned only a single, partial match; the 3' 76 bases (immediately upstream of the *FMO2* start codon) share 95% identity with accession XM_011565412.1, the previously identified putative FMO, Px011621. A BLAST search of the same region from the HS strain also returned a match to this same accession. Coupled with a 53 bp overlap with the 5' end of the *FMO2* coding region identified with the ROTH proximal promoter region, it was likely that the two regions are orthologous and so merited further investigation. To explore and identify the potential cis-acting factors at play the two promoter region sequences were analysed further using tools for the prediction of putative elements that might affect transcription of this gene.

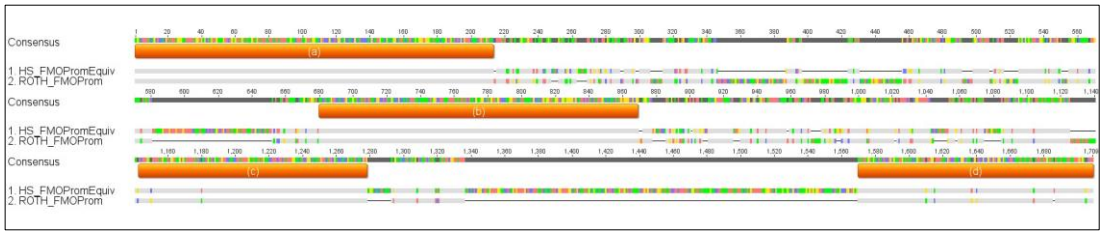


Figure 6.14. Alignment of proximal promoter regions for HS and ROTH strains. Total sequences share 63.8% sequence identity. Regions highlighted in orange and labelled (a) to (d) share high sequence identity between the strains. Percentage shared sequence identity: (a) = 100%, (b) = 100%, (c) = 98.5%, (d) = 95%.

The two promoter regions were considerably different, sharing only 63.8% pairwise identity, with many polymorphisms and significant indels present. The number of transcription factor binding sites differed by the HS strain having one extra in the sequence identified. Some of the transcription factor binding sites appeared to be conserved between the strains, although the order and composition of the transcription factor binding sites elsewhere differed between the two strains. There is a single nucleotide polymorphism, A-36T between the two strains (36 bp upstream from the *FMO2* start codon) responsible for the presence of a different transcription factor binding site at this location for each of the strains. The SNP codes for a TBP transcription factor binding site in HS, whereas ROTH has a TFIID at the same location.

The identification of the long inverted repeat in the HS strain is a signature of a transposable element. The distance between the complementary LIR 'stem' regions, in this case 441 bp suggest that this is not an active transposon but more likely a fossil of a transposable element [384, 385].

Further analysis using RepBase provided a weak score against a 'kolobok' transposable element at this position [386]. This is a DNA transposon and based on the length of the LTRs is unlikely to accurately represent the transposon that was once inserted into this position. Rather the length of the LTRs at this position is more indicative of a retrotransposon, that transposed via RNA intermediates. There is also no evidence of sequence encoding a transposon between the LTRs. A transposase is an enzyme that catalyses the movement of a transposable element by binding to the end of it and aiding its integration in another genomic location [387]. Together these findings suggest the transposon insertion at this position was mediated by another active transposon that recognised and exploited the LTRs, or alternatively the transposon at this position was subsequently remodelled with parts deleted.

Transposable elements have been previously shown to modify gene expression when inserted near genes. A prime example of their involvement in metabolic resistance in insects was demonstrated by the *Accord* element inserted upstream of the cytochrome P450, *CYP6G1*. This increased the expression of this P450 thus conferring DDT resistance in *D. melanogaster* [174]. However, while the case has been made for transposable elements having evolutionary selective retention associated with cytochrome P450 genes, implying they may have a functional role [388], there is evidence put forward by Wilding *et al.* suggesting that the presence of a transposable element closely associated with a putative detoxification gene does not guarantee it making a functional contribution. Through a reporter assay the authors found that the CuRE1 transposon inserted upstream of the *CYP9M10* P450 gene, capable of metabolising permethrin, did not confer the upregulation of the gene; the authors concluding that the transposon is likely in tight linkage disequilibrium with an as yet unidentified causal motif [389].

It was not possible to successfully amplify corresponding ROTH sequence identified for the first 255bp of the HS strain, therefore it was not possible to conclude if the ROTH promoter also has a corresponding long inverted repeat like HS. However, given the lack of sequence conservation between these regions of the promoter for these strains, it is unlikely. There are many possible reasons for the differential expression shown. One explanation could be related to loss or gain of transcription factor binding sites in HS strain, possibly historically caused by a transposable element insertion. It is also possible that a single nucleotide substitution could be all that's required to increased transcription of the detoxifying gene as demonstrated by Itokawa *et al.*, where a G-27A nucleotide change within a putative core promoter, TATA-box of *CYP9M10* confers an increase in pyrethroid resistance in *Culex quinpuefasciatus* [390]. Detailing differences in *CYP3A* (which plays a dominant role in xenobiotic detoxification) expression levels in human populations, Lamba *et al.* detail both a 60bp transposable element and a SNP conferring increased protein expression [391].

With so many transcription factor binding sites identified throughout the various species, the default transcription factor cut-off value used was 7 bp in length in order to avoid an overwhelming number of false positives. It should be noted though that this is an arbitrary number and might have resulted in influential transcription factor binding sites being missed if they are shorter in length. The majority of the transcription factor binding sites found in the two promoter regions (TBP and TFIID), are components of what were traditionally thought of as the basal machinery and that as a component of the transcription regulatory mechanism were considered invariant, simply responding to other enhancer binding factors, activators, repressors and coregulators [392]. However, by looking at homology and combinations of basal transcription factor binding sites including TBP and TFIID across species, Rabenstein *et al.* suggest that along with other known (and some as yet unidentified) transcription factor binding sites, these may play a more significant role in tissue and cell-specific patterns of gene transcription through being differentially combined [392]. Further complexes have been found by other researchers, such as that by Chung *et al*. when confirming that the *Accord* long terminal repeat retrotransposon does increase expression in *D. melanogaster* (in line with previous studies) of the cytochrome P450, *CYP6G1*, leading to resistance to a variety of insecticide classes. Additionally though, the authors found that the same upstream *Accord* transposon carries tissue specific enhancers resulting in *CYP6G1* expression in *D. melanogaster* gastric cecum, midgut, malpighian tubules and fat body [393].

With differing positions and combinations of transcription factor binding sites between the two strains, it is possible that the effects of one polymorphism might be affected by effects of a second polymorphism. This could result in the cumulative effects of the regulatory variants being poorly predicted by the marginal effects measured for any individual transcription factor binding site [394] and reinforces the need for a considered approach that would be needed if designing further functional experiments.

Without further experiments focused on specific *cis*-acting promoter elements to confirm or eliminate their contribution, it is not possible to conclude which elements are responsible for the overexpression. However, the manipulation of transcription factor binding sites can provide novel scope for therapeutic intervention such as the use of tamoxifen as oestrogen receptor positive breast cancer. Treatment with this compound inhibits the transcription of oestrogen-responsive genes by competitively binding to oestrogen receptors on target tissues producing a nuclear complex that inhibits DNA synthesis [395]. Upon confirmation of the role of a transcription factor binding site, perhaps an approach like this could be utilised in combating metabolic resistance to insecticides.

In functionally assessing the various predicted regulatory elements detailed in this chapter, it would be very interesting to see which particular elements, or combination of elements, (for example, the transcription factor binding sites present within the putatively unstable region of the stem-loop structure or simply the 5' region immediately upstream of *FMO2* CDS) are responsible for the *FMO2* overexpression. It is now easier to conduct such a functional assay as gene synthesis can be used to rapidly delete sequence regions or combinations of sequence regions and then, using the reporter assay, assess the effect of these changes on expression.

Finally, while the work outlined in this chapter has provided strong evidence of a *cis*-acting factor in *FMO2* overexpression, the possibility cannot be ruled out that a *trans*-acting factor also contributes. It would be interesting to explore this, in future, by crossing the HS and Roth strains and comparing the expression of the alleles of the *FMO2* gene in the F1 progeny with that of the parents. This approach was designed by Wittkopp *et al*. and allows *cis* and *trans* effects to be distinguished [396]. It is based on the premise that in F1 hybrids the two

alleles of a gene are in the same cellular environment (nucleus) and so exposed to same set of trans-acting factors. If the two alleles are expressed differently this is a signature of a *cis*-regulatory difference. In contrast a trans-regulatory difference manifests as conserved expression of the two alleles in F1s, despite differential expression of the gene in the parental homozygotes.

6.5 Summary

This chapter has provided experimental evidence that *FMO2* promoter regions from the resistant HS and susceptible ROTH *P. xylostella* strains confer differing levels of expression, with the HS promoter region driving significantly higher levels of expression in a dual luciferase reporter assay than ROTH.

Subsequent computational mining of the promoter sequences revealed a number of regulatory elements, including a predicted fossil of a transposable element, that differ between the two promoter sequences, although any one, or combination of the genetic variations identified might result in the overexpression of the *FMO2* gene shown by the HS strain.

As part of the preparation for the promoter assays the genome from the ROTH strain was sequenced via next generation sequencing. Further refinement of the NGS data and assembly already produced might prove valuable to the wider scientific community working on this pest species.

7 General Discussion

7.1 Candidate gene selection and validation

In this PhD thesis I present a number of lines of evidence that overexpression of a flavin-containing monooxygenase likely confers metabolic resistance to diamides in *P. xylostella*. In this chapter I summarise the data supporting this, placing this in the context of the wider literature. I discuss the relevance and implications of my results for control of this economically important pest species and finish by detailing opportunities for future work.

In chapter 4, results from microarrays performed on strains selected with different diamide compounds, chlorantraniliprole and flubendiamide, were compared to identify congruous genes and narrow the potential number of candidates. This revealed a handful of candidate genes that were consistently overexpressed in the resistant strains for further functional analysis. Microarrays have been used in a wide range of studies investigating the mechanisms underlying resistance to insecticides and have proved an effective means of identifying the genes involved [177, 296]. More recently RNA sequencing (RNAseq) has largely replaced the use of microarrays for transcriptome profiling. The former has several advantages over the latter and can be used to identify qualitative changes in genes of interest in addition to quantitative changes, furthermore it can also provide additional information on alternative splicing. However, RNAseq performs less well in the absence of a reference genome (based on unpublished data held in the Bass lab), which was the situation at the start of this study when gene expression was explored. Because a transcriptome sequence was used as the basis of microarray design it is possible that genes not present in these data, but relevant to resistance, could have been missed. Furthermore, because whole larvae were used to extract RNA it is also possible that genes expressed at high levels, but only in certain tissues could have been missed. Despite these limitations, by comparing several resistant strains, a core candidate gene set was identified and a range of approaches used to investigate the association of these genes with diamide resistance.

One of the approaches for functional validation used in this study was expression of *P. xylostella* candidate resistance genes in transgenic *Drosophila*. This allowed us to

demonstrate that overexpression of *FMO2* (and to a degree *XDH*) confers resistance to diamides.

All the genes tested in the GAL4/UAS system for this thesis were expressed under the control of actin. It might be possible that the ubiquitous overexpression had lethal or sub-lethal detrimental effects upon the fitness of the flies. Given the lethality demonstrated by the ubiquitous overexpression of the cytochrome P450, *CYP12A4* by Bogwitz *et al.* compared to tissue specific overexpression of the same gene conferring insecticide resistance [368], it is possible that a different result might have been obtained if the same genes were expressed within specific tissues. This could be explored in future works, but also emphasises the need to ideally use more than one approach to validate putative resistance genes.

7.2 *FMO2* involvement in resistance in *P. xylostella*

Through the course of this thesis evidence has been put forward for the major involvement of *FMO2* in conferring metabolic resistance to diamides. It is possible given the importance of this gene family in xenobiotic detoxification in mammals, that within insects, this group of monooxygenases play a more important role than appreciated, and may have been overlooked by other studies in favour of more well-known enzymes such as cytochrome P450s or 'mixed function monooxygenases' (MFMs). One such MFM was described by Lindigeit *et al.* who described an MFM 'flavoprotein' with a maximal activity temperature of 40-45^{o}C responsible for N-oxygenation of senecionine, and so was then named a senecionine N-oxygenase (SNO). Through the N-oxygenation of senecionine, this SNO is involved in the sequestration of senecionine N-oxide from plants as a defence mechanism [219]. Naumann *et al.* investigated the phenology of this SNO and suggested that it originated by the duplication of a gene encoding an FMO of unknown function [220]. Indeed, as has been found in this PhD, annotations for the same putative gene vary between FMO and SNO. It is true that in comparison to cytochrome P450s, FMOs are represented by significantly fewer genes in insects [375], with only two present in *D. melanogaster* and three described in *Bombyx mori* [211, 220]. Scharf *et al.* hypothesise that the two Drosophila FMOs play divergent physiological roles, and that insect FMOs are quite unique relative to their counterparts in other prokaryotic and eukaryotic taxa [355].

In the characterisation of an FMO (*FMO5*) from human and guinea pig sources, and an analysis of the evolution and functional divergence of FMOs by Overby *et al.* and Hao *et al*. respectively [318, 397], it seems that FMO genes are not truly orthologous between animals. Instead, FMO genes may have evolved independently in each lineage by gene duplication, with some being redundant and others evolving different functions and metabolizing different substrates. Both the resistant HS and susceptible ROTH strains still possess a functional *FMO2* gene, which is highly conserved in coding sequence between the two species, suggesting that it may have an important physiological role within this organism. This has yet to be elucidated, but the studies detailing the evolution of this group of genes suggests that their original function is in the detoxification of plant allelochemicals [220, 375]. The only base functions of FMOs in insects to be properly characterised are the SNOs, characterised by a high substrate specificity for pyrrolizidine alkaloids. The SNO analogue, PNO, from the tiger moth, *Grammia geneura* was expressed in *E. coli* by Sehlmeyer *et al.* in a direct comparison with the *Tyria jacobaeae* SNO [211]. The authors found that the high substrate specificity of the SNO from the specialist ragwort (*Jacobea vulgaris*) feeder, *T. jacobaeae*, was in contrast with the PNO from the generalist feeder, *G. geneura* which displayed noticeably expanded substrate specificity. Using a RT-PCR approach, a comparison of tissue-specific expression of SNO and an unknown FMO in *T. jacobaeae* was made revealing that SNO transcripts were detectable in the fat body, integument and head, with the FMO being detected in the integument and fat body [211]. The authors speculate that the PCR amplification results from the integument may have been through contamination of adhering fat tissue not removed during the dissection. This may of course be true, however, expression of FMO in the integument would concur with the results found through the LC-MS of assayed *P. xylostella* shown in section 3.3.2.2, showing that the HS strain of *P. xylostella* appears to metabolise topically dosed chlorantraniliprole.

7.2.1 Resistance stability

In this thesis, the phenotypic decline in resistance was used to explore changes in the expression of the genes of interest and help identify those candidate genes that are and are not contributing to diamide resistance. The results demonstrated the rapid decline in the expression of *FMO2* over the same period compared to the other candidate genes providing evidence of a causal role in resistance.

However, the stability of pesticide resistance also has important implications for insecticide resistance management strategies. Parrella and Trumbel found that the decline of resistance in the leafminer *Liriomyza trifolii* was sufficiently slow that even after 10 months without exposure to permethrin or chlorpyrifos, a resistant strain was still not susceptible to the field rate of either pesticide [398]. Ferguson also found that 3 strains of resistant field collected *L. trifolii* exhibited unstable resistance to cyromazine, abamectin and spinosad. On average, the resistance in the strains reverted to comparable levels of a susceptible strain in less than eight generations [399]. A similar pattern was described by Askari-Sarayazdi *et al.* in another leafminer, *Liriomyza sativae*. Again, a decline of 80% in resistance to chlorpyrifos was recorded over the course of 13 generations [400]. All of these experiments took place on closed populations, removing the possibility of immigration of susceptible genotypes which would likely speed up the decline in resistance [400]. Allele frequency, dominance and fitness of the resistant genotype are additive effects and could be possible reasons for the instability of resistance in a closed population [401, 402].

Similarly to a chlorantraniliprole resistant strain assayed by Wang *et al.*, which exhibited a 99% drop in resistance of 7 generations [246], in section 5.3.1 the unselected HS *P. xylostella* strain displayed a 98% reduction in the LD_{50} over 17 generations. The implication of these studies is that the cessation in use of a pesticide as part of an IRM strategy can result in a decline in resistance. However, at least in a closed population, such as would be present in glasshouses, a considerably longer rotational pause before returning to a pesticide with the same mode of action would be needed in order to be effectual.

It was not possible during the course of this resistance stability experiment to assess the resistance ratios of the selected and unselected lines to the other diamide compounds, but this would be an informative process in any future experiments; as would selection experiments with the other diamide compounds.

It would likely also prove informative if the individual generations of the selected and unselected lines underwent qPCR to confirm the trends shown already by each of the candidate genes. The use of further microarrays or other transcriptomic analysis (such as RNA-Seq) could serve to expand on this work to confirm those candidate genes already

identified or possibly even identify new ones through further identification of differentially expressed genes.

7.2.2 *FMO2* substrate specificity affects pesticide detoxification

Both bioassays of *P. xylostella* and transgenic *D. melanogaster* revealed differences in sensitivity to different diamide insecticides. While maintaining a level of resistance above that of the susceptible comparison, the resistant HS strain of *P. xylostella* has greater sensitivity to the phthalic diamide, flubendiamide, than it does the anthranilic diamides, chlorantraniliprole and cyantraniliprole. Variation in resistance ratios between compounds with the same mode of action has been detailed by Prabhaker *et al.* showing low levels of cross-resistance between neonicotinoid compounds in the whitefly, *Bemisia tabaci* [403]. In *in vitro* assays, Roditakis *et al.* demonstrated that the cytochrome P450, *CYP6CM1vQ*, conferring resistance to imidacloprid in *Bemisia tabaci*, metabolised the neonicotinoids, clothianidin and thiacloprid, but not acetamiprid or thiamethoxam [288].

The transgenic *D. melanogaster* expressing the *FMO2* gene also showed increased resistance to chlorantraniliprole (significant in one experiment, and marginally not significant in another), but addition of the gene conferred no additional resistance to flubendiamide. These results appear to demonstrate a structure activity relationship dictated by the *FMO2* gene that confers insecticide resistance. Working from rabbit lung and porcine liver FMOs, Nagata *et al.* calculated that the two isoforms demonstrated different substrate specificity, with the hepatic FMO having wider substrate specificity than the pulmonary FMO [215]. By testing a range of substrates with varying chemical structures and sizes, the authors found that differences in substrate size, in particular the length of alkyl side chains, were likely responsible for whether a compound was metabolised. From calculations made from the substrates that were or were not metabolised, Nagata *et al.* estimated the dimensional differences of the FMO substrate channels that appeared to be responsible for the variation in substrate specificities. Ziegler reiterates this point in a later review [358] which covers a publication by Guo *et al.* [214]. Guo *et al.* discovered that microsomes purified from various mammalian organs were able to catalyse different sized thiocarbamides, but each microsome preparation catalysed only up to a specific substrate size. Therefore, the overall size of the nucleophile appears to be a major factor limiting access to the 4a-

hydroperoxyflavin in different FMO isoforms [358]. Based on this work it is reasonable to suggest that the differences in the chemical structure and size of anthranilic and phthalic diamides likely underlie the differences observed in the ability of both transgenic flies expressing *FMO2* and resistant *P. xylostella* to metabolize the two types of chemistry. As detailed in the previous section, this finding has implications for *P. xylostella* control as it reveals that flubendiamide retains efficacy against this mechanism of resistance and could be used in place of the anthranilic diamides.

7.2.3 Testing for *FMO2* contribution to pesticide detoxification

As detailed above, several functional experiments provided evidence of the role of *FMO2* in resistance. However, experiments detailed in Chapter 5 investigating the use of PBO and methimazole (MEM) as inhibitors of cytochrome P450s and FMOs respectively failed to support the other work. Inhibitor+insecticide bioassays revealed that exposure of the resistant HS strain to PBO had no effect on the resistance status of this strain to chlorantraniliprole, as might have been expected. However, surprisingly MEM actually decreased the sensitivity of the HS strain to this insecticide by 4-fold. Explanation for this is not obvious. One explanation might be that MEM is acting as an activator of *FMO2* as discussed in section 5.4.2. However, taking into consideration results from all of the experiments, including those not directly involving synergistic compounds, it is possible that other mechanisms may explain the results. As detailed in section 5.1.2, MEM is often used as a primary synergist to differentiate the role of FMOs from those of cytochrome P450 enzymes. While it has been shown that MEM does have substrate specificity for some P450s [404], the overriding evidence is that this compound should inhibit FMO (whether it affects some P450s or not). Even if MEM does lower the effectiveness of some cytochrome P450s within the HS *P. xylostella* strain, the expectation would still remain that use of this synergist would increase its sensitivity.

Nace *et al.* investigated the effect of MEM as a competitive inhibitor on the neurotoxicity of 3,3'-iminodipropionitrile (IDPN) in rats. By experimenting with the addition of MEM in separate assays with IDPN and N-hydroxy 3,3'-iminodipropionitrile (HOIDPN), a putative neurotoxic metabolite, the authors found that MEM blocked or reduced (dependent on parent compound concentration) the effects of IDPN, and increased the effects of HOIDPN.

It was concluded that it was likely that FMO not only bioactivated IDPN, but was involved in the detoxification of the resulting metabolite HOIDPN [405]. If *FMO2* was responsible for both the catalysis of chlorantraniliprole into a intermediary toxic compound and then subsequent detoxification of that compound, the inhibition of *FMO2* by MEM would result in toxicity from the parent compound as it would be ineffectual against both the parent and intermediary compounds.

Given that the other experimental data put forward in this thesis implicate *FMO2* as a putative resistance gene, one explanation for the increased resistance displayed by HS following treatment with MEM is that *FMO2* is not only detoxifying chlorantraniliprole, but also a toxic secondary metabolite produced through chlorantraniliprole metabolism/activation by either a different enzyme, or another FMO isoform. This would explain why overexpression of the *FMO2* leads to resistance in the HS strain, with it detoxifying both chlorantraniliprole and a secondary toxic compound. The creation of transgenic *D. melanogaster* expressing the *FMO2* gene also displaying resistance to chlorantraniliprole implies it metabolises the parent compound. It has been noted by Guo *et al.* that MEM does not result in the universal inhibition of all FMO isoforms, and does have substrate specificity for some cytochrome P450s [404]. Additionally, Schlenk reviews that methimazole has been shown to be bioactivated to a cytochrome P450 inhibiting compound, sulfinic acid in microsomal preparations of rat liver FMO [406]. Inhibition of an unknown enzyme other than *FMO2*, that is producing a secondary toxic compound through the metabolism of chlorantraniliprole, could produce the results seen in this thesis.

The discussion in section 5.4.2 suggests that other methods for identifying the contribution of FMOs should be investigated such as alternative synergistic compounds. Another alternative would be the exploitation of the physicochemical property of FMOs to be susceptible to thermal-inactivation. Reporting on the induction and inhibition of pesticide metabolizing enzymes and the contributions of both FMOs and cytochrome P450s, in the sulfoxidation of phorate, Hodgson studied the relative contributions of the two enzymes. Working with microsomal preparations, P450 isozymes were inhibited using an antibody to NADPH-cytochrome P450 reductase and FMO activity was inhibited by heat treatment (50^{o}C for 1 min). The author reports that for liver microsomes, the total of FMO and P450 activities

is almost exactly equal to the activity of untreated microsomes. The author does note however that this methodology would be less useful for other microsomal preparations where *FMO2* is the primary isoform as this isoform tends to be less heat labile [361]. Conversely, Krueger *et al.* inactivated human *FMO2* by a temperature treatment of 45^{o}C for 5 minutes [407]. Grothusen *et al.* also suggest that a temperature of 50^{o}C would inactivate FMOs and that cytochrome P450s (of humans) seem to be quite stable to thermal treatment, although they have shown some differential sensitivity (13%-30% loss of activity) [408]. Thermal inactivation of FMOs would provide a direction for further investigation of these enzymes, although, obviously, the implications for in-vivo testing using this method may be limited. As detailed here, methodologies and substrates that specifically inhibit FMOs are not yet clearly elucidated. Development in this area would aid in the identification of possible FMO contributions, but care should be taken in future studies (particularly in the use of synergists) not to completely rely on the result of an inhibition assay without question.

7.3 *FMO2 cis*-regulatory elements

Identification and comparison of the 5' untranslated regions from the resistant HS and susceptible ROTH strains of *P. xylostella FMO2* revealed considerable lack of conservation between the promoter regions of the two strains. As a long-term laboratory susceptible strain, ROTH has been subjected to a considerable length of isolation from the natural environment and the physiological stresses this would confer. Thus, that differences exist between the strains is perhaps no great surprise given that this strain has not encountered xenobiotics other than those attributable to the host plant used for maintenance of the population. In a review of molecular mechanisms of metabolic detoxification, Li *et al*. describe that in cytochrome P450s, in most cases increased expression results from mutations and indels in *cis*-acting promoter sequences and/or *trans*-acting regulatory loci [172]. In another review, in this case of carboxyl/cholinesterases, Oakeshott *et al.* note clusters of polymorphisms in the promoter of *Est6* affecting regulation rather a single polymorphism [199].

The identification of multiple predicted regulatory elements (transcription factor binding sites, a long inverted repeat, and a transposable element) in the resistant strain increases the complexity of elucidating the true factor (or factors) responsible for *FMO2*

overexpression and the evolutionary steps by which these changes occurred. For example, in their study on overexpression of the P450, *CYP9M10*, Itokawa *et al*. conclude that the conservation of the *cis*-acting single nucleotide mutation they discovered, along with other *cis*-acting regulatory mutations could represent allelic progression of an insecticide resistance gene. This is where a detoxification gene could obtain higher levels of expression through the accumulation of sequential *cis*-acting regulatory elements. The authors go on to speculate that allele progression could be a common way to achieve a high level of resistance within the cytochrome P450, rather than a high level of resistance conferred by a single mutation [390].

Inverted repeats can define the boundaries of transposons, being self-complementary, long Inverted repeats can form stem-loops. These properties play an important role in genome instability and are regarded as sites that are typically not well conserved. Zou *et al.* made an *in-vivo* analysis of *cis*-acting expression determinants of the tobacco *psbA* 5'UTR. Separately introducing sequence deletions and base alterations in the stem-loop had negative impacts on translation, showed 2-3 fold decrease in mRNA stability and 1.5-6 fold reduction in translation efficiency. The implication of this is that the stem-loop itself may be somehow directly involved in a regulatory role [409].

In a review of molecular mechanisms of metabolic resistance to natural and synthetic xenobiotics, Li *et al*. postulate that large, rapid changes in expression can be mediated by transposable elements, for example, when an insect is under high environmental stress causing major mortality, for example an insecticide [172]. The authors suggest that conversely, mutational changes associated with allelochemical detoxification may be acquired more slowly as an accumulation of smaller genomic changes.

Shrader *et al*. found that genomic regions encompassing accumulations of transposable elements in the invasive ant, *Cardiocondyla obscurior* evolved faster than other regions containing SNPs, and gene/exon duplications and deletions [410]. In the case of *C. obscurior* this aids this invasive species in adapting to new environments, however, the same principle

could be related to a pest species such as *P. xylostella* in its ability to adapt to the new environmental stresses through the use of novel insecticides.

The prevalence and accessibility of NGS dictates that increasing numbers of examples of transposable elements will be identified. Making use of the human genome, Jordan *et al.* have assessed that almost 25% of the analysed promoter regions contain sequences derived from transposable elements, many of which have been experimentally characterised as *cis*-acting regulatory elements. The authors conclude that through the introduction and adoption by host loci of regulatory sequences, transposable elements may result in evolutionary changes beneficial to the host organism [411]. It is possible that such a process is at work in the resistant HS strain with the presence of three putative transcription factor binding sites located within the loop region of the HS strain that are not present in the ROTH strain.

Given the evidence generated by the dual luciferase reporter assay, and identification of elements in the promoter region that may affect the transcription of the *FMO2* gene, efforts should be made to elucidate the underlying *cis*-acting elements responsible for the overexpression putatively conferring resistance.

7.4 Multiple gene contributions

It should be acknowledged that the resistance demonstrated by the HS strain of *P. xylostella*, despite the identification the *FMO2* gene being the putative cause of resistance, may be caused by a combination of different genes.

There is significant evidence in the literature of multiple genes expressed concurrently contributing to a resistance status. A recent publication by Shi *et al.* speculate multiple cytochrome P450 genes simultaneously contribute to resistance in the spider mite, *Tetanychus cinnabarinus*. Through RNAi, the authors found that the silencing of six overexpressed P450 genes in a resistant strain had an even greater effect in sensitization of this strain to fenpropathrin, than silencing the genes individually [412]. However, this finding might be expected if any of these genes had important physiological functions and redundancy in role between them. In another recent study by Liu *et al.* involving the investigation of cross-resistance and fitness effects of cyantraniliprole resistance, selection of a population of *P. xylostella*, resulted in increases (although to a lesser extent) in resistance

to the other diamides, chlorantraniliprole and flubendiamide. Through synergist assays and crossings of susceptible and resistant strains to produce new lines, it was concluded that resistance to cyantraniliprole was controlled by multiple genes [279].

It has already been suggested in this chapter that the possibility exists that at least one other enzyme may be metabolically involved with the *FMO2* pathway, but there is a possibility that other candidate genes identified in this thesis are also implicated, particularly xanthine dehydrogenase (*XDH*). With varying degrees of significance, and while not to the degree of the *FMO2*, *XDH* has demonstrated in several of the experimental chapters an association with diamide resistance. As a secondary tier candidate in this thesis, and the comparative late identification of its gene sequence, *XDH* has not been investigated with the vigour applied to the *FMO2*. However, given its putative physiological role in the excretion of urea, it would be logical to imagine it in a pathway involved in the excretion of metabolites of xenobiotic compounds. Indeed, increased urea levels due to detoxification of chlorantraniliprole are reported by Bindu *et al.* in larvae of the silk moth, *Bombyx mori* [413]. It would be logical to conclude that an increase in the production of urea would facilitate the need for an increase in excretion.

From the results presented through this thesis, it would not be suggested that *XDH* is responsible for metabolising diamides directly, but it may be involved in a later stage of the detoxification pathway. Using the GAL4/UAS system already in place it should be possible to co-express the *FMO2* and *XDH* genes in a new *D. melanogaster* line. This would allow investigation on if co-expression increases the level of resistance beyond that in lines expressing the genes independently.

7.5 Current Relevance

The timing of this PhD study is particularly relevant; as discussed in section 1.5, *P. xylostella* is predominantly a pest in tropical and subtropical regions where brassica crops are grown year-round [83]. While *P. xylostella* can be a pest in the UK, the temperate climate and temporally intermittent growing of host crops for this species mean that typically it is not a predominant pest problem for the UK as populations are kept within economic thresholds. 2016, however has proved to be an exception to the rule. Currently operating 84 light-traps

spread country-wide throughout the UK and collecting daily information, in 2016 the Rothamsted insect survey noted indications of an exceptionally large immigration of *P. xylostella* from the continent beginning in late May and Early June. The numbers being caught in some locations were orders of magnitude over what would normally be expected. For example, at several locations, hundreds of individuals have been caught in a single night, with one notable trapping collecting 69 individuals in one night. This was obviously not the highest number, but was three times the number as has been caught at the same location in the previous 23 years combined [414]. Figure 7.01 shows the mean number of *P. xylostella* caught per trap, per year from 1966 to 2015. While there is natural variation in the population levels, a significant peak can be seen in 1996.

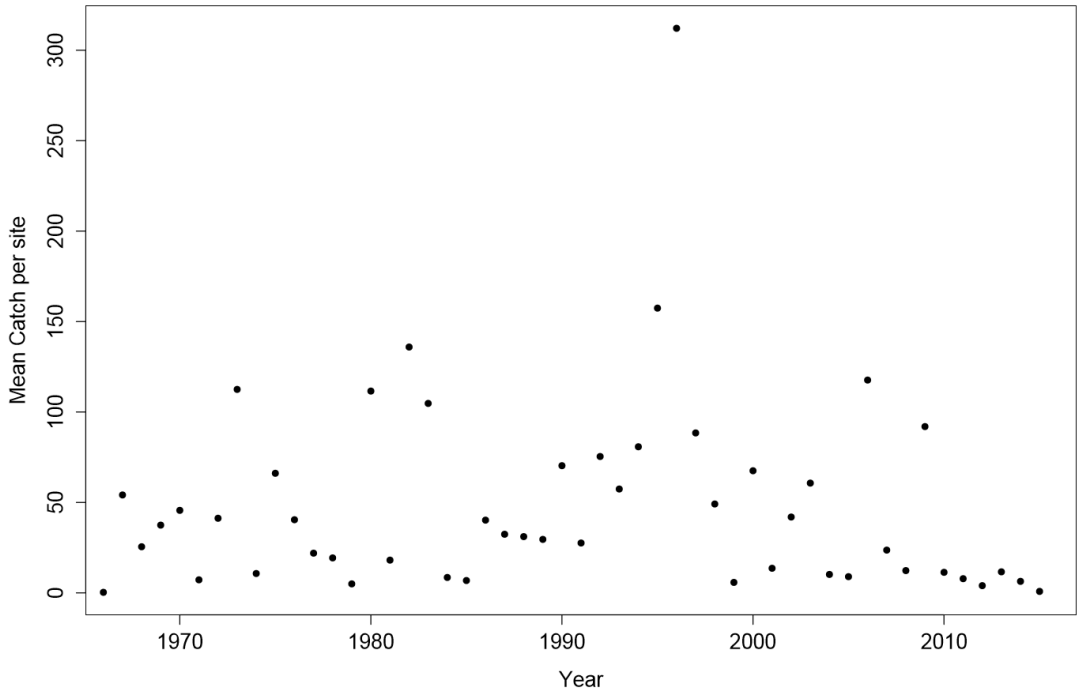


Figure 7.01. Mean number of *P. xylostella* caught across all RIS light traps per year from 1966 to 2015. There was an exceptional peak in excess of 300 caught in 1996. Source: RIS (private communication).

Counts for 2016 are not yet complete, but the data received so for indicates that the immigration numbers are following a similar (if not larger) influx. Generally, the numbers of *P. xylostella* fluctuate through the course of the year, with figure 7.02 demonstrating the daily fluctuations during the course of years where overall total counts for the year were high (excluding 1996). Figure 7.03 shows the same information including 1996. Individual daily

counts can be affected by the weather for example, but generalised peaks in abundance can still be seen at different sites at different times of the year. This can be representative of migration and/or the temporally intermittent (as it is in the UK), localised growth of host species. An influx of such large numbers early in the growing season as shown in 2016, mirroring that of 1996, can affect crop production for an entire season by boosting the baseline stock present in the environment affecting IPM practices. The agriculture and horticulture development board (AHDB) have stated that while there are resistance issues present in other parts of the world, as yet, there is no evidence that the moths migrating to the UK each year are highly resistant to the insecticides approved in the UK for this species [100]. This is reassuring and highlights the importance of phenotyping migrations (particularly uncommon mass migrations such as that this year) for resistance to the available insecticides so that IPM and IRM can be implemented to best effect.

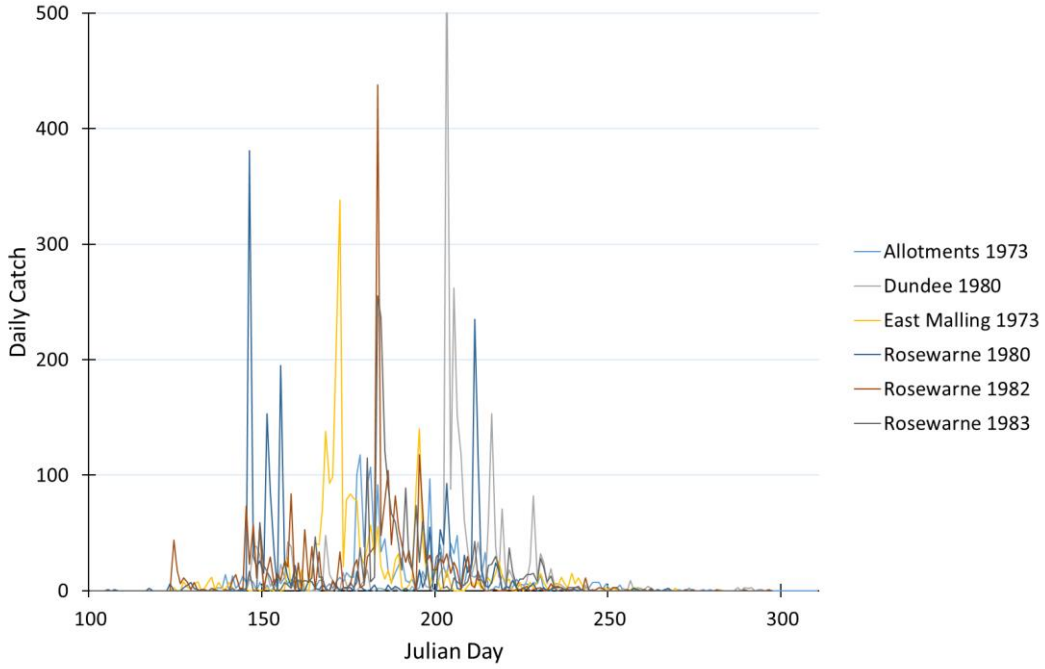


Figure 7.02. Daily catches for *P. xylostella* for the top trap sites for this species from the years with the highest annual site totals (excluding 1996).

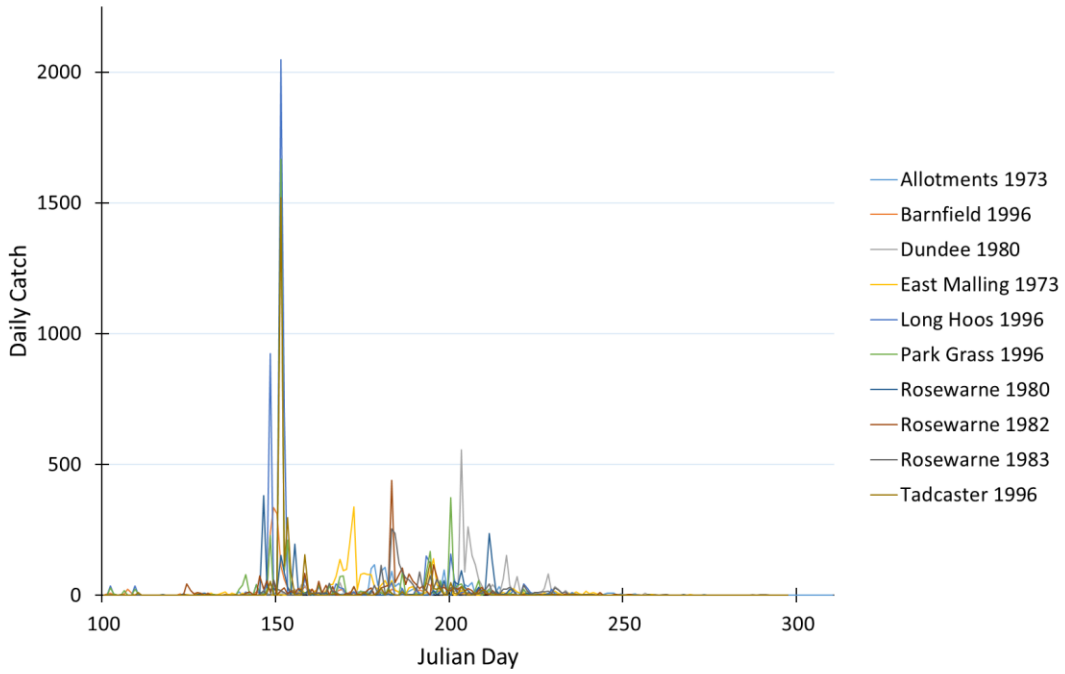


Figure 7.03. Daily catches for *P. xylostella* for the top 10 trap sites for this species from the years with the highest annual site totals. This figure includes sites from 1996, where large catches can be seen early in the year.

7.6 Implications for *P. xylostella* control and resistance management

Most work to date monitoring for resistance has been looking at target site resistance. This is particularly the case for *P. xylostella*'s resistance to diamides, for which only target-site resistance to diamides has been reported [145, 275]. However, work presented in this thesis indicates that metabolic resistance now needs to be considered and monitored for.

Steinbach *et al*. identified the G4946E mutation in populations from 10 countries across the globe [144]. The spread and resistance conferred by this mutation are almost certainly responsible for a great deal of resistance. However, the same study also reports that in some areas such as the Shandong province of China, 75% of the *P. xylostella* population are wildtype G4946 and 25% are heterozygous thus so far maintaining a level of diamide control. Given the conservation of the *FMO2* gene between the resistant and susceptible strain, the likelihood is that the *FMO2* is functioning (at a low level of expression) in its native role in field populations, probably in everyday allelochemical metabolism, but probably not (or least it has not been noted) metabolising xenobiotic compounds. This provides the worrying

prospect that any population of *P. xylostella* could hold potential scope to readily develop this mechanism of metabolic resistance. In an isolated population of *P. xylostella* where target-site resistance has yet to develop, either through genetic migration or convergent mutation, it is possible that *FMO2* metabolism could fill a resistance niche. It is also logical that *FMO2* metabolic detoxification can work alongside target-site mutations, acting additively or synergistically to increase resistance further.

As discussed above, I observed in both transgenic *D. melanogaster* expressing *FMO2* and the HS strain, differences in sensitivity to chlorantraniliprole and flubendiamide. This can be exploited in the management of populations of *P. xylostella* carrying this mechanism. Furthermore, further future characterisation of the substrate specificity of *FMO2* would be useful in identifying the most effective insecticides to use or rotate in control.

7.7 Project output

As already described in this chapter, identification of novel gene(s) conferring insecticide resistance provides an opportunity to develop tools with which to screen new putative insecticide chemistries. These could be used to assess the metabolic liability of a new insecticide and so rapidly understand how effective it might be against resistant insect populations.

Through the creation of transgenic lines of *D. melanogaster* expressing the detoxifying *FMO2* gene, this PhD project has provided such a tool. Relatively speaking, now the lines have been created, no particular specialist scientific equipment beyond the correct facilities for culture maintenance and assay work would be required to make informative use of this resource in the future. Proof that resources are commercially valuable to industrial agro-chemical companies is demonstrated by Syngenta having filed at least two patents for methods involving transgenic GAL4/UAS *D. melanogaster*. One patent is for transgenic *D. melanogaster* lines where the expression of a nuclear hormone receptor gene such as *dhr96* that can be manipulated to reduce expression of xenobiotic metabolising key genes as a method for screening insecticides. Genes such as *dhr96* are able to elicit changes in expression by acting as a 'master-switch' for genes downstream [415]. Similar to this first patent, Syngenta also have a patent aimed at insect lines harnessing the GAL4 /UAS system

focusing specifically on cytochrome P450 genes. Again, focusing primarily at the creation of lines that enable the under expression of these genes to levels less than that of a natural/field population [416]. Many potential insecticides are discounted at an early stage of testing as they do not result in an insecticidal effect. However, the theory of having an under-expressing line (compared to a wild-type population) is born of the possibility that some potential new compounds are discounted because the 'natural' level of metabolic detoxification of xenobiotic compounds found in a test insect population is enough for a population to survive the initial discriminating dose. While that particular compound may not be effective, it does not mean that the chemistry of the compound should be completely discounted.

FMO2 being implicated in insecticide resistance has implications for the screening of new candidate insecticide chemistries. Industrially, new compounds will be screened against known resistance mechanisms both in-vitro and in-vivo. Prior to this PhD, only a publication by Tian *et al.* makes reference to the possible implication of FMO in insecticide detoxification resulting in a resistant phenotype in the beet armyworm, *Spodopter exigua* [222]. The discovery of a second species (*P. xylostella*) in which *FMO2* has been investigated further as a gene conferring resistance to a different class of insecticide means that it may be more widespread than has been realised. It's similarity to cytochrome P450s in terms of metabolic functioning may have resulted in it previously having been overlooked (or masked by) concentration on cytochrome P450 genes. The broad substrate specificity of FMOs means that it is possible that given countermeasures such as synergists used against cytochrome P450s, pressure may be put on this group of genes to evolve to fill a void where insecticide detoxification by cytochrome P450s are rendered less effective.

The significance of human FMOs has increased as more compounds have been discovered that are substrates of these enzymes [359]. The results presented in this thesis and the advent and increasing widespread use of next generation and transcriptome sequencing predict that the discovery and significance of *FMO2* involvement in insects, particularly in insecticide detoxification, will likely be an exciting area of investigation in the future.

7.8 Further work

There are several areas where the results of this thesis could be expanded upon in future work.

- The HS strain shows significant, but varying degrees of resistance to the different diamide compounds with an apparent difference demonstrated between anthranilic and phthalic compounds. Further bioassays phenotyping this strain for cross-resistance to other compounds would be informative for IRM strategies, but might also serve to clarify substrate specificities for the putative resistance gene, *FMO2*.
- *FMO2* is implicated in the resistance shown in the HS strain of *P. xylostella*, however, while convincing, further evidence could be gathered. Results produced through synergist assays provided a complex picture of possible *FMO2* involvement. Testing alternative synergistic compounds may elucidate a clearer understanding of *FMO2* involvement as might techniques such as thermal inactivation of *FMO2*.
- It would be useful for resistance management studies to investigate the dominance of resistance seen in the HS strain. This could be done simply by crossing the HS strain with a lab susceptible strain such as ROTH. Such an experiment could also be extended to generate F2 individuals. These would be predicted to have every potential genotype and the association of *FMO2* overexpression with survivorship to a discriminating dose of chlorantraniliprole could be explored.
- An immediate study that could be carried out would be the LC-MS investigation and possible quantification of metabolites produced either by the HS *P. xylostella* strain, or by the GAL/UAS transgenic *D. melanogaster*. Gaddamidi *et al.* have detailed 19 metabolites produced through chlorantraniliprole metabolism in lactating goat which would provide a starting point on which to build [417].
- If possible, expression of *FMO2* in-vitro, for example in *E. coli*, or insect cell-line could prove a valuable resource especially in the screening of new compounds. Tomalski *et al.* identified and tracked both the parent compound and metabolites of imidacloprid over time through radio labelling [373]. The same technique could be applied in in-vitro assays or in-vivo, in whole organisms, not only leading to metabolite identification, but possibly also specific metabolic enzymes
- Should further evidence cement the *FMO2* as conferring the resistance seen, characterisation of this gene is not yet complete and efforts should especially be made to identify the substrate specificity. Not in terms of just what substrates it will catalyse, but the structural reasons underlying the specificities. Additionally,

attempts to elucidate the complete metabolic pathway involving the *FMO2* could be made through a combination of the approaches just mentioned, with in-vitro and in-vivo studies of metabolites, combined with molecular approaches of candidate gene transgenic co-expression, CRISPR and RNAi.

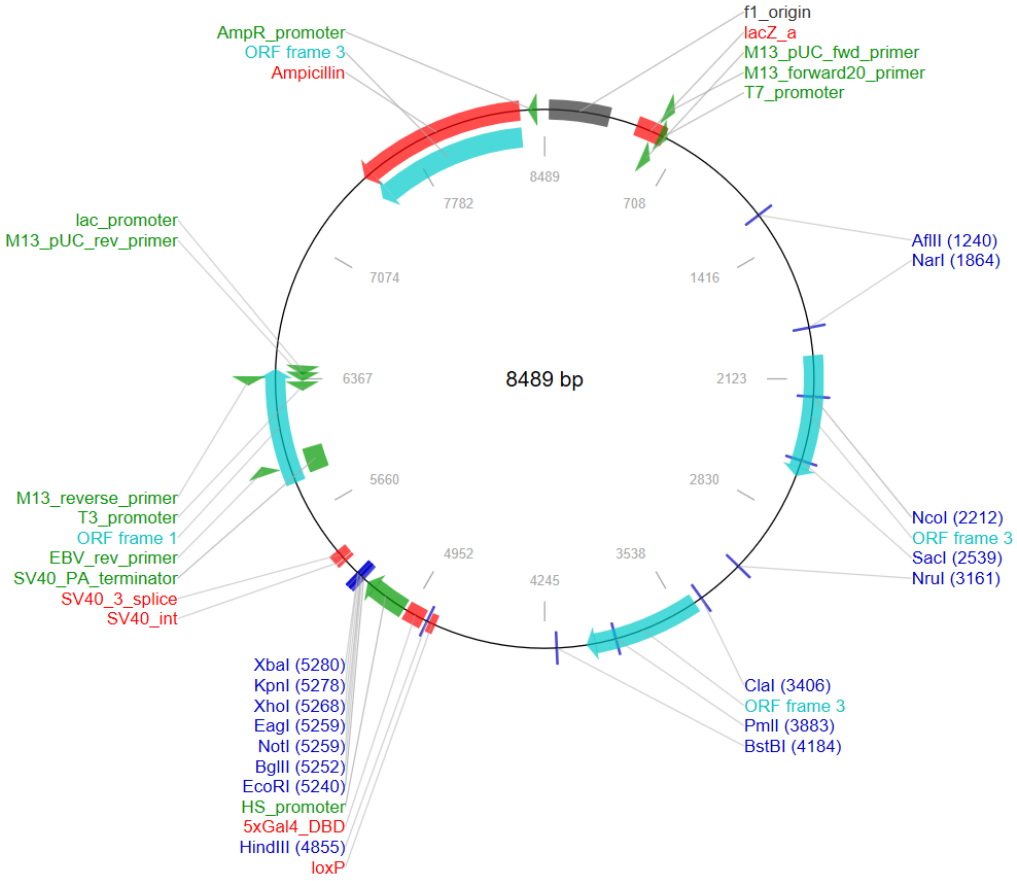
- Finally, the underlying genomic explanation for overexpression of the *FMO2* gene should be explored. It may be that the dual luciferase reporter assay has identified that the 5' UTR/promoter region is responsible for the overexpression, but the precise genetic changes involved still remain to be defined.

8 Appendix

8.1 Accessions to NCBI database

Sequence	Description	NCBI Accession Number
Px_HS_FMO2	FMO2 candidate gene CDS sequence	KY924612
Px_HS_SCD	SCD candidate gene CDS sequence	KY924613
Px_HS_CYP6BG1	CYP6BG1 candidate gene CDS sequence	KY924614
Px_HS_XDH	XDH candidate gene CDS sequence	KY924615
Px_HS_FMO2Prom	Putative HS promoter region for FMO2	KY924616
Px_ROTH_FMO2Prom	Putative ROTH promoter region for FMO2	KY924617

8.2 pUAST Plasmid vector map



9 References

1. Pinstrup-Andersen, P., *Food security: definition and measurement.* Food Security, 2009. **1**(1): p. 5-7.
2. Coates, J., *Build it back better: Deconstructing food security for improved measurement and action.* Global Food Security-Agriculture Policy Economics and Environment, 2013. **2**(3): p. 188-194.
3. Clay, E., *Food Security: Concepts and Measurement*, in *FAO Expert Consultation on Trade and Food Security*, FAO, Editor. 2002.
4. FAOSTAT. 2015 [cited 2016; Available from: http://faostat3.fao.org/browse/D/FS/E.
5. Evans, A., *The feeding of the nine billion: Global food security for the 21st century*. 2009, London: Chatham House.
6. *Food Security Overview*. 2016 [cited 2016; Available from: http://www.worldbank.org/en/topic/foodsecurity/overview#1.
7. Lobell, D.B. and M.B. Burke, *Why are agricultural impacts of climate change so uncertain? The importance of temperature relative to precipitation.* Environmental Research Letters, 2008. **3**(3): p. 8.
8. Parry, M.L., et al., *Effects of climate change on global food production under SRES emissions and socio-economic scenarios.* Global Environmental Change-Human and Policy Dimensions, 2004. **14**(1): p. 53-67.
9. Lobell, D.B., et al., *Prioritizing climate change adaptation needs for food security in 2030.* Science, 2008. **319**(5863): p. 607-610.
10. Oerke, E.C., *Crop losses to pests.* Journal of Agricultural Science, 2006. **144**: p. 31-43.
11. *Report of the World Food Conference*. 1974. Rome: United Nations.
12. *Food and Agriculture Organization of the United Nations. World food security: a reappraisal of the concepts and approaches. Director General's Report*. 1983. Rome: United Nations.
13. *World Bank Policy Study: Poverty and Hunger*. 1986.
14. *Food and Agriculture Organization of the United Nations. Rome declaration on world food security and World Food Summit plan of action*. in *World Food Summit*. 1996. Rome.
15. *The State of Food Insecurity in the World 2001*. 2002. Rome.
16. Bonnemaison.L, *Insect pests of crucifers and their control.* Annual Review of Entomology, 1965. **10**: p. 233-&.
17. Finch S; Thompson AR, *Pests of Cruciferous Crops*, in *Vegetable Crop Pests*, R.G. McKinlay, Editor. 1992, Springer. p. 87-137.
18. Srinivasan R; Shelton AM; Collins HL. *Proceedings of the sixth international workshop on managementof the diamondback moth and other crucifer insect pests*. 2011. Thailand.
19. Kogan, M., *Integrated pest management: Historical perspectives and contemporary developments.* Annual Review of Entomology, 1998. **43**: p. 243-270.
20. Eilenberg, J., A. Hajek, and C. Lomer, *Suggestions for unifying the terminology in biological control.* Biocontrol, 2001. **46**(4): p. 387-400.
21. DA, N., *Biological control, integrated pest management and conceptual models.* Biocontrol News and Information, 1996. **17**: p. 35N-44N.
22. Caltagirone, L.E., *Landmark examples in classical biological-control.* Annual Review of Entomology, 1981. **26**: p. 213-232.
23. Tothill, J.D., T.H.C. Taylor, and R.W. Paine, *The Coconut Moth in Fiji: a History of Its Control by Means of Parasites*. 1930, London: Imperial Bureau of Entomology.

24. Cowie, R.H., *Can snails ever be effective and safe biocontrol agents?* International Journal of Pest Management, 2001. **47**(1): p. 23-40.
25. Elliott, N., R. Kieckhefer, and W. Kauffman, *Effects of an invading coccinellid on native coccinellids in an agricultural landscape.* Oecologia, 1996. **105**(4): p. 537-544.
26. Simberloff, D. and P. Stiling, *How risky is biological control?* Ecology, 1996. **77**(7): p. 1965-1974.
27. Shine, R., *The ecological impact of invasive cane toads (bufo marinus) in australia.* Quarterly Review of Biology, 2010. **85**(3): p. 253-291.
28. Phillips, B.L. and R. Shine, *Adapting to an invasive species: Toxic cane toads induce morphological change in Australian snakes.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(49): p. 17150-17155.
29. van Lenteren, J., *Measures of success in biological control of arthropods by augmentation of natural enemies*, in *Measures of success in biological control*, G.G.W. S, Editor. 2000, Kluwer Academic: Dordrecht, The Netherlands. p. 77-103.
30. Symondson, W.O.C., K.D. Sunderland, and M.H. Greenstone, *Can generalist predators be effective biocontrol agents?* Annual Review of Entomology, 2002. **47**: p. 561-594.
31. Collier, T. and R. Van Steenwyk, *A critical evaluation of augmentative biological control.* Biological Control, 2004. **31**(2): p. 245-256.
32. Doutt, R.L. and K.S. Hagen, *Periodic colonization of chrysopa-californica as a possible control of mealybugs.* Journal of Economic Entomology, 1949. **42**(3): p. 560-561.
33. Trumble, J.T. and J.P. Morse, *Economics of integrating the predaceous mite phytoseiulus-persimilis (acari, phytoseiidae) with pesticides in strawberries.* Journal of Economic Entomology, 1993. **86**(3): p. 879-885.
34. Tylianakis, J.M., R.K. Didham, and S.D. Wratten, *Improved fitness of aphid parasitoids receiving resource subsidies.* Ecology, 2004. **85**(3): p. 658-666.
35. Bianchi, F., C.J.H. Booij, and T. Tscharntke, *Sustainable pest regulation in agricultural landscapes: a review on landscape composition, biodiversity and natural pest control.* Proceedings of the Royal Society B-Biological Sciences, 2006. **273**(1595): p. 1715-1727.
36. FA, F., *Insect injuries to the seed and root of Indian corn*. 1896, University of Illinois Agricultural Experiment Station. p. 209-296.
37. Spencer, J.L., et al., *Behaviour and ecology of the western corn rootworm (Diabrotica virgifera virgifera LeConte).* Agricultural and Forest Entomology, 2009. **11**(1): p. 9-27.
38. Schroeder, J.B., S.T. Ratcliffe, and M.E. Gray, *Effect of four cropping systems on variant western corn rootworm (Coleoptera : Chrysomelidae) adult and egg densities and subsequent larval injury in rotated maize.* Journal of Economic Entomology, 2005. **98**(5): p. 1587-1593.
39. Boucher, T.J., et al., *Managing the pepper maggot (Diptera : Tephritidae) using perimeter trap cropping.* Journal of Economic Entomology, 2003. **96**(2): p. 420-432.
40. Cavanagh, A., et al., *Using Trap Crops for Control of Acalymma vittatum (Coleoptera: Chrysomelidae) Reduces Insecticide Use in Butternut Squash.* Journal of Economic Entomology, 2009. **102**(3): p. 1101-1107.
41. Finch, S. and R.H. Collier, *Host-plant selection by insects - a theory based on 'appropriate/inappropriate landings' by pest insects of cruciferous plants.* Entomologia Experimentalis Et Applicata, 2000. **96**(2): p. 91-102.
42. Bruce, T.J.A., L.J. Wadhams, and C.M. Woodcock, *Insect host location: a volatile situation.* Trends in Plant Science, 2005. **10**(6): p. 269-274.
43. Nottingham, S.F., et al., *Behavioral and electrophysiological responses of aphids to host and nonhost plant volatiles.* Journal of Chemical Ecology, 1991. **17**(6): p. 1231-1242.

44. Blight, M.M., et al., *Antennal perception of oilseed rape, Brassica napus (Brassicaceae), volatiles by the cabbage seed weevil Ceutorhynchus assimilis (Coleoptera: Curculionidae).* Journal of Chemical Ecology, 1995. **21**(11): p. 1649-1664.
45. Reed, D.W., K.A. Pivnick, and E.W. Underhill, *Identification of chemical oviposition stimulants for the diamondback moth, plutella-xylostella, present in 3 species of brassicaceae.* Entomologia Experimentalis Et Applicata, 1989. **53**(3): p. 277-286.
46. Badenes-Perez, F.R., B.A. Nault, and A.M. Shelton, *Dynamics of diamondback moth oviposition in the presence of a highly preferred non-suitable host.* Entomologia Experimentalis Et Applicata, 2006. **120**(1): p. 23-31.
47. Badenes-Perez, F.R., A.M. Shelton, and B.A. Nault, *Evaluating trap crops for diamondback moth, Plutella xylostella (Lepidoptera : Plutellidae).* Journal of Economic Entomology, 2004. **97**(4): p. 1365-1372.
48. Holden, M.H., et al., *Designing an effective trap cropping strategy: the effects of attraction, retention and plant spatial distribution.* Journal of Applied Ecology, 2012. **49**(3): p. 715-722.
49. Shelton, A.M. and E. Badenes-Perez, *Concepts and applications of trap cropping in pest management.* Annual Review of Entomology, 2006. **51**: p. 285-308.
50. Hall, F.R. and J.W. Barry, *An overview of biorational pest-control agents*, in *Biorational Pest Control Agents: Formulation and Delivery*, F.R. Hall and J.W. Barry, Editors. 1995. p. 1-18.
51. Rosell, G., et al., *Biorational insecticides in pest management.* Journal of Pesticide Science, 2008. **33**(2): p. 103-121.
52. Richardson, R.H., J.R. Ellison, and W.W. Averhoff, *Autocidal control of screwworms in north-america.* Science, 1982. **215**(4531): p. 361-370.
53. De la Fuente, J., et al., *Autocidal control of ticks by silencing of a single gene by RNA interference.* Biochemical and Biophysical Research Communications, 2006. **344**(1): p. 332-338.
54. Gong, P., et al., *A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly.* Nature Biotechnology, 2005. **23**(4): p. 453-456.
55. Fryxell, K.J. and T.A. Miller, *Autocidal biological-control - a general strategy for insect control-based on genetic-transformation with a highly conserved gene.* Journal of Economic Entomology, 1995. **88**(5): p. 1221-1232.
56. Ross, A.F., *Localized acquired resistance to plant virus infection in hypersensitive hosts.* Virology, 1961. **14**(3): p. 329-&.
57. Raffa, K.F. and A.A. Berryman, *The role of host plant-resistance in the colonization behavior and ecology of bark beetles (coleoptera, scolytidae).* Ecological Monographs, 1983. **53**(1): p. 27-49.
58. Dreyer, D.L. and B.C. Campbell, *chemical basis of host-plant resistance to aphids.* Plant Cell and Environment, 1987. **10**(5): p. 353-361.
59. Bruce, T.J.A., et al., *The first crop plant genetically engineered to release an insect pheromone for defence.* Scientific Reports, 2015. **5**: p. 9.
60. *IRAC mode of action classification scheme. Version 8.1*. 2016.
61. Sparks, T.C. and R. Nauen, *IRAC: Mode of action classification and insecticide resistance management.* Pesticide Biochemistry and Physiology, 2015. **121**: p. 122-128.
62. O'Conner-Marer, P.J., *The safe and effective use of pesticides (Pesticide application conpendium 1)*. 2nd ed. 2000: Agriculture & Natural Resources.
63. Wang, X.L. and Y.D. Wu, *High Levels of Resistance to Chlorantraniliprole Evolved in Field Populations of Plutella xylostella.* Journal of Economic Entomology, 2012. **105**(3): p. 1019-1023.

64. Zhao, J.Z., et al., *Monitoring and characterization of diamondback moth (Lepidoptera : Plutellidae) resistance to spinosad.* Journal of Economic Entomology, 2002. **95**(2): p. 430-436.
65. Sawicki, R.M. and I. Denholm, *Management of resistance to pesticides in cotton pests.* Tropical Pest Management, 1987. **33**(4): p. 262-272.
66. Denholm, I. and M.W. Rowland, *Tactics for managing pesticide resistance in arthropods - theory and practice.* Annual Review of Entomology, 1992. **37**: p. 91-112.
67. Tabashnik, B.E., *Managing resistance with multiple pesticide tactics - theory, evidence, and recommendations.* Journal of Economic Entomology, 1989. **82**(5): p. 1263-1269.
68. Comins, H.N., *Tactics for resistance management using multiple pesticides.* Agriculture Ecosystems & Environment, 1986. **16**(2): p. 129-148.
69. Curtis, C.F., *Theoretical-models of the use of insecticide mixtures for the management of resistance.* Bulletin of Entomological Research, 1985. **75**(2): p. 259-265.
70. Pan, Z.Q., et al., *Western Corn Rootworm (Coleoptera: Chrysomelidae) Dispersal and Adaptation to Single-Toxin Transgenic Corn Deployed With Block or Blended Refuge.* Environmental Entomology, 2011. **40**(4): p. 964-978.
71. Kliot, A. and M. Ghanim, *Fitness costs associated with insecticide resistance.* Pest Management Science, 2012. **68**(11): p. 1431-1437.
72. Tabashnik, B.E., T.J. Dennehy, and Y. Carriere, *Delayed resistance to transgenic cotton in pink bollworm.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(43): p. 15389-15393.
73. Hackett, S.C. and M.B. Bonsall, *Type of fitness cost influences the rate of evolution of resistance to transgenic Bt crops.* Journal of Applied Ecology, 2016. **53**(5): p. 1391-1401.
74. Gassmann, A.J., Y. Carriere, and B.E. Tabashnik, *Fitness Costs of Insect Resistance to Bacillus thuringiensis.* Annual Review of Entomology, 2009. **54**: p. 147-163.
75. Keiding, J., *Persistence of resistant populations after relaxation of selection pressure.* World Review of Pest Control, 1967. **6**(4): p. 115-&.
76. Shanahan, G., *The sheep blowfly's tolerance to insecticides.* The agricultural gazette of New South Wales, 1967. **78**: p. 444-445.
77. McKenzie, J.A., M.J. Whitten, and M.A. Adena, *The effect of genetic background on the fitness of diazinon resistance genotypes of the australian sheep blowfly, lucilia-cuprina.* Heredity, 1982. **49**(AUG): p. 1-9.
78. Georghiou, G.P., *Management of Resistance in Arthropods*, in *Pest resistance to pesticides*, G.P. Georghiou and T. Saito, Editors. 1983, Springer. p. 769-792.
79. Bull, D., *Growing Problem: Pesticides and the Third World Poor*. 1982: OXFAM.
80. Castle, S.J., et al., *Field evaluation of different insecticide use strategies as resistance management and control tactics for Bemisia tabaci (Hemiptera : Aleyrodidae).* Bulletin of Entomological Research, 2002. **92**(6): p. 449-460.
81. Georghiou, G.P., *Principles of insecticide resistance management.* Phytoprotection, 1994. **75**: p. 51-59.
82. Hallman, G.J. and D.L. Denlinger, *Introduction: temperature sensitivity and integrated pest management*. Temperature sensitivity in insects and application in integrated pest management, ed. H. GJ and D. DL. 1998: Westview Press. 1-5.
83. Grzywacz, D., et al., *Current control methods for diamondback moth and other brassica insect pests and the prospects for improved management with lepidopteran-resistant Bt vegetable brassicas in Asia and Africa.* Crop Protection, 2010. **29**(1): p. 68-79.

84. Marchioro, C.A. and L.A. Foerster, *Development and Survival of the Diamondback Moth, Plutella xylostella (L.) (Lepidoptera: Yponomeutidae) as a Function of Temperature: Effect on the Number of Generations in Tropical and Subtropical Regions.* Neotropical Entomology, 2011. **40**(5): p. 533-541.
85. Talekar, N.S. and A.M. Shelton, *Biology, Ecology, and Management of the Diamondback Moth.* Annual Review of Entomology, 1993. **38**: p. 275-301.
86. Wittstock, U., et al., *Glucosinolate hydrolysis and its impact on generalist and specialist insect herbivores.* Integrative Phytochemistry: from Ethnobotany to Molecular Ecology, 2003. **37**: p. 101-125.
87. Falk, K.L. and J. Gershenzon, *The desert locust, Schistocerca gregaria, detoxifies the glucosinolates of Schouwia purpurea by desulfation.* Journal of Chemical Ecology, 2007. **33**(8): p. 1542-1555.
88. Ratzka, A., et al., *Disarming the mustard oil bomb.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(17): p. 11223-11228.
89. Renwick, J.A.A., *Chemical ecology of oviposition in phytophagous insects.* Experientia, 1989. **45**(3): p. 223-228.
90. Hillyer, R.J. and Thorstei.Aj, *Influence of host plant or males on ovarian development or oviposition in diamondback moth plutella maculipennis (curt).* Canadian Journal of Zoology, 1969. **47**(5): p. 805-&.
91. Renwick, J.A.A., *The chemical world of crucivores: lures, treats and traps.* Entomologia Experimentalis Et Applicata, 2002. **104**(1): p. 35-42.
92. Zalucki, M.P., et al., *Estimating the Economic Cost of One of the World's Major Insect Pests, Plutella xylostella (Lepidoptera: Plutellidae): Just How Long Is a Piece of String?* Journal of Economic Entomology, 2012. **105**(4): p. 1115-1129.
93. Sarfraz, M., A.B. Keddie, and L.M. Dosdall, *Biological control of the diamondback moth, Plutella xylostella: A review.* Biocontrol Science and Technology, 2005. **15**(8): p. 763-789.
94. Loehr, B., et al., *Impact of an exotic parasitoid on Plutella xylostella (Lepidoptera : Plutellidae) population dynamics, damage and indigenous natural enemies in Kenya.* Bulletin of Entomological Research, 2007. **97**(4): p. 337-350.
95. Poelking, A., *Diamondback moth in the philippines and its control with diadegma-semiclausum.* Diamondback Moth and Other Crucifer Pests, 1992: p. 271-278.
96. Kirk, A.A., et al., *Variability in Plutella and its natural enemies: implications for biological control*. Management of Diamondback Moth and Other Crucifer Pests, ed. N.M. Endersby and P.M. Ridland. 2004. 71-77.
97. Furlong, M.J., et al., *Quantitative evaluation of the biotic mortality factors affecting diamondback moth in south-east Queensland, Australia*. Management of Diamondback Moth and Other Crucifer Pests, ed. N.M. Endersby and P.M. Ridland. 2001. 185-193.
98. Furlong, M.J., et al., *Experimental analysis of the influence of pest management practice on the efficacy of an endemic arthropod natural enemy complex of the diamondback moth.* Journal of Economic Entomology, 2004. **97**(6): p. 1814-1827.
99. Liu, X.X., et al., *Natural Enemies Delay Insect Resistance to Bt Crops.* Plos One, 2014. **9**(3).
100. *Agriculture and Horticulture Development Board: Control options for diamondback moth*. 2016 30/08/2016]; Available from: https://croprotect.com/pests/diamondback-moth.
101. Ankersmit, G.W., *Ddt-Resistance in Plutella-Maculipennis (Curt) (Lep) in Java.* Bulletin of Entomological Research, 1953. **44**(3): p. 421-425.
102. Kirsch, K. and H. Schmutterer, *Low Efficacy of a Bacillus-Thuringiensis (Berl) Formulation in Controlling the Diamondback Moth, Plutella-Xylostella (L), in the*

Philippines. Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie, 1988. **105**(3): p. 249-255.
103. Tabashnik, B.E., et al., *Field Development of Resistance to Bacillus-Thuringiensis in Diamondback Moth (Lepidoptera, Plutellidae).* Journal of Economic Entomology, 1990. **83**(5): p. 1671-1676.
104. Iqbal, M., et al., *Evidence for resistance to Bacillus thuringiensis (Bt) subsp kurstaki HD-1, Bt subsp aizawai and abamectin in field populations of Plutella xylostella from Malaysia.* Pesticide Science, 1996. **48**(1): p. 89-97.
105. Wright, D., *Biological control of Plutella xylostella: a global perspective*, in *Proceedings of the international symposium*, D. Bordat and A.A. Kirk, Editors. 2004: Montpellier, France. p. 9-14.
106. Wang, X.L., et al., *Baseline Susceptibility of the Diamondback Moth (Lepidoptera: Plutellidae) to Chlorantraniliprole in China.* Journal of Economic Entomology, 2010. **103**(3): p. 843-848.
107. Xia, Y., et al., *Resistance monitoring for eight insecticides in Plutella xylostella in central China.* Crop Protection, 2014. **63**: p. 131-137.
108. Zhao, J.Z., et al., *Monitoring of diamondback moth (Lepidoptera : Plutellidae) resistance to spinosad, indoxacarb, and emamectin benzoate.* Journal of Economic Entomology, 2006. **99**(1): p. 176-181.
109. Ishiwata, S., *On a new type of severe flacherie (sotto disease) (original in Japanese).* Dainihon Sansi Kaiho, 1901. **114**: p. 1-5.
110. Berliner, E., *Ueber die schlaffsucht der Ephestia kuhniella und Bac. thuringiensis n. sp.* Journal of Applied Entomology, 1915. **2**: p. 21-56.
111. Hannay, C.L. and P. Fitzjames, *The protein crystals of bacillus-thuringiensis berliner.* Canadian Journal of Microbiology, 1955. **1**(8): p. 694-&.
112. Monro, R.E., *Protein turnover and formation of protein inclusions during sporulation of bacillus thuringiensis.* Biochemical Journal, 1961. **81**(2): p. 225-&.
113. McConnell, E. and A.G. Richards, *The production by bacillus thuringiensis berliner of a heat-stable substance toxic for insects.* Canadian Journal of Microbiology, 1959. **5**(2): p. 161-168.
114. McConnell, E. and L.K. Cutkomp, *Studies with bacillus-thuringiensis in relation to the european corn borer.* Journal of Economic Entomology, 1954. **47**(6): p. 1074-1082.
115. Hall, I.M. and L.A. Andres, *Field evaluation of commercially produced bacillus-thuringiensis berliner used for control of lepidopterous larvae on crucifers.* Journal of Economic Entomology, 1959. **52**(5): p. 877-880.
116. de Maagd, R.A., A. Bravo, and N. Crickmore, *How Bacillus thuringiensis has evolved specific toxins to colonize the insect world.* Trends in Genetics, 2001. **17**(4): p. 193-199.
117. Marroquin, L.D., et al., *Bacillus thuringiensis (Bt) toxin susceptibility and isolation of resistance mutants in the nematode Caenorhabditis elegans.* Genetics, 2000. **155**(4): p. 1693-1699.
118. Zhong, C.H., et al., *Characterization of a Bacillus thuringiensis delta-endotoxin which is toxic to insects in three orders.* Journal of Invertebrate Pathology, 2000. **76**(2): p. 131-139.
119. Sansinenea, E., *Discovery and Description of Bacillus thuringiensis*, in *Bacillus thuringiensis Biotechnology*, E. Sansinenea, Editor. 2012, Springer: Netherlands. p. 3-18.
120. Betz, F.S., B.G. Hammond, and R.L. Fuchs, *Safety and advantages of Bacillus thuringiensis-protected plants to control insect pests.* Regulatory Toxicology and Pharmacology, 2000. **32**(2): p. 156-173.

121. Mendelsohn, M., et al., *Are Bt crops safe?* Nature Biotechnology, 2003. **21**(9): p. 1003-1009.
122. Shelton, A.M., et al., *Resistance of diamondback moth (lepidoptera, plutellidae) to bacillus-thuringiensis subspecies in the field.* Journal of Economic Entomology, 1993. **86**(3): p. 697-705.
123. Harvey-Samuel, T., et al., *Pest control and resistance management through release of insects carrying a male-selecting transgene.* Bmc Biology, 2015. **13**.
124. Krafsur, E.S., *Sterile insect technique for suppressing and eradicating insect populations: 55 years and counting.* Journal of Agricultural Entomology, 1998. **15**(4): p. 303-317.
125. Benedict, M.Q. and A.S. Robinson, *The first releases of transgenic mosquitoes: an argument for the sterile insect technique.* Trends in Parasitology, 2003. **19**(8): p. 349-355.
126. Vreysen, M.J.B., et al., *Glossina austeni (Diptera : Glossinidae) eradicated on the Island of Unguja, Zanzibar, using the sterile insect technique.* Journal of Economic Entomology, 2000. **93**(1): p. 123-135.
127. Jin, L., et al., *Engineered Female-Specific Lethality for Control of Pest Lepidoptera.* Acs Synthetic Biology, 2013. **2**(3): p. 160-166.
128. Thomas, D.D., et al., *Insect population control using a dominant, repressible, lethal genetic system.* Science, 2000. **287**(5462): p. 2474-2476.
129. Harris, A.F., et al., *Field performance of engineered male mosquitoes.* Nature Biotechnology, 2011. **29**(11): p. 1034-U109.
130. Harris, A.F., et al., *Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes.* Nature Biotechnology, 2012. **30**(9): p. 828-830.
131. Carvalho, D.O., et al., *Suppression of a Field Population of Aedes aegypti in Brazil by Sustained Release of Transgenic Male Mosquitoes.* Plos Neglected Tropical Diseases, 2015. **9**(7).
132. Han, P.F., et al., *RNAi-Mediated Knockdown of Serine Protease Inhibitor Genes Increases the Mortality of Plutella xylostella Challenged by Destruxin A.* Plos One, 2014. **9**(5).
133. Lee, D.W., Y. Kim, and Y.H. Koh, *RNA interference of PBAN receptor suppresses expression of two fatty acid desaturases in female Plutella xylostella.* Journal of Asia-Pacific Entomology, 2011. **14**(4): p. 405-410.
134. Bautista, M.A., et al., *RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, Plutella xylostella, reduces larval resistance to permethrin.* Insect Biochemistry and Molecular Biology, 2009. **39**(1): p. 38-46.
135. Yang, G., et al., *Characterization of a myrosinase cDNA from Brassica parachinensis and its defense role against Plutella xylostella after suppression.* Insect Science, 2012. **19**(4): p. 461-471.
136. You, M.S., et al., *A heterozygous moth genome provides insights into herbivory and detoxification.* Nature Genetics, 2013. **45**(2): p. 220-225.
137. Tang, W.Q., et al., *DBM-DB: the diamondback moth genome database.* Database-the Journal of Biological Databases and Curation, 2014.
138. Jouraku, A., et al., *KONAGAbase: a genomic and transcriptomic database for the diamondback moth, Plutella xylostella.* Bmc Genomics, 2013. **14**: p. 16.
139. He, W.Y., et al., *Developmental and insecticide-resistant insights from the de novo assembled transcriptome of the diamondback moth, Plutella xylostella.* Genomics, 2012. **99**(3): p. 169-177.

140. Wei, S.J., et al., *Genetic Structure and Demographic History Reveal Migration of the Diamondback Moth Plutella xylostella (Lepidoptera: Plutellidae) from the Southern to Northern Regions of China.* Plos One, 2013. **8**(4): p. 14.
141. Endersby, N.M., et al., *Microsatellites reveal a lack of structure in Australian populations of the diamondback moth, Plutella xylostella (L.).* Molecular Ecology, 2006. **15**(1): p. 107-118.
142. Caprio, M.A. and B.E. Tabashnik, *Allozymes used to estimate gene flow among populations of diamondback moth (lepidoptera, plutellidae) in hawaii.* Environmental Entomology, 1992. **21**(4): p. 808-816.
143. Kim, I., et al., *Mitochondrial COI gene sequence-based population genetic structure of the diamondback moth, Plutella xylostella, in Korea.* Korean Journal of Genetics, 2003. **25**(2): p. 155-170.
144. Steinbach, D., et al., *Geographic spread, genetics and functional characteristics of ryanodine receptor based target-site resistance to diamide insecticides in diamondback moth, Plutella xylostella.* Insect Biochemistry and Molecular Biology, 2015. **63**: p. 14-22.
145. Troczka, B., et al., *Resistance to diamide insecticides in diamondback moth, Plutella xylostella (Lepidoptera: Plutellidae) is associated with a mutation in the membrane-spanning domain of the ryanodine receptor.* Insect Biochemistry and Molecular Biology, 2012. **42**(11): p. 873-880.
146. Feyereisen, R., *Molecular biology of insecticide resistance.* Toxicology Letters, 1995. **82-3**: p. 83-90.
147. Strycharz, J.P., et al., *Resistance in the highly DDT-resistant 91-R strain of Drosophila melanogaster involves decreased penetration, increased metabolism, and direct excretion.* Pesticide Biochemistry and Physiology, 2013. **107**(2): p. 207-217.
148. Scott, J.G., *Handbook of Pest Management in Agriculture*, ed. D. Pimental. 1991, Roca, Raton: CRC.
149. Casida, J.E., *Pest Toxicology: The Primary Mechanisms of Pesticide Action.* Chemical Research in Toxicology, 2009. **22**(4): p. 609-619.
150. Zhu, F., et al., *Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides.* Scientific Reports, 2013. **3**: p. 8.
151. Kramer, K.J. and S. Muthukrishnan, *Insect chitinases: Molecular biology and potential use as biopesticides.* Insect Biochemistry and Molecular Biology, 1997. **27**(11): p. 887-900.
152. Van Leeuwen, T., et al., *Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(12): p. 4407-4412.
153. Sawicki, R.M. and A.W. Farnham, *Genetics of resistance to insecticides of ska strain of musca domestica .3. location and isolation of factors of resistance to dieldrin.* Entomologia Experimentalis Et Applicata, 1968. **11**(2): p. 133-&.
154. Katagi, T., *Relation between penetration rates of pesticides and partition coefficients in topical application to Spodoptera litura.* Journal of Pesticide Science, 2001. **26**(2): p. 165-168.
155. Liu, N.N., et al., *Behavioral change, physiological modification, and metabolic detoxification: mechanisms of insecticide resistance.* Acta Entomologica Sinica, 2006. **49**(4): p. 671-679.
156. Lin, Y.Y., et al., *Cuticular penetration of beta-cypermethrin in insecticide-susceptible and resistant strains of Bactrocera dorsalis.* Pesticide Biochemistry and Physiology, 2012. **103**(3): p. 189-193.

157. Vannini, L., T.W. Reed, and J.H. Willis, *Temporal and spatial expression of cuticular proteins of Anopheles gambiae implicated in insecticide resistance or differentiation of M/S incipient species.* Parasites & Vectors, 2014. **7**: p. 11.
158. Balabanidou, V., et al., *Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in Anopheles gambiae.* Proceedings of the National Academy of Sciences of the United States of America, 2016. **113**(33): p. 9268-9273.
159. Georghiou, G.P., *The evolution of resistance to pesticides.* Annual Review of Ecology and Systematics, 1972. **3**: p. 133-168.
160. Guedes, N.M.P., et al., *Flight take-off and walking behavior of insecticide-susceptible and - resistant strains of Sitophilus zeamais exposed to deltamethrin.* Bulletin of Entomological Research, 2009. **99**(4): p. 393-400.
161. Haddi, K., et al., *Metabolic and Behavioral Mechanisms of Indoxacarb Resistance in Sitophilus zeamais (Coleoptera: Curculionidae).* Journal of Economic Entomology, 2015. **108**(1): p. 362-369.
162. Tome, H.V.V., et al., *Azadirachtin avoidance by larvae and adult females of the tomato leafminer Tutu absoluta.* Crop Protection, 2013. **46**: p. 63-69.
163. Fornadel, C.M., et al., *Analysis of Anopheles arabiensis Blood Feeding Behavior in Southern Zambia during the Two Years after Introduction of Insecticide-Treated Bed Nets.* American Journal of Tropical Medicine and Hygiene, 2010. **83**(4): p. 848-853.
164. Russell, T.L., et al., *Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania.* Malaria Journal, 2011. **10**: p. 10.
165. Moiroux, N., et al., *Changes in Anopheles funestus Biting Behavior Following Universal Coverage of Long-Lasting Insecticidal Nets in Benin.* Journal of Infectious Diseases, 2012. **206**(10): p. 1622-1629.
166. Reddy, M.R., et al., *Outdoor host seeking behaviour of Anopheles gambiae mosquitoes following initiation of malaria vector control on Bioko Island, Equatorial Guinea.* Malaria Journal, 2011. **10**: p. 10.
167. Wang, C.L., M.E. Scharf, and G.W. Bennett, *Behavioral and physiological resistance of the German cockroach to gel baits (Blattodea : Blattellidae).* Journal of Economic Entomology, 2004. **97**(6): p. 2067-2072.
168. Gerold, J.L. and J.J. Laarman, *Selection of some strains of anopheles atroparvus with different behavioural responses to contacts with ddt.* Nature, 1964. **204**(495): p. 500-&.
169. Gerold, J.L. and J.J. Laarman, *Behavioural responses to contact with ddt in anopheles atroparvus.* Nature, 1967. **215**(5100): p. 518-&.
170. Gordon, H.T., *Nutritional factors in insect resistance to chemicals.* Annual Review of Entomology, 1961. **6**: p. 27-&.
171. Feyereisen, R., *Insect P450 enzymes.* Annual Review of Entomology, 1999. **44**: p. 507-533.
172. Li, X., M.A. Schuler, and M.R. Berenbaum, *Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics.* Annual Review of Entomology, 2007. **52**: p. 231-53.
173. Li, X.C., M.R. Berenbaum, and M.A. Schuler, *Plant allelochemicals differentially regulate Helicoverpa zea cytochrome P450 genes.* Insect Molecular Biology, 2002. **11**(4): p. 343-351.
174. Daborn, P.J., et al., *A single P450 allele associated with insecticide resistance in Drosophila.* Science, 2002. **297**(5590): p. 2253-2256.

175. Daborn, P., et al., *DDT resistance in Drosophila correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid.* Molecular Genetics and Genomics, 2001. **266**(4): p. 556-563.
176. Li, A.Y., et al., *Resistance to Coumaphos and Diazinon in Boophilus microplus (Acari : Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism.* Journal of Medical Entomology, 2003. **40**(4): p. 482-490.
177. Sun, W., et al., *Genome-wide analysis of phenobarbital-inducible genes in Drosophila melanogaster.* Insect Molecular Biology, 2006. **15**(4): p. 455-464.
178. Ingham, V.A., et al., *Dissecting the organ specificity of insecticide resistance candidate genes in Anopheles gambiae: known and novel candidate genes.* Bmc Genomics, 2014. **15**.
179. Cui, F., et al., *Carboxylesterase-mediated insecticide resistance: Quantitative increase induces broader metabolic resistance than qualitative change.* Pesticide Biochemistry and Physiology, 2015. **121**: p. 88-96.
180. Daborn, P.J., et al., *Evaluating the insecticide resistance potential of eight Drosophila melanogaster cytochrome P450 genes by transgenic over-expression.* Insect Biochemistry and Molecular Biology, 2007. **37**(5): p. 512-519.
181. Catania, F., et al., *World-wide survey of an Accord insertion and its association with DDT resistance in Drosophila melanogaster.* Molecular Ecology, 2004. **13**(8): p. 2491-2504.
182. Amichot, M., et al., *Point mutations associated with insecticide resistance in the Drosophila cytochrome P450 Cyp6a2 enable DDT metabolism.* European Journal of Biochemistry, 2004. **271**(7): p. 1250-1257.
183. Feyereisen, R., *Insect P450 inhibitors and insecticides: challenges and opportunities.* Pest Management Science, 2015. **71**(6): p. 793-800.
184. Scott, J.G., *Cytochromes P450 and insecticide resistance.* Insect Biochemistry and Molecular Biology, 1999. **29**(9): p. 757-777.
185. Chiu, T.L., et al., *Comparative molecular modeling of Anopheles gambiae CYP6Z1, a mosquito P450 capable of metabolizing DDT.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(26): p. 8855-8860.
186. Scott, J.G. and G.D. Wheelock, *Characterization of a cytochrome-p450 responsible for pyrethroid resistance in the housefly.* Acs Symposium Series, 1992. **505**: p. 16-30.
187. Karunker, I., et al., *Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of Bemisia tabaci (Hemiptera : Aleyrodidae).* Insect Biochemistry and Molecular Biology, 2008. **38**(6): p. 634-644.
188. Edi, C.V., et al., *CYP6 P450 Enzymes and ACE-1 Duplication Produce Extreme and Multiple Insecticide Resistance in the Malaria Mosquito Anopheles gambiae.* Plos Genetics, 2014. **10**(3): p. 12.
189. Gao, X., et al., *Identification and Characterization of the Gene CYP340W1 from Plutella xylostella and Its Possible Involvement in Resistance to Abamectin.* International Journal of Molecular Sciences, 2016. **17**(3).
190. Konno, T., E. Hodgson, and W.C. Dauterman, *Studies on methyl parathion resistance in heliothis-virescens.* Pesticide Biochemistry and Physiology, 1989. **33**(2): p. 189-199.
191. Hayes, J.D., J.U. Flanagan, and I.R. Jowsey, *Glutathione transferases*, in *Annual Review of Pharmacology and Toxicology*. 2005, Annual Reviews: Palo Alto. p. 51-88.
192. Ranson, H., et al., *Evolution of supergene families associated with insecticide resistance.* Science, 2002. **298**(5591): p. 179-181.
193. Chiang, F.M. and C.N. Sun, *Glutathione transferase isozymes of diamondback moth larvae and their role in the degradation of some organophosphorus insecticides.* Pesticide Biochemistry and Physiology, 1993. **45**(1): p. 7-14.

194. Sonoda, S. and H. Tsumuki, *Studies on glutathione S-transferase gene involved in chlorfluazuron resistance of the diamondback moth, Plutella xylostella L. (Lepidoptera : Yponomeutidae).* Pesticide Biochemistry and Physiology, 2005. **82**(1): p. 94-101.
195. Ranson, H., et al., *Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector Anopheles gambiae.* Biochemical Journal, 2001. **359**: p. 295-304.
196. Kostaropoulos, I., et al., *Glutathione S-transferase in the defence against pyrethroids in insects.* Insect Biochemistry and Molecular Biology, 2001. **31**(4-5): p. 313-319.
197. Mitchell, C.L., et al., *The Mechanisms Underlying alpha-Amanitin Resistance in Drosophila melanogaster: A Microarray Analysis.* Plos One, 2014. **9**(4): p. 16.
198. Lumjuan, N., et al., *The role of the Aedes aegypti Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides.* Insect Biochemistry and Molecular Biology, 2011. **41**(3): p. 203-209.
199. Oakeshott, J.G., et al., *Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family.* Bioessays, 1999. **21**(12): p. 1031-1042.
200. Rooker, S., et al., *Coamplification of esterase A and B genes as a single unit in Culex pipiens mosquitoes.* Heredity, 1996. **77**: p. 555-561.
201. Raymond, M., et al., *An overview of the evolution of overproduced esterases in the mosquito Culex pipiens.* Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 1998. **353**(1376): p. 1707-1711.
202. Hemingway, J. and S. Karunaratne, *Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism.* Medical and Veterinary Entomology, 1998. **12**(1): p. 1-12.
203. Jornvall, H., J.O. Hoog, and B. Persson, *SDR and MDR: completed genome sequences show these protein families to be large, of old origin, and of complex nature.* Febs Letters, 1999. **445**(2-3): p. 261-264.
204. Kavanagh, K., et al., *The SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes.* Cellular and Molecular Life Sciences, 2008. **65**(24): p. 3895-3906.
205. Oppermann, U.C.T. and E. Maser, *Molecular and structural aspects of xenobiotic carbonyl metabolizing enzymes. Role of reductases and dehydrogenases in xenobiotic phase I reactions.* Toxicology, 2000. **144**(1-3): p. 71-81.
206. Oppermann, U., et al., *Short-chain dehydrogenases/reductases (SDR): the 2002 update.* Chemico-Biological Interactions, 2003. **143**: p. 247-253.
207. Menzel, R., et al., *Cytochrome P450s and short-chain dehydrogenases mediate the toxicogenomic response of PCB52 in the nematode Caenorhabditis elegans.* Journal of Molecular Biology, 2007. **370**(1): p. 1-13.
208. Hulsmeyer, M., et al., *Crystal structure of cis-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase from a PCB degrader at 2.0 angstrom resolution.* Protein Science, 1998. **7**(6): p. 1286-1293.
209. Hao, D.C. and P.G. Xiao, *Prediction of sites under adaptive evolution in flavin-containing monooxygenases: Selection pattern revisited.* Chinese Science Bulletin, 2011. **56**(12): p. 1246-1255.
210. Hodgson, E. and P.E. Levi, *Pesticides: An important but underused model for the environmental health sciences.* Environmental Health Perspectives, 1996. **104**: p. 97-106.
211. Sehlmeyer, S., et al., *Flavin-Dependent Monooxygenases as a Detoxification Mechanism in Insects: New Insights from the Arctiids (Lepidoptera).* Plos One, 2010. **5**(5).

212. Ziegler, D.M., *Flavin-containing monooxygenases - enzymes adapted for multisubstrate specificity.* Trends in Pharmacological Sciences, 1990. **11**(8): p. 321-324.
213. Taylor, K.L. and D.M. Ziegler, *Studies on substrate-specificity of the hog liver flavin-containing monooxygenase - anionic organic sulfur-compounds.* Biochemical Pharmacology, 1987. **36**(1): p. 141-146.
214. Guo, W.X.A., L.L. Poulsen, and D.M. Ziegler, *Use of thiocarbamides as selective substrate probes for isoforms of flavin-containing monooxygenases.* Biochemical Pharmacology, 1992. **44**(10): p. 2029-2037.
215. Nagata, T., D.E. Williams, and D.M. Ziegler, *Substrate specificities of rabbit lung and porcine liver flavin-containing monooxygenases - differences due to substrate size.* Chemical Research in Toxicology, 1990. **3**(4): p. 372-376.
216. Lawton, M.P., et al., *A nomenclature for the mammalian flavin-containing monooxygenase gene family based on amino-acid-sequence identities.* Archives of Biochemistry and Biophysics, 1994. **308**(1): p. 254-257.
217. Ziegler, D.M., *An overview of the mechanism, substrate specificities, and structure of FMOs.* Drug Metabolism Reviews, 2002. **34**(3): p. 503-511.
218. Hodgson, E., et al., *Flavin-containing monooxygenase and cytochrome P450 mediated metabolism of pesticides: from mouse to human.* Pesticides and the Future, 1998: p. 231-243.
219. Lindigkeit, R., et al., *The two faces of pyrrolizidine alkaloids: The role of the tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids.* European Journal of Biochemistry, 1997. **245**(3): p. 626-636.
220. Naumann, C., T. Hartmann, and D. Ober, *Evolutionary recruitment of a flavin-dependent monooxygenase for the detoxification of host plant-acquired pyrrolizidine alkaloid-def ended arctiid alkaloids in the moth Tyria jacobaleae.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(9): p. 6085-6090.
221. Wang, L.Z., et al., *Independent Recruitment of a Flavin-Dependent Monooxygenase for Safe Accumulation of Sequestered Pyrrolizidine Alkaloids in Grasshoppers and Moths.* Plos One, 2012. **7**(2).
222. Tian, X.R., X.X. Sun, and J.Y. Su, *Biochemical mechanisms for metaflumizone resistance in beet armyworm, Spodoptera exigua.* Pesticide Biochemistry and Physiology, 2014. **113**: p. 8-14.
223. ffrench-Constant, R.H., et al., *Why are there so few resistance-associated mutations in insecticide target genes?* Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 1998. **353**(1376): p. 1685-1693.
224. IRAC. *Established Insecticide Target Site Mutations (ver 2.0)*. 2016; Available from: http://www.irac-online.org/documents.
225. Toda, S., et al., *Two amino acid substitutions in acetylcholinesterase associated with pirimicarb and organophosphorous insecticide resistance in the cotton aphid, Aphis gossypii Glover (Homoptera : Aphididae).* Insect Molecular Biology, 2004. **13**(5): p. 549-553.
226. Khajehali, J., et al., *Acetylcholinesterase point mutations in European strains of Tetranychus urticae (Acari: Tetranychidae) resistant to organophosphates.* Pest Management Science, 2010. **66**(2): p. 220-228.
227. Andrews, M.C., et al., *Identification of mutations conferring insecticide-insensitive AChE in the cotton-melon aphid, Aphis gossypii Glover.* Insect Molecular Biology, 2004. **13**(5): p. 555-561.
228. Martinez-Torres, D., et al., *A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, Myzus*

persicae (Sulzer) (Hemiptera : Aphididae). Insect Molecular Biology, 1999. **8**(3): p. 339-346.
229. Williamson, M.S., et al., *Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides.* Molecular and General Genetics, 1996. **252**(1-2): p. 51-60.
230. Haddi, K., et al., *Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (Tuta absoluta).* Insect Biochemistry and Molecular Biology, 2012. **42**(7): p. 506-513.
231. Zhu, F., et al., *Insecticide Resistance and Management Strategies in Urban Ecosystems.* Insects, 2016. **7**(2).
232. *Arthropod Pesticide Resistance Database*. 2016 30/08/2016]; Available from: http://www.pesticideresistance.org.
233. Wang, X.L., et al., *Dominant Inheritance of Field-Evolved Resistance to Fipronil in Plutella xylostella (Lepidoptera: Plutellidae).* Journal of Economic Entomology, 2016. **109**(1): p. 334-338.
234. Baxter, S.W., et al., *Mis-Spliced Transcripts of Nicotinic Acetylcholine Receptor alpha 6 Are Associated with Field Evolved Spinosad Resistance in Plutella xylostella (L.).* Plos Genetics, 2010. **6**(1).
235. Wang, X., et al., *A point mutation in the glutamate-gated chloride channel of Plutella xylostella is associated with resistance to abamectin.* Insect Molecular Biology, 2016. **25**(2): p. 116-125.
236. Wang, X.L., et al., *Two novel sodium channel mutations associated with resistance to indoxacarb and metaflumizone in the diamondback moth, Plutella xylostella.* Insect Science, 2016. **23**(1): p. 50-58.
237. Sonoda, S., et al., *Frequencies of the M918I mutation in the sodium channel of the diamondback moth in China, Thailand and Japan and its association with pyrethroid resistance.* Pesticide Biochemistry and Physiology, 2012. **102**(2): p. 142-145.
238. Sonoda, S., C. Igaki, and H. Tsumuki, *Alternatively spliced sodium channel transcripts expressed in field strains of the diamondback moth.* Insect Biochemistry and Molecular Biology, 2008. **38**(9): p. 883-890.
239. Schuler, T.H., et al., *Toxicological, electrophysiological, and molecular characterisation of knockdown resistance to pyrethroid insecticides in the diamondback moth, Plutella xylostella (L.).* Pesticide Biochemistry and Physiology, 1998. **59**(3): p. 169-182.
240. Endersby, N.M., et al., *Widespread pyrethroid resistance in Australian diamondback moth, Plutella xylostella (L.), is related to multiple mutations in the para sodium channel gene.* Bulletin of Entomological Research, 2011. **101**(4): p. 393-405.
241. Guo, L., et al., *Functional analysis of a point mutation in the ryanodine receptor of Plutella xylostella (L.) associated with resistance to chlorantraniliprole.* Pest Management Science, 2014. **70**(7): p. 1083-1089.
242. Ku, C.C., et al., *Glutathione transferase isozymes involved in insecticide resistance of diamondback moth larvae.* Pesticide Biochemistry and Physiology, 1994. **50**(3): p. 191-197.
243. Huang, H.S., et al., *Molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the diamondback moth, Plutella xylostella.* Insect Biochemistry and Molecular Biology, 1998. **28**(9): p. 651-658.
244. Sonoda, S., *Molecular analysis of pyrethroid resistance conferred by target insensitivity and increased metabolic detoxification in Plutella xylostella.* Pest Management Science, 2010. **66**(5): p. 572-5.

245. Pu, X., et al., *Characterisation of abamectin resistance in a field-evolved multiresistant population of Plutella xylostella.* Pest Management Science, 2010. **66**(4): p. 371-8.
246. Wang, X.L., et al., *Characterisation of field-evolved resistance to chlorantraniliprole in the diamondback moth, Plutella xylostella, from China.* Pest Management Science, 2013. **69**(5): p. 661-665.
247. Troczka, B., et al., *Resistance to diamide insecticides in diamondback moth, Plutella xylostella (Lepidoptera: Plutellidae) is associated with a mutation in the membrane-spanning domain of the ryanodine receptor.* Insect Biochemistry and Molecular Biology, 2012. **42**(11): p. 873-80.
248. Tohnishi, M., et al., *Flubendiamide, a novel insecticide highly active against lepidopterous insect pests.* Journal of Pesticide Science, 2005. **30**(4): p. 354-360.
249. Jeanguenat, A., *The story of a new insecticidal chemistry class: the diamides.* Pest Management Science, 2013. **69**(1): p. 7-14.
250. Nauen, R., *Insecticide mode of action: return of the ryanodine receptor.* Pest Management Science, 2006. **62**(8): p. 690-692.
251. Lahm, G.P., et al., *Insecticidal anthranilic diamides: A new class of potent ryanodine receptor activators.* Bioorganic & Medicinal Chemistry Letters, 2005. **15**(22): p. 4898-4906.
252. Cordova, D., et al., *Anthranilic diamides: A new class of insecticides with a novel mode of action, ryanodine receptor activation.* Pesticide Biochemistry and Physiology, 2006. **84**(3): p. 196-214.
253. Lahm, G.P., D. Cordova, and J.D. Barry, *New and selective ryanodine receptor activators for insect control.* Bioorganic & Medicinal Chemistry, 2009. **17**(12): p. 4127-4133.
254. Sigma-Aldrich, *Flubendiamide chemical structure.* 2016; Available from: http://www.sigmaaldrich.com.
255. Sigma-Aldrich. *Chlorantraniliprole chemical structure*. 2016; Available from: http://www.sigmaaldrich.com.
256. Sigma-Aldrich, *Cyantraniliprole chemical structure.* 2016; Available from: http://www.sigmaaldrich.com.
257. IRAC, *MoA Classification*. 2011, Available from http://www.irac-online.org.
258. Sattelle, D.B., D. Cordova, and T.R. Cheek, *Insect ryanodine receptors: molecular targets for novel pest control chemicals.* Invertebrate Neuroscience, 2008. **8**(3): p. 107-119.
259. IRAC diamide working group. *The mode of actionof diamides and other lepidopteran insecticides and general rotation rocommendations*, in *6th International DBM Conference*. 2010.
260. Zhang, R.M., et al., *Lethal and sublethal effects of cyantraniliprole on Bactrocera dorsalis (Hendel) (Diptera: Tephritidae).* Pest Management Science, 2015. **71**(2): p. 250-256.
261. Zhang, R.M., S.Y. He, and J.H. Chen, *Monitoring of Bactrocera dorsalis (Diptera: Tephritidae) Resistance to Cyantraniliprole in the South of China.* Journal of Economic Entomology, 2014. **107**(3): p. 1233-1238.
262. Tiwari, S. and L.L. Stelinski, *Effects of cyantraniliprole, a novel anthranilic diamide insecticide, against Asian citrus psyllid under laboratory and field conditions.* Pest Management Science, 2013. **69**(9): p. 1066-1072.
263. Scott, I.M., J.H. Tolman, and D.C. MacArthur, *Insecticide resistance and cross-resistance development in Colorado potato beetle Leptinotarsa decemlineata Say (Coleoptera: Chrysomelidae) populations in Canada 2008-2011.* Pest Management Science, 2015. **71**(5): p. 712-721.

264. Reding, M.E. and A.B. Persad, *Systemic Insecticides for Control of Black Vine Weevil (Coleoptera: Curculionidae) in Container- and Field-Grown Nursery Crops.* Journal of Economic Entomology, 2009. **102**(3): p. 927-933.
265. Uchiyama, T. and A. Ozawa, *Rapid development of resistance to diamide insecticides in the smaller tea tortrix, Adoxophyes honmai (Lepidoptera: Tortricidae), in the tea fields of Shizuoka Prefecture, Japan.* Applied Entomology and Zoology, 2014. **49**(4): p. 529-534.
266. Sial, A.A. and J.F. Brunner, *Selection for resistance, reversion towards susceptibility and synergism of chlorantraniliprole and spinetoram in obliquebanded leafroller, Choristoneura rosaceana (Lepidoptera: Tortricidae).* Pest Management Science, 2012. **68**(3): p. 462-468.
267. Che, W.N., et al., *Insecticide Resistance Status of Field Populations of Spodoptera exigua (Lepidoptera: Noctuidae) From China.* Journal of Economic Entomology, 2013. **106**(4): p. 1855-1862.
268. Muthusamy, R., M. Vishnupriya, and M.S. Shivakumar, *Biochemical mechanism of chlorantraniliprole resistance in Spodoptera litura (Fab) (Lepidoptera: Noctuidae).* Journal of Asia-Pacific Entomology, 2014. **17**(4): p. 865-869.
269. Lai, T.C. and J.Y. Su, *Assessment of resistance risk in Spodoptera exigua (Hubner) (Lepidoptera: Noctuidae) to chlorantraniliprole.* Pest Management Science, 2011. **67**(11): p. 1468-1472.
270. He, Y., J. Zhang, and J. Chen, *Effect of Synergists on Susceptibility to Chlorantraniliprole in Field Populations of Chilo suppressalis (Lepidoptera: Pyralidae).* Journal of Economic Entomology, 2014. **107**(2): p. 791-796.
271. Demkovich, M., et al., *Effect of Piperonyl Butoxide on the Toxicity of Four Classes of Insecticides to Navel Orangeworm (Amyelois transitella) (Lepidoptera: Pyralidae).* Journal of Economic Entomology, 2015. **108**(6): p. 2753-2760.
272. Roditakis, E., et al., *First report of Tuta absoluta resistance to diamide insecticides.* Journal of Pest Science, 2015. **88**(1): p. 9-16.
273. Troczka, B.J., et al., *Stable expression and functional characterisation of the diamondback moth ryanodine receptor G4946E variant conferring resistance to diamide insecticides.* Scientific Reports, 2015. **5**.
274. Yan, H.H., et al., *Flubendiamide resistance and Bi-PASA detection of ryanodine receptor G4946E mutation in the diamondback moth (Plutella xylostella L.).* Pesticide Biochemistry and Physiology, 2014. **115**: p. 73-77.
275. Guo, L., et al., *Novel mutations and mutation combinations of ryanodine receptor in a chlorantraniliprole resistant population of Plutella xylostella (L.).* Scientific Reports, 2014. **4**.
276. Ninsin, K.D. and T. Tanaka, *Synergism and stability of acetamiprid resistance in a laboratory colony of Plutelia xylostella.* Pest Management Science, 2005. **61**(8): p. 723-727.
277. Hu, Z.-d., et al., *Biochemical Mechanism of Chlorantraniliprole Resistance in the Diamondback Moth, Plutella xylostella Linnaeus.* Journal of Integrative Agriculture, 2014. **13**(11): p. 2452-2459.
278. Yu, S.J. and S.N. Nguyen, *Insecticide susceptibility and detoxication enzyme activities in permethrin-selected diamondback moths.* Pesticide Biochemistry and Physiology, 1996. **56**(1): p. 69-77.
279. Liu, X., et al., *Cross-resistance, mode of inheritance, synergism, and fitness effects of cyantraniliprole resistance in Plutella xylostella.* Entomologia Experimentalis Et Applicata, 2015. **157**(3): p. 271-278.
280. Collins, H., *Techniques for Rearing Plutella xylostella*. 2012, Personal Communication.

281. Kibbe, W.A., *OligoCalc: an online oligonucleotide properties calculator.* Nucleic Acids Res, 2007. **35 (webserver issue)**(May 25).
282. Liu, X., et al., *Resistance Selection and Characterization of Chlorantraniliprole Resistance in Plutella xylostella (Lepidoptera: Plutellidae).* Journal of Economic Entomology, 2015. **108**(4): p. 1978-1985.
283. Klein, J. and L. Alder, *Applicability of gradient liquid chromatography with tandem mass spectrometry to the simultaneous screening for about 100 pesticides in crops.* Journal of Aoac International, 2003. **86**(5): p. 1015-1037.
284. Ferrer, C., et al., *Determination of pesticide residues in olives and olive oil by matrix solid-phase dispersion followed by gas chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry.* Journal of Chromatography A, 2005. **1069**(2): p. 183-194.
285. Alptekin, S., et al., *Induced thiacloprid insensitivity in honeybees (Apis mellifera L.) is associated with up-regulation of detoxification genes.* Insect Molecular Biology, 2016. **25**(2): p. 171-180.
286. Bass, C., et al., *Mutation of a nicotinic acetylcholine receptor beta subunit is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae.* Bmc Neuroscience, 2011. **12**: p. 11.
287. Abbott, W.S., *A method of computing the effectiveness of an insecticide.* Journal of Economic Entomology, 1925. **18**: p. 265-267.
288. Roditakis, E., et al., *Assessment of the Bemisia tabaci CYP6CM1vQ transcript and protein levels in laboratory and field-derived imidacloprid-resistant insects and cross-metabolism potential of the recombinant enzyme.* Insect Science, 2011. **18**(1): p. 23-29.
289. Zhang, S.Z., et al., *Susceptibility of field populations of the diamondback moth, Plutella xylostella, to a selection of insecticides in Central China.* Pesticide Biochemistry and Physiology, 2016. **132**: p. 38-46.
290. Nauen, R., et al., *Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants.* Pesticide Biochemistry and Physiology, 2003. **76**(2): p. 55-69.
291. Yang, Y.L., et al., *Molecular characterization and RNA interference of three midgut aminopeptidase N isozymes from Bacillus thuringiensis-susceptible and -resistant strains of sugarcane borer, Diatraea saccharalis.* Insect Biochemistry and Molecular Biology, 2010. **40**(8): p. 592-603.
292. Doichuanngam, K. and R.A. Thornhill, *Penetration, excretion and metabolism of c-14 malathion in susceptible and resistant strains of plutella-xylostella.* Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology, 1992. **101**(3): p. 583-588.
293. Sternburg, J. and C.W. Kearns, *Metabolic fate of ddt when applied to certain naturally tolerant insects.* Journal of Economic Entomology, 1952. **45**(3): p. 497-505.
294. Ahmed, H. and B.G. Gardiner, *Activation of malathion by locust bodywall.* Nature, 1968. **217**(5130): p. 776-&.
295. Puinean, A.M., et al., *Characterisation of imidacloprid resistance mechanisms in the brown planthopper, Nilaparvata lugens Stal (Hemiptera: Delphacidae).* Pesticide Biochemistry and Physiology, 2010. **97**(2): p. 129-132.
296. Zhu, Y.C. and R. Luttrell, *Altered gene regulation and potential association with metabolic resistance development to imidacloprid in the tarnished plant bug, Lygus lineolaris.* Pest Management Science, 2015. **71**(1): p. 40-57.
297. Lu, Y., P. Huggins, and Z. Bar-Joseph, *Cross species analysis of microarray expression data.* Bioinformatics, 2009. **25**(12): p. 1476-1483.

298. Rhodes, D.R., et al., *Large-scale meta-analysis of cancer microarray data identifies common transcriptional profiles of neoplastic transformation and progression.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(25): p. 9309-9314.
299. Parmigiani, G., et al., *A cross-study comparison of gene expression studies for the molecular classification of lung cancer.* Clinical Cancer Research, 2004. **10**(9): p. 2922-2927.
300. Daves, M.H., et al., *Meta-analysis of multiple microarray datasets reveals a common gene signature of metastasis in solid tumors.* Bmc Medical Genomics, 2011. **4**.
301. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method.* Methods, 2001. **25**(4): p. 402-408.
302. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing.* Journal of the Royal Statistical Society Series B-Methodological, 1995. **57**(1): p. 289-300.
303. Smyth, G.K., J. Michaud, and H.S. Scott, *Use of within-array replicate spots for assessing differential expression in microarray experiments.* Bioinformatics, 2005. **21**(9): p. 2067-2075.
304. Wang, X., et al., *Characterisation of field-evolved resistance to chlorantraniliprole in the diamondback moth, Plutella xylostella, from China.* Pest Management Science, 2013. **69**(5): p. 661-665.
305. de Hoon, M.J.L., et al., *Open source clustering software.* Bioinformatics, 2004. **20**(9): p. 1453-1454.
306. Saldanha, A.J., *Java Treeview-extensible visualization of microarray data.* Bioinformatics, 2004. **20**(17): p. 3246-3248.
307. Garber, M.E., et al., *Diversity of gene expression in adenocarcinoma of the lung.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(24): p. 13784-13789.
308. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(25): p. 14863-14868.
309. Bautista, M.A.M., T. Tanaka, and T. Miyata, *Identification of permethrin-inducible cytochrome P450s from the diamondback moth, Plutella xylostella (L.) and the possibility of involvement in permethrin resistance.* Pesticide Biochemistry and Physiology, 2007. **87**(1): p. 85-93.
310. Lai, T., J. Li, and J. Su, *Monitoring of beet armyworm Spodoptera exigua (Lepidoptera: Noctuidae) resistance to chlorantraniliprole in China.* Pesticide Biochemistry and Physiology, 2011. **101**(3): p. 198-205.
311. Jensen, R.A., *Enzyme Recruitment in Evolution of New Function.* Annual Review of Microbiology, 1976. **30**: p. 409-425.
312. Etienne, W., et al., *Comparison of mRNA gene expression by RT-PCR and DNA microarray.* Biotechniques, 2004. **36**(4): p. 618.
313. Morey, J.S., J.C. Ryan, and F.M. Van Dolah, *Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR.* Biological Procedures Online, 2006. **8**: p. 175-193.
314. Hartmann, T. and D. Ober, *Biosynthesis and metabolism of pyrrolizidine alkaloids in plants and specialized insect herbivores.* Biosynthesis: Aromatic Polyketides, Isoprenoids, Alkaloids, 2000. **209**: p. 207-243.
315. Hartmann, T., et al., *Acquisition, transformation and maintenance of plant pyrrolizidine alkaloids by the polyphagous arctiid Grammia geneura.* Insect Biochemistry and Molecular Biology, 2005. **35**(10): p. 1083-1099.

316. Hartmann, T., et al., *Specific recognition, detoxification and metabolism of pyrrolizidine alkaloids by the polyphagous arctiid Estigmene acrea.* Insect Biochemistry and Molecular Biology, 2005. **35**(5): p. 391-411.
317. Cashman, J.R., *Structural and catalytic properties of the mammalian flavin-containing monooxygenase.* Chemical Research in Toxicology, 1995. **8**(2): p. 165-181.
318. Hao, D., et al., *Molecular phylogeny, long-term evolution, and functional divergence of flavin-containing monooxygenases.* Genetica, 2009. **137**(2): p. 173-187.
319. Jakoby, W.B. and D.M. Ziegler, *The Enzymes of Detoxication.* Journal of Biological Chemistry, 1990. **265**(34): p. 20715-20718.
320. Persson, B., et al., *The SDR (short-chain dehydrogenase/reductase and related enzymes) nomenclature initiative.* Chemico-Biological Interactions, 2009. **178**(1-3): p. 94-98.
321. Oppermann, U.C.T., I. Belai, and E. Maser, *Antibiotic resistance and enhanced insecticide catabolism as consequences of steroid induction in the gram-negative bacterium Comamonas testosteroni.* Journal of Steroid Biochemistry and Molecular Biology, 1996. **58**(2): p. 217-223.
322. Cochran, D.G., *Excretion in Insects*, in *Insect Biochemistry and Function*, D.J. Candy and B.A. Kilby, Editors. 1975, Chapman and Hall, London. p. 177-281.
323. Hochstein, P., L. Hatch, and A. Sevanian, *Uric-Acid - Functions and Determination.* Methods in Enzymology, 1984. **105**: p. 162-166.
324. Parks, D.A. and D.N. Granger, *Xanthine-Oxidase - Biochemistry, Distribution and Physiology.* Acta Physiologica Scandinavica, 1986. **126**: p. 87-99.
325. irzykiewicz, H., *Xanthine Oxidase of the Clothes Moth, Tineola Bisselliella, and Some Other Insects.* Australian Journal of Biological Sciences, 1955. **8**: p. 369–377.
326. Komoto, N., *A deleted portion of one of the two xanthine dehydrogenase genes causes translucent larval skin in the oq mutant of the silkworm (Bombyx mori).* Insect Biochemistry and Molecular Biology, 2002. **32**(6): p. 591-597.
327. Sant'Anna, M.R.V., et al., *Gene silencing in phlebotomine sand flies: Xanthine dehydrogenase knock down by dsRNA microinjections.* Insect Biochemistry and Molecular Biology, 2008. **38**(6): p. 652-660.
328. Beedham, C., *The role of non-P450 enzymes in drug oxidation.* Pharmacy World & Science, 1997. **19**(6): p. 255-263.
329. Slansky, F., *Xanthine Toxicity to Caterpillars Synergized by Allopurinol, a Xanthine Dehydrogenase Oxidase Inhibitor.* Journal of Chemical Ecology, 1993. **19**(11): p. 2635-2650.
330. Thompson, S.N., *Trehalose - The insect 'blood' sugar.* Advances in Insect Physiology, Vol 31, 2003. **31**: p. 205-285.
331. Stock, M., et al., *Putative Sugar Transporters of the Mustard Leaf Beetle Phaedon cochleariae: Their Phylogeny and Role for Nutrient Supply in Larval Defensive Glands.* Plos One, 2013. **8**(12).
332. Tugnait, M., et al., *N-oxygenation of clozapine by flavin-containing monooxygenase.* Drug Metabolism and Disposition, 1997. **25**(4): p. 524-527.
333. Ripp, S.L., et al., *Oxidation of cysteine S-conjugates by rabbit liver microsomes and cDNA-expressed flavin-containing mono-oxygenases: Studies with S-(1,2-dichlorovinyl)-L-cysteine, S-(1,2,2-trichlorovinyl)-L-cysteine, S-allyl-L-cysteine, and S-benzyl-L-cysteine.* Molecular Pharmacology, 1997. **51**(3): p. 507-515.
334. Hughes, T.T., et al., *Drosophila as a genetic model for studying pathogenic human viruses.* Virology, 2012. **423**(1): p. 1-5.
335. Napoli, C., C. Lemieux, and R. Jorgensen, *Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans.* Plant Cell, 1990. **2**(4): p. 279-289.

336. Bernstein, E., et al., *Role for a bidentate ribonuclease in the initiation step of RNA interference.* Nature, 2001. **409**(6818): p. 363-366.
337. Sen, G.L. and H.M. Blau, *A brief history of RNAi: the silence of the genes.* Faseb Journal, 2006. **20**(9): p. 1293-1299.
338. Kim, Y.H., et al., *RNA interference: Applications and advances in insect toxicology and insect pest management.* Pesticide Biochemistry and Physiology, 2015. **120**: p. 109-117.
339. Wan, P.J., et al., *RNAi suppression of the ryanodine receptor gene results in decreased susceptibility to chlorantraniliprole in Colorado potato beetle Leptinotarsa decemlineata.* Journal of Insect Physiology, 2014. **63**: p. 48-55.
340. Yang, Y., et al., *RNAi mediated knockdown of the ryanodine receptor gene decreases chlorantraniliprole susceptibility in Sogatella furcifera.* Pesticide Biochemistry and Physiology, 2014. **108**: p. 58-65.
341. Zhang, H., et al., *The identification and characterisation of a new deltamethrin resistance-associated gene, UBL40, in the diamondback moth, Plutella xylostella (L.).* Gene, 2013. **530**(1): p. 51-56.
342. Mukherjee, K., et al., *Neuron-specific protein interactions of Drosophila CASK-beta are revealed by mass spectrometry.* Frontiers in Molecular Neuroscience, 2014. **7**: p. 10.
343. Zimmer, C.T. and R. Nauen, *Cytochrome P450 mediated pyrethroid resistance in European populations of Meligethes aeneus (Coleoptera: Nitidulidae).* Pesticide Biochemistry and Physiology, 2011. **100**(3): p. 264-272.
344. Foley, J.A., et al., *Solutions for a cultivated planet.* Nature, 2011. **478**(7369): p. 337-342.
345. Gompel, N. and E. Schroder. *Drosophila Germline Transformation*. 2015; Available from: http://gompel.org/wp-content/uploads/2015/12/Drosophila-transformation-with-chorion.pdf.
346. Kozak, M., *Point mutations define a sequence flanking the aug initiator codon that modulates translation by eukaryotic ribosomes.* Cell, 1986. **44**(2): p. 283-292.
347. Nishikawa, T., T. Ota, and T. Isogai. *A program for identifying the initiation codons in cDNA sequences*. 2016; Available from: http://atgpr.dbcls.jp/.
348. Salamov, A.A., T. Nishikawa, and M.B. Swindells, *Assessing protein coding region integrity in cDNA sequencing projects.* Bioinformatics, 1998. **14**(5): p. 384-390.
349. Han, W.S., et al., *Residual toxicity and sublethal effects of chlorantraniliprole on Plutella xylostella (Lepidoptera: Plutellidae).* Pest Management Science, 2012. **68**(8): p. 1184-1190.
350. Guo, L., et al., *Sublethal and transgenerational effects of chlorantraniliprole on biological traits of the diamondback moth, Plutella xylostella L.* Crop Protection, 2013. **48**: p. 29-34.
351. Ribeiro, L.M.S., et al., *Fitness costs associated with field-evolved resistance to chlorantraniliprole in Plutella xylostella (Lepidoptera: Plutellidae).* Bulletin of Entomological Research, 2014. **104**(1): p. 88-96.
352. Krueger, S.K. and D.E. Williams, *Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism.* Pharmacology & Therapeutics, 2005. **106**(3): p. 357-387.
353. Usmani, K.A., W.C.G. Chen, and A.J.M. Sadeque, *Identification of Human Cytochrome P450 and Flavin-Containing Monooxygenase Enzymes Involved in the Metabolism of Lorcaserin, a Novel Selective Human 5-Hydroxytryptamine 2C Agonist.* Drug Metabolism and Disposition, 2012. **40**(4): p. 761-771.
354. Yeniceli, D., et al., *Development of a CD-MEKC method for investigating the metabolism of tamoxifen by flavin-containing monooxygenases and the inhibitory*

effects of methimazole, nicotine and DMXAA. Electrophoresis, 2013. **34**(3): p. 463-470.
355. Scharf, M.E., et al., *Catalytic activity and expression of two flavin-containing monooxygenases from Drosophila melanogaster.* Archives of Insect Biochemistry and Physiology, 2004. **57**(1): p. 28-39.
356. Lattard, V., et al., *Cloning, sequencing, and tissue-dependent expression of flavin-containing monooxygenase (FMO) 1 and FMO3 in the dog.* Drug Metabolism and Disposition, 2002. **30**(2): p. 119-128.
357. Wyatt, M.K., et al., *Identification of amino acid residues associated with modulation of flavin-containing monooxygenase (FMO) activity by imipramine: Structure/function studies with FMO1 from pig and rabbit.* Biochemistry, 1998. **37**(17): p. 5930-5938.
358. Ziegler, D.M., *Recent Studies on the Structure and Function of Multisubstrate Flavin-Containing Monooxygenases.* Annual Review of Pharmacology and Toxicology, 1993. **33**: p. 179-199.
359. Cashman, J.R. and J. Zhang, *Human flavin-containing monooxygenases*, in *Annual Review of Pharmacology and Toxicology*. 2006. p. 65-100.
360. Furnes, B. and D. Schlenk, *Evaluation of xenobiotic N- and S-oxidation by variant flavin-containing monooxygenase 1 (FMO1) enzymes.* Toxicological Sciences, 2004. **78**(2): p. 196-203.
361. Hodgson, E., *Induction and inhibition of pesticide-metabolizing enzymes: roles in synergism of pesticides and pesticide action.* Toxicology and Industrial Health, 1999. **15**(1-2): p. 6-11.
362. Bass, C., et al., *Gene amplification and microsatellite polymorphism underlie a recent insect host shift.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(48): p. 19460-19465.
363. Riveron, J.M., et al., *Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector Anopheles funestus.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(1): p. 252-257.
364. Kane, J.F., *Effects of rare codon clusters on high-level expression of heterologous proteins in escherichia-coli.* Current Opinion in Biotechnology, 1995. **6**(5): p. 494-500.
365. Gustafsson, C., S. Govindarajan, and J. Minshull, *Codon bias and heterologous protein expression.* Trends in Biotechnology, 2004. **22**(7): p. 346-353.
366. Zhang, S.P., G. Zubay, and E. Goldman, *Low-usage codons in escherichia-coli, yeast, fruit-fly and primates.* Gene, 1991. **105**(1): p. 61-72.
367. Burgess-Brown, N.A., et al., *Codon optimization can improve expression of human genes in Escherichia coli: A multi-gene study.* Protein Expression and Purification, 2008. **59**(1): p. 94-102.
368. Bogwitz, M.R., et al., *Cyp12a4 confers lufenuron resistance in a natural population of Drosophila melanogaster.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(36): p. 12807-12812.
369. Dong, X.L., et al., *Proteomic and Properties Analysis of Botanical Insecticide Rhodojaponin III-Induced Response of the Diamondback Moth, Plutella xyllostella (L.).* Plos One, 2013. **8**(7).
370. Lee, D.W., et al., *RNA interference of pheromone biosynthesis-activating neuropeptide receptor suppresses mating behavior by inhibiting sex pheromone production in Plutella xylostella (L.).* Insect Biochemistry and Molecular Biology, 2011. **41**(4): p. 236-243.

371. Gong, L., et al., *Testing Insecticidal Activity of Novel Chemically Synthesized siRNA against Plutella xylostella under Laboratory and Field Conditions.* Plos One, 2013. **8**(5).
372. Rinkevich, F.D. and J.G. Scott, *Limitations of RNAi of a6 nicotinic acetylcholine receptor subunits for assessing the in vivo sensitivity to spinosad.* Insect Science, 2013. **20**(1): p. 101-108.
373. Tomalski, M., et al., *Metabolism of Imidacloprid in Workers of Reticulitermes flavipes (Isoptera: Rhinotermitidae).* Annals of the Entomological Society of America, 2010. **103**(1): p. 84-95.
374. Bull, D.L. and R.S. Patterson, *Characterization of pyrethroid resistance in a strain of the german-cockroach (dictyoptera, blattellidae).* Journal of Economic Entomology, 1993. **86**(1): p. 20-25.
375. Langel, D. and D. Ober, *Evolutionary recruitment of a flavin-dependent monooxygenase for stabilization of sequestered pyrrolizidine alkaloids in arctiids.* Phytochemistry, 2011. **72**(13): p. 1576-1584.
376. Wang, R. and Y.D. Wu, *Dominant fitness costs of abamectin resistance in Plutella xylostella.* Pest Management Science, 2014. **70**(12): p. 1872-1876.
377. Khakame, S.K., X.L. Wang, and Y.D. Wu, *Baseline Toxicity of Metaflumizone and Lack of Cross Resistance Between Indoxacarb and Metaflumizone in Diamondback Moth (Lepidoptera: Plutellidae).* Journal of Economic Entomology, 2013. **106**(3): p. 1423-1429.
378. Morra, R., et al., *Functional analysis of the cis-acting elements responsible for the induction of the Cyp6a8 and Cyp6g1 genes of Drosophila melanogaster by DDT, phenobarbital and caffeine.* Insect Molecular Biology, 2010. **19**(1): p. 121-130.
379. Pang, R., et al., *Identification of promoter polymorphisms in the cytochrome P450 CYP6AY1 linked with insecticide resistance in the brown planthopper, Nilaparvata lugens.* Insect Molecular Biology, 2014. **23**(6): p. 768-778.
380. Lewis, S.M. and C.K. Cratsley, *Flash signal evolution, mate choice, and predation in fireflies*, in *Annual Review of Entomology*. 2008, Annual Reviews: Palo Alto. p. 293-321.
381. Bao, W.D., K.K. Kojima, and O. Kohany, *Repbase Update, a database of repetitive elements in eukaryotic genomes.* Mobile DNA, 2015. **6**.
382. Cariño, F.A., et al., *Constitutive overexpression of the cytochrome P450 gene CYP6A1 in a house fly strain with metabolic resistance to insecticides.* Insect Biochemistry and Molecular Biology, 1994. **24**: p. 411-418.
383. Li, X.C., M.A. Schuler, and M.R. Berenbaum, *Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics.* Annual Review of Entomology, 2007. **52**: p. 231-253.
384. Gordenin, D.A., et al., *Inverted dna repeats - a source of eukaryotic genomic instability.* Molecular and Cellular Biology, 1993. **13**(9): p. 5315-5322.
385. Zhao, G.Y., et al., *Evidence for Active Maintenance of Inverted Repeat Structures Identified by a Comparative Genomic Approach.* Plos One, 2007. **2**(2).
386. Meng, Q.S., et al., *A systematic identification of Kolobok superfamily transposons in Trichomonas vaginalis and sequence analysis on related transposases.* Journal of Genetics and Genomics, 2011. **38**(2): p. 63-70.
387. Heffron, F., et al., *Dna-sequence analysis of the transposon tn3 - 3 genes and 3 sites involved in transposition of tn3.* Cell, 1979. **18**(4): p. 1153-1163.
388. Chen, S. and X.C. Li, *Transposable elements are enriched within or in close proximity to xenobiotic-metabolizing cytochrome P450 genes.* Bmc Evolutionary Biology, 2007. **7**.

389. Wilding, C.S., et al., *A cis-regulatory sequence driving metabolic insecticide resistance in mosquitoes: Functional characterisation and signatures of selection.* Insect Biochemistry and Molecular Biology, 2012. **42**(9): p. 699-707.
390. Itokawa, K., et al., *A single nucleotide change in a core promoter is involved in the progressive overexpression of the duplicated CYP9M10 haplotype lineage in Culex quinquefasciatus.* Insect Biochemistry and Molecular Biology, 2015. **66**: p. 96-102.
391. Lamba, J.K., et al., *Genetic contribution to variable human CYP3A-mediated metabolism.* Advanced Drug Delivery Reviews, 2002. **54**(10): p. 1271-1294.
392. Rabenstein, M.D., et al., *TATA box-binding protein (TBP)-related factor 2 (TRF2), a third member of the TBP family.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(9): p. 4791-4796.
393. Chung, H., et al., *Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the Drosophila melanogaster insecticide resistance gene Cyp6g1.* Genetics, 2007. **175**(3): p. 1071-1077.
394. Marks, A.R., U.S. Neill, and American Society for Clinical Investigation, *Science In Medicine: The JCI Textbook Of Molecular Medicine*. 2007: Jones & Bartlett Learning.
395. Tobias, K.M. and S.A. Johnston, *Veterinary Surgery: Small Animal: 2-Volume Set*. 2013: Elsevier Health Sciences.
396. Wittkopp, P.J., B.K. Haerum, and A.G. Clark, *Evolutionary changes in cis and trans gene regulation.* Nature, 2004. **430**(6995): p. 85-88.
397. Overby, L.H., et al., *Characterization of flavin-containing monooxygenase-5 (fmo5) cloned from human and guinea-pig - evidence that the unique catalytic properties of fmo5 are not confined to the rabbit ortholog.* Archives of Biochemistry and Biophysics, 1995. **317**(1): p. 275-284.
398. Parrella, M.P. and J.T. Trumble, *Decline of resistance in liriomyza-trifolii (diptera, agromyzidae) in the absence of insecticide selection pressure.* Journal of Economic Entomology, 1989. **82**(2): p. 365-368.
399. Ferguson, J.S., *Development and stability of insecticide resistance in the leafminer Liriomyza trifolii (Diptera : Agromyzidae) to cyromazine, abamectin, and spinosad.* Journal of Economic Entomology, 2004. **97**(1): p. 112-119.
400. Askari-Saryazdi, G., et al., *Selection for chlorpyrifos resistance in Liriomyza sativae Blanchard: Cross-resistance patterns, stability and biochemical mechanisms.* Pesticide Biochemistry and Physiology, 2015. **124**: p. 86-92.
401. Wang, J., et al., *Analysis on life table of organophosphate-resistant strains of Culex pipiens pallens.* Entomologia Sinica, 1998. **5**(2): p. 159-165.
402. Roush, R.T. and J.A. McKenzie, *Ecological genetics of insecticide and acaricide resistance.* Annual Review of Entomology, 1987. **32**: p. 361-380.
403. Prabhaker, N., et al., *Assessment of cross-resistance potential to neonicotinoid insecticides in Bemisia tabaci (Hemiptera : Aleyrodidae).* Bulletin of Entomological Research, 2005. **95**(6): p. 535-543.
404. Guo, Z.Y., et al., *Orphenadrine and methimazole inhibit multiple cytochrome P450 enzymes in human liver microsomes.* Drug Metabolism and Disposition, 1997. **25**(3): p. 390-393.
405. Nace, C.G., et al., *Effect of methimazole, an FMO substrate and competitive inhibitor, on the neurotoxicity of 3,3'-iminodipropionitrile in male rats.* Fundamental and Applied Toxicology, 1997. **37**(2): p. 131-140.
406. Schlenk, D., *Occurrence of flavin-containing monooxygenases in non-mammalian eukaryotic organisms.* Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology, 1998. **121**(1-3): p. 185-195.

407. Krueger, S.K., et al., *Identification of active flavin-containing monooxygenase isoform 2 in human lung and characterization of expressed protein.* Drug Metabolism and Disposition, 2002. **30**(1): p. 34-41.
408. Grothusen, A., et al., *A convenient method to discriminate between cytochrome P450 enzymes and flavin-containing monooxygenases in human liver microsomes.* Archives of Toxicology, 1996. **71**(1-2): p. 64-71.
409. Zou, Z., C. Eibl, and H.U. Koop, *The stem-loop region of the tobacco psbA 5 ' UTR is an important determinant of mRNA stability and translation efficiency.* Molecular Genetics and Genomics, 2003. **269**(3): p. 340-349.
410. Schrader, L., et al., *Transposable element islands facilitate adaptation to novel environments in an invasive species.* Nature Communications, 2014. **5**.
411. Jordan, I.K., et al., *Origin of a substantial fraction of human regulatory sequences from transposable elements.* Trends in Genetics, 2003. **19**(2): p. 68-72.
412. Shi, L., et al., *Collaborative contribution of six cytochrome P450 monooxygenase genes to fenpropathrin resistance in Tetranychus cinnabarinus (Boisduval).* Insect Molecular Biology, 2016. **25**(5): p. 653-665.
413. Bindu, P., et al., *Toxicological effect of chlorantraniliprole on fat body and haemolymph metabolism in the final instar larvae of silk insect, bombyx mori L.* International Journal for Science and Nature, 2015. **6**(2): p. 177-182.
414. *RIS Remarks*. 2016, Rothamsted Research Insect Survey.
415. Shah, S.M. and J.D. Windass, *Methods for screening insecticides*. 2005, Google Patents.
416. Shah, S.M., R.J. Lind, and J.D. Windass, *Methods*. 2004, Google Patents.
417. Gaddamidi, V., et al., *Metabolism of C-14 Chlorantraniliprole in the Lactating Goat.* Journal of Agricultural and Food Chemistry, 2011. **59**(4): p. 1316-1323.