Drosophila, Metabolomics and Insecticide Action

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Author's Declaration

The research reported within this thesis is my own work except where otherwise stated, and has not been submitted for any other degree

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Abstract

The growing problem of insecticide resistance is jeopardising current pest control strategies and current insecticide development pipelines are failing to provide new alternatives quickly enough. Metabolomics offers a potential solution to the bottleneck in insecticide target discovery. As a proof of concept, metabolomics data for permethrin exposed *Drosophila melanogaster* was analysed and interpreted. Changes in the metabolism of amino acids, glycogen, glycolysis, energy, nitrogen, NAD^+, purine, pyrimidine, lipids and carnitine were observed along with markers for acidosis, ammonia stress, oxidative stress and detoxification responses. Many of the changed metabolites and pathways had never been linked to permethrin exposure before. A model for the interaction of the observed changes in metabolites was proposed.

From the metabolic pathways with the largest changes, candidate genes from tryptophan catabolism were selected to determine if the perturbed pathways had an effect on survival when exposed to permethrin. Using QPCR it was found that all genes in the entire pathway were downregulated by permethrin exposure with the exception of *vermilion* suggesting an active response to try and limit flux through tryptophan catabolism during permethrin exposure. Knockdown of the tryptophan catabolising genes *vermilion*, *cinnabar* and *CG6950* in *Drosophila* using whole fly RNAi resulted in changes in susceptibility to permethrin for both topical and oral routes of exposure. Knockdown of the candidate genes also caused changes in susceptibility when the insecticides fenvalerate, DDT, chlorpyriphos and hydramethylnon were orally administered. These results show that tryptophan catabolism knockdown has an effect on surviving insecticides with a broad range in mode of action. Symptoms that occur in *Drosophila* during exposure to the different insecticides were also noted.

To gain further understanding into the mechanisms affecting survival, tissue specific knockdown was performed revealing tissue and gender specific changes in survival when *vermilion*, *cinnabar* and *CG6950* are knocked down. Metabolomics was performed on the knockdown strains to determine the efficacy of the knockdowns on tryptophan catabolism and to identify any knock-on effects. The results indicate that tryptophan metabolite induced

perturbations to energy metabolism and glycosylation also occur in *Drosophila* along with apparent changes in the absorption of ectometabolites. As the knockdown of *vermilion*, *cinnabar* and *CG6950* tended to result in reduced susceptibility to insecticides, they would make poor targets for insecticidal compounds, however, they may be the first examples of genes that are not directly involved in insecticide metabolism or cuticle synthesis that increase insecticide tolerance in *Drosophila*.

As the first metabolomics data set showed evidence for oxidative stress during permethrin exposure, preliminary work was begun for identifying the tissue specificity and timing of oxidative stress in both Dipterans and Lepidopterans using *Drosophila* and *Bombyx mori* as models. In *Drosophila* oxidative stress did not begin immediately suggesting that the insecticide itself is not a cause, however, a rapid increase in oxidative stress occured over a six hour period after a day of oral exposure implicating catabolites of permethrin. *Bombyx* were highly susceptible to permethrin showing oxidative stress in the Malpighian tubule and silk gland when exposed.

This study has shown that metabolomics is highly effective at identifying pathways which modulate survival to insecticide exposure. It has also brought insight into how insecticide induced pathology may cause death. Data has also been generated which could help characterize the putative transaminase CG6950.

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Definitions/Abbreviations

CNS = Central nervous system

CYP = Cytochrome P450

DDT = dichlorodiphenyltrichloroethane

DNA = Deoxyribonucleic acid

GST = Gutathione S-Transferase

Kyn = Kynurenine

LD_{50} = Lethal dose (50%)

LT_{50} = Lethal time (50%)

NMR = Nuclear Magnetic Resonance

PCR = Polymerase Chain Reaction

QPCR = Quantitative PCR

RNA = Ribonucleic acid

RNAi = RNA interference

roGFP = Redox Sensitive Green Fluorescent protein

ROS = Reactive Oxygen Species

Trp = Tryptophan

UAS = Upstream Activating Sequence

XA = Xanthurenic acid

Chapter 1 - Introduction

1 Arthropods as pests

1.1 The success of arthropods

Arthropods are a highly successful phylum of invertebrates occupying a broad range of aquatic and terrestrial parasitic, herbivorous and carnivorous niches including those that have an impact to human activity. Of the ~8.7 million species estimated to exist on earth (Mora et al., 2011) ~6.1 million (~70%) are arthropods (Hamilton et al., 2013). This work will mainly focus on the arthropod class Insecta as it contains the majority of major pest species and the impacts of mite, tick and sea lice pests are also discussed.

Insects are capable of exploiting niches with extremes of temperature ranging from above 60°C (Wehner et al., 1992) to below -60°C (Baust and Miller, 1970), scarcity of water (Cloudsley-Thompson, 2001), high salinity (Herbst and Bradley, 1988) and low oxygen (Nagell and Landahl, 1978). The lifetime fecundity of insect females, although varying between individual species, can range from hundreds, eg. *Manduca sexta*, to over a million, eg. *Apis mellifera*, eggs meaning lone fertilized females can found entire populations (Awad et al., 2013). Insects are also highly adaptable being able to quickly establish themselves in new geographical ranges or adjust to changes in local climate (Musolin, 2007; Roy and Brown, 2015) and exploit new food sources and host ranges (de la Paz Celorio-Mancera et al., 2013; Henniges-Janssen et al., 2011). This versatility has inevitably led to the occupation of niches that are detrimental to the human economy, food supply and heath.

1.2 Pests as a destroyer of property

During their life cycle insects need to consume organic materials to accumulate biomass while finding or making safe refuges to carry out various stages of their development and proliferation. This means that irreplaceable fabrics, documents, furniture and zoological collections of historic importance are constantly under threat from insect pests that eat animal or plant products and remains eg. *Anobium punctatum*, *Stegobium paniceum* and various members of

the families Dermestidae and Tineidae (Brimblecombe and Lankester, 2013; Rajendran and Hajira Parveen, 2005). Structures and buildings are susceptible to damage by boring and tunnelling species, like *Alphitobius diaperinus* which causes damage to insulation and wooden structures when it seeks a place to pupate causing annually over $16 million in damages to the poultry industry in the US state of Virginia alone (Geden and Carlson, 2001; Turner Jr., 1985). Termites and carpenter ants each cause multiple million dollars of damage to buildings annually in the US and can make them structurally unsound due to their nesting habits (Hansen, 1993; Su and Scheffrahn, 1998) however, the economic impacts of those and species occupying similar niches globally has not been documented.

Insect pests also cause losses for harvests which are of economic importance. In North America carpenter ants alone render over 10% of spruce, balsam fir and white cedar timber unfit for sale (Hansen, 1993) while, even with insecticide use, insect pests of cash crops such as tobacco, cotton, cacao, coffee and sugarcane cause over 10% pre-harvest yield losses costing multiple billions of dollars annually (Oliveira et al., 2014). Additional post-harvest losses may then be caused by stored product pests (Rajendran and Hajira Parveen, 2005) like *Ephestia elutella* and *Lasioderma serricorne* which globally destroy approximately 1% of stored tobacco costing over $300 million annually (Ryan, 1999).

1.3 Pests as a threat to food security

1.3.1 The problem of a growing human population

The human population has exceeded 7 billion and is expected to surpass 10 billion by the year 2100 (median estimate) however, predicted global food production using current agricultural methods is expected to only be able to support at most a population of 8 billion or even less if developing countries switch to more nutritious diets (Sakschewski et al., 2014). Even when including the predicted average 0.66% (0.85-0.49% and even less for cereal crops) annual increase in yield to food crops from improved cultivars, technology and logistics (Bouwman, 1997), the predicted carrying capacity of the planet may fall short unless losses from pest species can be reduced.

1.3.2 The scale of yields lost to arthropod pests

Arthropod pests are in direct competition with humanity for the consumption of the same food sources. It is estimated that in first world countries between 9-16% of pre-harvest food crops are lost to arthropod pests annually (Culliney, 2014; Oliveira et al., 2014) while for individual crops and third world countries this number can easily exceed 50% (Abate et al., 2000; Culliney, 2014). There is then a further 3-10% loss post-harvest due to arthropod stored product pests (Culliney, 2014; Rajendran and Hajira Parveen, 2005). Mite infestations, although not as common as insect infestations, can cause equal damage to crops and are often treated with the same pesticides (Culliney, 2014; Hummel et al., 2009) however, average annual food losses globally able to be attributed solely to mites has not been reviewed.

Livestock is also subject to losses caused by parasitic arthropods. Parasitic myiasis inducing and blood feeding arthropods cause livestock yearly weight gain reductions of up to 18% and reduce productivity of non meat commodities, like leather, milk and wool (Kamut and Jezierski, 2014). This occurs though both blood loss and decreased grazing as a result of irritation and the triggering of defence or avoidance behaviours (Kamut and Jezierski, 2014). The Dipteran families *Culicidae*, *Ceratopogonidae*, *Muscidae*, *Oestridae*, *Simuliidae* and *Tabanidae* each cause annual losses worth several $100 million with *Stomoxys calcitrans* alone costing the North American cattle industry $1 billion (Kamut and Jezierski, 2014). Blood feeding arthropod ectoparasites like cattle lice cause the US dairy industry estimated losses worth over $125 million (Kaufman, 2002) while cattle ticks in Brazil cause losses worth over $2 billion (Guerrero et al., 2006). The productivity of egg laying poultry is also affected by parasitism with infestations of parasitic mites like *Dermanyssus gallinae* causing reductions in productivity of 2-15% (Cencek, 2003). Arthropod parasites also impact on aquaculture with sea lice, parasitic copepods, globally causing approximately 9% losses to farmed fish (Johnson et al., 2004).

In addition to direct damage to food sources arthropod pests can also act as vectors for diseases of crops and livestock that further reduce yields. Insects, especially sap sucking species, and mites are known to transmit numerous plant pathogenic virus, bacteria, spiroplasm, phytoplasma, fungal and nematode

species (Hummel et al., 2009; Nagaraju et al., 2015; Škorić et al., 2014; Zhao et al., 2014). Haematophagous insects, mites, ticks and sea lice also transmit many viruses, bacteria and helminth associated diseases of livestock (Johnson et al., 2004; Kamut and Jezierski, 2014; Miller et al., 2013). In the USA over $14.6 billion in food crop yields can be attributed to honey bee pollinators (Morse and Calderone, 2000) so diseases spread by parasitic mites cause indirect yield losses to food crops by colony collapse disorder (Forsgren et al., 2009).

1.4 Vectors of disease as a threat to human health

1.4.1 The variety of arthropod transmitted diseases

Diseases with arthropod vectors are not limited to just farm animals with over half the human population at risk of several fatal and debilitating human diseases of global importance (WHO, 2014). There are numerous bacterial, viral, protozoan and filarial diseases spread by the bites and faeces of haematophagous insects, mites and ticks (Badiaga and Brouqui, 2012; Dantas-Torres et al., 2012; Krenn and Aspock, 2012; Watt and Parola, 2003; WHO, 2014). As these diseases are not the focus of this work, the distribution and impact of only a few examples namely malaria, dengue fever, Chagas disease, lyme borreliosis, scrub typhus and bubonic plague are briefly discussed.

1.4.1.1 Malaria

Malaria is an *Anopheles* mosquito transmitted disease caused by various protozoans of the genus *Plasmodium* with over 3.4 billion people at risk of infection and ~627,000 deaths from ~207 million cases in 2012 (WHO, 2014). Although once a serious disease, malaria incidence is steadily declining with the number of deaths globally decreasing due to international prevention and control strategies (Hay et al., 2004; WHO, 2014) while novel vaccines and transmission inhibiting pharmaceuticals (Baragana et al., 2015; Moorthy and Okwo-Bele, 2015) may even make eradication possible.

1.4.1.2 Dengue fever

The dengue virus is transmitted by *Aedes* mosquitoes and causes dengue fever with more than 100 million people infected annually and over 2.5 billion people at risk of infection (WHO, 2014). Dengue fever has no vaccine or cure but is seldomly fatal with only 2.5% mortality (WHO, 2014) however, it is highly debilitating resulting in a high economic cost from hospitalization and absenteeism (Halasa et al., 2012). Each case of dengue causes a direct loss of 7.2 man-days productivity but with the inclusion of the labour involved in the care and treatment of patients the figure is raised to 30.5 man-days lost productivity (Halasa et al., 2012).

1.4.1.3 Chagas disease

Chagas disease caused by *Trypanosoma cruzi* and is spread through the faeces of triatomine bugs meaning infection can occur both through direct contact with the vector and through food contamination (WHO, 2014). Although only ~10 million people are infected at any one time the disease is problematic to control because there are over 150 species of triatomine bugs that can act as vectors which are easily transmitted and are found globally living in close proximity to humans and there are also numerous animal reservoirs for the disease (WHO, 2014).

1.4.1.4 Lyme borreliosis

Lyme borreliosis is a bacterial disease spread by *Ixodes* ticks and is prevalent throughout the northern hemisphere with over 50,000 cases annually in Europe (Dantas-Torres et al., 2012) while in the USA there are 7.9 cases per 100,000 people (WHO, 2014).

1.4.1.5 Scrub typhus

Scrub typhus caused by the bacterium *Orientia tsutsugamushi* is a re-emerging disease that currently infects ~1 million people a year however over 1 billion people are at risk and antibiotic resistant strains are emerging (Watt and Parola, 2003). The disease has a high mortality rate, with 10-20% of patients dying even with treatment (Astrup et al., 2014), and is spread by several vectors including fleas, lice, ticks and trombiculid mites (Watt and Parola, 2003).

1.4.1.6 Bubonic plague

The bacteria *Yersinia pestis* causes bubonic plague and is transmitted though the bites of fleas and lice (Badiaga and Brouqui, 2012; Perry and Fetherston, 1997). Bubonic plague is a re-emerging disease with epidemics being able to spread rapidly causing up to 60% mortality and wiping out 10-15% of affected populations (Perry and Fetherston, 1997). The use of antibiotics and public health measures have reduced the number of annual plague cases to ~1,600 globally however, rodent hosts that act as endemic reservoirs are widespread on all continents except Australia making eradication impossible (Perry and Fetherston, 1997) and multidrug resistant strains have also emerged that are unaffected by all current antibiotic treatments (Galimand et al., 1997) meaning large outbreaks may occur in the near future.

1.4.2 The risks posed to non endemic countries by disease and vector transmission and climate change

Many countries where certain arthropod vectored diseases are absent are at risk of those diseases establishing themselves and becoming endemic. When native species can act as vectors for foreign diseases there is a risk posed by infected travellers, whether human or animal, inoculating local populations. Examples of this has occurred with Crimean-Congo hemorrhagic fever in Europe which is spread by *Hyalomma* ticks that are distributed globally up to 50° degrees latitude (Maltezou and Papa, 2010). The introduction of new vectors that occupy different niches can allow the spread of diseases into ranges that were previously unable to be occupied. An example of this is the introduction of the cold and drought tolerant mosquito species *Aedes albopictus* to Europe allowing dengue, which was previously limited to south European climates suitable for *Aedes aegypti*, to spread further north (Medlock et al., 2012). There can also be the introduction of both vectors and diseases to areas previously not at risk as occurred in 1999 with the West Nile Virus becoming established in North America, quickly adapting to be vectored by *Culex* mosquitoes that had been introduced by shipping centuries earlier (Fonseca et al., 2004; Kilpatrick, 2011). Global warming and climate change which are indirect consequences of human activity have the potential to alter ranges that are habitable for vectors (Rogers and Randolph, 2000) while the creation of favourable vector breeding conditions

outside native ranges can be a direct result of human activity (Nawrocki and Craig, 1989).

2 Control methods for pest species

2.1 Monitoring

In order to implement any effective integrated pest management strategy monitoring is required to provide both a criteria for when to apply which pest control programs and to supply feedback on how effective such programs are. Parameters monitored include the number of pest species present, pest species distribution, pest population densities, the prevalance of resistance associated alleles, the abundance of natural predators and pathogens, the potential impact of pest damage to yield or health if left uncontrolled, estimating yield losses due to damage already done, costs taken to control the pest and the environmental impact of control strategies (Archer and Bynum, 1993; Aukema et al., 2000; Harrington et al., 2013; Henson and Stark, 1959; Kovach et al., 1992; Liu and Bjelland, 2014; Poligui et al., 2014; Wondji et al., 2012).

2.2 Quarantine

As there are many habitats where opportunistic invasive species would thrive, there is a need to quarantine transported goods and products that can carry dormant or active stages of pest species. Methods of quarantine include both regional and international import and transport bans, destruction of potentially contaminated materials and decontamination in the form of fumigation, steam cleaning and irradiation of materials (Ferrier, 2010; Reaser et al., 2008; Scheel, 2009). Examples of materials which require quarantine are wooden packing materials that are at risk of harbouring wood boring species, vehicles and building materials which can carry both insect and plant pests and food products which can carry crop pests (Ferrier, 2010; Reaser et al., 2008; Scheel, 2009). The effectiveness of quarantine is however limited by trade volumes, the frequency of contamination by pest species and the interception rate of quarantined items being transported (Bacon et al., 2012).

2.3 Habitat destruction

One method to control pest populations is to remove locations that facilitate breeding making the habitat unsuitable for large pest populations. This is commonly practiced with mosquito control programs where breeding sites are drained (Yohannes et al., 2005). A similar tactic is applied to crop pests by crop rotation (Sexson and Wyman, 2005) and destruction of host plants after harvest (Yang et al., 2005) removing the reliable supply of host plants needed to establish large pest populations. Tillage of the soil buries plant remains removing the availability to terrestrial herbivore attack while preserving soil nitrogen and does damage to or exposes to predation any subterranean stages of development for some pest species however, it also reduces the diversity of natural predators making the benefits of tillage both crop and pest species specific (Blumberg and Crossley, 1983).

2.4 Physical barriers

A common strategy to prevent pest attack is to place physical barriers that the pest cannot overcome between pest populations and any materials or people that are at risk. Since ancient times harvested grains and seeds have been stored in containers or structures that are sealed with mud or dung preventing external pest attack (Karthikeyen et al., 2009). The construction of physical barriers has proven highly effective against termites where aluminium particles and mesh of the correct size can prevent the subterranean spread and attack of wooden structures (Su and Scheffrahn, 1998) while the use of plastic collars around wooden posts can prevent damage done by *Alphitobius diaperinus* beetle larvae and also be used for trapping (Geden and Carlson, 2001). Physical barriers also have an important role in disease prevention with mosquito nets, which may also be impregnated with insecticide, being a highly effective and low cost approach to malaria control (Alonso et al., 1991; Bradley et al., 1986).

2.5 Baited traps

Volatile chemicals play an important role for insects to find food sources and mates which are highly species specific, often causing strong chemotaxis (Bruce et al., 2005; Sakurai et al., 2011). Chemical attractants can therefore be used

to lure pest species away from potential mates or into traps from which they cannot escape. The use of baited traps has proven highly successful for several Lepidopteran and Coleopteran species eg. (Beroza and Knipling, 1972; Weslien and Lindelöw, 1990). The planting of preferred hosts as trap crops can lure pest species away from the crops of interest reducing damage, facilitating efficient chemical population control or encourage natural predators (Hokkanen, 1991). For subterranean pest species bait tubes sunk into the ground can be used for both monitoring and control (Su and Scheffrahn, 1998).

2.6 Biological control methods

Like all species, invertebrate pests are subject to predation, disease, parasitism and intraspecific competition which can be utilised by humanity to control pest populations. Natural predators can provide effective control of some pest species like the predatory mite *Amblyseius swirskii* against spider mites, whiteflies and thrips (Messelink et al., 2010) with arthropod predator populations able to be encouraged by modifying farming practices (Blumberg and Crossley, 1983; Hokkanen, 1991). Similar degrees of pest control can also be achieved using parasitic wasps (Zeddies et al., 2001), fungi (Fang et al., 2012), bacteria (Goldberg and Margalit, 1977) and viruses (Arthurs et al., 2005).

The competition for mates and breeding sites within a pest species can also be exploited by the continued release of sterile adults (usually males) that then outnumber and displace fertile competitors and the mated females produce no viable offspring (Vreysen et al., 2000). The “sterile insect technique” has mostly been conducted with irradiation sterilized males, e.g. (Vreysen et al., 2000) but transgenic pest species offer the opportunity for higher efficacy by having constructs that cause female-specific lethality which are linked to genes that confer a selective advantage, usually insecticide resistance, to male progeny (Schetelig and Handler, 2013). This means that the majority of surviving males after insecticide applications will be unable to produce female offspring further diminishing the reproductive capacity of the population while increasing competition for any wild type males.

2.7 Insecticides

Although other control methods can be effective, they often have the disadvantages of taking several months or generations of the pest to reduce pest populations (Vreysen et al., 2000) and may not prevent immediate damage (Arthurs et al., 2005) so the first and primary response to arthropod pest populations is usually the application of pesticides (referred to as insecticides if used on insects and acaricides if used on mites and ticks even if they are the same compound). An insecticide can be considered any compound (proteins included) that causes insect mortality or greatly reduced fitness, preferably reducing pest populations selectively within a short timescale. Insecticides are even used as a precautionary measure in quarantine procedures to avoid the potential transmission of pest species (Gratz et al., 2000; Scheel, 2009).

2.7.1 History of insecticidal agents

2.7.1.1 Traditional pest control methods

Many of the traditional control methods for arthropod pests used by nomadic hunter gatherers and rural farmers of third world countries are believed to have ancient origins (Hakbijl, 2002). The use of solarisation (baking in the sun) and smoke curing to kill grain pests and storage under vegetable oils or with insect repellent or toxic leaves, like neem, to prevent post-harvest losses are still commonly practiced in African countries (Golob et al., 2007). The use of ash, sand and diatomaceous earths to kill both stored product pests and repel arthropod parasites of the human body have records dating back to the Old Kingdom of ancient Egypt but is believed to be even older possibly dating as far back as the stone age (Hakbijl, 2002).

2.7.1.2 The era of inorganic pesticides

The more intensive farming practices that occurred during the 1800s led to the need for efficient insecticides. These compounds tended to be highly toxic inorganics such as elemental sulphur (1822), mercuric chloride (1822), various sulphur compounds (1860) and lead and calcium arsenates (1892) often solvated in kerosene (1865) or petroleum (1877) (O'Kane, 1932). Although not an inorganic pesticide, hydrocyanic acid (HCN) was developed for use as a fumigant

in 1886 (O'Kane, 1932). The problem with these compounds was that they were not selective also being detrimental to not only farm workers and consumers (Frost, 1960) but the crops themselves (Ginsburg, 1926) let alone other non-target species. Despite the disadvantages, some inorganic pesticides were still used until the mid 1950s (Frost, 1960).

2.7.1.3 The era of insecticide discovery

Beginning with the discovery of the insecticidal properties of DDT (Läuger et al., 1944) which was heralded as the first synthetic "organic" insecticide, winning Paul Müller the Nobel prize in 1948, there was an era until the mid 1990s where many new families of compounds with insecticidal properties and different modes of action were discovered (Sparks, 2013). The advantage of these new insecticides were low costs (Wirtz et al., 2009) and far greater selectivity (Casida, 2010; Casida and Durkin, 2013) compared to the inorganic insecticides. The availability of these insecticides enabled systematic disease vector eradication programs to be started (Andrews, 1950), a process that is still continuing (WHO, 2014).

2.7.1.4 No new commercialized modes of action

There is a problem however, in that no new mode of action has been commercialized for the last twenty years and it has been over thirty years since an insecticide was approved by World Health Organization for vector control (David et al., 2013). The research and development costs of developing new insecticides against known targets are also increasing with incremental numbers of compounds having to be screened for each product developed (Sparks, 2013), a problem well known in pharmacology (Sharma and Tan, 2013). The identification of new targets using traditional methods is made difficult by the mode of action often only being discovered after an insecticide targeting it has already been developed, not being able to design sufficiently selective or effective compounds targeting obvious pathways and the prohibitively high costs of random screening (Cong et al., 2012). There is also the problem of the increasing number of pest species with resistant populations (Hardy, 2014; Sparks, 2013) and the increasing frequency of new resistant populations arising (Hardy, 2014), combined with legislation restricting the use of existing

insecticides e.g. (Gross, 2013), limiting the choice of options for treating pest outbreaks. This means the discovery of both new modes of action and new marketable insecticides is a priority.

2.7.2 Modes of action

2.7.2.1 Neurotoxins

Of the 26 modes of action classified by the Insecticide Resistance Action Committee (http://www.irac-online.org/modes-of-action/) 11 function by targeting components of the CNS. The insecticides with these modes of action function through the disruption of normal neurotransmission by either preventing neural excitation (receptor, chloride and sodium channel antagonists) or induce hyperexcitation leading to the depletion of neurotransmitters (Feng et al., 1992) (receptor, chloride and sodium channel agonists and antagonists of neurotransmitter recycling pathways, like acetylcholine esterase). It is estimated that approximately 74% of all insecticides sold fall into this category (Sparks, 2013) with the carbamates and organophosphates inhibiting acetylcholinesterase (Casida and Quistad, 2004; Watt and Parola, 2003), neonicotinoids and spinosyns being agonists of the acetylcholine receptor (Watson et al., 2010; Zhang et al., 2000) and the pyrethroids and organochlorines which are agonists of the voltage gated sodium channel (Breckenridge et al., 2009; O'Reilly et al., 2006).

2.7.2.2 Cuticle abrasives and lipid sorbtives

The integrity of the insect cuticle is vital to prevent unsustainable water loss (Noh et al., 2015). Sorbtive dusts whether naturally occurring, like ashes, chalk and diatomaceous earths, or manmade, like silica aerogels, strip lipids from the insect cuticle while other crystalline silicate dusts damage the cuticle by abrasion resulting in increased water loss that leads to death (Golob, 1997; Hakbijl, 2002). Although slow acting, insecticidal dusts are often chemically inert offering a long term protection that other insecticides cannot (Golob, 1997).

2.7.2.3 Mitochondrial inhibitors

The electron transport chain of aerobic respiration, which takes place in mitochondria, is indispensible for eukaryotic life so all four mitochondrial complexes, ATP synthase and the proton gradient are targets for insecticides with each being considered a separate mode of action. The insecticides rotenone, pyflubumide, hydramethylnon, phosphine and chlorfenapyr are just some examples of compounds that target oxidative phosphorylation in insects (http://www.irac-online.org/modes-of-action/).

2.7.2.4 Chitin synthesis disrupters

The polysaccharide chitin is an integral part of the insect cuticle conferring tensile strength and flexibility (Kramer and Koga, 1986; Lillywhite and Maderson, 1988). As chitin is part of the exoskeleton of insects it has roles in movement, growth, desiccation and acts as a physical barrier against infection and damage (Kramer and Koga, 1986). Since mammals do not need an exoskeleton, which is vital to insects, chitin synthesis makes a good selective target for insecticides (Matsumura, 2010; Uchida et al., 1985). There are many families of chitin synthesis inhibiting insecticides (Merzendorfer, 2013) however the targets of most are still unidentified with the exceptions of the pyrimidine nucleoside peptides which inhibit chitin synthase (Merzendorfer, 2013) and benzoylureas which are known to target the sulfonylurea receptor (Matsumura, 2010). Disruption of chitin synthesis is usually only lethal if exposed in pre-adult stages of development, although some are ovicidal, resulting in abortive molting, slippage of cuticle plates, an inability to shed pieces of old cuticle and malformation of the cuticle (Matsumura, 2010; Merzendorfer, 2013; Uchida et al., 1985), with survivors that manage reach adulthood still showing numerous disabilities (Belinato et al., 2009).

2.7.2.5 Growth regulators

Similar to chitin synthesis inhibition, the perturbing of hormone signalling pathways involved in ecdysis also have detrimental effects on the success of molting (Tarlochan S. Dhadialla et al., 1998). Analogs of the juvenile hormone suppress molting, preventing adulthood from being reached as well as inducing sterility (Tarlochan S. Dhadialla et al., 1998). The analogs of the molting

hormone ecdysone cause ceasation of feeding and premature fatal molting while adults suffer from impaired fertility (Tarlochan S. Dhadialla et al., 1998). Currently there are no known compounds that act as antagonists for the juvenile hormone and ecdysone receptors. As there is crosstalk between the two signalling pathways (Kayukawa et al., 2014) it is likely the two modes of action would interact however this has never been investigated.

2.7.2.6 Antifeedants

In insects, the presence of phagostimulants (Schoonhoven and Van Loon, 2002) and phagorepellants (Ishikawa, 1966) in potential food sources are detected by receptors in the maxilla and processed by the central nervous system in combination with internal indicators of nutrition in the form of mechanosensors (Bernays and Chapman, 1973) and endogenous factors, like neuroactive peptides (Morooka et al., 2012), resulting in a decision of whether to feed or not. Chemicals that inhibit these hunger inducing pathways can be used as insecticides that result in reduced damage to materials and crops used as food sources because pest species are either forced to relocate to other hosts or suffer mortality due to starvation (Isman, 2002; Nachman and Smagghe, 2011).

2.7.2.7 Other modes of action

Other recognised modes of action include non-specific multisite targeting insecticides, like the borates, and various taxon specific pesticides e.g. etoxazole, cryomazine and the tetronic acid derivatives. There are also proteins with insecticidal properties, the most famous being the Cry and Cyt toxin of *Bacillus thuringiensis* (Bravo et al., 2007), often originating from pathogenic bacteria and fungi, plants and arachnids (Kelemu et al., 2004; King and Hardy, 2013; Ortiz-Urquiza et al., 2009; Tao et al., 2006). These insecticidal proteins can be directly expressed in transgenic crops conferring resistance against pests (Tianpei et al., 2015) or fused to carrier proteins that facilitate uptake by the gut (Down et al., 2006).

As neuropeptides play an important timing dependent role in homeostasis, neuropeptide mimics have potential as insecticidal agents. One approach is to make degradation resistant cyclic peptides containing the functional motifs (Gilon et al., 1997) however, small cyclic peptides that can efficiently pass the

epithelial barriers still need to be designed. Alternatively, the use of chemical analogs that can overcome the absorption and degradation problems also has potential (Nachman and Smagghe, 2011).

2.7.3 Market prevalence

The global insecticide market is estimated to be worth approximately €7.8 billion with Japan, the USA, India, Brazil and China being the largest consumers (Wirtz et al., 2009). Until recently the neonicotinoids had the largest market share of 26% (Sparks, 2013) however there may be a drop in sales caused by recent changes in regulation (Gross, 2013). The other largest single family insecticide groups by sales are the pyrethroids (17%), the organophosphates (13%), the carbamates (6%), avermectins (6%) and fiproles (5%) (Sparks, 2013). Insecticides that target insect growth account for 5% while other non-pyrethroid sodium channel modulators make up 12% of the market (Sparks, 2013). Dedicated acaricides and biopesticides constitute a small portion of the market with 4% and 3.5% of sales respectively (Glare et al., 2012; Sparks, 2013).

3 The pyrethroids and permethrin

3.1 History of pyrethroids

The use of dried and powdered *Chrysanthenum roseum* and *Chrysanthenum cinerariaefolium* flowers, then known as “Persian Insect Powder”, for insect control has been practiced for around two millennia (Housset and Dickmann, 2009) with active cultivation of *C. cinerariaefolium* since 1694 (Katsuda, 2012). It was only around 1840 that the insecticidal activity of “Pyrethrum Powder” was confirmed and applied as a mosquito repellent in 1890 (Katsuda, 2012). By 1908 petroleum extracts of dried *C. cinerariaefolium* flowers were prepared and used as oil based sprays for indoors (Katsuda, 2012) with the first recorded agricultural use occurring in 1923 at a French vineyard (Glassford, 1930) however, the low photostability and high costs made this an exception. The active compounds in pyrethrum, pyrethrin I and II were first isolated in 1924 by Staudinger and Ruzicka and several analogs with exchanged alcohol motifs made however these also lacked photostability (Arlt et al., 1981).

Before the beginning of World War II over 70% of *C. cinerariaefolium* was produced by Japan (Glassford, 1930; Katsuda, 2012) which led to research into the chemical synthesis of pyrethroids during the war when import was not possible (Housset and Dickmann, 2009). The first synthetic pyrethroid was allethrin developed in 1949 (Schechter et al., 1951) however, it was only during the late 1960s that the Elliot group at Rothamsted identified that the isobutenyl and furan motifs of pyrethrins were responsible for the lack of photostability (Casida, 2010). Independently phenothrin had been developed where the alcohol group of pyrethrin I was replaced with 3-phenoxybenxyl alcohol which conferred increased photostability (Fujimoto et al., 1973) and this was then improved by the substitution of the methyls of the isobutenyl group with chlorides leading to the creation of permethrin in 1972 (Elliott et al., 1973). The photostablity of permethrin allowed the widescale use of pyrethroids in outdoor environments and paved the way for discovering other members of the pyrethroid family of compounds including the more toxic cypermethrin and deltamethrin which has stereospecific crystallization (Casida, 2010).

3.2 Chemical synthesis

There are many routes to synthesize derivatives of chrysanthemic acid which are the pharmacore of most pyrethroids (reviewed in Arlt *et al.*, 1981 (Arlt et al., 1981)). The original route to synthesize permethric acid was based on the sysnthesis of chrysanthemic acid which involved the reaction of 2,5-dimethyl-2,4-hexadiene or the chlorinated derivative with a diazoacetic ester (usually ethyl) (Arlt et al., 1981). The 1,1-Dichloro-4-methyl-1,3-pentadien was synthesised from 3-methyl-1-butene by the addition of carbon tetrachloride (CCl_4) (Arlt et al., 1981). Modern industry uses the prenol route of synthesis (**Figure 1-1**) (UK Patent, 1979, 17 Jan, GB2000764 A) (or variation thereof) using prenol and 1,1,1-triethoxyethane as starting materials. Under basic conditions the reagents undergo Johnson-Claisen orthoester rearrangement to form the olefin ethyl 3,3-dimethyl-4-pentenoate which is then subjected to haloalkane addition by CCl_4 using $FeCl_2$ and amines as catalysts to form ethyl 4,6,6,6-tetrachloro-3,3-dimethylhexanoate. Cyclization then occurs under alkaline conditions by side chain dehydrohalogenation before using sodium hydroxide to hydrolyse the ester forming permethric acid. Permethric acid is then

chlorinated using thionyl chloride in n-hexane and reacted with 3-phenoxybenxyl alcohol before the resulting permethrin is precipitated.

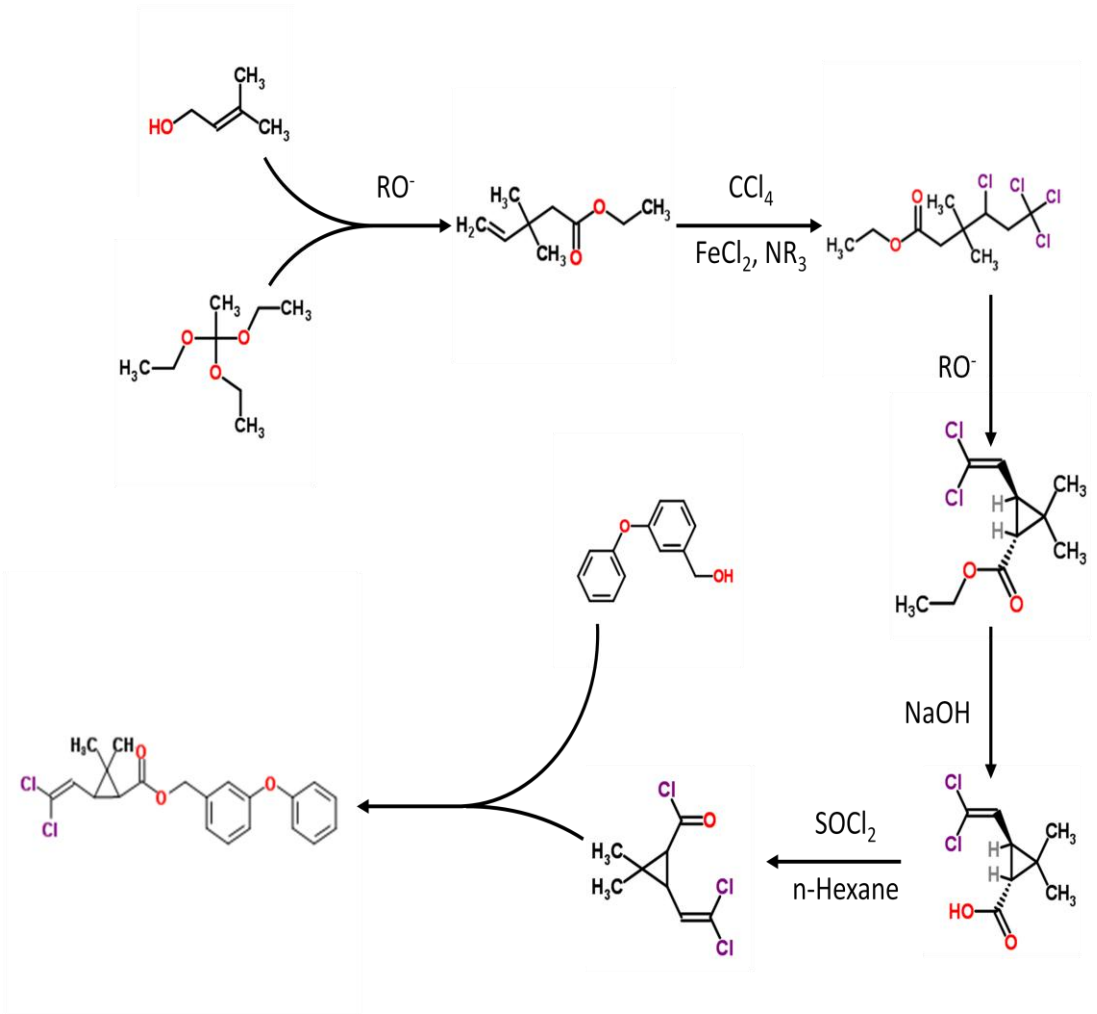

Figure 1-1. The prenol route of permethrin synthesis. R = alkyl group.

3.3 Primary target

The primary target of permethrin and other pyrethroids is the voltage gated sodium channel (O'Reilly et al., 2006). The voltage gated sodium channel consists of four domains with internal homology, each having six membrane spanning helixes with an elongated linker between S5 and S6 called a P-loop that acts as an ion selectivity filter (Goldin, 2003; O'Reilly et al., 2006). When binding to the voltage gated sodium channel, pyrethroids assume a horseshoe conformation interacting with S5 helix of domain II and the S6 helix of domain III (O'Reilly et al., 2006). The phenyl rings of the alcohol motif interacts with the Phe^{1534}, and Leu^{925} residues while Met^{918} interacts with the ether oxygen (O'Reilly et al., 2006). Thr^{929} interacts with the oxygen of the ester bond stabilizing the orientation of the hydrophobic front of the acid group to interact with Leu^{932}

(O'Reilly et al., 2006). It is hypothesised that the binding of pyrethroids stabilises the interface of the S5 and S6 helices (O'Reilly et al., 2006) which are important for the slow inactivation of voltage gated sodium channels (Goldin, 2003) resulting in the channels having a higher probability of being in the open conformation.

3.4 Known secondary targets

Permethrin and other pyrethroids are known to have many secondary targets however many have only ever been investigated in non-target species or only tested with a single pyrethroid. Permethrin is known to act as an antifeedant (Armstrong and Bonner, 1985) and inhibit acetylcholine receptors (Abbassy et al., 1983; Kiss and Osipenko, 1991) and acetylcholinesterase (Badiou et al., 2008; Sellami et al., 2014). The inhibition of Na-K-ATPase (He et al., 1998), Ca^{2+} and Mg^{2+} ATPases (Luo and Bodnaryk, 1988) and voltage gated calcium channels (Yan et al., 2011) by permethrin leads to calcium deregulation. It is known from other pyrethroids that ornithine aminotransferase and lactate dehydrogenase (Bakry et al., 2011) may also be inhibited.

There are several permethrin and pyrethroid secondary targets that have only ever been investigated in vertebrates including the serotonin receptor (Oortgiesen et al., 1989), benzodiazepine receptor (Devaud and Murray, 1988), mitochondrial complex I (Gassner et al., 1997), voltage gated chloride channels (Burr and Ray, 2004), GABA gated chloride channels (Bloomquist et al., 1986), ryanodine receptors and Inositol (1,4,5)-triphosphate receptors (Wang et al., 2009a). It is also known from mammalian studies that permethrin and some catabolites are highly liposoluble causing them accumulate in the cell and mitochondrial membranes resulting in a decrease in membrane fluidity (Vadhana et al., 2011) and that changes in membrane fluidity are often associated with altered permeability and changes in the activity of membrane bound proteins and therefore ROS generation (Braguini et al., 2004; Vadhana et al., 2011).

3.5 Symptoms of permethrin exposure

The symptoms for permethrin poisoning are more detailed for mammalian models than invertebrates. Known symptoms include sensitivity to external stimuli, fine tremor progressing to whole body tremor and prostration, incoordination, hyperactivity, paralysis, increased body temperature, aggressive behaviour, agitation, irritation to the eyes and skin, dizziness, headache, nausea, fatigue, vomiting, weakness, chest tightness, parasthesia, palpitation, blurred vision and increased sweating (Breckenridge et al., 2009; He et al., 1989). Insects are also known to show restlessness (hyperactivity), incoordination, prostration, and paralysis (Gammon et al., 1981). In Diptera ataxia and fluid loss from the mouth and anus has been observed (Adams and Miller, 1980; Gerolt, 1976) while in Lepidoptera a shortening and curving of the body is known to occur (Zhang et al., 2008b). There is however a knowledge gap for invertebrate symptoms when exposed to permethrin (and other pyrethroids) with many orders never having been investigated.

3.6 Uncertainty over the exact cause of death

Despite the numerous studies investigating the efficacies of permethrin and other neurotoxic insecticides against various invertebrate pests, the actual pathologies leading to death are still unknown.

3.6.1 Controversies with current theories

3.6.1.1 Differences between vertebrate and insect respiration under pyrethroid induced paralysis

One hypothesis as to how pyrethroids and other neurotoxic insecticides induce mortality is by asphyxiation. Unlike mammals however, where paralysis of the central nervous system disables gas exchange (respiratory failure) (Cha et al., 2014) and stops the heart and circulatory systems resulting in hypoxic injury (ischemia)(Sayim et al., 2005) and ultimately death, arthropods can perform gas exchange while paralysed (Gerolt, 1976; Zheng et al., 2013) however, the shift from discontinuous gas exchange to continuous gas exchange results in higher water loss (Gerolt, 1976). This lack of hypoxia in arthropods is highlighted by lactic acid increases, a marker of anaerobic metabolism, not being found in most

invertebrate studies using neurotoxic insecticides, organophosphates being an exception due to inhibition of the TCA cycle enzymes (Forcella et al., 2007; Surendra Nath, 2000).

3.6.1.2 Strength of the evidence for depletion of energy reserves

The other popular hypothesis is that the continuous excitation of nerves and muscles results in a depletion of energy stores resulting in death by exhaustion. There is evidence for this in that the induction of the mobilization of energy reserves has a synergistic effect on some neurotoxic insecticides (Ahmed et al., 2015; Kodrik et al., 2010) however, there are some neurotoxins like permethrin that are not affected by increases in the mobilization of glucose reserves (Ahmed et al., 2015). There is further controversy with this theory because survival against pyrethroid exposure is inversely proportional to ability to survive starvation stress (Polanco et al., 2011) suggesting that the quantity of stored energy reserves is not related to survival, and through mobilization, may even be detrimental.

3.6.2 Evidence for other potential pathologies

There is also evidence from the literature of other potentially pathologic changes that occur during permethrin exposure, all of which could cause mortality. When paralysed by pyrethroids, insects experience increased water loss due to the switch from discontinuous gas exchange to continuous gas exchange (Gerolt, 1976). This would lead to death by desiccation unless the insect can regain sufficient coordination to find a drinkable fluid and consume it. Other potentially fatal pathologies associated with pyrethroids are ammonia stress (Reddy and Bhagyalakshmi, 1994) and oxidative stress (Terhzaz et al., 2015). Apoptosis, which is observed in the central nervous system (Roma et al., 2013), although not immediately fatal, would result in irreparable damage to coordination and cognitive processes leading to a loss of fitness that would make the organism more susceptible to other life shortening events like predation.

4 The problem of resistance and few new insecticides

As stated in **Section 2.7.1**, many insecticides have been used as agents of pest control for decades giving pest species many opportunities to develop resistance while the lack of new insecticides to replace ineffective ones means resistance is a growing problem. Resistance arises from a combination of natural variation, mutation and the selective pressure applied to populations of pest species that are exposed to insecticides but with an insufficient dose or coverage to cause total elimination of the population. There are many factors that can result in conditions that would favour the emergence of resistance including the method of insecticide application, age and storage conditions of the insecticide formulation and frequency of application.

The most common application method for non-systemic insecticides is fumigation and residual spraying however, the nozzle and insecticide formulation used has an impact on the size of the spray droplets (Al-Sarar et al., 2006; Henriet and Baur, 2009). The size of droplets affects the spread, the losses to drift, the area covered and the localized concentrations of the insecticide while the insecticide formulation affects the spread and precipitation of the active ingredient once contacting a surface (Al-Sarar et al., 2006; Henriet and Baur, 2009) with typically only <0.03% of the applied insecticide ever reaching target organisms (Pimentel and Burgess, 2012). This means there is a trade-off between leaving areas without insecticide application where pests can hide but guaranteeing mortality in treated areas and having an even coverage of a greater area but having higher chances that individual pests will experience a sub-lethal dose (Al-Sarar et al., 2006; Henriet and Baur, 2009). As some pests can sense and will avoid high doses of insecticide it has been found that the development of resistance is encouraged by uneven spray densities (Al-Sarar et al., 2006).

The concentration of insecticides applied diminishes over time due to weathering, and decay (Al-Sarar et al., 2006) meaning additional applications are required to stop concentrations falling to a sub-lethal dose with increased frequencies of application causing reduced damage to crops (Story and Sundstrom, 1986) but also the increased occurrence of resistance (Goka, 1999).

The development of resistance can also be encouraged by applying low doses that only induce mortality in some susceptible genotypes (McDonald et al., 1987), as often accidentally occurs when pesticides are stored for long periods of time in conditions that result in their decomposition (Xue et al., 2008) or stereoisomerisation, resulting in the formation of less active stereoisomers (Liu et al., 2005).

4.1 Mechanisms of resistance to insecticides

Resistance can be caused by a change in the number or regulation of resistance associated genes, modification of detoxifying proteins, changes in target site susceptibility or changes in behaviour. Increased expression of resistance genes can be caused by the duplication of the genes (Bariami et al., 2012), mutations in the promoter causing a loss of inhibitor binding (Maitra et al., 1996) or transposons can be inserted into the promoter region (Chung et al., 2007) or inserted into the promoters or coding sequences of the regulatory genes (Sabourault et al., 2001). Mutations in the coding sequences and exons of resistance associated proteins can result in them having a higher affinity to pesticides than their natural substrates resulting in sequestration (Kostaropoulos et al., 2001) or selective degradation of pesticides (Yamamoto et al., 2009) or can result in increased enzymatic activity (Campbell et al., 1998) or gaining novel functions (Aminetzach et al., 2005).

The proteins involved in non target site insensitivity mediated resistance function either by limiting the dose of pesticide that reaches the target proteins or are actively involved in the degradation. The absorbed dose of pesticide can be limited by reducing cuticle penetration and gut absorption through the overexpression of drug efflux pumps like P-glygoprotein (Lanning et al., 1996) or increasing the thickness of the epithelial barrier (Lin et al., 2012). When pesticides do penetrate the cuticle or gut they are sequestered either by specialized proteins (Shemshedini and Wilson, 1990) or by enzymes with a high binding affinity (Campbell et al., 1998).

Insecticide degradation occurs in three phases. Phase I detoxification occurs by enzymatic cleavage or redox of functional groups, often making the compound more hydrophilic. In Phase II the remaining insecticide metabolites are

conjugated before excretion by Phase III detoxification systems (Berenbaum and Johnson, 2015). Carboxylesterases are a family of serine hydrolases capable of cleaving the ester, amide and thioester bonds (Aranda et al., 2014; Jackson et al., 2013) which are commonly found in pesticides due to the increase in lipophilicity they confer to the compound. Cytochrome oxidoreductases are a group of metalloenzymes involved in the oxidation of functional groups of compounds with the cytochrome P450 monooxygenase and cytochrome C oxygenase families often associated with insecticide resistance, some of which are NADH or NADPH dependent (Nikou et al., 2003). Glutathione-S-Transferases (GSTs) are a family of enzymes that use GSH in reduction and substitution reactions. GSTs play an important part in insecticide resistance by solubilising lipophilic compounds for excretion (Berenbaum and Johnson, 2015), preventing oxidative damage and repairing lipids (Jiu et al., 2015). Other compounds used for Phase II conjugation of insecticide catabolites are glucuronate, glucose, alanine, glycine, glutamine, glutamate and serine (Shono et al., 1978). The last step of insecticide degradation involves the excretion of conjugated insecticide catabolites by ATP-binding cassette superfamily transporters like the multidrug resistance-associated protein (Chahine and O'Donnell, 2011).

Mutations in the coding sequence of the primary targets of insecticides can cause target site insensitivity by producing protein variants that do not bind the insecticide as efficiently (Williamson et al., 1993). This means higher doses are required to achieve the concentrations needed to cause sufficient target binding for mortality to occur resulting in increased resistance. Changes in behaviour can also cause resistance by avoidance behaviour where chemosensory neurons allow the pest species to avoid insecticide treated surfaces resulting in reduced efficacy despite there being no physiological resistance within the population (Sungvornyothin et al., 2001). Although not yet demonstrated in insects, it is also known that resistant and susceptible populations of pests show large differences in metabolic profiles when exposed to insecticides (Ali et al., 2014) suggesting the possibility of metabolic compensation being used to counteract pathologies induced by the insecticide. These compensatory pathways could theoretically cause increased resistance in a polygenic manner.

4.2 Resistance to permethrin

Resistance to permethrin can be caused by the upregulation of GSTs which act to sequester permethrin lowering the dose available to bind to the primary target (Kostaropoulos et al., 2001). In insects Phase I of permethrin degradation occurs by carboxylesterases cleaving the ester bond between the permethric acid and the 3-phenoxybenxyl alcohol groups, resulting in inactivation of voltage gated sodium channel agonism (Shono et al., 1978), and cytochrome P450 oxidoreductases, like the CYP9J family in the mosquito *Aedes aegypti* (Bariami et al., 2012) which hydroxylate the ortho and para carbons of the phenyl rings in the 3-phenoxybenxyl group and the methyls of the cyclopropane motif (Shono et al., 1978). In some organisms zeta-class GSTs dechorinate the pyrethric acid group (Yamamoto et al., 2009) while alcohol dehydrogenases and aldehyde dehydrogenases convert 3-phenoxybenxyl alcohol to 3-phenoxybenxylic acid. These catabolites of permethrin are then conjugated to glucose, alanine, glycine, glutamine, glutamate and serine (Shono et al., 1978) before rapid excretion to avoid adduct formation (Noort et al., 2008).

The mutations of the voltage gated sodium channel that confer resistance through target insensitivity are often the residues of the permethrin binding site Phe^{1534}, Leu^{925}, Met^{918}, Thr^{929} and Leu^{932} or nearby residues like Gly^{923}, Thr^{929}, Phe^{979}, Leu^{982}, Ile^{1011}, Val^{1016}, Gly^{1535} and Phe^{1538} (Davies and Williamson, 2009). The mutations usually convert hydrophilic residues into hydrophobic ones and hydrophobic residues into either hydrophilic or more bulky and sterically hindering residues like the L1014F mutation responsible for the knockdown resistance phenotype despite being comparatively far from the binding site (Davies and Williamson, 2009). The Val410M/L mutation is the only known mutation outside the pyrethroid binding pocket which causes increased resistance while in vitro studies suggest that mutations in Leu^{914} would cause a slight decrease in sensitivity to pyrethroids although this has yet to be observed in wild populations (Davies and Williamson, 2009). Interestingly, these mutations are in or near the residues that have been found to be responsible for the insensitivity of mammals to pyrethroids (Met^{918}, Cys^{933}, Ile^{936} and Phe^{1530}) with the M918T super-knockdown resistance causing mutation making insects up to 500 times more resistant (Davies and Williamson, 2009).

4.3 Cross resistance and Insensitivity to synergists

There is a further complication posed by resistance in that some mutations conferring resistance to one insecticide can also increase resistance to other insecticides through cross-resistance, even when the other insecticide has a different mode of action, which results in the resistant pest population being harder to control. This can be due to the substrate promiscuity of some insecticide detoxification enzymes where expression increases associated with resistance to one insecticide can confer resistance to other insecticides that are also substrates (Le Goff et al., 2003). Cross-resistance can also be caused by mutations causing target site insensitivity if insecticides with the same mode of action share a common binding pocket as is the case with permethrin and DDT (O'Reilly et al., 2006).

In order to suppress insecticide resistance chemicals called synergists are added to pesticide formulations to increase insecticide absorption and transport, impair excretion, increase activity against the target or inhibit degradation (Bernard and Philogene, 1993). The most commonly used synergist is piperonyl butoxide because it inhibits CYP450s, some GSTs (Chahine and O'Donnell, 2011), acetylcholine esterase and carboxylesterases (Khot et al., 2008) however, it can also be counterproductive because it also upregulates some CYP450s and GSTs (Chahine and O'Donnell, 2011). Unfortunately, some resistant pest populations have become insensitive to current synergists (Kranthi et al., 1997; Zhang et al., 2008a) meaning that new alternatives for both insecticide and synergist are needed.

5 Omics as a tool for insecticide research

The “omics” fields of research are high throughput and capable of quickly producing large quantities of data giving a deeper insight into how organisms function and provide a broad picture of the changes that occur under xenobiotic stress (Roat et al., 2014; Zhang et al., 2012b). This “omics” offers a new way to overcome conventional limitations in target discovery (Rabinowitz et al., 2011) which could be applied to insecticide research.

5.1 Genomics

Genomics is the study of the genome and is performed using technologies derived from Sanger sequencing (Sanger et al., 1977) or next generation sequencing techniques (Metzker, 2010). The genome sequence of an organism can give insight into the existence, diversity and numbers of xenobiotic detoxification gene families and therefore the metabolic capabilities to defend against xenobiotic stress, such as insecticides, as has been found in the case of the honey bee *apis mellifera* which has low numbers of xenobiotic detoxification genes explaining the low tolerance of the species to insecticides (Berenbaum and Johnson, 2015; Claudianos et al., 2006). Genomics can also provide information about "population genomics" to determine the prevalence of resistance associated alleles and gene flow between pest populations (Catania et al., 2004; Franck et al., 2007).

5.2 Transcriptomics

Transcriptomics is the study of the transcriptome as not all genes are expressed equally despite all eukaryotic life having only two copies of each chromosome, with both usually having an equal copy number of genes. Differences in the expression levels of genes are the result of differences in promoter strength and regulatory mechanisms. This means the concentrations of RNA for a gene is not always proportional to the copy number of the gene (Catania et al., 2004) and gene regulation can be altered to adapt to environmental conditions (Zhang et al., 2012b). For determining transcript levels of certain genes there are several oligo hybridization microarray technologies available e.g. (Lockhart et al., 1996) however, next generation sequencing technologies are offering opportunities to determine the existence of transcripts and their expression levels even in the absence of a sequenced genomic template (Wang et al., 2009b; Zhang et al., 2012b).

Transcriptomics can give insight into the regulation of xenobiotic detoxification genes (Liang et al., 2015; Zhang et al., 2012b), novel transcripts of xenobiotic detoxification genes (Zhang et al., 2012b) and the differences between susceptible and resistant strains (Lv et al., 2015). As next generation sequencing allows for the changes in gene expression to be determined without

the genome having to be sequenced, it is a powerful tool for the identification of candidate genes in non-model organisms (Zhang et al., 2012b; Zhu et al., 2012).

5.3 Proteomics

Proteomics is the study of proteins as the amounts of protein present is not always directly proportional to the level of transcriptional activity because translation is subject to the strength of the ribosomal binding site (Brewster et al., 2012). Other factors that can affect protein concentrations are ribosomal drop off caused by small regulatory RNAs (Eulalio et al., 2008) and protein degradation caused by the innate protein stability or ubiquitination (Hochstrasser, 1995). In insecticide research proteomic techniques are mainly used for determining enzyme concentration changes on insecticide exposure (Roat et al., 2014) or between different strains (Konus et al., 2013) and for purifying proteins for functional characterization.

Core to proteomics is 2D-PAGE gel electrophoresis, and derivatives thereof, which separates proteins by charge and then mass (O'Farrell, 1975) which can be denaturing or non-denaturing. This allows quantification and identification of proteins using mass spectroscopy e.g. (Roat et al., 2014), including any post-translational modification (Küster and Mann, 1998), and the purification of proteins to investigate protein-protein interactions (Rigaut et al., 1999) and for functional characterization (Tang et al., 2013). Structural characterization using NMR, X-ray crystallography or cryo-electron microscopy allows the computational investigation of insecticide binding and substrate specificity while also offering the potential for developing leads for new active compounds (DiMaio et al., 2015; Jhoti et al., 2007; O'Reilly et al., 2006).

5.4 Metabolomics

Metabolomics is the study of metabolites and compounds present in an organism and their flux caused by metabolism and transport. As enzymes have different activities which may be subject to allosteric regulation it is hard to predict flux from genomic data, however, the profiles of individual metabolites can be correlated to differences in both the genome (Hoi et al., 2014) and

transcriptome (Chintapalli et al., 2013). The metabolic profile of an organism has a strong influence on the phenotype (Kamleh et al., 2008) and changes in the metabolic profile can be indicative of factors detrimental to the health of the organism (Rabinowitz et al., 2011). As such metabolomics has great potential as a tool for delivering the more exact metabolic routes of pesticide action which will aid novel insecticide development.

Metabolomics is performed using various methods of chromatographic separation before using mass spectroscopy or nuclear magnetic resonance (NMR) to determine the identity of the separated compounds. Chromatography, whether gas, high pressure liquid, or capillary electrophoresis, separates compounds based on physical properties determined by the mobile and stationary phases used (Issaq et al., 2008). Further separation of co-eluting compounds can be achieved by feeding the output of one chromatography system into a second system using mobile and stationary phases with different properties (Issaq et al., 2008).

Mass spectroscopy relies on moving charged particles being deflected by both magnetic fields and electrical fields proportional to the mass to charge ratio of the particle. As most small ionized compounds only have a single charge the accurate mass can be determined while fragmentation of the parent ion caused by the ionization process produces a unique pattern of charged fragments that can be used for compound identification. Tandem mass spectroscopy functions by inducing a second round of fragmentation producing a fragmentation pattern for each fragment of the parent compound allowing for easier compound identification (McLafferty, 1981). Tandem mass spectroscopy can be performed on linear ion trap systems by sending fragments into a second system (McLafferty, 1981) while orbitrap systems can perform the task in the same machine using a radio pulse (Hu et al., 2005; Michalski et al., 2011).

Nuclear magnetic resonance (NMR) spectroscopy relies on the aligned spins of atoms with an uneven number of nucleons in a strong magnetic field oscillating when exposed to a perpendicular radio frequency electromagnetic pulse (Günther, 2013). From the Fourier transform of the emitted radio signal the chemical connectivity of ^{1}H, ^{13}C, ^{14}N, ^{15}N and ^{31}P atoms can be determined. The use of 2D-NMR where a second radio pulse of a different frequency is applied at

an angle to the first allows complete resolution of the bonding within a compound (Fiala and Sklenář, 2007). The use of NMR for metabolomics has better quantification and less ambiguity about metabolite identity than mass spectroscopy but lower sensitivity so fewer metabolites are detected (Ellinger et al., 2013).

With mass spectroscopy based metabolomics the separation of compounds with similar retention times can sometimes be achieved by derivitization where chemical groups, like an additional chiral center, are added onto the compounds, or only specific types of compound, to change the physical properties and therefore retention time (Nagao et al., 2013). Metabolites related to a specific metabolite can be identified and the flux through metabolising pathways determined by heavy isotope labeling which causes a shift in the mass of fragments containing heavy isomers from the labeled compound (Rabinowitz et al., 2011). Data processing for mass spectroscopy data from metabolomics studies involves noise removal, peak identification, alignment and normalization (Katajamaa and Orešič, 2007) however, the most difficult step is the correct annotation of putative metabolites (Dunn et al., 2013).

Metabolomics offers the potential to identify metabolic bottlenecks and compensatory pathways caused by disease (Rabinowitz et al., 2011) or chemical exposure (Takei et al., 2010) and has already had success at identifying new targets for pharmacology (Rabinowitz et al., 2011). This means that metabolomics also has the potential application for understanding how insecticides function and in the identification of targets that, when inhibited, would impair survival and for which new insecticide synergists could be developed.

6 *Drosophila*, the model insect

The vinegar fly, *Drosophila melanogaster* (originally *Drosophila ampelophora*), was first used as a model organism in 1901 by W. E. Castle for experiments looking at the effects of inbreeding on fertility (Castle et al., 1906). *Drosophila* was originally chosen as a model because it has a short lifecycle and was easy to feed all year round (Castle et al., 1906) however, *Drosophila* was first chosen as

a developmental model for having comparatively large eyes which could be used to investigate Lamarckian inheritance (Payne, 1910). It was the identification of mutations with a visible phenotype (Morgan, 1911) that began use of *Drosophila* as a genetic model.

The success of *Drosophila* as a model for genetic studies was made possible by the development of balancer chromosomes (Wallace and King, 1951), efficient chemical mutagenesis (Nasrat et al., 1954) and the visible banding patterns of polytene chromosomes (Judd et al., 1972). The Nobel Prize winning use of genetics for developmental studies performed by Nusslein-Volhard and Weischaus in the 1980's (Nusslein-Volhard and Wieschaus, 1980) identified many key developmental genes that are important in other model systems (Merchant, 2012). Availability of transgenics (Rubin and Spradling, 1982), a sequenced and annotated genome (Adams et al., 2000), tissue specific transcriptomic and metabolomic data sets (Chintapalli et al., 2013; Chintapalli et al., 2007) and commercially available RNAi lines for almost every gene (Green et al., 2014; Ni et al., 2009) have made *Drosophila* a model of choice for many fields of research.

6.1 Life cycle

The Drosphila life cycle has been detailed on numerous occasions e.g. (Castle et al., 1906; Powell, 1997) with exact timings being temperature dependent. Eggs take two to three days to mature in the female but once fertilised hatch within 20 hours of being laid (Powell, 1997). The polarity of the developing embryo is determined by concentration gradients of maternally deposited RNAs, like those of the *nanos* and *bicoid* genes (Johnston and Nüsslein-Volhard, 1992). The nucleus undergoes 13 rounds of division before migrating to the surface of the egg at 90 minutes after fertilization (Campos-Ortega and Hartenstein, 2013; Johnston and Nüsslein-Volhard, 1992) before cellularizing by 3 hours after being laid (Johnston and Nüsslein-Volhard, 1992). Gastrulation then occurs involving germ band elongation, germ band shortening and dorsal closure and head involution (Campos-Ortega and Hartenstein, 2013).

Larvae are continuous feeders molting at 25 hours after hatching and then again 24 hours later (Powell, 1997). Third instar larvae feed for 48 hours before

entering a wandering stage to find suitable locations to pupate (Castle et al., 1906; Powell, 1997), going through 15 distinct developmental stages which occur over a 105 hour period (Bainbridge and Bownes, 1981). Adults become sexually mature within 39 hours of eclosion with the first eggs laid 2-3 days after eclosion and are able to lay up to 430 eggs within the lifespan (Castle et al., 1906). The adult lifespan depends on genotype but, within strains commonly used for research, a lifespan of 27-45 days can be expected (Oxenkrug, 2010). In total the whole lifecycle occurs within 11 days under ideal conditions but can take as long as 21 days (Castle et al., 1906).

6.2 Physiology

6.2.1 Anatomy

Drosophila melanogaster is a Dipteran insect and therefore has all the anatomical features associated with that order (Chapman, 1998). Adults are usually 2.4-2.6mm long (genotype and nutrition dependent) having two wings and a vestigial pair of haltiers, six legs, a head, thorax and abdomen with eight abdominal segments. The eyes are pigmented with ommochromes formed from tryptophan catabolism (Ryall and Howells, 1974) and drosopterins formed from guanosine metabolism (Kim et al., 2013; Wiederrecht et al., 1984)

6.2.1.1 Development of the Malpighian Tubules

The Malpighian tubule, a tissue associated with xenobiotic detoxification (Yang et al., 2007), consists of 484 principal and 110 stellate cells each with specialized transport functions (Halberg et al., 2015). The Malpighian tubules form from the embryonic hindgut creating a primordial bud of 20-25 cells at 6.25 hours after fertilization (Denholm et al., 2003; Skaer, 1989). At 7.75 hours after fertilization a single cell forms the distal tips which encourage proliferation of the stem cells that form the principal cells (Skaer, 1989), while stem cells of a posterior mesodermal origin, which have a shared origin with those of the caudial visceral mesoderm, migrate to the forming tubules and differentiate into the stellate cells (Denholm et al., 2003). By 15 hours after fertilization the tip cells disappear and the Malpighian tubules stop growing (Skaer, 1989)

6.2.1.2 Development of the fat body

The fat body, another xenobitoic detoxification gene expressing tissue (Perry et al., 2011), forms from the inner mesoderm starting at embryonic stage 11-14 of the Bownes scale of embryonic development (Bownes, 1975) and merging into a single layer by embryonic stage 15-16 (Zhang and Xi, 2014). The fat body forms primarily in the abdomen and during pupation undergoes “fat body remodelling” where the majority of cells become spherical at around 6 hours after pupation and lose adhesion by 14 hours persisting as an energy source during the early adult stage (Zhang and Xi, 2014). After two days post-eclosion the spherical fat cells apoptose (Zhang and Xi, 2014).

6.2.1.3 Development of the CNS

The central nervous system contains the targets of neurotoxic insecticides, including the pyrethroids, and consists of the brain and ventral nerve cord. Development of the central nervous system has four phases, first neuroectoderm cells are formed from the ectoderm in regions determined by the polarity gene expression of the blastoderm (Technau et al., 2014). During gastrulation the neuroectoderm cells delaminate to form central nervous system progenitor cells that proliferate to form a chain of ganglion mother cells (Technau et al., 2014). These ganglion mother cells then either differentiate to form the larval central nervous system or undergo quiescence to form adult central nervous system (Technau et al., 2014).

6.2.1.4 Development of the gut

The gut not only acts as an organ for the absorption of nutrients but also acts as a barrier to prevent pathogen invasion (Kuraishi et al., 2011) and has an early role in xenobiotic detoxification (Perry et al., 2011). During the slow elongation of the germ band the stomodeum forms from ectodermal cells at the anteroventral tip and elongates until it joins the endodermal anterior midgut continuing to elongate due to a recruitment of ectodermal cells (Campos-Ortega and Hartenstein, 2013). Intestinal stem cells that have formed divide daily differentiating into absorptive enterocytes or secondary enteroendocrine cells (Zeng and Hou, 2015).

6.2.2 Relevance as a model for other insects

Even though the *Drosophila* genus only contains one member that is directly a pest species of undamaged fruit, *Drosophila suzukii* (Birmingham et al., 2011), it shares many of the basic anatomical and metabolic features shared by other Dipteran, Coleopteran and Lepidopteran pests. The mosquito *Anopheles gambiae* genome shares over 60% homology with that of *Drosophila* (Gilleard et al., 2005) which is why *Drosophila* was suited as a platform for developing *Anopheles* transgenesis (Catteruccia et al., 2000). *Drosophila* has proven to be a good model for both the "sterile-male technique" of population control (Heinrich and Scott, 2000) and in the study of invertebrate host-pathogen interactions (Bier and Guichard, 2012). This means *Drosophila* is an ideal candidate for the study of the modes of action and resistance mechanisms of known pesticides and could facilitate the discovery of potential targets and identify chemicals for new insecticidal agents.

6.2.3 Relevance as a model for human disease

Drosphila can also be used for the study of human conditions since 75% of human genetic disease related genes have a functional *Drosophila* ortholog (Pandey and Nichols, 2011). *Drosophila* has already successfully been used to simulate several human renal and neurodegerative diseases (Bilen and Bonini, 2005; Pandey and Nichols, 2011; Wang et al., 2004).

6.3 Transgenics

One of the main advantages of *Drosophila* is the large genetic toolbox available for transgenics which, when combined with the short generation time allows for the quick and precise manipulation of the gene of interest. Some of the available techniques are described in **Section 6.3.2**.

6.3.1 Transformation

6.3.1.1 Embryo injection

The first transgenic *Drosophila* were created by Rubin and Spradling using a P-element transposon with a rosy marker introduced into the germline using an embryo injection technique (Rubin and Spradling, 1982). Transformation with this method required the eggs used to be freshly laid and under 40 minutes old and kept at 10-18°C (Rubin and Spradling, 1982). The eggs are washed with water and then 95% alcohol before they are aligned and injected with a plasmid mix from the anterior (Rubin and Spradling, 1982). Eggs that have reached stage 2 of the Bownes scale of embryonic development (Bownes, 1975) before injection are rejected (Rubin and Spradling, 1982). Of the injected eggs that are viable approximately 20-50% have transformed cells but successful germline transformation is only 1-3% (Rubin and Spradling, 1982). As transformation currently represents the major bottleneck in *Drosophila* transgenics there have been attempts to automate the process, however, like with all traumatic transformation methods there is some loss of viability (Zappe et al., 2006).

6.3.1.2 Electroporation

There is a protocol in the literature for the introduction of genetic material into *Drosophila* eggs using elecroporation which mentions preliminary evidence of the technique being able to be used for transformation (Kamdar et al., 1995). It has recently been shown that electroporation can successfully be applied to transform the eggs of the silkmoth *Bombyx mori* being able to process over 600 eggs quickly in a single batch and having similar transformation efficiencies to embryo injection (Guo et al., 2004). This suggests that eletroporation may one day also become a viable alternative to embryo injection in *Drosophila*.

6.3.2 Applied transgenic systems in *Drosophila*

6.3.2.1 UAS/GAL4

Core to *Drosophila* transgenics is the UAS/GAL4 system allowing the interchangeable tissue specific expression of transgenes in different tissues or expression of different transgenes in the same tissue simply by setting up a cross between a strain expressing the GAL4 transcription factor under the control of a

desired promoter with a strain that contains the transgene under control of the UAS promoter. The UAS/GAL4 system is an orthogonal transcription factor and modified binding site that was extracted from yeast and applied to mammalian cell culture (Webster et al., 1988) before being imported into the *Drosophila* model (Fischer et al., 1988). Since then several other orthogonal transcription factor/promoter systems have been developed (del Valle Rodriguez et al., 2012).

6.3.2.2 RNA interference (RNAi)

RNA interference allows the silencing of any gene of choice by expressing short double stranded RNA sequences that match the sequence of the transcript (Yang et al., 2000). RNAi functions by the expressed small interfereing RNAs, that act like endogenous microRNAs, being processed by the argonaute proteins before being loaded into the RISC complex which then cleaves any RNA transcripts that match the loaded siRNA sequence (Eulalio et al., 2008). The siRNAs produced by RNAi can also reduce protein translation from matching transcripts by ribosome drop-off (Eulalio et al., 2008).

6.3.2.3 PhiC31 integrase and other recombinases

Site specific recombinases like phiC31 (Bateman et al., 2006), FLP (Golic and Lindquist, 1989) and Cre (Siegal and Hartl, 1996) allow for the site specific insertion of genetic elements once a landing site has been created using a transposable element (Green et al., 2014). There are even orthogonal recombination sites available for some recombinases allowing for the rapid assembly of more complex genetic constructs which could be inserted into *Drosophila* (Colloms et al., 2014).

6.3.2.4 Conditional promoters and repressors

The use of conditional promoters allows for the investigation of genes that are lethal at specific stages in development (Heinrich and Scott, 2000) or to use the same genotype for both the control and experimental group. The Heat shock protein 70 promoter is temperature sensitive increasing expression when the organism is exposed to heat shocks of temperatures above 37°C (Rio and Rubin, 1985). The *lac* operator-repressor system enables gene expression to be controlled by the addition of lactose or the analog IPTG (Isopropyl ß-D-1-

thiogalactopyranoside)(Wakiyama et al., 2011). The tetracycline operator-repressor system is analogous to the *lac* operator-repressor system except uses tetracycline or doxycycline ligands to induce expression (Bieschke et al., 1998). The UAS/GAL4 system can be modified for conditional expression by expressing a temperature sensitive variant of the GAL80 repressor protein or fusing the GAL4 protein to a ligand dependent switching domain (McGuire et al., 2004). Negative selective markers can be constructed by coupling a toxic or lethal gene to a conditionally expressing element.

6.3.2.5 Gene Targeting

Gene targeting is the site specific and seamless manipulation of the genome to insert, replace or delete genes. Gene targeting in *Drosophila* for insertions and substitutions relies on the insertion of the gene targeting construct into a donor strain using a transposable element before crossing to a strain expressing a recombinase and rare cutting nuclease which can be tissue specific (Chen et al., 2015). The gene targeting construct is circularized and released from the genome by the recombinase acting on two flanking recombinase sites in the gene targeting construct before it is linearized by a cleavage site for the rare cutting nuclease (Rong and Golic, 2000). The linearized gene targeting construct then can undergo homologous recombination with the target locus, however, gene targeting efficiency is a lot higher if there is a double strand break in the target locus (Xue et al., 2014). There are many methods to induce a double strand break including TALENs and zinc finger nucleases (Gaj et al., 2013), however, the RNA guided nuclease Cas9 is likely to replace the other methods due to the simplicity with which the target sequence can be adjusted by changing the guide RNA used.

6.3.2.6 Genetically encoded reporters

Genetically encoded reporters, usually under the control of the UAS/GAL4 system, can be used to investigate biological parameters *in vivo*. Some examples include roGFP which detects intracellular redox changes (Albrecht et al., 2011) and calcium reporters (Riemensperger et al., 2012).

6.3.2.7 Non chromosomally integrated stable transgenic elements

It has been found that tethering the centromere specific histone CENH3 to a region of DNA can induce the formation of an artificial centromere that segregates along with the chromosomes during mitosis (Mendiburo et al., 2011). Although yet to be utilized, this system offers the potential for creating stable genetic constructs that are not integrated into the chromosome.

7 Rationale for the project

The main points motivating this research project were:

There is an urgent need for new insecticides and synergists.

There is a lack of information as to how commonly used insecticides induce death on the molecular level, a question that could be answered using metabolomics.

Perturbations in metabolites caused by insecticide exposure are probably the result of insecticide induced metabolic lesions. Closer investigation might identify lesions responsible for death and weakly inhibited pathways that affect survival which could be future targets for insecticides or synergists.

To restore metabolic homeostasis during insecticide exposure pathways that are beneficial or detrimental to the organism will show changes in regulation. Chemicals that disable compensatory metabolic pathways that aid survival or increase flux down detrimental pathways could be used as synergists.

There is a potential to impact on insecticide survival by further impairing metabolic lesions only weakly affected by the insecticide and by disabling compensatory metabolic pathways that aid survival.

Therefore it can be hypothesised that on exposure to permethrin, metabolic changes that impact on survival will occur which can be detected using metabolomics. Through the introduction of metabolic lesions by the RNAi

knockdown of genes involved in these pathways affected by permethrin exposure an alteration in the tolerance or susceptibility towards permethrin will be caused which would point to potential synergist targets or anti-targets. Also, if the survival affecting mechanisms though which the perturbed pathways function are not specific to permethrin exposure it can be predicted that the knockdown of the same genes would affect survival when exposed to insecticides other than permethrin. Finally, performing metabolomics on the knockdowns for genes of interest could identify specific mechanisms responsible for the changes in survival.

As an indication as to whether this could be achieved, a report by **Metabolon** listing a metabolic profile of *Drosophila* exposed to permethrin was generated to act as a starting point for the project.

8 Research goals and objectives

In order to prove/disprove the hypothesis the following goals and objectives were researched:

To identify metabolic pathways being perturbed in *Drosophila melanogaster* by permethrin exposure.

To relate the altered metabolic pathways to genes and cellular processes.

To explain the metabolic changes using what is known about permethrin.

To identify genes that might influence survival against permethrin exposure.

To validate the effects candidate genes have on survival against exposure to permethrin and other insecticides using transgenic *Drosophila melanogaster*.

To identify metabolic pathways altered by candidate genes which cause differences in survival and to relate them to those altered by permethrin.

Chapter 2 - Materials and Methods

1 Materials

1.1 Chemicals, reagents and kits

1.1.1 Chemicals

Absolute ethanol (Fisher Scientific (UK))

Acetone (Fisher Scientific (UK))

Agarose AMP (Multi Purpose Agarose) (Roche Diagnostics)

Ampicillin (Sigma-Aldrich)

Boric acid (Fisher Scientific (UK))

$CaCl_2$ (Fisher Scientific (UK))

Chloroform (Fisher Scientific (UK))

Chlorpyriphos (Sigma-Aldrich)

DDT (Sigma-Aldrich)

Diaminoethanetetraacetic acid disodium salt (Fisher Scientific (UK))

Fenvalerate (Sigma-Aldrich)

Hydramethylnon (Sigma-Aldrich)

Isopropanol (Fisher Scientific (UK))

K-acetate (Fisher Scientific (UK))

Kanamycin (Sigma-Aldrich)

KCl (Fisher Scientific (UK))

KH_2PO_4 (Fisher Scientific (UK))

LiCl (Fisher Scientific (UK))

Methanol (Fisher Scientific (UK))

N-ethyl maleimide (Sigma-Aldrich)

$Na_2HPO_4.7H_2O$ (Fisher Scientific (UK))

NaCl (Fisher Scientific (UK))

NaH_2PO_4 (Fisher Scientific (UK))

NaOH (Sigma-Aldrich)

Paraformaldehyde (Fisher Scientific (UK))

Permethrin (Sigma-Aldrich)

Sodium dodecyl sulphate (Sigma-Aldrich)

Sucrose (Fisher Scientific (UK))

Tris base (Fisher Scientific (UK))

Triton-X100 (Sigma-Aldrich)

1.1.2 Reagents

10x Loading Dye (Invitrogen)
1kbPlus DNA Ladder (Invitrogen)
Bovine serum albumin (Invitrogen)
Brilliant III 2x SYBR Green QPCR Master Mix (Agilent)
Concentrated HCl (16.7M) (Sigma-Aldrich)
dNTP Mix (Invitrogen)
DreamTaq Green PCR Master Mix (Fisher Scientific (UK))
Ethidium bromide (Sigma-Aldrich)
H_2O_2 (30% wt) (Sigma-Aldrich)
Herculase Reaction Buffer (Stratagene)
IDTE Buffer (Integrated DNA Technologies)
Mineral oil (Sigma-Aldrich)
Mulberry leaf powder (Silkworm Store (UK))
NEBuffer 3 (New England Biolabs)
NEBuffer 4 (New England Biolabs)
Oligo(dT) primers (Invitrogen)
Primers (Integrated DNA Technologies)
Schneider's *Drosophila* Medium (GIBCO) (Invitrogen)
SOC Medium (Invitrogen)
Taqman probes (Integrated DNA Technologies)
Taqman Universal PCR 2x Mastermix (Invitrogen)
Trizol Reagent (Invitrogen)

1.1.3 Enzymes

BglII (New England Biolabs)
Calf-intestinal alkaline phosphatase (CIP) (New England Biolabs)
Herculase II Fusion DNA Polymerase (Stratagene)
NotI-HF (New England Biolabs)
PstI-HF (New England Biolabs)

1.1.4 Kits

Amplex®Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen)
Bio-Rad Protein Assay (Bradford Assay) (Bio-Rad)
Calcium Phosphate Transfection Kit (Invitrogen)
PureLink®RNA Minikit (Invitrogen)
QIAprep®Spin Miniprep Kit (250) (QIAGEN)
QIAquick Gel Extraction Kit (50) (QIAGEN)
Quick Ligation Kit (New England Biolabs)
RNase Free DNase Set (50) (QIAGEN)
RNeasy® Minikit (50) (QIAGEN)

SuperScript II Reverse Transcriptase Kit (Invitrogen)

T4 DNA Ligation Kit (Invitrogen)

1.2 Equipment

Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems)

Beckman Coulter J2-HS High Speed Refrigerated Centrifuge (Beckman-Coulter)

Canon A40 PowerShot camera, with Canon KOOD 25mm orange filter (Canon)

E25 Incubator Shaker Series (Excellin)

Glass Capillaries for Nanoject II Auto-injector, 7", #3-000-203-G/XL (Drummond Scientific Company)

Heraeus Pico 17 centrifuge (Thermo Electron Corporation)

High Performance Ultraviolet Transilluminator (Ultra Violet Products.LLC)

LEICA MZ6 microscope (Leica Microsystems)

Leica Wild M3C microscope (Leica)

Leitz 180570 Wild Photo Port with 100/100 Split and Built-in Iris (Leitz)

Leitz 376110 Extension Tube (Leitz)

Leitz 404207 Phototube Adapter (Leitz)

Leitz-Wetzlar 519740 Periplan 10x red dot eyepiece (Leitz)

MicrosonTM Ultrasonic Cell Disrupter (MISONIX)

Mini-Sub Cell®GT gel tank (BioRad)

MIR-254 SANYO Incubator (SANYO)

Mithras LB940 plate reader (EG&G BERTHOLD)

Moving-Coil Microelectrode Puller Model 753 (Campden Instruments Limited)

NanojectII microinjector (Drummond Scientific Company)

NanoVue Spectrophotometer (Fisher Scientific)

Nex-C3 camera (Sony)

PCRSprint Thermal Cycler PCR heating block (Hybaid)

Power Pac 300 (BioRad)

R-259 microwave (SHARP)

Sigma 204 centrifuge (Sigma Laborzentrifugen, Germany)

Universal visualizing light source for fluorescent stereo microscopy (BLS)

Waterbath (GRANT JBI)

Wild Heerbrugg 246910 Apochromat 1x lens (Leica)

1.3 Solutions and buffers

1.3.1 0.5M EDTA

0.5M EDTA buffer was made by dissolving 18.6g diaminoethanetetraacetic acid disodium salt in distilled water before adjusting the pH to 8.0 using 1M NaOH solution and making up to 100ml.

1.3.2 1M Tris solution

1M Tris solution was made by dissolving 12.114g Tris base in distilled water before adjusting the pH to 7.5 using concentrated HCl and making up to 100ml.

1.3.3 Tris-EDTA (TE) buffer

TE buffer was made by mixing 100µl 1M Tris solution with 20µl 0.5M EDTA buffer and making up to 10ml with distilled water.

1.3.4 Tris-borate-EDTA (TBE) buffer

5x TBE buffer was made by dissolving 54g Tris base, 27.5g Boric Acid and 20ml 0.5M EDTA buffer in 960ml distilled water before adjusting the pH to 8 using 1M NaOH solution and making up to 1 litre. 0.5x TBE buffer was made by a 1 in 10 dilution of 5x TBE buffer.

1.3.5 Phosphate buffered saline (PBS)

10x PBS (0.1M) was made by dissolving 80g NaCl, 2g KCl, 14.4g Na_2HPO_4 and 2.4g KH_2PO_4 in 960ml distilled water before adjusting the pH to 7.4 using concentrated HCl and making up to 1 litre. 1x PBS was made by a 1in 10 dilution of 10x PBS using distilled water. PBS with 20mM N-ethyl maleimide was made using 5ml 10x PBS, 1ml 1M N-ethyl maleimide and 44ml distilled H_2O.

1.3.6 Formaldehyde solution

20% w/v formaldehyde solution was made by dissolving 4g paraformaldehyde in 20 ml distilled water and heating at 70°C before cooling to room temperature. 2% w/v formaldehyde solution was made by a 1in 10 dilution of 20% w/v formaldehyde solution using distilled water.

1.3.7 Primer solutions

Primer stock solutions (100µM) were made by dissolving the lyophilized primer in distilled water, using 10µl distilled water for every nMole of primer, centrifuging at 6k rpm for 15 seconds at 4°C and stored at -20°C until needed. Working

solutions of primers were made by dissolving 6.6µl stock solution in 93.4µl distilled water.

Taqman probes were ordered with FAM dye, ROX passive reference and NFQ-MGB quencher. Taqman probes were resuspended in IDTE Buffer according to the supplied protocol and split into five equal aliquots before being stored at -20°C.

1.3.8 Sodium phosphate buffer

0.25M sodium phosphate buffer was made by dissolving 3.9g $NaH_2PO_4.H_2O$ and 25.93g $Na_2HPO_4.7H_2O$ in 480ml distilled water before adjusting the pH to 7.4 using concentrated HCl and making up to 500ml. 0.05M sodium phosphate buffer was made by a 1 in 5 dilution using distilled water.

1.3.9 Genome extraction buffer

Genome extraction buffer was made by mixing 5ml 1M Tris solution, 10ml 0.5M EDTA, 116.88mg NaCl and 250mg Sodium dodecyl sulphate and making up to 50ml with distilled water.

1.4 Materials provided by the University of Glasgow Media Preparation Services

The food vials and food bottles containing standard *Drosophila* medium, bottles of L-Broth or L-Agar and 1ml vials of 40% glycerol, 2% peptone solution (for long term frozen storage of bacteria strains) were all prepared at the beginning of each week by the University of Glasgow Media Preparation Services.

2 *Drosophila* rearing

2.1 Strains

2.1.1 Driver lines

Table 2-1. List of driver lines used

Name	Genotype	Source	Number	Reference
Actin-Gal4/CyO	*y*^{1}*w*$^{-}$;P{*act5c*-GAL4}25FO1/CyO, *y*$^{+}$;+;+	Bloomington[1]	4414	-
Actin-Gal4/CyO-GFP	*y*^{1}*w*$^{-}$;P{*act5c*-GAL4}25FO1/CyO-GFP, *y*$^{+}$;+;+	Bloomington[1] No longer held but kept In-house	-	-
c564	*w*$^{-}$;+;P{*c564*};+	Prof. Shoichiro Kurata[2]	-	(Harrison et al., 1995)
Elav-Gal4	*w*$^{-}$;+;P{*elav*-GAL4};+	Bloomington[1]	8760	-
Tsp42-Gal4	*w*$^{-}$;+;P{*tsp42*-GAL4};+	In-house	-	(Overend et al., 2012)
Tub-GAL4:UAS-Dcr/Tm3Sb	*w*$^{-}$;+;P{*tub*-GAL4:UAS-Dicer}/Tm3*Sb*;+	Dr. Eric Spana[3]	-	-
Uo-GAL4	*w*$^{-}$;P{*uo*-GAL4}/CyO;+;+	In-house	-	(Terhzaz et al., 2010)

1 - Bloomington Stock Centre, Indiana, USA; 2 - Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan; 3 - Duke University, USA

2.1.2 Expressor lines

Table 2-2. List of expressor lines used

Name	Genotype	Source	Number	Reference
105854	*y*$^{-}$*w*$^{-}$;P{*KK101938*}*VIE-260B*;+;+	VDRC[1]	105854	-
107798	*y*$^{-}$*w*$^{-}$;P{*KK108195*}*VIE-260B*;+;+	VDRC[1]	107798	-
108093	*y*$^{-}$*w*$^{-}$;P{*KK101085*}*VIE-260B*;+;+	VDRC[1]	108093	-
108365	*y*$^{-}$*w*$^{-}$;P{*KK104374*}*VIE-260B*/CyO;+;+	VDRC[1]	108365	-
110229	*y*$^{-}$*w*$^{-}$;P{*KK102925*}*VIE-260B*;+;+	VDRC[1]	110229	-
11322	*w*^{1118}; P{*GD1236*}*v11322*};+;+	VDRC[1]	11322	-
22322	*w*^{1118};P{*GD12085*}*v22322*/CyO;+;+	VDRC[1]	22322	-
24231	*w*^{1118};P{*GD13838*}*v24231*;+;+	VDRC[1]	24231	-

24232	*w*^{1118};+;P{*GD13838*}*v24232*;+	VDRC[1]	24232	-
40686	*w*^{1118};+;P{*GD12901*}*v40686*;+	VDRC[1]	40686	-
B28	w^{-};P{UAS-W::eYFP};+;+	In-house (Jon Day)	-	unpublished
F36	w^{1};P{UAS-Cat.A};+;+	Bloomington[2]	24621	-
UAS-mito-roGFP	w^{-};P{UAS-mito-roGFP}/CyO;+;+	This work	-	-
UAS-roGFP	w^{-};P{UAS-roGFP}/CyO;+;+	This work	-	-

1 – Vienna *Drosophila* Research Centre, Austria; 2 - Bloomington Stock Centre, Indiana, USA

2.1.3 Other lines

Table 2-3. List of other lines used

Name	Genotype	Source	Number	Reference
CS	+;+;+;+	In-house	-	-
CS(w^{-})	w^{h};+;+;+	In-house	-	-
Oregon R	+;+;+;+	In-house	-	-
VDRCKKControl	*y*$^{-}$*w*$^{-}$;P{*KC26*}*VIE*;+;+	Dr. Edward Green[1]	-	-
Vermilion^{-}	*v*^{1};+;+;+	Bloomington[2]	137	-
Cinnabar^{-}	+;*da*^{1}*pr*^{1}*cn*^{1}/SM5;+;+	Bloomington[2]	273	-
Cardinal^{-}	+;+;*cd*^{1};+	Bloomington[2]	3052	-

1- Duke University, USA; 2 - Bloomington Stock Centre, Indiana, USA

2.2 Crosses

Table 2-4. List of crosses used

Name	Male parent	Female Parent	Progeny Genotype used	Applications
107798/+	107798	CS	*y*$^{-}$*w*$^{-}$ /+;P{*KK108195*}*VIE-260B*/+;+;+	Parental control for topical application survival assays
11322/+	11322	CS	*w*^{1118}/+; P{*GD1236*}*v11322*/+;+; +	Parental control for topical application survival assays
22322/+	22322	CS	*w*^{1118}/+;P{*GD12085*}*v22322* /+;+;+	Parental control for topical application survival assays
Act >11322	Actin-Gal4/CyO	11322	*y*$^{-}$*w*$^{-}$/*w*^{1118};P{*act5c*-GAL4}25FO1/P{*GD1236*}*v11322*;+;+	Whole fly *cn*RNAi strain for topical application survival assays

Act>105854	**Actin-Gal4/CyO**	**105854**	***y*$^{-}$*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KK101938*}*VIE-260B*;+;+**	**Whole fly *cn*RNAi strain for topical application and feeding survival assays**
Act>105854(GFP)	**Actin-Gal4/CyO-GFP)**	**105854**	***y*$^{-}$*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KK101938*}*VIE-260B*;+;+**	**Whole fly *cn*RNAi strain for metabolic profiling**
Act>107798	**Actin-Gal4/CyO**	**107798**	***y*^{1}*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KK108195*}*VIE-260B*;+;+**	**Whole fly *v*RNAi strain for topical application and feeding survival assays**
Act>107798(GFP)	**Actin-Gal4/CyO-GFP**	**107798**	***y*^{1}*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KK108195*}*VIE-260B*;+;+**	**Whole fly *v*RNAi strain for metabolic profiling**
Act>108093	**Actin-Gal4/CyO**	**108093**	***y*^{1}*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KK101085*}*VIE-260B*;+;+**	**Whole fly *CG6950*RNAi for feeding survival assays and metabolic profiling**
Act>108093(GFP)	**Actin-Gal4/CyO-GFP**	**108093**	***y*^{1}*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KK101085*}*VIE-260B*;+;+**	**Whole fly *CG6950*RNAi for metabolic profiling**
Act>108365	**Actin-Gal4/CyO**	**108365**	***y*^{1}*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KK104374*}*VIE-260B*;+;+**	***CG2791*RNAi strain tested for viability**
Act>110229	**Actin-Gal4/CyO**	**110229**	***y*^{1}*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KK102925*}*VIE-260B*;+;+**	**Whole fly *CG1673*RNAi strain for topical application survival assays**
Act>24231	**Actin-Gal4/CyO**	**24231**	***y*$^{-}$*w*$^{-}$/*w*^{1118};P{*act5c*-GAL4}25FO1/P{*GD13838*}*v24231*;+;+**	***CG8199*RNAi strain tested for viability**
Act>24232	**Actin-Gal4/CyO**	**24232**	***y*$^{-}$*w*$^{-}$/*w*^{1118};P{*act5c*-GAL4}25FO1/+;P{*GD13838*}*v24232*/+;+**	***CG8199*RNAi strain tested for viability**
Act>40686	**Actin-Gal4/CyO**	**40686**	***y*$^{-}$*w*$^{-}$/*w*^{1118};P{*act5c*-GAL4}25FO1/+;P{*GD12901*}*v40686*/+;+**	***CG17691*RNAi strain tested for viability**
Act>B28	**Actin-Gal4/CyO**	**B28**	***y*$^{-}$*w*$^{-}$/*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{UAS-W::eYFP};+;+**	**Whole fly overexpression of white for topical application survival assays**

Act>F36	**Actin-Gal4/CyO**	**F36**	***y*$^{-}$*w*$^{-}$/w^{1};P{*act5c*-GAL4}25FO1/P{UAS-Cat.A};+;+**	**Whole fly overexpression of catalase for topical application survival assays**
Act>VDRCKKControl	**Actin-Gal4/CyO**	**VDRCKK Control**	***y*$^{-}$*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KC26*}*VIE*;+;+**	**Control for KK lines in whole fly related topical application and feeding survival assays**
Act>VDRCKKControl(GFP)	**Actin-Gal4/CyO-GFP**	**VDRCKK Control**	***y*$^{-}$*w*$^{-}$;P{*act5c*-GAL4}25FO1/P{*KC26*}*VIE*;+;+**	**Control for KK lines in whole fly related metabolic profiling**
Actin-GAL4 x UAS-mito-roGFP	**Actin-Gal4/CyO**	**UAS-mito-roGFP**	***y*$^{-}$*w*$^{-}$/w^{-};P{*act5c*-GAL4}25FO1/P{UAS-mito-roGFP};+;+ and *y*$^{-}$*w*$^{-}$/w^{-};P{UAS-mito-roGFP}/CyO;+;+**	**Intermediate strains in generating stable whole fly mito-roGFP expressing line**
Actin-GAL4 x UAS-roGFP	**Actin-Gal4/CyO**	**UAS-roGFP**	***y*$^{-}$*w*$^{-}$/w^{-};P{*act5c*-GAL4}25FO1/P{UAS-roGFP};+;+ and *y*$^{-}$*w*$^{-}$/w^{-};P{UAS-roGFP}/CyO;+;+**	**Intermediate strains in generating stable whole fly roGFP expressing line**
Actin-GAL4, UAS-mito-roGFP/CyO	**UAS-mito-roGFP/CyO**	**Actin-Gal4/UAS-mito-roGFP**	**w^{-};P{*act5c*-GAL4}25FO1,P{UAS-mito-roGFP}/Cyo;+;+**	**Whole fly mito-roGFP expression with both P elements on chromosome 2 for measuring ROS production**
Actin-GAL4, UAS-roGFP/CyO	**UAS-roGFP/CyO**	**Actin-Gal4/UAS-roGFP**	**w^{-};P{*act5c*-GAL4}25FO1,P{UAS-roGFP}/Cyo;+;+**	**Whole fly roGFP expression with both P elements on chromosome 2 for measuring ROS produced by permethrin**
Actin-GAL4/+	**Actin-Gal4/CyO**	**CS**	***y*^{1}*w*$^{-}$/+;P{*act5c*-GAL4}25FO1/+;+;+**	**Parental control for whole fly related topical application survival assays**
B28/+	**B28**	**CS**	**w^{-}/+;P{UAS-W::eYFP}/+;+;+**	**Parental control for topical application survival assays**
c564/+	**c564**	**CS**	***w*$^{-}$/+;+;P{*c564*}/+;+**	**Parental control for fat body related topical application survival assays**
c564>105854	**c564**	**105854**	***y*$^{-}$*w*$^{-}$/*w*$^{-}$;P{*KK101938*}*VIE-260B*/+;P{*c564*}/+;+**	**Fat body *cn*RNAi strain for topical application survival assays**

c564>107798	**c564**	**107798**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK108195*}*VIE-260B*/+;P{*c564*}/+;+**	**Fat body *v*RNAi strain for topical application survival assays**
c564>22322	**c564**	**22322**	***w*^{1118}*/w*$^{-}$;P{*GD12085*}*v22322*/+; P{*c564*}/+;+**	**Fat body *CG6950*RNAi strain for topical application survival assays**
c564>VDRCKK Control	**c564**	**VDRCKK Control**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KC26*}*VIE*/+;P{*c564*} /+;+**	**Control for KK lines in fat body related topical application survival assays**
Elav>105854	**Elav-Gal4**	**105854**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK101938*}*VIE-260B*/+;P{*elav*-GAL4}/+;+**	**CNS *cn*RNAi strain for topical application survival assays**
Elav>107798	**Elav-Gal4**	**107798**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK108195*}*VIE-260B*/+;P{*elav*-GAL4}/+;+**	**CNS *v*RNAi strain for topical application survival assays**
Elav>22322	**Elav-Gal4**	**22322**	***w*^{1118}*/w*$^{-}$;P{*GD12085*}*v22322*/+; P{*elav*-GAL4}/+;+**	**CNS *CG6950*RNAi strain for topical application survival assays**
Elav>VDRCKK Control	**Elav-Gal4**	**VDRCKK Control**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$; P{*KC26*}*VIE*/+;P{*elav*-GAL4}/+;+**	**Control for KK lines in CNS related topical application survival assays**
Elav-GAL4/+	**Elav-Gal4**	**CS**	***w*$^{-}$/+;+;P{*elav*-GAL4}/+;+**	**Parental control for CNS related topical application survival assays**
F36/+	**F36**	**CS**	**w^{1}/+;P{UAS-Cat.A}/+;+;+**	**Parental control for topical application survival assays**
Tsp42>105854	**Tsp42-Gal4**	**105854**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK101938*}*VIE-260B*/+;P{*tsp42*-GAL4}/+;+**	**Midgut *cn*RNAi strain for topical application survival assays**
Tsp42>107798	**Tsp42-Gal4**	**107798**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK108195*}*VIE-260B*/+;P{*tsp42*-GAL4}/+;+**	**Midgut *v*RNAi strain for topical application survival assays**
Tsp42>22322	**Tsp42-Gal4**	**22322**	***w*^{1118}*/w*$^{-}$;P{*GD12085*}*v22322*/+; P{*tsp42*-GAL4}/+;+**	**Midgut *CG6950*RNAi strain for topical application survival assays**
Tsp42>roGFP	**Tsp42-Gal4**	**UAS-roGFP**	***w*$^{-}$; P{UAS-roGFP}/+;P{*tsp42*-GAL4}/+;+**	**Midgut dissection practice**

Tsp42>VDRCKKControl	**Tsp42-Gal4**	**VDRCKK Control**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$; P{*KC26*}*VIE*/+;P{*tsp42*-GAL4}/+;+**	**Control for KK lines in midgut related topical application survival assays**
Tsp42-GAL4/+	**Tsp42-Gal4**	**CS**	***w*$^{-}$/+;+;P{*tsp42*-GAL4}/+;+**	**Parental control for midgut related topical application survival assays**
Tub>105854	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**105854**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK101938*}*VIE-260B*/+; P{*tub*-GAL4:UAS-Dicer}/+;+**	***v*RNAi strain tested for viability**
Tub>107798	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**107798**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK108195*}*VIE-260B*/+;P{*tub*-GAL4:UAS-Dicer}/+;+**	***v*RNAi strain tested for viability**
Tub>108365	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**108365**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK104374*}*VIE-260B*/+;P{*tub*-GAL4:UAS-Dicer}/+;+**	***CG2791*RNAi strain tested for viability**
Tub>110229	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**110229**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$; P{*KK102925*}*VIE-260B*/+;P{*tub*-GAL4:UAS-Dicer}/+;+**	***CG1673*RNAi strain tested for viability**
Tub>11322	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**11322**	***w*^{1118}*/ w*$^{-}$; P{*GD1236*}*v11322*/+; P{*tub*-GAL4:UAS-Dicer}/+;+**	***v*RNAi strain tested for viability**
Tub>22322	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**22322**	***w*^{1118}*/ w*$^{-}$;P{*GD12085*}*v22322*/+; P{*tub*-GAL4:UAS-Dicer}/+;+**	**Whole fly *CG6950*RNAi strain for topical application survival assays**
Tub>24231	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**24231**	***w*^{1118}*/w*$^{-}$;P{*GD13838*}*v24231*/+; P{*tub*-GAL4:UAS-Dicer}/+;+**	***CG8199*RNAi strain tested for viability**
Tub>24232	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**24232**	***w*^{1118}*/w*$^{-}$;+;P{*tub*-GAL4:UAS-Dicer}/P{*GD13838*}*v24232*;+**	***CG8199*RNAi strain tested for viability**
Tub>40686	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**40686**	***w*^{1118}*/w*$^{-}$;+;P{*tub*-GAL4:UAS-Dicer}/P{*GD12901*}*v40686*;+**	***CG17691*RNAi strain tested for viability**
Tub-GAL4/+	**Tub-GAL4:UAS-Dcr/Tm3Sb**	**CS**	***w*$^{-}$/+;+;P{*tub*-GAL4:UAS-Dicer}/+;+**	**Parental control for whole fly related topical application survival assays**

Uo>105854	**Uo-GAL4**	**105854**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*uo*-GAL4}/P{*KK101938*}*VIE-260B*;+;+**	**Malpighian tubule *cn*RNAi strain for topical application survival assays**
Uo>107798	**Uo-GAL4**	**107798**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*uo*-GAL4}/P{*KK108195*}*VIE-260B*;+;+**	**Malpighian tubule *v*RNAi strain for topical application survival assays**
Uo>11322	**Uo-GAL4**	**11322**	***w*^{1118}/+;P{*uo*-GAL4}/P{*GD1236*}*v11322*;+;+**	**Malpighian tubule *cn*RNAi strain for topical application survival assays**
Uo>22322	**Uo-GAL4**	**22322**	***w*^{1118}/+;P{*uo*-GAL4}/P{*GD12085*}*v22322*;+;+**	**Malpighian tubule *CG6950*RNAi strain for topical application survival assays**
Uo>Cat	**Uo-GAL4**	**F36**	***w*$^{-}$/w^{1};P{*uo*-GAL4}/P{UAS-Cat.A};+;+**	**Malpighian tubule overexpression of catalase for topical application survival assays**
Uo>VDRCKKControl	**Uo-GAL4**	**VDRCKK Control**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$/+;P{*uo*-GAL4}/P{*KC26*}*VIE*;+;+**	**Control for KK lines in Malpighian tubule related topical application survival assays**
Uo-GAL4/+	**Uo-GAL4**	**CS**	***w*$^{-}$/+;P{*uo*-GAL4}/+;+;+**	**Parental control for Malpighian tubule related topical application survival assays**
w^{-};107798/+	**107798**	**CS(w^{-})**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*KK108195*}*VIE-260B*/+;+;+**	**Candidate parental control for topical application survival assays**
w^{-};Actin-GAL4/+	**Actin-GAL4/CyO**	**CS(w^{-})**	***y*$^{-}$*w*$^{-}$*/w*$^{-}$;P{*act5c*-GAL4}25FO1/+;+;+**	**Candidate parental control for topical application survival assays**

The Actin-GAL4, UAS-roGFP/CyO stable line was generated by crossing female Actin-GAL4/UAS-roGFP and male UAS-roGFP/CyO F1 progeny and selecting fluorescent larvae using a Universal visualizing light source for fluorescent stereo microscopy (BLS) attached to a LEICA MZ6 microscope (Leica Microsystems). From the F2 progeny individuals with fluorescence and the CyO balancer chromosome were self crossed to establish the stable line. The same strategy was used to generate the Actin-GAL4, UAS-mito-roGFP/CyO stable line

2.3 Diet and rearing conditions

Table 2-5. Recipe of standard *Drosophila* medium

Ingredient	Amount
Wheat germ	10 g/L
Soya flour	5.25 g/L
Maize Meal	15 g/L
Yeast	35 g/L
Treacle	30 g/L
Sucrose	15 g/L
Glucose	30 g/L
Agar	10 g/L
Propionic acid	5 ml/L
Methylparaben	10 ml/L
Water	Up to No. of Litres needed

All parental fly lines (**Table 2-1, 2-2 and 2-3**) were kept in food vials containing 7ml standard *Drosophila* medium (**Table 2-5**) and tipped to a new vial every 14 to 21 days. All parental lines were kept at 23°C, 50% humidity on a 12:12 light dark cycle because slightly reduced temperature reduced mortality and maintenance. Flies used for crosses (**Table 2-4**) were kept in food vials containing 7ml standard *Drosophila* medium and tipped to a new vial every 3 days with 1-3 day old F1 progeny being harvested for experimentation. All progeny strains used for topical application and feeding assays were kept at 26°C, 50% humidity on a 12:12 light dark cycle (MIR-254, SANYO Incubator). The Actin-GAL4, UAS-roGFP/CyO line was maintained as a parental line. Parents of the Act>VDRCKKControl(GFP), Act>107798(GFP), Act>105854(GFP) and Act>108093(GFP) crosses to be used for metabolic profiling were transferred to new food vials on a daily basis to synchronize larval growth.

3 *Drosophila* dissections and extractions

3.1 RNA extraction

TipOne graduated, filter pipette tips (StarLab) were used for all RNA handling.

3.1.1 Whole fly

Batches of five male and five female 5-9 day old flies were put in 1.5ml Eppendorf tubes, chilled on ice and 200µl Trizol reagent (Invitrogen) added before being ground with a pestle. The pestle was thoroughly washed, then rinsed once with distilled water and once with 70% ethanol before use and between each sample. The samples were kept on ice and sonicated with three one second pulses using a MicrosonTM Ultrasonic Cell Disrupter (MISONIX) at power setting 3.5. The sonicator tip was wiped with 70% ethanol then sonicated for one second in three batches of fresh distilled water before wiping and use. This process was repeated between each sample.

A further 800µl Trizol reagent was added and the samples mixed by inverting the Eppendorf tube six times before incubating at room temperature for five minutes. 0.2ml chloroform was added and mixed by inverting six times before a three minute incubation and centrifugation for 15 minutes at 4°C and 13,000rpm. 0.5ml from the upper aqueous layer was transferred to a new 1.5ml Eppendorf tube and 0.5ml RNase free isopropanol added. The Eppendorf tube was inverted six times then incubated on ice for ten minutes and then centrifuged for 10 minutes at 13,000rpm and 4°C before removing the supernatant and adding 1ml RNase free 75% ethanol to wash the RNA pellet. After centrifugation for 5 minutes at 13,000rpm and 4°C the supernatant was removed and the RNA pellet air dried before resuspension in 87.5µl RNase free distilled water.

DNA digestion was performed using the RNase Free DNase Set (50) (QIAGEN) according to Appendix: E of the RNeasy Mini Handbook (QIAGEN). To the 100µl of sample 350µl of either RLT Buffer (RNeasy® Minikit (50) (QIAGEN)) or Lysis Buffer (PureLink®RNA Minikit (Invitrogen)) was added (depending on kit brand available) and mixed by inverting the Eppendorf tube six times before adding 250µl of RNase free ethanol. The solution was mixed three times by pipetting before transferring to the spin column of either the RNeasy® Minikit (50) (QIAGEN) or PureLink®RNA Minikit (Invitrogen). The sample was then processed as of Step 3 of the RNeasy® Minikit (50) (QIAGEN) RNA cleanup protocol or the PureLink®RNA Minikit (Invitrogen) Purifying RNA from Animal and Plant Cells: Binding, Washing and Elution protocol with the exception that the columns were

given five minutes to equilibrate when first adding the RNA containing solution and before eluting with RNase free distilled water. The sample RNA was eluted with 30µl RNase free distilled water and RNA concentration determined using a NanoVue Spectrophotometer (Fisher Scientific) by comparing the sample to the RNase free distilled water used for elution as a blank. RNA samples were stored at -80°C for later use in QPCR.

3.1.2 Dissected tissue

Batches of 15 male and 15 female 5-9 day old flies were chilled on ice before dissection (see details under **Sections 3.1.2.1-3.1.2.4**) under Schneider's *Drosophila* Medium (GIBCO, Invitrogen) before transferring the tissue to 1.5ml Eppendorf tubes and suspending in 100µl Trizol reagent. The samples were centrifuged for 30 seconds at 4°C and 13,000rpm before rotating the Eppendorf tubes 180° around the vertical axis and repeating to ensure no tissue had adhered to the walls of the Eppendorf tube above the meniscus of the Trizol reagent. Tissue samples were stored at -80°C for later RNA extraction and used within a week.

Tissue samples were kept on ice and sonicated with six one second pulses using a MicrosonTM Ultrasonic Cell Disrupter (MISONIX) at power setting 3. The sonicator tip was cleaned as stated in **Section 3.1.1**. A further 600µl Trizol reagent was added and the samples mixed by inverting the Eppendorf tube six times before incubating at room temperature for five minutes. 140µl chloroform was added and mixed by inverting six times before a three minute incubation and centrifugation for 15 minutes at 4°C and 13,000rpm. 350µl from the upper aqueous layer was transferred to a new 1.5ml Eppendorf tube and 0.5ml RNase free ethanol added. The Eppendorf tube was inverted six times before transferring to the spin column of either the RNeasy® Minikit (50) (QIAGEN) or PureLink®RNA Minikit (Invitrogen). The sample was then processed as of Step 3 of the RNeasy® Minikit (50) (QIAGEN) RNA cleanup protocol or the PureLink®RNA Minikit (Invitrogen) Purifying RNA from Animal and Plant Cells: Binding, Washing and Elution protocol with the exception that the columns were given five minutes to equilibrate when first adding the RNA containing solution and before eluting with 30µl RNase free distilled water. Water containing

eluted RNA was then transferred back into the spin column for a second round of elution. RNA concentration was determined as stated in **Section 3.1.1**.

3.1.2.1 Malpighian Tubule dissection

Malpighian tubules were harvested in one of three ways. Flies to be dissected were held by the thorax horizontally using a forceps and the dorsal side of abdominal segment A6 or A7 torn using a second pair of forceps. The anus, genital entrance and rear abdominal segments were then slowly pulled away from the fly pulling the gut with it until all four Malpighian tubules were removed from the body cavity. The ureters were then cleaved from the gut using forceps and the tubules collected.

If ureters of the Malpighian tubules became detached from the gut before the tubules were removed from the body cavity, the abdomen was grasped from the side at abdominal segment A2 and the whole abdomen removed from the thorax and carefully slid along the gut before collecting the anterior pair of tubules wrapped around the foregut. The foregut was then pulled off from the thorax and the anterior part of the fly disposed of before pulling the gut out fully from the posterior end. The dorsal anterior cuticle edge was then held with a pair of forceps while a second pair was slid from the anterior end along the interior sides of the body cavity before gently removing any remaining reproductive organs and tangled anterior pair of tubules which were then collected.

If the posterior end of the abdomen failed to tear and the whole abdomen came loose, then the foregut was immediately pulled off from the thorax and the anterior part of the fly disposed. The anus and genital opening was cleaved off, the dorsal anterior cuticle edge held and a second pair of forceps was slid from the anterior end along the interior sides of the body cavity to gently remove the whole gut, reproductive organs and Malpighian tubules. The tubules were then detached from the gut and reproductive organs and collected. All Malpighian tubules were transferred directly into Eppendorf tubes containing 100µl Trizol reagent using a thin drawn glass rod with a diameter approximately twice the width of a Malpighian tubule.

3.1.2.2 Midgut dissection

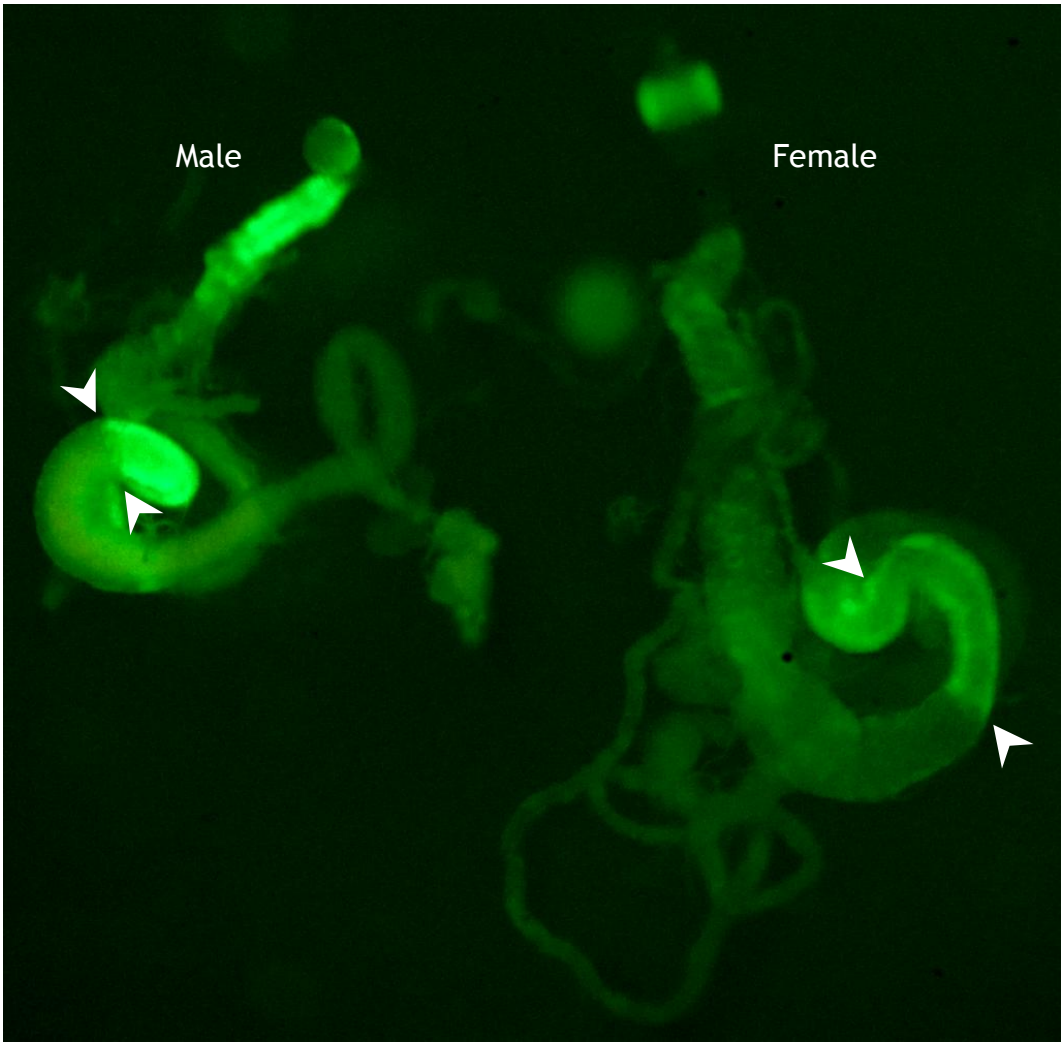

Figure 2-1. Regions of gut expressing Tsp42>roGFP. Dissected fly guts in situ expressing roGFP to highlight regions where the Tsp42-GAL4 driver line expresses. Typical cleavage points are highlighted with white arrows.

Flies to be dissected were held by the thorax horizontally using a forceps and decapitated using a cleaving motion to avoid pulling the gut out. The abdomen was grasped from the side at abdominal segment A2 and the whole abdomen detached from the thorax being careful to avoid pulling the gut out of the abdomen. The foregut was then pulled from the thorax freeing the abdomen and the anus and genital opening cleaved off. The dorsal anterior cuticle edge was held before a second pair of forceps was slid from the anterior end along the interior sides of the body cavity to gently remove the whole gut, reproductive organs and Malpighian tubules. The reproductive organs were removed and discarded before cleaving and collecting the Tetraspanin 42 expressing region of the midgut, shown in **Figure 2-1**. To ensure the Tetraspanin 42 expressing region of the gut was collected practice dissections were carried out on Tsp42>roGFP flies and visualised under a Universal

visualizing light source for fluorescent stereo microscopy attached to a LEICA MZ6 microscope. Generally the midgut was cleaved at approximately two anterior ureter lengths from where the ureters attached to the gut and at a constriction in the gut found just after the first loop in the gut from the ureters. Midguts were transferred to the walls of Eppendorf tubes containing 100µl Trizol reagent using forceps and suspended in a drop of Schneider's *Drosophila* Medium that came with the forceps by surface tension. The centrifugation step used in **Section 3.1.2** would then move the tissue into the Trizol reagent.

3.1.2.3 Fat body dissection

Flies to be dissected were held by the thorax horizontally using a forceps and the abdomen grasped from the dorsal part at abdominal segment A2 peeling the upper cuticle of the abdomen to the anus. Segment A1 or any fat body from under where segment A1 resided was collected and the gut and ventral part of the abdomen pulled from the thorax. The dorsal cuticle of the abdomen was freed from the anus and had the fat body peeled from the cuticle in an anterior to posterior direction. The ventral cuticle had all organs removed, collecting the fat body around the spermatheca of females, and all fat body peeled off the ventral cuticle.

If the whole abdomen detached the anus and genital opening were cleaved off and the abdomen filleted by tearing apart along the ventral part of the abdomen. The fat body was then peeled from the cuticle in an anterior to posterior direction.

All the fat body was transferred in Schneider's *Drosophila* Medium to Eppendorf tubes using a 200µl pipette tip with the end truncated by a pair of scissors, in 30µl batches (to reduce the amount of fat body lost to adherence to the pipette tip). The fat body in Schneider's *Drosophila* Medium was centrifuged for 1 minute at 4°C and 13,000rpm before removing the supernatant and resuspending in 100µl Trizol reagent.

3.1.2.4 Brain dissection

Flies to be dissected were held by the thorax horizontally using a forceps and the head removed from the body at the neck. While still holding the remains of

the neck the proboscis was grabbed high up using a forceps and removed. Both pairs of forceps were then used to tear along the hypotomal sulcus towards each eye. The anterior cuticle of the head was then torn at the eye socket just below the antenna and peeled off. The brain was then scooped away from the remaining cuticle and cleaned of trachea and retina remains. Brains were transferred to the walls of Eppendorf tubes containing 100µl Trizol reagent using forceps and suspended in a drop of Schneider's *Drosophila* Medium that came with the forceps by surface tension. The centrifugation step used in **Section 3.1.2** would then move the tissue into the Trizol reagent.

3.2 Metabolite sample preparation and data processing

3.2.1 Preparation of samples for Metabolon report generated before the project

The work in this section was performed by **Ms. Louise Henderson**. Samples were prepared and used by **Metabolon** to generate the report upon which this PhD project was based. Third instar feeding CS larvae were orally exposed in batches of 100 to an approximately 10% lethal dose of 10µg/ml permethrin that had been dissolved in molten standard *Drosophila* medium and cooled in 10ml aliquots. Seven day old adult CS flies were segregated according to gender and exposed by contact by being placed in batches of 100 into glass vials coated with a 1µg/vial sub lethal dose of permethrin and closed with a buzz plug soaked in 5% w/v sucrose solution to provide food and humidity. At 0, 2, 12 and 24 hours after exposure began all surviving individuals were collected and flash frozen using liquid nitrogen. Experiments were performed with four biological replicates. The samples were shipped on dry ice to Metabolon Inc. for metabolomic analysis with their proprietary pipeline, and subsequent data processing as detailed in Weiner *et al*. (Weiner et al., 2012).

3.2.2 Preparation of samples for metabolomics of RNAi knockdowns

Feeding third instar larvae of the same size were sorted using a Universal visualizing light source for fluorescent stereo microscopy attached to a LEICA MZ6 microscope selecting against the GFP containing *CyO-GFP* balancer chromosome. Larvae were washed in distilled water and placed in shallow PBS

(pH 7.4) for half an hour to remove traces of *Drosophila* medium. Each sample had N=10 with four replicates. Samples were prepared by flash freezing the larvae in liquid nitrogen before homogenization in 250µl 3:1:1 methanol:chloroform:water solution and sonication with three one second pulses using a Microson™ Ultrasonic Cell Disrupter at power setting 3.5. The samples were then centrifuged at 13k rpm for five minutes at 4°C and the supernatant transferred to a new Eppendorf tube before freezing in liquid nitrogen and stored at -80°C until all samples for the metabolomics run had been collected. Samples were sent to the Glasgow Polyomics Facility and metabolomics was performed using LC-MS^2 with an Orbitrap in both positive and negative modes. The resulting IDEOM format Excel file was then analysed manually.

The metabolomics data was sorted according to p-value for the t-test comparing the significance of changes in metabolite concentrations relative to the control strain and all metabolites with p>0.1 across all three knockdown strains were deleted before sorting according to metabolic pathway. The remaining 431 metabolites then had the putative metabolite identities validated using a combination of %retention time error, mass error and the literature to determine if the metabolite might be a *Drosophila* endometabolite, product of the gut flora or present in the standard *Drosophila* diet. If the %retention time error was >25% or the mass error >2ppm the putative annotation of the metabolite was compared against the raw mass spectroscopy data to determine if misannotation had occurred.

4 QPCR

All quantitative (Q)-PCR was performed in MicroAmp Fast Reaction Tubes (8Tubes/Strip) (Invitrogen) using MicroAmp Optical 8-Cap Strips (Invitrogen) and run on an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). Quadruplicates of each QPCR run were performed and the average knockdown or change in expression for the gene of interest calculated. Primers and Taqman probes used are listed in **Table 2-6**.

Table 2-6. Primers used for QPCR

Gene	Forward Primer	Probe (Taqman only)	Reverse Primer	Annealing temperature
α-tubulin	AGGGTATGGAGGAGGGAGATGTC		TGCGATTGGAAGCGTAAACAC	56°C
α-tubulin (taqman)	CCTCGAAATCGTAGCTCTACAC	/56-FAM/TCACACGCG/ZEN/ACAAGGAAAATTCACAGA/3IABkFQ/	ACCAGCCTGACCAACATG	
rp49	ATCGGGCCAGCATACAG		TCCGACCAGGTTACAAGAA	56°C
vermilion	TCGATGAAACCAAGACGCTGGAGA		GAAACCAGATGCGGGTGCCAGG	56°C
kfase	GTGCAAAGACCTTGACCGGG		AGTCAGTTCCCTGCCATGCTG	56°C
cinnabar	CGGTTATTGGAGCAGGACTTG	/56-FAM/CATGCGGGC/ZEN/AAAGTTCAAGGCT/3IABkFQ/	TGCGAAAGAGCCAGGTTAATAC	
cardinal	TGCTCAGCTGCATGGTGT		CGGCCAGACTGGTCTTGTA	56°C
CG6950	GTGCCCCGCTTTGTTCCCCT		TGCGGCAGAGCTCGGCTATC	56°C
white	CCCAGGAAACATTTGCTCAAG	/56-FAM/CCTATCCGG/ZEN/GCGAACTTTTGGC/3IABkFQ/	GGATGGCGATACTTGGATGC	
dCD98	CGTCGGGATGAGGTTAAGTTC	/56-FAM/ATGTCGATC/ZEN/TTGGCATCTCCGTTCTG/3IABkFQ/	TCGTTGGCGTACTTCATGAC	
CG1673	AGTTTAAGGTTAGCGAGGCG	/56-FAM/TGGAGCTCT/ZEN/TTGGTGCGGGAA/3IABkFQ	GGTAGCTGATCCTGTTCACG	
CG8199	CTTTGTCACCATCTCCAGTCC	/56-FAM/CTTTCCACC/ZEN/CAATGCCCCAGG /3IABkFQ	GCTCCCTCACCAAAGTAACAG	
CG17691	GTATGGGAACTGGAAAACGAATG	/56-FAM/AACGCAATG/ZEN/GATTTGGCTCTAGACGA/3IABkFQ	CCCGTAGATTTACTGAACACCG	

4.1 cDNA synthesis

RNA samples were used to make cDNA by adding 1µg of RNA for whole fly RNA samples or 250ng of RNA for tissue specific RNA samples, 1µl Oligo(dT) (500µg/ml), 1µl 10nM dNTP Mix, 4µl 5X First-Strand Buffer, 2µl 0.1M DTT, 1µl RNaseOUT and making up to 19µl using RNase free distilled water in a 0.2ml PCR tube (StarLab). The solution was mixed by pipetting. The tubes were heated to 65°C for five minutes using a PCRSprint Thermal Cycler PCR heating block

(Hybaid), chilled on ice and 0.9µl Superscript II RT added. The solution was mixed by pipetting. The tubes were heated to 42°C for 50 minutes and then 70°C for 15 minutes using the PCR heating block before holding at 4°C. The tubes of sample cDNA were either stored at -20°C for later use or chilled on ice for immediate use in QPCR.

4.2 SybrGreen QPCR

QPCR using primers was performed with 5µl Brilliant III 2x SYBR Green QPCR Master Mix, 1µl forward primer, 1µl reverse primer, 1µl cDNA and 2µl RNase free distilled water per well. QPCR runs were set up to include samples, an α-tubulin ladder, duplicate blanks with the cDNA and primers replaced with distilled water and duplicate primer blanks where each primer pair was in a tube where the template cDNA was replaced with distilled water. Each sample consisted of eight tubes with half using primers for the gene of interest and the remainder using primers for α-tubulin. The temperature cycle setup used was 95°C for 10 minutes for one cycle, 95°C for 30 seconds followed by 56°C for 30 seconds and 72°C for one minute for 40 cycles before performing a melt curve using 95°C for 15 seconds, 56°C for one minute and 95°C for 15 seconds. The software for the QPCR machine had the passive reference set to none.

4.3 Taqman QPCR

QPCR using Taqman Primers was performed with 5µl Taqman®Gene Expression 2x Mastermix, 0.5µl Taqman probe, 2µl sample cDNA and 2.5µl distilled water. The temperature cycle setup used was 50°C for two minutes followed by 95°C for ten minutes for one cycle before 95°C for 15 seconds and 60°C for 1 minute for 30 cycles. The software for the QPCR machine had the quencher set to NFQ-MGB passive reference set to ROX.

4.4 Data processing

All QPCR data was processed using the StepOne Software v2.1. Differences in cycle threshold values between samples were normalized using the values for the housekeeping gene *α-tubulin*. The difference in cycle threshold values of the gene of interest were used to determine the percent knockdown of the RNAi

fly strains compared to the lowest parental control or the regulation of genes on permethrin exposure. Graphs of the QPCR results were plotted in Graphpad Prism 5 (GraphPad Software Inc., USA) and two-tailed t-tests performed to determine significance.

5 Survival assays

5.1 Topical

Crosses for each strain used six males and six virgin females per vial with the parentals transferred to fresh food vials every third day and individuals being replaced if deaths occurred. Adult F1 progeny were segregated according to gender within three days of eclosing and aged in fresh food vials between four to six days so that all adult F1 progeny used were aged 5-9 days old.

The microinjector (NanojectII, Drummond Scientific Company) was set to apply its maximum (69nl) per application. Microinjector needles were made using Glass Capillaries for Nanoject II Auto-injector, 7", #3-000-203-G/XL (Drummond Scientific Company) pulled by a Moving-Coil Microelectrode Puller Model 753 (Campden Instruments Limited) with the following settings, pre-pull heating time: 16, initial pull force: 3, main pull force: 66, main pull delay: 5, heater control: 338 and 4 coils on the heater element with an approximately 3mm diameter hole. Permethrin was diluted in acetone to give 0 (control), 1, 2, 5, 6, 8, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5 and 30 ng/fly doses.

Adult F1 progeny were immobilised on a gas pad emitting CO_2, the permethrin was applied to the thoracic notum and the acetone allowed to dry. 10, 20, 25 or 30 flies treated with the same topical permethrin concentration were placed in fresh food vials and immediately incubated at 26°C, 50% humidity on a 12:12 light dark cycle because pyrethroids have a temperature dependent toxicity (Sparks *et al.*, 1982). If the concentration was lower than the LD_0 or higher than the LD_{100} it was not repeated. A minimum of six different concentrations were used and a minimum of 2 technical replicates (total 50 flies) and 3 biological replicates were made for each concentration of permethrin solution used. The number of surviving flies was recorded at 24 and 48 hours.

5.1.1 Permethrin treated flies for gene expression studies

CS males applied with 2ng/fly and females with 5ng/fly doses (approximately LD_{10}) and both genders treated with an acetone control were removed from the food vials at four hours after application, when the symptoms of poisoning started, and used for whole fly RNA extraction as in **Section 3.1.1**.

5.2 Feeding

Permethrin (200mg/ml in EtOH), fenvalerate (50mg/ml in EtOH), DDT (200mg/ml in acetone), chlorpyriphos (200mg/ml in EtOH) and hydramethylnon (25mg/ml in 3:1 EtOH:acetone) insecticide stock solutions were made. Stock solutions and an ethanol control were diluted in ethanol and 10µl/ml added to 1% agar, 3% sucrose solutions that were distributed into empty food vials for the *Drosophila* medium. F1 progeny flies between 5-9 days old were segregated according to gender and starved for four hours to synchronize feeding before being transferred to vials for feeding survival assays at 12:30 A.M.. Doses used were determined experimentally using the control strain so that three doses were equally spaced between a dose that caused 16% mortality and a dose that caused 84% mortality on the same day. Doses used are listed in **Table 2-7**. Each dose tested had N=30 with two technical replicates and three biological replicates. The vial was placed in a horizontal orientation to prevent mortality though adherence to the agar while in an incapacitated state. Mortality data was recorded hourly between 8:30 A.M. and 18:30 P.M. each day until all flies were dead.

Table 2-7. Feeding assay dose ranges tested

Insecticide	Male	Female
Permethrin	35-220 µg/ml	280-370 µg/ml
Fenvalerate	1.3-9.5 µg/ml	90-210 µg/ml
DDT	1-18 µg/ml	28-105 µg/ml
Chlorpyriphos	0.02-0.035 µg/ml	0.03-0.06 µg/ml
Hydramethylnon	11-75 µg/ml	18-103 µg/ml

5.3 Contact

CS parental flies with six males and six females per vial were transferred to fresh food vials every third day with individuals being replaced if deaths occurred. Adult F1 progeny were segregated according to gender within three days of eclosing and aged in fresh food vials between four and six days so that all adult F1 progeny used were aged 5-9 days old.

Permethrin was dissolved in acetone and 100 µl of the required concentration applied to the sides of 20ml glass vials (approx area of 40cm^2) giving final concentrations of permethrin per vial of 0 (control), 5, 10,15, 20, 25, 30, 35 and 40 µg/vial.

20 sexually segregated adult F1 progeny were transferred to each concentration of poison coated glass vials with a minimum of 2 replicates made for each concentration of vial coating. The vials were kept at 26°C, 50% humidity on a 12:12 light dark cycle. Flies were fed using a 5% sucrose solution held in 1ml syringes (BD Plastipak) with a wick made of tissue paper inserted into the nozzle. The feeder was then inserted through a tightly fitting hole in the buzz plug used to stopper the glass vial where the flies were contained. The number of surviving flies was recorded after 24, 48 and 72 hours.

5.4 Data processing

5.4.1 Topical application

The average weight per fly of each gender of each strain was determined by placing a known number of 50 or more flies in a preweighed universal tube and reweighing it. The difference in weight was then divided by the number of flies to give the average weight per fly. The doses for the topical application survival assays were converted from ng/fly to ng/mg by dividing the dose by the average weight.

Microsoft Excel was used to convert the mortalities for each strain at 24 hours to a percent, outlying values identified and removed in an objective fashion using Grubb's test (Grubbs, 1950) and corrected for control mortality due to the solvent using the Schneider-Orelli variant of Abbott's formula (Abbott, 1925;

Schneider - Orelli, 1947). Corrected mortalities were converted to probits (Bliss, 1957) using Microsoft Excel's NORMINV(probability, mean, standard_dev) function with the mean set to 5 and the standard deviation set to 1.

Using Graphpad Prism 5 (GraphPad Software Inc., USA), the log of the corrected dose was plotted along the x axis against the corresponding probit value for each replicate and dose tested, with each gender of each strain being treated as separate data sets. A linear regression analysis was performed on every data set configured to display the x intercept and 95% confidence intervals where y = 5. The x intercept was then converted into the 50% lethal dose (LD_{50}) value using Microsoft Excel with the formula =10^(x intercept). The same formula was used to convert the 95% confidence intervals into ng/mg values.

The LD_{50}s of each replicate were used to perform ANOVA using the Tukey's range test as a post test in Graphpad Prism 5. The similarity of the LD_{50}s and overlap of 95% confidence intervals from all replicates were compared using a rearrangement (**Formula 1**) of the Litchfield & Wilcoxon method (Litchfield & Wilcoxon, 1949) for opposite genders of each strain and for the same genders of different strains. The LD_{50} of any driver-expressor crosses had to be significantly different from both parental controls and for both statistical methods to be in agreement for a change in permethrin susceptibility to be recognized.

Formula 1: $P<x = (10^{(SQRT(((LOG10((95\%ULLD/LD_{50}L)^s))^2)+((LOG10((LD_{50}H/95\%LHLD)^s))^2))))<(LD_{50}H/LD_{50}L)$

***P* = probability**

s = Stringency factor

x = 0.05 when s=1, 0.01 when s=1.31 and 0.001 when s=1.68

95%ULLD = Upper 95% confidence interval of the lower LD_{50}

95%LHLD = Lower 95% confidence interval of the higher LD_{50}

LD_{50}L = Lower LD_{50} value being compared

LD_{50}H = Higher LD_{50} value being compared

5.4.2 Feeding

Using Microsoft Excel, feeding survival bioassay data was corrected for control mortality using Abbott's formula before using trimmed-Probit analysis (Bliss, 1957) to linearize the cumulative Gaussian distribution. No more than the outer 3% of the data set was trimmed. Probits were plotted against the log of the number of hours since the assay had started using Graphpad Prism 5 to determine the 50% lethal time (LT_{50}). The LT_{50}s for individual replicates were then plotted against the log of the dose to produce toxicity responses. The LT_{50} of 24 hours was used to determine the 50% lethal dose (LD_{50}) at 24 hours for permethrin. Statistical significance between the toxicity responses of different genotypes was determined by testing if the slopes and intercepts differed using the linear regression analysis of Graphpad Prism 5.

5.4.3 Contact

Data processing for contact survival assays was the same as topical application survival assays except the dose was plotted in µg/vial and the linear regression of the probit analysis used to determine the LD_0.

6 Generation of *UAS-rogfp* fly lines

6.1 Transformation of plasmids into *E. coli*

1µl of plasmid or ligation product was added to a tube containing 50µl of One Shot®TOP10 chemically competent *E.coli* (Invitrogen) and incubated on ice for 30 minutes. The cells were then heat shocked in a waterbath (GRANT JBI) at 37°C for two minutes, before adding 250µl SOC medium (Invitrogen) media and incubated in a shaking incubator (E25 Incubator Shaker Series, Excellin) at 37°C for one hour at 160rpm. The transformed *E.coli* were plated on antibiotic containing L-Agar plates by pipetting 125µl bacterial culture per plate and incubated for 12 hours at 37°C.

6.2 Plasmid Extraction

Transformed *E. coli* colonies were selected and each inoculated in 5ml L-Broth and incubated for 12 hours at 37°C at 160rpm. 500µl aliquots were taken from each inoculation and mixed in vials of 40% glycerol, 2% peptone solution before flash freezing in liquid nitrogen and storage at -80°C for future use. Plasmid DNA was extracted from bacterial cultures by miniprep. 1.5ml of bacterial culture was centrifuged (Heraeus Pico 17 centrifuge, Thermo Electron Corporation) at 13k rpm for three minutes before removing the supernatant and adding another 1.5ml of bacterial culture and centrifuging again. After removal of the supernatant the pellet was processed as for Step 2 of the Quick-StartProtocol of the QIAprep®Spin Miniprep Kit (250) (QIAGEN) miniprep kit. This process was repeated for the bacterial cultures derived from each colony. Plasmid DNA concentration was determined using a NanoVue Spectrophotometer (Fisher Scientific) by comparing the sample to the elution buffer (EB) from the miniprep kit as a blank. DNA samples were stored at -20°C for later use.

6.3 Maxiprep

Pipette tips were used to scrape the surface of frozen transformed *E. coli* glycerol stocks and used to inoculate a 250ml conical flask containing 100ml of L-Broth mixed with antibiotic and incubated for 12 hours at 37°C at 160rpm. Cultures were then processed according to the Bench Protocol of the QIAGEN Plasmid Maxi Kit (10) (QIAGEN) maxiprep kit using a Beckman Coulter J2-HS High Speed Refrigerated Centrifuge (Beckman-Coulter). DNA samples were stored at -20°C for later use.

6.4 Restriction Digest

Plasmids from each transformed *E. coli* colony were analysed to confirm the identity and purity of the plasmid DNA received on the filter paper using a restriction digest before gel electrophoresis. The restriction digest was performed in a 1.5ml Eppendorf tube by adding 5µl of sample, 2µl NEBuffer, and 1µl of each restriction enzyme before making up to 20 µl with distilled H_2O and incubating at 37°C in a water bath for one hour. Three controls were

performed per sample using the same procedure with two having only one of each of the restriction enzymes and one used no restriction enzymes.

6.5 Gel Electrophoresis

A 1% agarose TBE gel was made adding 0.6g agarose in 60ml 0.5M TBE buffer, heating in a microwave until just boiling, then mixing and repeating until all agarose was dissolved. The gel was left to cool then, while still liquid, 6µl ethidium bromide added and mixed well. The gel was poured into a gel plate (BioRad) that had the ends capped by a vice and a 16 tooth comb inserted. Once the gel had set, the comb was removed and the gel plate transferred to a Mini-Sub Cell®GT (BioRad) filled with 0.5M TBE buffer so that the gel was just covered. Samples and controls from the restriction digest had 2µl 10x loading dye added and mixed by pipetting before pipetting 10µl into separate wells of the gel. 10µl 1kb Plus DNA Ladder solution (12µl 1kb Plus DNA Ladder, 88µl TE buffer and 20µl 6x loading dye per 120µl volume) was used as a comparison for fragment sizes. The gel was run using a Power Pac 300 (BioRad) power supply at 150V until the loading dye was two thirds down the gel. The gel was visualized using a High Performance Ultraviolet Transilluminator (Ultra Violet Products.LLC) and a picture taken using a mounted camera (Canon A40 PowerShot, with Canon KOOD 25mm orange filter).

6.6 Gel Extraction

After photographing the gel the band containing the roGFP2 gene was excised using a clean surgical blade (Swann-Morton®) and stored in a 1.5ml Eppendorf tube at -20°C overnight. The DNA fragment was then extracted from the gel using a QIAquick Gel Extraction Kit (50) (QIAGEN) following the protocols of the kit. Fragment DNA concentration was determined using a NanoVue Spectrophotometer as for plasmid extraction for immediate use or storage at -20°C.

6.7 Ligation

Ligation was performed using a Quick Ligation Kit (New England Biolabs) according to kit instructions before immediate transformation as stated in **Section 6.1**.

6.8 Maintenance of the plasmid pra305

A filter paper containing pra305 plasmid DNA (pEGFP-N1 with *eGFP* gene mutated to *roGFP* and upstream human $E_{1\alpha}$ subunit of pyruvate dehydrogenase mitochondrial targeting sequence that expresses as a fusion protein) (Hanson et al., 2004), kindly donated by Prof. S. James Remington, was transferred to a 1.5ml Eppendorf tube and washed with 50µl TE buffer. 50µl of One Shot®TOP10 chemically competent *E.coli* (Invitrogen) were added to the Eppendorf tube before being placed on ice for 30 minutes and then treated as **Section 6.1** and plated on 30µg/ml kanamycin LB agar plates. The plasmid was extracted as **Section 6.2**, identity verified by digestion in NEBuffer 4 using PstI-HF and NotI-HF according to **Section 6.4** and run on a gel as stated in **Section 6.5**.

6.9 Cloning strategy

The segment of the pra305 plasmid containing the roGFP gene was mutated using high fidelity PCR to include a BglII restriction site. High fidelity PCR was carried out using Herculase Enhanced DNA Polymerase according to product instructions using an annealing temperature of 64.6°C. The primers used were TGCCTGAGATCTATGAGGAAGATGCTCGCC (forward primer to include mitochondrial targeting sequence), CCGGTCAGATCTATGGTGAGCAAGGGCGAG (forward primer to exclude the mitochondrial targeting sequence) and AGAGTCGCGGCCGCTTTACTTGTACAGCTCGT (reverse primer). The resulting PCR products were run on a gel as in **Section 6.5** and extracted as in **Section 6.6**.

Restriction digests were performed on the roGFP PCR products and pUAST plasmid as in **Section 6.4** using the restriction enzymes BglII and NotI-HF with NEBuffer 3. The 8.896kb pUAST fragment was CIP (New England Biolabs) treated according to manufacturer instructions, run on a gel as stated in **Section**

6.5, gel extracted as in **Section 6.6** before ligation to one of the two roGFP fragments (0.888kb and 0.731kb respectively) according to **Section 6.7**.

The ligation transformed *E.coli* were plated on 100µg/ml ampicillin in L-Agar plates by pipetting 125µl bacterial culture per plate and incubated for 12 hours at 37°C. Five colonies were selected from the pUASTmito-roGFP and five colonies from the pUASTroGFP plates followed by amplification and miniprep as described in **Section 6.2**. Plasmid concentration was determined using a NanoVue Spectrophotometer before storage at -20°C for transformation into S2 cells and then whole *Drosophila*.

Plasmids from each colony were analysed to confirm the identity and purity of the plasmid DNA from the transformations using a restriction digest (**Section 6.4**) before gel electrophoresis (**Section 6.5**). The restriction digest was performed using NEBuffer 3, BglII and NotI-HF.

6.10 Verification of plasmid sequence

The minipreps of pUASTmito-roGFP plasmids from two colonies, the pUASTroGFP plasmids from two colonies and pra305 plasmid were sent to GATC Biotech (www.gatc-biotech.com) for sequencing to ensure no mutations had occurred during the high fidelity PCR. The primers used for sequencing were GCACCATCTTCTTCAAGGAC (Forward) and TTCACCTTGATGCCGTTC (Reverse). Sequence alignment was performed using the Ensemble software (Ensemble Software, UK) on the .abi format files from GATC Biotech and the roGFP sequence that was sent with the pra305 plasmid (**Appendix I**).

6.11 Validation of constructs

Maxipreps were prepared of pUAST, pUASTmito-roGFP and pUASTroGFP. A Costar® 6 well cell culture plate (Corning) of *Drosophila* S2 cells (Life Invitrogen) in Schneider's *Drosophila* medium was kindly provided by **Ms. Dominika Korzekwa** and either pUAST (negative control), pUASTmito-roGFP or pUASTroGFP cotransfected with pDES-GAL4 (kept in-house) using a Calcium Phosphate Transfection Kit (Invitrogen) according to the kit instructions. Cells were visually inspected for fluorescence 24 hours after transfection using a

Universal visualizing light source for fluorescent stereo microscopy attached to a LEICA MZ6 microscope.

Both pUASTmito-roGFP and pUASTroGFP had 50µl of maxiprep shipped on dry ice to BestGene (BestGene Inc., USA) for transformation into *Drosophila* and ten lines of UAS-roGFP and nine lines of UAS-mito-roGFP were received. Lines were visually inspected for off target autofluorescence using a Universal visualizing light source for fluorescent stereo microscopy attached to a LEICA MZ6 microscope. The line 12888-1-1M-Ch2 was used to generate a stable *actin* driven whole fly roGFP expressing line (stable *c564*, *elav*, *uo* and *tsp42* driven lines were also made but not used) and 12888-2-3M-Ch2 was used to generate a stable *actin* driven whole fly mito-roGFP expressing line (**Section 2.2**).

Functionality of the roGFP reporter was determined by grinding groups of 10 feeding third instar larvae (the pigments of adults interfered with the assay) in Eppendorf tubes with 100µl PBS using a pestle and sonicating as in **Section 3.1.1**. Homogenates were made up with PBS to 176µl for determining the fully reduced state of the probe and 193µl for determining the fully oxidized state of the probe before transferring to a clear bottomed opaque 96 well plate.

A Mithras LB940 plate reader (EG&G BERTHOLD) was used to excite the samples at 405nm followed by 492nm while detecting fluorescence at 530nm. The plate reader was set to cycle though the two excitation frequencies four times with a one second delay between each excitation, to prevent the 405nm emission reducing the 492nm emission.

To fully reduce and oxidize the probes 20µl of 0.1M DTT (10mM final concentration) and 3µl of 30% wt/v H_2O_2 (25µM final concentration) were added to the samples respectively before incubating at 4°C for 15 minutes and adding 4µl of 1M N-ethyl maleimide and mixing thoroughly followed by another 10 minute incubation. A second set of fluorescence readings were then taken using the plate reader to determine if a shift in the ratio of emission intensities had occurred.

7 ROS quantification using roGFP

Standard *Drosophila* medium was melted in a microwave and pipetted in approximately 3ml aliquots to cover the base of 5cm diameter petri dishes. Petri dishes of permethrin containing food were made by adding 127µg/ml permethrin dissolved in alcohol (a moderate dose for males used in the feeding survival assays) and vortexing thoroughly.

Early third instar *Drosophila* larvae were transferred in groups of 100 (max 200 per plate) using forceps to fresh food containing petri dishes for 24 hours. Larvae were then split equally between fresh food control and 127µg/ml permethrin containing food petri dishes. At 6, 9, 24, 36 and 24 hours after transfer to the experimental petri dishes four technical replicates of 10 larvae were removed and transferred to Eppendorf tubes containing 100µl PBS with 20mM N-ethyl maleimide, ground using a pestle and sonicated as in **Section 3.1.1**. An additional 100µl PBS with 20mM N-ethyl maleimide was added and the 405nm and 492nm fluorescent emissions read using a Mithras LB940 plate reader as in **Section 6.11**. Positive controls treated with DTT and H_2O_2 were prepared as in **Section 6.11**. All time points were assayed in quadruplicate and mortality at 24, 30 and 48 hours noted.

8 *Bombyx* rearing

8.1 Diet preparation

M_{100} chow (100% reconstituted mulberry leaf powder) was prepared by mixing 18mg mulberry leaf powder (Silkworm Store) with 48ml distilled H_2O in a 10cm diameter petri dish and heated in a microwave for 20 seconds. The diet was cut using a scalpel and never allowed to come into contact with frass. M_{100} chow was discarded if not used within a week.

8.2 Diagnosis for grasserie

Newly arrived *Bombyx Mori* eggs or larvae (Silkworm Store) were quarantined and a PCR based diagnostic for the presence of Bombyx mori nucleopolyhedrovirus (BmNPV), a common and fatal disease-causing baculovirus

of silkworm stocks, performed in accordance to Khurad *et al*., 2004 (Khurad et al., 2004). Only progeny of batches that were baculovirus negative were used for experimentation.

8.2.1 Extraction of genomic DNA

Ten first instar larvae, single fourth or fifth instar larvae or approximately 50µl of frass was homogenized in 400µl of genome extraction buffer using a pestle before adding an additional 400µl of genome extraction buffer. The solution was cooked at 65°C using a Dri-Block DB-2A (Techne) for 30 minutes mixing every 10 minutes. The solution was cooled on ice and 176µl 2:5 5M K-acetate:6M LiCl solution added and mixed thoroughly before incubating on ice for 30 minutes. Samples were then centrifuged at 13k rpm for 15 minutes at 4°C before transferring the supernatant to a new Eppendorf tube and adding 700µl isopropanol, mixing and incubating on ice for 10 minutes. Samples were then centrifuged at 13k rpm for 15 minutes at 4°C, the supernatant discarded and the DNA pellet washed with 1ml 70% ethanol before centrifuging again at 13k rpm for five minutes at 4°C. The supernatant was discarded and the pellet air dried for 30 minutes before resuspending in 50µl distilled water. DNA concentrations were determined using a NanoVue Spectrophotometer by comparing the sample to the distilled water used for elution as a blank. DNA samples were used immediately or stored at -20°C for later use.

8.2.2 PCR diagnosis for BmNPV

PCR was performed using 10µl DreamTaq Green PCR Master Mix, 1 µl forward primer, 1µl reverse primer, 1µl genomic DNA and 7µl of distilled water. The primers used and PCR conditions were as described in Khurad *et al*., 2004 (Khurad et al., 2004). Primers were CCAAACGACTATGACGCAAATTAATTTT and TTGTTAAATTGGCCCACCACACTTTGT and the PCR reaction mixture heated at 94°C for five minutes for one cycle, 94°C for 30 seconds followed by 52°C for 30 seconds and 72°C for one minute repeated for 35 cycles, held at 72°C for three minutes using a PCR heating block and then held at 4°C. PCR products were run on a gel as described in **Section 6.5** and the presence of a 473bp band was indicative of BmNPV infection in the batch of *Bombyx*. Infected batches were

disposed of according to laboratory proceedure and all rearing equipment thoroughly sterilized.

8.3 *Bombyx* stock and maintenance

Bombyx Mori eggs were hatched and reared at 26°C, 50% humidity on a 16:8 light dark cycle. Larvae were fed on M_{100} chow (100% reconstituted mulberry leaf powder) and reared in 10cm Petri dishes (STERILIN) changed every second day. As the race (strain) of the silkworms was not known, the moltinism, voltinism, chorion colour, serosa colour, larval markings, haemolymph colour, cocoon colour, cocoon shape, phagy and adult phenotypic markers were observed. Larvae were harvested for experimentation when weighing 1g. Instar and mortality data was also recorded.

Adult moths were mated for four hours before disengaging the male and males were not allowed to mate with more than two females. Mated females were held by the wings in a vertical position and gently shaken to encourage urination before being placed on a paper surface and confined with a medium weighing boat to lay eggs. Eggs were either left for four days after laying before being put into long term storage at 4°C (minimum 20 days, maximum 2 years) or had the diapause interrupted using the hot acid dip method. The hot acid dip method was performed on eggs under 20 hours old by bathing them in 16% HCl at 46°C for 6 minutes before rinsing with distilled water until the pH was neural and air drying.

9 Measurement of H_2O_2 in acutely exposed *Drosophila* and *Bombyx*

9.1 Permethrin application

9.1.1 Drosophila

Topical application was performed on 7-day-old CS strain *Drosophila* as in the topical application survival bioassays using control, LD_{10}, LD_{50} and LD_{80} doses of permethrin (0, 2, 5 and 6ng/fly for males and 0, 6, 8 and 12.5ng/fly for females). Ten flies were removed at 1, 2, 6, 12 and 24 hours and placed in 1.5ml Eppendorf tubes before adding 100µl 0.05M phosphate buffer and flash

freezing in liquid nitrogen. The frozen samples were stored at -80°C until all samples for the peroxide assay had been collected. Samples were stored for no longer than three days.

Contact survival bioassays were performed on 7-day-old CS strain *Drosophila* using a control of 0µg/vial and 5µg/vial doses of permethrin. Ten flies were removed at 96 hours and then processed like 7-day-old CS topical application samples.

9.1.2 Bombyx

Topical survival bioassays were performed on 208 final instar *Bombyx* larvae using 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4ng/µl permethrin in acetone on larvae weighing between 0.7-1.9g at 24 and 48 hours to identify the LD_{50} and dose response. Application of 1µl to the thoracic notum was performed using a pipette without CO_2 pad immobilization. Based on the regression line of the probit analysis, doses of 0 (control), 2 (LD_{20}) and 3.5 (LD_{60})ng/g were selected for use in protein and peroxide assays.

Bombyx were topically applied on the thoracic notum with an equal number of males and females in each group and processed at different time points. Whole larvae samples were prepared at 48 hours after exposure by putting 10 larvae in a 50ml falcon tube, filling it with liquid nitrogen three times and then homogenizing the larvae under liquid nitrogen using a Polytron® PT1200B homogenizer with 92/Polytron® PT-DA 1212/2 bit (Kinematica AG). The liquid nitrogen was then allowed to evaporate and the sample suspended in 30ml 0.05M phosphate buffer before centrifuging at 4k rpm for 6 minutes (Sigma 204 centrifuge, Sigma Laborzentrifugen, Germany). The middle aqueous layer was then transferred to an Eppendorf tube and centrifuged at 13k rpm for 3 minutes at 4°C and transferred to a new Eppendorf before flash freezing in liquid nitrogen and storage at -80°C until all samples for the peroxide assay had been collected, samples were stored for no longer than three days.

Groups of ten larvae were anaesthetized on ice before decapitation with a scalpel blade collecting head samples at 48 hours after exposure. The bodies were dissected collecting Malpighian tubules at 12, 24, 36, 48, 60 and 72 hours

after exposure, cuticle from between the star and moon spots at 12 and 48 hours after exposure and midgut and silk glands at 48 hours after exposure. 100µl 0.05M phosphate buffer was added, the samples were flash frozen in liquid nitrogen and stored at -80°C until all samples for the peroxide assay had been collected, samples were stored for no longer than three days.

9.2 Sample preparation

The samples were defrosted on ice and ground with a pestle. The pestle was thoroughly washed then rinsed once with distilled water and once with 70% ethanol before use and between each sample. The samples were kept on ice. Samples were then sonicated using a Microson™ Ultrasonic Cell Disrupter (MISONIX) as in **Section 3.1.1**. Samples were sonicated with four one second pulses before centrifuging at 13k rpm for three minutes. The supernatant was drawn into a pipette without disturbing the pellet leaving two millimetres of fluid above the pellet surface and transferred into a fresh Eppendorf tube. The Eppendorf tube was spun at 13k rpm for 3 minutes and the supernatant transferred to a third Eppendorf tube, avoiding disturbing any lipids in the pellet that had precipitated out. The samples were kept on ice and immediately processed using the protein and peroxide assay kits.

9.3 Bradford assay

Samples were diluted 1 in 100 using 0.05M phosphate buffer, to avoid sample viscosity from preventing proper mixing, and their protein content measured using a Bio-Rad Protein Assay (Bradford Assay) (Bio-Rad) following kit instructions with the exception that the 50µl sample was added to the 96 well plate before the 200µl Bradford reagent to get better mixing. Technical replicates were performed in triplicate and samples diluted with phosphate buffer to a protein concentration of 15µg/µl for peroxide assays. All readings were made using 96 well plates and a Mithras LB940 plate reader. Standards of bovine serum albumin (Invitrogen) (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 45µg/ml) were used.

9.4 Peroxide assay

All peroxide assays were carried out using an Amplex®Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen). Phosphate buffer supplied with the kit was used in sample preparation. Assays were performed on 50µl of sample following the kit instructions with technical replicates performed in triplicate. All assay readings were made using 96 well plates and a Mithras LB940 plate reader and standards of H_2O_2 (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 45µM/ml).

9.5 Data processing

Data was processed using Graphpad Prism 5, readings for both protein and peroxide assay samples were compared relative to the standard curves to obtain values in µg and µM respectively. Results from the peroxide assays were then compared relative to each other using AVOVA with the Tukey's Range Test as a post test.

10 Photography

Photos of the decapitated heads of tryptophan catabolism mutants and knockdowns and of insecticide exposure symptoms in live *Drosophila* were taken using a Nex-C3 camera (Sony) attached to a Leica Wild M3C microscope with a Wild Heerbrugg 246910 Apochromat 1x lens (Leica) by a Leitz 180570 Wild Photo Port with 100/100 Split and Built-in Iris with Leitz 404207 Phototube Adapter, Leitz-Wetzlar 519740 Periplan 10x red dot eyepiece and Leitz 376110 Extension Tube (Leitz). Eye colour was quantified using Corel Paintshop Photo Pro X3 (Corel, UK) using the dropper tool set to a 11x11 pixel sample size taking 12-13 measurements for each eye of two different fly heads.

Chapter 3 - Metabolic changes in *Drosophila* on permethrin exposure

1 Introduction

Exposure to compounds causes a shift in metabolites that can be diagnostic for the specific compound (Taylor et al., 2010) and informative as to how the compound interacts with the organism in question and what occurs in the organism to maintain homeostasis. In this way metabolomics can identify bottlenecks or increased flux though pathways caused by the compound or the active regulation of pathways by the organism in response to the compound, for example detoxification pathways, to reduce potential pathologies even if there is no obvious phenotype. By targeting these altered pathways it is possible to reduce or increase the effect the compound has (Jackson, 1993). The use of metabolomics in target identification has already been successfully applied in pharmacology (Rabinowitz et al., 2011) and in the validation of genetic lesions in the genetically tractable model organism *Drosophila melanogaster* (Kamleh et al., 2008).

The damage and health problems inflicted by insect pests has caused humanity to become reliant on efficient insecticides. This has resulted in the problem that all currently known insecticide modes of action have resistant pest populations, with some populations being resistant to multiple modes of action, and the emergence of resistance in previously susceptible populations becoming more frequent (Hardy, 2014). The magnitude of resistance can be suppressed to some extent by adding synergists that increase permeation of the insecticide or suppress degradation pathways; however, there are now resistant pest populations insensitive to existing synergists (Zhang et al., 2008a). This means there is an urgent need to find new targets to allow the development of novel insecticides and synergists that will prevent existing insecticides from becoming ineffective.

The primary target of the pyrethroid insecticides is the voltage gated sodium channel (O'Reilly et al., 2006); however there has been no systematic study of secondary targets while those that have been identified (listed in **Ch. 1, Section 3.4**) have not been validated in target organisms. Studies investigating changes

in physiological parameters caused by pyrethroid exposure often only investigate a select choice of compounds without studying associated metabolites (Saleem and Shakoori, 1993) or quantify crude fractions consisting of numerous similar compounds (Saleem and Shakoori, 1987), with the majority of studies carried out in non-target organisms that were relevant to medical or environmental questions. Although high throughput metabolomics approaches have been applied to vertebrate (Liang et al., 2013) and aquatic arthropod (Taylor et al., 2010) models of pyrethroid exposure there have been no metabolomics studies carried out on models relevant to insect pest species. Metabolomics was therefore used to identify metabolic pathways altered in *Drosophila melanogaster* by the pyrethroid permethrin and to identify any permethrin induced lesions in metabolism and metabolic changes that would aid or impair survival.

2 Source of the data set

In conjunction with Pfizer Veterinary Discovery, a commercial metabolomic analysis had been commissioned before the start of this project. Samples were prepared by **Ms. Louise Henderson** according to **Ch. 2, Section 3.2.1** to generate the report by **Metabolon**, which was made available at the start of the project. A major first goal of this project was to analyse and interpret the raw data from this analysis with the aim of identifying metabolic pathways which seemed particularly affected. Such analysis could identify both the changes induced by permethrin, and the steps taken by the insect to defend against the xenobiotic. All interpretation of the data was performed personally, parts of which have been published in Brinzer *et al.*, 2015 (Brinzer et al., 2015).

3 Results and discussion

The metabolomic analysis had data for larvae orally exposed to a ~LD_{10} dose of permethrin and adults exposed to a sublethal dose through contact at 2, 12 and 24 hours of exposure. The adult data set showed far fewer metabolic changes than larvae indicating either larvae are more metabolically active than adults or the dose used on adults was below the effective concentration to trigger certain changes. It was experimentally determined (**Ch. 2, Section 5.3**) using contact survival assays that the LD_{50} for adult Canton S flies was 7 µg/vial (3.2-10.1

95%CI) for males and 20.2 µg/vial (16.6-24.6 95%CI) for females confirming that the dose used to generate the adult data set (1 µg/vial) was too low. The use of multiple time points enabled the determination of flux for some metabolites and the identification of progressively worsening restrictions in metabolism. Metabolites detected from pathways that changed significantly (ANOVA and random forest) on permethrin exposure are listed in **Tables 3-1** to **3-10** and the physiological implications covered below.

3.1 Amino acid metabolism

Permethrin exposed *Drosophila* larvae showed an increase in the majority of free amino acids (**Table 3-1**). This is consistent with what has been reported in other invertebrate and vertebrate species (**Appendix II**) on exposure to permethrin and other pyrethroids, probably as a result of enhanced protein degradation (Reddy and Bhagyalakshmi, 1994). In vertebrates protein oxidation is thought to be the cause for the increase in protein degradation and is found to primarily occur in the liver, a xenobiotic detoxification tissue, as a result of necrosis (Parthasarathy and Joseph, 2011) however, the causes for and tissue specificity of protein degradation during pyrethroid exposure have never been investigated in invertebrates although permethrin exposure is known to trigger apoptosis in neurons (Roma et al., 2013). The protein degradation origin of the free amino acids is supported by the increase of several N-Acetyl-amino acids, which are only produced post-translationally by protein N-acetylation.

Table 3-1. Permethrin effects on amino acid metabolism in larvae

Larvae			Fold Change after exposure		
Metabolic Subpathway	Metabolite	Pathway/Function	2h	12h	24h
Gly, Ser and Thr	glycine		1.62	1.54	1.33
Gly, Ser and Thr	sarcosine	Choline→glycine	2.23	3.18	1.35
Gly, Ser and Thr	dimethylglycine	Choline→glycine	0.43	0.47	0.55
Gly, Ser and Thr	serine		1.93	1.67	1.32
Gly, Ser and Thr	N-acetylserine	Degradation product of N-acetylated proteins	1.29	1.23	1.41
Gly, Ser and Thr	threonine		1.87	1.93	1.61
Gly, Ser and Thr	2-aminobutyrate	Product of 2-oxobutyrate and ammonia	1.53	2.04	1.99

Larvae			Fold Change after exposure		
Metabolic Subpathway	Metabolite	Pathway/Function	2h	12h	24h
Gly, Ser and Thr	allo-threonine	Glycine↔allo-threonine	1.52	4.15	3.91
Gly, Ser and Thr	betaine	Choline→glycine	1.13	1.18	1.13
Ala and Asp	alanine		0.98	1.08	1.07
Ala and Asp	β-alanine	Uracil→β-alanine and substrate for catecholamine synthesis	0.54	0.71	0.6
Ala and Asp	aspartate		1.45	1.15	0.95
Ala and Asp	asparagine		2.31	2.29	1.91
Glu	glutamate	Amino acid and neurotransmitter	2.17	1.86	1.71
Glu	glutamine		1.03	1.1	1.08
Glu	GABA	Glutamate→succinate, neurotransmitter	0.81	0.58	0.29
Glu	N-acetylglutamate		0.51	0.41	0.44
His	histidine		1.32	1.49	1.34
His	histamine	Histidine→histamine, neurotransmitter	0.98	0.95	0.54
Lys	lysine		1.03	1	0.87
Lys	2-aminoadipate	Lysine→acetyl-CoA, competitive inhibitor of glutamate and cysteine transport	2.69	4.48	2.57
Lys	pipecolate	Lysine→acetyl-CoA	1.9	2.19	1.87
Carnitine	N-6-trimethyllysine	Protein→N-6-trimethyllysine→carnitine	1.66	1.88	1.75
Lys	N2-acetyllysine	Modified histone degradation product	1.69	1.82	3.36
Lys	N6-acetyllysine	Modified histone degradation product	1.94	1.74	1.66
Phe and Tyr	phenylalanine		1.53	1.31	1.24
Phe and Tyr	tyrosine		4.18	8.39	0.95
Phe and Tyr	L-DOPA	Tyrosine→dopamine, neurotransmitter precursor	6.7	13.73	1.96
Phe and Tyr	N-acetylphenylalanine	Excreted product of phenylalanine	1.33	1.27	1.44
Trp	Kynurenic acid	Tryptophan→kynurenine→kynurenic acid	2.27	5.52	4.08
Trp	kynurenine	Tryptophan→kynurenine→XA	1.59	1.83	1.2
Trp	Tryptophan		1.36	1.15	0.98
Trp	3-hydroxykynurenine	Tryptophan→kynurenine→XA	1.24	1.67	1.3
Val, Leu and Ile	3-methyl-2-oxobutanoate	Valine synthesis and degradation metabolite	6.78	8.3	8.3
Val, Leu and Ile	3-methyl-2-oxopentanoate	Isoleucine synthesis and degradation metabolite	2.78	2.73	3.64
Val, Leu and Ile	Isoleucine		2.02	1.52	1.14
Val, Leu and	Leucine		1.86	1.56	1.14

Larvae			Fold Change after exposure		
Metabolic Subpathway	Metabolite	Pathway/Function	2h	12h	24h
Ile					
Val, Leu and Ile	N-acetylleucine	Degradation product of N-acetylated proteins	1.24	1.35	0.79
Val, Leu and Ile	Valine		1.48	1.36	1.11
Val, Leu and Ile	4-methyl-2-oxopentanoate	Leucine synthesis and degradation metabolite	2.91	2.33	3.41
Cys, Met and SAM	cysteine		1.62	1.7	1.53
Cys, Met and SAM	methionine sulfoxide	Oxidised product of methionine, caused by ROS	2.18	3.21	3.57
Cys, Met and SAM	methionine		1.34	1.02	0.92
Cys, Met and SAM	homocysteine	Methionine→cysteine	1.92	3.58	4.03
Cys, Met and SAM	homoserine	Produced by bacteria in gut	0.83	0.66	1.02
Cys, Met and SAM	5'methyladenosine (MTA)	Methionine→SAM→5'methyladenosine	2.16	2.76	3.15
Urea, Arg and Pro	arginine	amino acid and urea cycle metabolite	1.28	1.09	1.02
Urea, Arg and Pro	N-acetylarginine	Product of arginine during ammonia stress	2.39	3.16	4.86
Urea, Arg and Pro	ornithine	Urea cycle metabolite	1.53	2.77	1.58
Urea, Arg and Pro	urea	Urea cycle metabolite	2.08	4.2	4.8
Urea, Arg and Pro	proline		0.99	0.9	0.84
Urea, Arg and Pro	N-acetylproline	Degradation product of N-acetylated proteins	1.79	2.84	1.55
Urea, Arg and Pro	spermidine	Ornithine→GABA	0.55	0.41	0.44
Glutathione	5-oxoproline	Cysteine→Glutathione→5-oxoproline	1.13	1.24	1.33
Glutathione	γ-glutamylvaline	Cysteine→Glutathione→5-oxoproline	3.18	3.14	2.59
Glutathione	γ-glutamylleucine	Cysteine→Glutathione→5-oxoproline	2.96	2.59	2.88
Glutathione	γ-glutamyisoleucine	Cysteine→Glutathione→5-oxoproline	2.73	2.55	2.06
Glutathione	γ-glutamyphenylalanine	Cysteine→Glutathione→5-oxoproline	2.1	2.18	2.45
Glutathione	γ-glutamyalanine	Cysteine→Glutathione→5-oxoproline	1.56	1.38	1.84

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

Glycine, serine, aspartate, asparagine, glutamate, phenylalanine, tryptophan, valine, leucine, isoleucine, methionine and arginine elevate rapidly on exposure reaching a peak around 2 hours after exposure before showing a decreasing trend as time progresses which would be consistent with a burst of protein degradation in response to a xenobiotic stimulus. Threonine, tyrosine, cysteine and histidine only peak around 12 hours after exposure indicating either a delay in pathway regulation, other perturbed metabolites acting as a feedstock for synthesis, inhibition of catabolic pathways or a delayed degradation of proteins rich in those amino acids. The concentrations of alanine, glutamine and lysine showed no change suggesting either that synthesis is more tightly regulated, catabolism has no proximate bottlenecks or is highly redundant or the rate of reincorporation into proteins is high. The only amino acid to show a decrease was proline which could be explained by an inhibition of synthesis from ornithine, increased conversion into glutamate or prolyl 4-hydroxylase activity feeding proline directly into succinate for the TCA cycle.

Adults showed a different amino acid profile on permethrin exposure (**Table 3-2**). Only threonine, aspartate, phenylalanine, glutamine, alanine, proline increased on exposure while histidine decreased indicating either that adults are less sensitive to permethrin-induced protein degradation or that the dose of permethrin used was too low to induce protein degradation. The increase in glutamine and alanine indicates reduced utilization of these amino acids by energy metabolism in adults compared to larvae. Proline is increased in adults while histidine is depleted unlike what is observed in larvae indicating different pressures on the utilization of those amino acids at different stages in the life cycle.

Table 3-2. Permethrin effects on amino acid metabolism in adults

Adult			Fold Change (Male)			Fold Change (Female)		
Metabolic Subpathway	Metabolite	Pathway/Function	2h	12h	24h	2h	12h	24h
Gly, Ser and Thr	sarcosine	Choline→glycine	0.97	0.74	2.13	0.48	0.64	2.84
Gly, Ser and Thr	threonine		1.03	1.18	1.1	0.84	1.14	1.36
Gly, Ser and Thr	betaine	Choline→glycine	1.02	1.2	0.72	0.78	0.68	0.55
Ala and Asp	alanine		0.99	1.17	0.92	1.01	1.1	1.06

Adult			Fold Change (Male)			Fold Change (Female)		
Metabolic Subpathway	Metabolite	Pathway/Function	2h	12h	24h	2h	12h	24h
Ala and Asp	aspartate		0.77	1.91	1.26	0.46	1.29	0.65
Glu	N-acetylglutamate		1.06	0.58	0.72	0.97	0.62	0.67
His	histidine		0.67	0.71	0.7	0.86	0.95	1.35
His	histamine		1.05	0.79	0.95	0.66	0.92	0.57
Phe and Tyr	phenylalanine		0.95	1.23	0.95	0.82	1.04	1.02
Trp	xanthurenic acid	Tryptophan→xanthurenic acid, ommochrome precursor, antioxidant	0.96	1.12	1.05	1.15	1.01	0.92
Trp	kynurenine	Tryptophan→kynurenine→ xanthurenic acid	0.97	0.86	1.14	0.94	1	1.62
Trp	3-hydroxykynurenine	Tryptophan→kynurenine→ xanthurenic acid	1.18	1.54	0.98	0.92	1.01	0.62
Cys, Met and SAM	methionine sulfoxide	Oxidised product of methionine, caused by ROS	0.65	0.47	0.55	0.62	0.58	0.71
Cys, Met and SAM	homocysteine	Methionine→cysteine	0.62	0.52	0.55	0.66	0.52	0.73
Urea, Arg and Pro	dimethylarginine	Modified histone degradation product	0.84	0.8	1.01	0.75	0.73	1.12
Urea, Arg and Pro	proline		0.98	4.15	1.12	0.98	2.05	1.18
Glutathione	5-oxoproline	Cysteine→Glutathione→ 5-oxoproline	0.89	1.24	1.27	1.1	1.01	1.15

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

3.1.1 Glycine, serine and threonine

It has been assumed that the increased free amino acids are used solely for protein synthesis upon permethrin challenge but the metabolomics data for larvae shows an increase in the catabolites of most amino acids indicating an increase in flux through degradation pathways. As seen in **Figure 3-1**, glycine is probably being metabolised via threonine (as indicated by the time offset between the fold changes of threonine and glycine peaking) using sarcosine and allo-threonine as sinks. The conversion of glycine to sarcosine goes via the putative glycine methyltransferase *CG6188*, a gene known to be upregulated in DDT resistant *Drosophila* strains (Misra et al., 2013). This would stimulate the consumption of methionine for cysteine synthesis though the production of S-adenosylhomocysteine. Interestingly the *Anopheles gambiae* homolog of *CG6188* (*AGAP002198*) has shown up in an in-silico model of *Anopheles*

metabolism as a bottleneck that may be a potential insecticidal target (Adebiyi et al., 2015). In larvae there is a depletion of dimethylglycine observed indicating a lesion at betaine-homocysteine methyltransferase, an enzyme whose orthologs are known to be inhibited by high methionine and cysteine concentrations. An upregulation of glycine-sarcosine methyl transferase activity could also explain the decrease in dimethylglycine however both enzymes have never been investigated in Diptera.

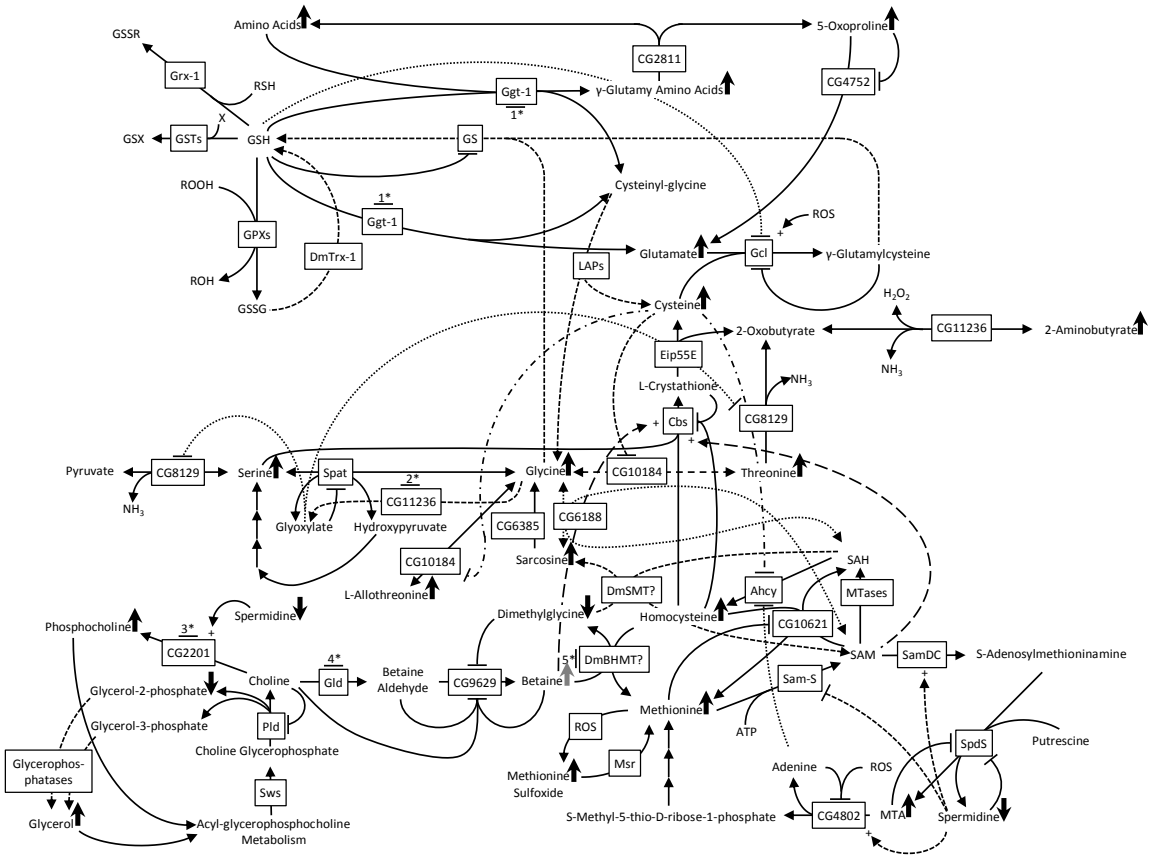

Figure 3-1. Summary of glycine, serine, threonine, methionine, cysteine and glutathione metabolism in larvae. Metabolites detected that were significantly altered on permethrin exposure have a black arrow at the upper right, ↑ = $p<0.05$ - an increase, ↓ = $p<0.05$ - a decrease. Metabolites that just missed the significance cutoff, $0.05<p<0.1$, have a grey arrow at the upper right. The boxes are enzymes involved in the reactions represented by arrows showing directionality, adjacent bars represent inhibition from metabolites leading to it, adjacent (+) represent stimulation from metabolites leading to it. Enzymes with names ending in a (?) have never been investigated in *Drosophila*. 1* = Inhibited by glycine, serine, methionine sulfoxide and glutathione, 2* = Inhibited by serine, glyoxylate, 2-oxobutyrate and cysteine, 3* = Inhibited by phosphocholine, choline, betaine, serine and methionine, 4* = Inhibited by betaine aldehyde, dimethylglycine and glycine, 5* = Inhibited by choline, betaine aldehyde, dimethylglycine, methionine, adenosylhomocysteine (SAH) and cysteine. Figure modified from (Brinzer et al., 2015).

3.1.2 Methionine and cysteine

There is evidence in larvae (**Table 3-1**) that methionine is being metabolised via S-adenosylmethionine to form methyl-5'-thioadenosine and homocysteine. The accumulation of methyl-5'-thioadenosine in the methionine recycling pathway of the larval data set indicates a lesion at MTA phosphorylase. Homocysteine together with serine is fed into cysteine for glutathione metabolism which is known to be upregulated in insects on pyrethroid exposure (Lin et al., 2014). Raised concentrations of γ-glutamyl amino acids (2-3.2-fold at 4, 12 and 24 h) indicates an increase in gamma-glutamyl transpeptidase activity on permethrin exposure as has been observed in mammalian models exposed to pyrethroids (Mongi et al., 2011).

3.1.3 Lysine

Lysine is being degraded via the pipecolate pathway, as indicated by increased concentrations of pipecolate and not saccharopine (**Table 3-1**), with an observed accumulation of 2-aminoadipate (4.48-fold at 12 hours). This lesion is potentially caused by 2-aminoadipate competing for kynurenine tranaminase binding which also uses kynurenates, other amino acids and oxoacids, all of which also are increased on permethrin exposure, as substrates (Han et al., 2010). Alternatively a lesion at the α-ketoglutarate dehydrogenase complex which catabolises step in the pathway after transamination could also explain the increase in 2-aminoadipate. The increase in N-6-trimethyllysine indicates that lysine is also being channelled into carnitine synthesis which explains the lack of lysine accumulation in larvae as there is metabolic redundancy.

3.1.4 Tryptophan

Tryptophan catabolism in larvae shows accumulations of the neurotoxic 3-hydroxykynurenine (1.67-fold at 12 hours) and the neuroprotective kynurenic acid (5.52-fold at 12 hours).

3.1.5 Valine, leucine and Isoleucine

Larvae have a large increase in catabolites of the branched chain amino acids (3-methyl-2-oxobutanoate (8.3-fold at 12 hours), 4-methyl-2-oxopentanoate, 3-methyl-2-oxopentanoate and isovaleric acid) indicating metabolic lesions at the branched-chain α-ketoacid dehydrogenase complex and isovaleryl-CoA dehydrogenase.

3.1.6 Phenylalanine and tyrosine

There is evidence in larvae that potentially toxic increases in phenylalanine are being avoided by degradation via both N-acetylphenylalanine, a metabolite that is rapidly excreted and tyrosine (as indicated by the time offset between the fold changes of tyrosine and phenylalanine peaking). The high tyrosine concentrations observed (up to 8.39 fold at 12 hours) can be explained by the influx of tyrosine synthesized from phenylalanine, by the impairment of dopamine metabolism by permethrin (Elwan et al., 2006) and by competition with phenylalanine and aspartate for aspartate transaminase binding.

L-DOPA (13.7-fold at 12 hours) is the amino acid catabolite with the largest increase in larvae and indicates a potential lesion at DOPA decarboxylase induced by permethrin treatment. L-DOPA transport could also be disrupted by competitive binding to the transporter by other amino acids (Kageyama et al., 2000) preventing access to enzymes involved in catabolising pathways.

3.1.7 Histidine

In larvae the increase in histidine and decrease in histamine suggests a lesion at histidine decarboxylase. As histamine is a neurotransmitter, the depletion is able to be explained by the uncontrolled release of neurotransmitters caused by permethrin induced hyper excitation of neurons (Feng et al., 1992).

3.1.8 Glutamate, glutamine, proline and alanine

If the free amino acids in larvae are from protein degradation an increase in all amino acids would be expected however, this is not seen for proline, alanine and glutamine indicating a high utilization of those amino acids. It is known that

proline (Scaraffia and Wells, 2003) and glutamine (Bédard et al., 1993) are used by insect cells to synthesize glutamate that is then used for energy generating pathways. N-acetylglutamate is the only N-acetyl amino acid to be depleted in larvae indicating either an inhibition of N-acetylglutamate synthase activity, possibly by methionine sulfoxide inhibition, or increased L-aminoacylase activity feeding it into glutamate. The lack of alanine accumulation in larvae is probably due to the high redundancy in alanine metabolism offered by the large number of reactions that it is a substrate for.

3.1.9 Aspartate, asparagine and arginine

In larvae there is an accumulation of urea cycle metabolites indicating that asparagine and aspartate are being fed into the urea cycle to make arginine which is being converted into urea and N-acetylarginine.

3.2 Energy metabolism

3.2.1 Glycogen metabolism and glycolysis

The most significant changes in energy metabolites were observed in larvae and not adult flies. It has been observed that exposure to pyrethroids causes a decrease in glycogen content in other species (**Appendix II**) eg. (Saleem et al., 1998) so larvae having increased concentrations of maltotetraose, maltotriose, maltose, mannose, sorbitol and panose, which are products of glycogen utilization, indicate that a similar mobilization of glycogen energy reserves is occurring after permethrin exposure in *Drosophila* larvae (**Table 3-3**). Interestingly it has been found that the overexpression of glycogen branching enzyme, an enzyme that makes glycogen more branched and quicker to metabolize, causes increased resistance to deltamethrin in mosquitoes (Xu et al., 2008) suggesting that the speed of glycogen mobilization has an important impact on survival. In both larval and adult (**Tables 3-3** and **3-4**) data sets there is an observed increase in glucose and glycerate with adults having the largest increases in glucose-6-phosphate and pyruvate (**Table 3-4**). Larvae also show an increase in fructose (5.7-fold at 12 hours) indicating permethrin causes increased flux though glycolysis, as occurs in other organisms exposed to pyrethroids (**Appendix II**) eg. (Saleem et al., 1998). β-Alanine is known to

reduce glucose metabolism in insects (Jacobs, 1968) so the β-alanine depletion may be contributing to glucose mobilization. There is a decrease in lactate seen in both larvae and adults suggesting a lesion at lactate dehydrogenase which has been observed with exposure to permethrin and other pyrethroids in some species (**Appendix II**) eg. (Azmi et al., 2002). As lactate is the primary marker for anaerobic respiration in terrestrial invertebrates, it cannot be determined if permethrin is causing a limitation to the oxygen supply for aerobic respiration.

Table 3-3. Permethrin effect on energy metabolism in larvae

Larvae			Fold Change after exposure		
Subpathway	**Metabolite**	**Pathway/Function**	**2h**	**12h**	**24h**
Aminosugars metabolism	N-acetylglucosamine	Chitin monomer	2.05	2.97	1.98
Fructose and mannose metabolism	fructose		4.98	5.71	5.15
Fructose and mannose metabolism	mannitol	Antioxidant from diet	0.16	0.04	0.2
Fructose and mannose metabolism	mannose		1.95	1.62	1.75
Fructose and mannose metabolism	sorbitol	Glycogen→glucose	2.1	2.47	1.85
Starch and sucrose metabolism	panose	Glycogen→glucose	2.26	1.97	2.56
Starch and sucrose metabolism	maltotetraose	Glycogen→glucose	2.06	1.03	1.38
Starch and sucrose metabolism	maltotriose	Glycogen→glucose	3.25	2.26	3.02
Starch and sucrose metabolism	maltose	Glycogen→glucose	2.77	2.96	3.76
Glycolysis	glucose	Glucose→pyruvate	1.77	1.73	1.79
Glycolysis	glucose-6-phosphate	Glucose→pyruvate	1.11	1.22	1.7
Glycolysis	fructose-6-phosphate	Glucose→pyruvate	1.5	1.95	2.12
Glycolysis	pyruvate		1	0.76	1.02
Glycolysis	lactate	Pyruvate↔lactate	0.62	0.58	0.66
Pentose	erythronate	Dephosphorylated 4-	1.5	1.3	1.38

Larvae			Fold Change after exposure		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h
phosphate pathway		phosphoerythronate from fructose-6-phosphate			
Pentose phosphate pathway	glycerate	glycerol→pyruvate, inducer of glycolysis	1.52	1.53	1.93
Pentose phosphate pathway	Gluconate		0.9	0.95	1.77
Pentose phosphate pathway	ribose		1.12	1.06	1.31
Pentose phosphate pathway	xylulose	Fructose-6-phosphate/glucuronic acid↔xylitol	0.67	0.89	1.24
Pentose and glucuronate interconversion	xylitol	Fructose-6-phosphate/glucuronic acid↔xylitol	2.26	3.05	2.43
Pentose and glucuronate interconversion	glucarate	UDP-glucuronate→ glucarate↔xylitol	2.8	3.07	3.2
Pentose and glucuronate interconversion	phenylglucopyranoside	UDP-glucose conjugate of phenol	2.44	2.14	2.76
Kreb's cycle	citrate	Kreb's cycle metabolite	1.25	0.45	0.79
Kreb's cycle	trans-aconitate	Kreb's cycle metabolite	5.9	7.43	6.84
Kreb's cycle	α-ketoglutarate	Used for transamination and oxidation reactions	1.5	1.1	1.62
Kreb's cycle	succinate	Kreb's cycle metabolite	0.72	0.63	0.44
Kreb's cycle	fumarate	Kreb's and urea cycle metabolite	1.83	1.32	1.04
Kreb's cycle	malate	Kreb's cycle metabolite	0.98	0.4	0.14
Oxidative phosphorylation	phosphate		1.93	2.19	1.75

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

Table 3-4. Permethrin effect on energy metabolism in adults

Adult			Fold Change (Male)			Fold Change (Female)		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h	2h	12h	24h
Fructose and mannose metabolism	mannitol	Antioxidant from diet	0.4	0.78	0.27	0.28	0.42	0.49
Fructose and mannose metabolism	sorbitol	Glycogen→ glucose	0.78	0.85	1.51	0.85	0.58	1.39
Pentose phosphate pathway	glycerate	glycerol→ pyruvate, inducer of glycolysis	1.07	1.76	1.2	0.99	1.19	1.17
Glycolysis	glucose-6-phosphate	Glucose→pyruvate	0.86	2.35	1.05	0.76	0.92	2.12
Glycolysis	glucose	Glucose→pyruvate	1.09	1.07	1.17	1.11	1.22	1.05
Glycolysis	3-phospho-glycerate	Glucose→pyruvate	1.04	2.97	1.06	1.18	1.03	1.44
Glycerolipid metabolism	Glycerone	glycerol→pyruvate	0.92	1.12	5.1	1.43	0.94	0.85
Glycolysis	pyruvate		1.4	2.91	1.38	1.66	1.59	1.34
Glycolysis	lactate	Pyruvate↔lactate	0.63	0.85	0.86	0.87	0.75	0.61
Pentose and glucuronate interconversion	xylitol	Fructose-6-phosphate/glucuronic acid↔xylitol	0.95	1.04	1.08	1.09	0.89	1.07

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

3.2.2 The Kreb's (TCA) cycle

The Kreb's (TCA) cycle (**Figure 3-2**) shows many permethrin induced metabolite concentration changes, the largest increase being in trans-aconitate (7.43-fold at 12 hours), indicating an inhibition of aconitase or acidic reaction conditions (Ambler and Roberts, 1948; Gardner, 2002; Nulton-Persson and Szweda, 2001). There is a depletion of succinate and malate but an accumulation of fumarate and α-ketoglutarate. The observed pattern of metabolites can be explained by a lesion at the α-ketoglutarate dehydrogenase complex or depletion of mitochondrial thiamine pyrophosphate resulting in a depletion of mitochondrial succinate, fumarate and malate while cytosolic fumarate accumulates due to the urea cycle consuming aspartate. The high concentrations of trans-aconitate would prevent cytosolic fumarate re-entering the TCA cycle through the inhibition of fumarate hydratase.

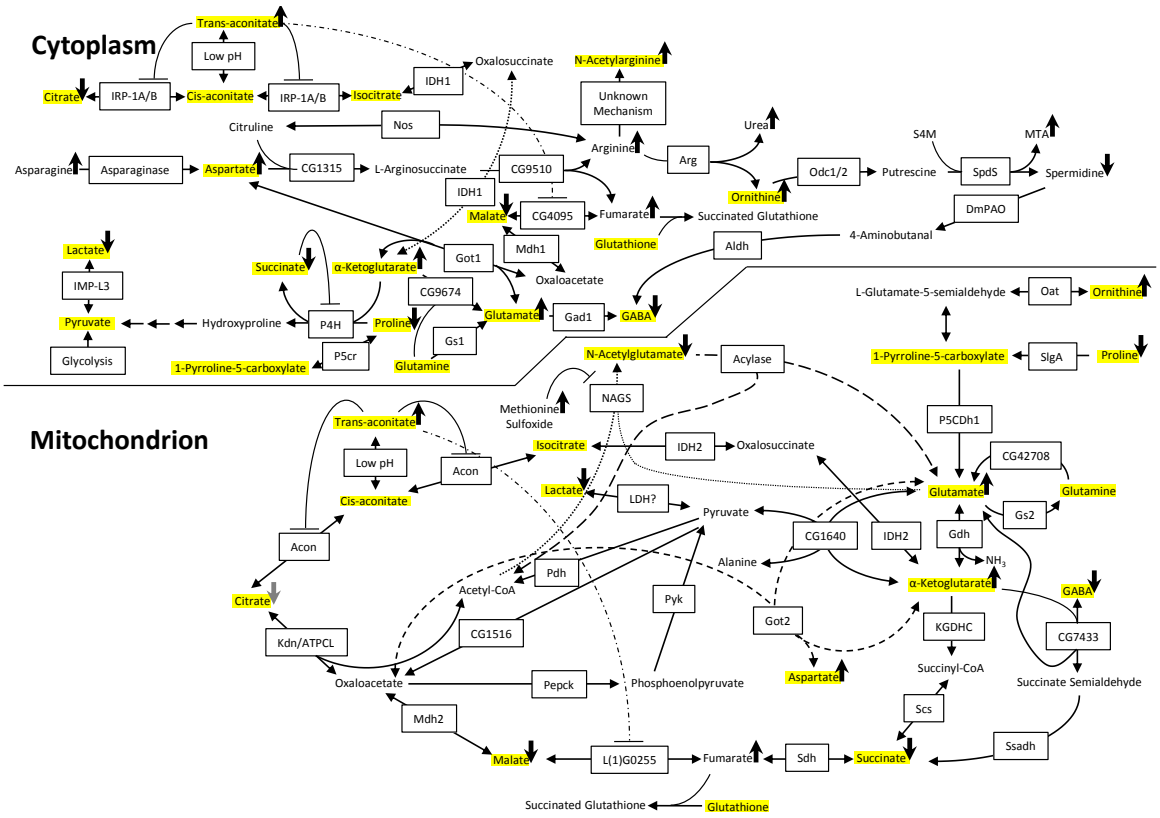

Figure 3-2. Diagram of Kreb's (TCA) cycle and nitrogen metabolism in larvae. Metabolites detected that were significantly altered on permethrin exposure have a black arrow at the upper right, ↑ = $p<0.05$ - an increase, ↓ = $p<0.05$ - a decrease. Metabolites that just missed the significance cutoff, $0.05<p<0.1$, have a grey arrow at the upper right. Metabolites highlighted in yellow are transported across the mitochondrial membrane. The boxes are enzymes involved in the reactions represented by arrows showing directionality, adjacent bars represent inhibition from metabolites leading to it, adjacent (+) represent stimulation from metabolites leading to it. Enzymes with names ending in a (?) have never been investigated in *Drosophila*. Figure modified from (Brinzer et al., 2015).

3.2.3 Role of the urea cycle in energy generation

Insect cells can utilize aspartate as an energy source when other TCA feedstocks are limiting (Öhman et al., 1995) and the observed lesion in the larval α-ketoglutarate dehydrogenase complex during permethrin exposure would greatly impair the capacity of the cell to generate energy by the TCA cycle. As fumarate is produced as a by-product of the urea cycle, there is the potential to use the urea cycle for some energy generation; however, the exact capacity to do so in insects has never been investigated. Interestingly honey bees (*Apis mellifera*) that have non-functional alleles of cytosolic fumarase, which prevents urea cycle generated fumarate entering the TCA cycle, have a reduced maximum energy output (Harrison et al., 1996) indicating the urea cycle can make a considerable contribution to energy needs.

3.2.4 Relevance of energy metabolism to insecticides

During times of high energy demand insects use proline for glutamate synthesis which is then fed into energy generating pathways (Scaraffia and Wells, 2003). The importance of this pathway for insect survival is demonstrated by the mortality observed in the Colorado potato beetle (*Leptinotarsa decemlineata*) when Δ^1-Pyrroline-5-carboxylate dehydrogenase, the second enzyme involved in the conversion of proline to glutamate, is knocked down by RNAi (Wan et al., 2014). The utilization of proline for succinate or glutamate production, a stimulated urea cycle, increased glycogen utilization and elevated glucose has been observed with exposure to malathion and DDT but no alterations of the other free amino acids occurred (Mansingh, 1965), indicating that all neuroexcitatory insecticides could result in energy metabolism perturbations but the increase in free amino acids are unique to pyrethroids.

3.3 Nitrogen metabolism

In other organisms (**Appendix II**) eg. (Reddy and Bhagyalakshmi, 1994) it has been shown that pyrethroid exposure causes an upregulation of aspartate transaminase, alanine transaminase and glutamate dehydrogenase, enzymes that facilitate the conversion of glutamate, alanine, α-ketoglutarate and oxaloacetate into aspartate. In larvae the urea cycle has an accumulation of arginine, ornithine and urea (**Table 3-1**) indicating increased aspartate utilization combined with the potential inhibition of ornithine aminotransferase which is a known secondary target of pyrethroids (Bakry et al., 2011). An alternative explanation for the increased ornithine concentrations would be an impairment of ornithine transport into the mitochondria where it is metabolised. Free amino acids produce ammonia under physiological conditions by deamination (Stadtman, 1993) which has to be detoxified by the urea cycle. High concentrations of ammonia saturate the catalytic capacity of arginase resulting on the accumulation of arginine derived guanido compounds like N-acetylarginine, a toxic compound and known marker of ammonia stress (Meert et al., 1991). Increased N-acetylarginine in larvae after 4, 12 and 24 hours permethrin exposure may indicate ammonia stress or a potential lesion at arginase.

3.4 NAD^+ metabolism

There is an overall increase in the salvage pathway of NAD^+ metabolism with nicotinate and nicotinate riboside having increased concentrations on permethrin exposure (**Table 3-5** and **Table 3-6**), but unlike most NAD^+ metabolism perturbations which result in increases of nicotinamide mononucleotide (NMN), a depletion was observed. This pattern of metabolites is consistent with an increase in NMN being salvaged via the nicotinate route or nicotinamide mononucleotide adenyltransferase. It has been shown that expression of *Drosophila* nicotinamidase, which converts nicotinamide to nicotinate, in neurons has a neuroprotective effect against oxidative stress (Balan et al., 2008) indicating that upregulation of this pathway is to reduce the effects of permethrin induced oxidative stress and other pathologies. NAD^+ metabolism can also affect gene regulation via Sir2 mediated histone deacetylation causing transcriptional silencing, as has been demonstrated in mammalian systems (Revollo et al., 2004), which would cause downstream responses to permethrin challenge.

Table 3-5. Permethrin effect on NAD^+ metabolism in larvae

Larvae			Fold Change after exposure		
Subpathway	**Metabolite**	**Pathway/Function**	**2h**	**12h**	**24h**
Nicotinate and nicotinamide metabolism	NMN	NMN↔NAD^+	0.99	0.46	0.39
Nicotinate and nicotinamide metabolism	NAD^+	Cofactor	2.26	2.42	2.88
Nicotinate and nicotinamide metabolism	nicotinate	Part of NAD^+ salvage pathway in *Drosophila*	1.4	1.3	1.47
Nicotinate and nicotinamide metabolism	nicotinate ribonucleoside	Part of NAD^+ salvage pathway in *Drosophila*	1.01	1.11	2.09

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

Table 3-6. Permethrin effect on NAD^+ metabolism in adults

Adult			Fold Change (Male)			Fold Change (Female)		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h	2h	12h	24h
Nicotinate and nicotinamide metabolism	NAD^+	Cofactor	1.43	1.05	1.24	1.52	0.73	2.55
Nicotinate and nicotinamide metabolism	nicotinamide riboside	Degradation product of NMN	1.07	0.98	0.9	1.01	1.08	0.99

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

3.5 Purine and pyrimidine metabolism

3.5.1 Purines

Larvae show an increase in purine and pyrimidine metabolism (**Table 3-7**), most likely driven by RNA degradation. It has been shown that pyrethroid exposure causes a decrease in total RNA in several species (**Appendix II**) eg. (Saleem and Shakoori, 1987). The presence of 2',3'-NMPs and their catabolites (2'AMP, 3'AMP and 2',3'-cUMP) and the modified nucleotide pseudouridine in the larval data set indicate that RNA degradation also occurs in *Drosophila* larvae during permethrin exposure. There is evidence in larvae that adenosine is being channelled via inosine and hypoxanthine towards the uric acid pathway, as indicated by the elevation in allantoin. Permethrin induces cGMP formation through acetylcholine release caused by excessive neuroexcitation (Bodnaryk, 1982), a property shared with other excitatory neurotoxic insecticides (Bodnaryk, 1976, 1977). This process would explain the guanine depletion seen in the larval data and offer a route for the reduction of the elevation in guanosine observed. The adult data set shows no consistent alterations of purine metabolism with the exception of a decrease in allantoin indicating a reduction in flux through the uric acid pathway (**Table 3-8**).

Table 3-7. Permethrin effect on purine and pyrimidine metabolism in larvae

Larvae			Fold Change after exposure		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h
Purine metabolism	hypoxanthine	Adenosine→ hypoxanthine	1.71	2.06	1.78
Purine metabolism	inosine	Adenosine→ hypoxanthine	1.28	1.24	1.07
Purine metabolism	2'-AMP	RNA degradation product	2.78	2.22	1.21
Purine	3'-AMP	RNA degradation product	1.95	2.36	1.42

Larvae			Fold Change after exposure		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h
metabolism					
Purine metabolism	AMP		1.5	1.06	0.82
Purine metabolism	guanine	guanine→cGMP	0.63	0.6	0.64
Purine metabolism	guanosine		1.47	1.35	1.05
Purine metabolism	uric acid	Hypoxanthine→allantoin	1.1	1.51	0.93
Purine metabolism	allantoin	Hypoxanthine→allantoin	2.22	2.57	3.35
Pyrimidine metabolism	cytidine		1.59	1.74	1.51
Pyrimidine metabolism	thymine		1.27	1.05	1.49
Pyrimidine metabolism	3-aminoisobutyrate	Thymine→succinyl-CoA, regulator of lipid metabolism	1.36	1.47	1.5
Pyrimidine metabolism	uracil	Uracil→β-alanine	2.24	1.85	2.31
Pyrimidine metabolism	uridine	Cytidine→uracil/UDP-glucuronate	1.27	1.12	0.84
Pyrimidine metabolism	pseudouridine	Modified base from RNA degradation	1.69	1.19	1.21
Pyrimidine metabolism	cUMP	RNA degradation product	1.18	1.44	1.21

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

3.5.2 Pyrimidines

Pyrimidine metabolism in the larva shows a slight increase in the nucleic acid thymine indicating increased DNA turnover, probably as a result of pyrethroid induced DNA damage (Patel et al., 2006) which has been observed in several species (**Appendix II**) eg. (Mckee and Knowles, 1986). The released thymine is being catabolised to 3-amino-isobutyric acid which accumulates after exposure. The pyrimidines thymine and 3-amino-isobutyric acid stimulate fatty acid metabolism in mice (Note et al., 2003) and *Drosophila* (Strub et al., 2008) indicating that the increase in both metabolites may be one of the factors causing the increase in lipid metabolism observed. An inhibition of 4-aminobutyrate aminotransferase activity can be ruled out as the enzyme also catabolises GABA and ß-alanine. There is evidence uridine is being utilised to both replenish uracil and form UDP-glucuronate (**Table 3-3**) which is involved in

the phase II detoxification of permethrin (Shono et al., 1978). Cytidine shows different degradation kinetics compared to the other nucleic acids despite flowing into uracil, with peak concentrations only being reached around 12 hours post permethrin exposure, indicating a lesion in the degradation pathway. As there is no increase in β-alanine it can be deduced the uracil is accumulating inside organelles and not the cytoplasm. The adult data set (**Table 3-8**) shows a decrease in 2',3'-cUMP which means that there is less RNA degradation in adults than in larvae. In adults there is a difference between genders in that females show an accumulation of all nucleic acids except uracil with time while males do not. As females show no change in downstream metabolites, it can be concluded that the nucleotides originate from DNA and are being recycled for DNA or RNA synthesis.

Table 3-8. Permethrin effect on purine and pyrimidine metabolism in adults

Adult			Fold Change (Male)			Fold Change (Female)		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h	2h	12h	24h
Purine metabolism	xanthosine	Xanthosine→allantoin	0.78	0.88	1.95	0.56	0.48	0.77
Purine metabolism	adenine	Adenosine→ hypoxanthine	0.91	0.89	0.95	1.18	0.96	1.5
Purine metabolism	AMP		1.02	0.95	0.84	0.99	0.82	1.39
Purine metabolism	guanosine		1.05	1.09	0.97	1	1.04	1.43
Purine metabolism	allantoin	Hypoxanthine→allantoin	0.38	0.71	0.76	0.68	0.57	0.53
Pyrimidine metabolism	cytidine		0.97	1.16	1.11	0.84	1.14	2.22
Pyrimidine metabolism	thymine		1	1	1	0.81	0.57	2.76
Pyrimidine metabolism	2',3'-cUMP	RNA degradation product	0.67	0.98	0.96	0.77	0.8	0.67

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

3.6 Fatty acid, lipid, sterol and carnitine metabolism

3.6.1 Fatty acids

Lipid peroxides are produced during pyrethroid exposure in many species (**Appendix II**) eg. (Terhzaz et al., 2015) and degraded by phospholipases (Grossmann and Wendel, 1983) releasing free fatty acids, lysolipids and glycerophosphoalcohols. An increase in these compounds and their catabolites is observed in larvae exposed to permethrin (**Table 3-9**), as has been seen pyrethroid exposed water fleas (Taylor et al., 2010). The accumulation of free

fatty acids and dicarboxylate products of ω-oxidation in both larvae and adults (**Table 3-10**) may indicate a metabolic defect in β-oxidation as occurs in mammals exposed to pyrethroids (Jin et al., 2014; Liang et al., 2013). In mammals it has been observed that cypermethrin exposure upregulates the expression of acyl-CoA oxidase, the cytosolic equivalent of the mitochondrial acyl-CoA dehydrogenase involved in β-oxidation, and carnitine palmitoyltransferase I (Jin et al., 2014). This suggests a lesion either at acyl-CoA dehydrogenase or at the carnitine-reliant shuttle for acyl-CoAs into the mitochondria. Interestingly it has been found that in *Drosophila* free fatty acid concentrations and the expression of argininosuccinate lyase, from the urea cycle, are interlinked (Heinrichsen et al., 2014) suggesting that the free fatty acid increases seen in larvae may be partially causative for the increase in flux seen in the urea cycle.

Table 3-9. Permethrin effect on fatty acid, lipid, sterol and carnitine metabolism in larvae

Larvae			Fold Change after exposure		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h
Medium chain fatty acid	linolenic acid		1.61	1.36	1.49
Medium chain fatty acid	caproic acid		1.58	1.65	1.52
Medium chain fatty acid	caprylic acid		1.36	1.3	1.1
Medium chain fatty acid	capric acid		1.33	1.47	1.2
Medium chain fatty acid	lauric acid		1.68	2.04	1.44
Medium chain fatty acid	5-dodecenoic acid		1.6	1.82	1.27
Long chain fatty acid	myristic acid		1.38	1.48	1.31
Long chain fatty acid	myristoleic acid		1.41	1.65	1.48
Long chain fatty acid	pentadecanoic acid		1.27	1.38	2.07
Long chain fatty acid	palmitic acid		1.52	1.89	1.7
Long chain fatty acid	palmitoleic acid		1.57	2.43	1.89
Long chain fatty acid	margaric acid		3.29	4.66	4.12
Long chain fatty acid	10-heptadecenoic acid		2.05	2.56	2.06
Long chain fatty acid	stearic acid		1.5	2.08	1.76
Long chain fatty acid	oleic acid		1.79	2.58	1.95
Long chain fatty acid	linoleic acid		1.73	1.86	1.67
Long chain fatty acid	nonadecanoic acid		1.61	2.73	3.52
Long chain fatty acid	10-nondecenoic acid		1.95	2.49	1.74
Long chain fatty acid	arachidic acid		1.39	1.79	1.73
Long chain fatty acid	eicosenoic acid		2.18	3.64	2.22
Long chain fatty acid	dihomo-linoleic acid		2.23	2.53	1.94

Larvae			Fold Change after exposure		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h
ω-Oxidation	decanedioic acid	End product of ω-oxidation	1.55	1.48	1
ω-Oxidation	dodecanedioic acid	End product of ω-oxidation	2.01	2.03	1.99
ω-Oxidation	tetradecanedioic acid	End product of ω-oxidation	1.51	2.2	2.69
ω-Oxidation	hexadecanedioic acid	End product of ω-oxidation	0.86	1.15	2.03
Fatty acid metabolism	isovaleric acid	By-product of BCAA catabolism from 3-Methylbutanoyl-CoA	4.33	6.34	3.39
Carnitine metabolism	carnitine	Involved in the transport of fatty acids into the mitochondria	1.34	1.21	1.77
Glycerolipid metabolism	choline phosphate	Glycerophospholipid catabolite	1.01	1.29	1.23
Glycerolipid metabolism	ethanolamine	Glycerophospholipid catabolite	1.5	1.37	1.42
Glycerolipid metabolism	phosphoethanolamine	Glycerophospholipid catabolite	1.58	1.63	1.48
Glycerolipid metabolism	glycerol	Glycerophospholipid catabolite	1.34	1.52	1.35
Glycerolipid metabolism	glycerol-2-phosphate	Glycerophospholipid catabolite, minor product	0.74	0.79	0.71
Glycerolipid metabolism	Citicoline	Choline→phosphatidylcholine	1.58	1.38	0.78
Glycerolipid metabolism	myo-inositol	Glycerophospholipid catabolite	1.71	1.52	1.86
Glycerolipid metabolism	inositol-1-phosphate	Glycerophospholipid catabolite	2.25	2.11	2.06
Lysolipid	1-palmitoylglycerophospho-ethanolamine		2.15	2.94	1.94
Lysolipid	2-palmitoylglycerophospho-ethanolamine		2.82	4.69	2.02
Lysolipid	1-palmitoleoylglycerophospho-ethanolamine		2.08	4.94	3.33
Lysolipid	2-palmitoleoylglycerophospho-ethanolamine		1.97	10.98	5.57
Lysolipid	1-stearoylglycerophospho-ethanolamine		1.69	2.21	1.26
Lysolipid	1-oleoylglycerophosphoetha		2.01	3.89	3.02

Larvae			Fold Change after exposure		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h
	nolamine				
Lysolipid	2-oleoylglycerophosphoethanolamine		3.3	6.74	4.55
Lysolipid	1-linoleoylglycerophospho-ethanolamine		2.63	3.84	4
Lysolipid	2-linoleoylglycerophospho-ethanolamine		3.54	5.75	5.81
Lysolipid	1-palmitoylglycerophosphocholine		1.03	0.11	1.05
Lysolipid	1-palmitoleoylglycerophosphocholine		0.54	0.27	2.4
Lysolipid	2-palmitoleoylglycerophosphocholine		0.75	0.6	2.36
Lysolipid	1-oleoylglycerophosphocholine		0.53	0.18	2.57
Lysolipid	1-linoleoylglycerophosphocholine		0.7	0.32	2.58
Lysolipid	2-linoleoylglycerophosphocholine		1.74	0.47	4.88
Lysolipid	1-palmitoylglycerophosphoinositol		2.75	3.79	2.58
Lysolipid	1-palmitoleoylglycerophosphoinositol		1.75	8.48	5.65
Lysolipid	1-stearoylglycerophosphoinositol		2.83	3.57	4.05
Lysolipid	1-oleoylglycerophosphoinositol		2.49	5.75	4.52
Lysolipid	1-linoleoylglycerophosphoinositol		2.11	5.35	6.38
Sterol/steroid metabolism	7-dehydroxycholersterol	Ecdysone precursor	1.33	1.38	1.91
Sterol/steroid metabolism	β-sitosterol	Cholesterol precursor	1.42	1.56	1.53
Sterol/steroid metabolism	campesterol	Cholesterol precursor	1.68	1.56	1.39
Sterol/steroid metabolism	ergosterol	Cholesterol precursor	1.51	1.49	1.64

Orange = $p<0.05$ - an increase, **Blue** = $p<0.05$ - a decrease, **Grey** = $0.05<p<0.10$

Table 3-10. Permethrin effect on fatty acid, lipid, sterol and carnitine metabolism in adults

Adult			Fold Change (Male)			Fold Change (Female)		
Subpathway	Metabolite	Pathway/Function	2h	12h	24h	2h	12h	24h
ω-Oxidation	2-hydroxyglutarate		0.63	0.6	0.86	0.79	0.69	0.72
ω-Oxidation	dodecanedioic acid	End product of ω-oxidation	0.86	1.23	1.4	0.98	2.37	1.73
ω-Oxidation	tetradecanedioic acid	End product of ω-oxidation	0.89	1.23	2.1	1.01	1.9	2.39
ω-Oxidation	hexadecanedioic acid	End product of ω-oxidation	1.13	1.47	1.52	1.04	1.72	2.04
Carnitine metabolism	carnitine	Involved in the transport of fatty acids into the mitochondria	1.06	1.2	0.97	1.01	1.15	1.53
Carnitine metabolism	acetylcarnitine		1.06	0.6	0.99	0.85	0.56	0.96
glycerolipid metabolism	myo-inositol	Glycerophospholipid catabolite	0.8	0.65	1.13	0.98	0.85	0.66
glycerolipid metabolism	glycerol-2-phosphate	Glycerophospholipid catabolite, minor product	1.01	1.4	0.89	0.98	1.37	0.67
Mevalonate metabolism	mevalonate		0.86	0.63	0.67	0.76	0.82	0.55
Sterol/steroid metabolism	7-dehydroxycholersterol	Ecdysone precursor	1.09	2.06	0.86	0.59	1.43	1.03
Sterol/steroid metabolism	β-sitosterol	Cholesterol precursor	0.74	0.95	0.62	0.81	0.64	0.75
Sterol/steroid metabolism	campesterol	Cholesterol precursor	0.71	0.78	0.6	0.97	0.73	0.91

Orange = p<0.05 - an increase, Blue = p<0.05 - a decrease, Grey = 0.05<p<0.10

3.6.2 Lipids

Lysolipids (monoacylphosphoglycerides) are intermediates in the synthesis and degradation of phospholipids (diacylphosphoglycerides). As pyrethroids cause lipid peroxidation (Terhzaz et al., 2015), the changes in lysolipids seen in larvae are probably derived from the degradation of lipid peroxides. Lysolipids derived from choline phospholipids show a weak trend of depletion on permethrin exposure (up to 0.11-fold at 12 hours) while ethanolamine and inositol phospholipid derived lysolipids show enrichment (up to 11-fold and 8.5-fold at 12 hours respectively). Pyrethroid exposure stimulates acetylcholine release by neurons (Feng et al., 1992) while inhibiting acetylcholine esterase (Badiou et al., 2008; Sellami et al., 2014; Singh and Agarwal, 1987), a phenomenon also been observed in mammals (Hossain et al., 2005; Yousef et al., 2006). The

acetylcholine that is not reabsorbed is catabolised via betaine feeding into glycine metabolism. This process, shown in **Figure 3-1**, would lead to a depletion of choline unless supplied by the catabolism of choline phospholipids and choline lysolipids, potentially explaining the observed reduction in choline lysolipids.

The increase in ethanolamine lysolipids may reflect a decrease in ethanolamine phosphoglyceride content which would explain the reduced membrane fluidity caused by pyrethroid exposure seen in mammalian cell cultures (Gabbianelli et al., 2002; Vadhana et al., 2011). In *Drosophila*, easily shocked (*eas*) mutants lack ethanolamine kinase resulting in a reduction of ethanolamine phosphoglyceride content in the membrane and display a hyper excitable neural phenotype (Pavlidis et al., 1994). This implies that continued degradation of ethanolamine phosphoglycerides caused by permethrin exposure has the potential to make nerves more susceptible to permethrin-induced hyper excitation under conditions of chronic exposure. Although ethanolamine phosphoglycerides may be having a protective effect against permethrin exposure, the increased concentrations of ethanolamine and phosphoethanolamine seen in larvae are potentially having pathological impacts as they have been linked to the inhibition of mitochondrial respiration (Modica-Napolitano and Renshaw, 2004) and stimulation of acetylcholine synthesis (Bostwick et al., 1992) in mammalian systems.

3.6.3 Sterols

In larvae an increase in several closely linked sterol precursors of ecdysone (1.3-1.9-fold at 2, 12 and 24 hours) was observed (**Table 3-9**). Adults show a depletion in beta-sitosterol and campesterol but still have an accumulation of 7-dehydroxycholersterol (**Table 3-10**). This indicates a lesion at one of the cytochrome p450 monooxygenases involved in ecdysone synthesis as has been demonstrated in the tick *Rhipicephalus sanguineus* where permethrin exposure inhibits ecdysone secretion (Roma et al., 2012). The accumulation of cholesterol in deltamethrin exposed rats (Yousef et al., 2006) may indicate that other sterol metabolizing enzymes are also impaired. The impairment of ecdysone synthesis would have a negative impact on the growth and survival of

larvae under chronic exposure which is why the pathway is already considered an insecticide target (Jia et al., 2013; Luan et al., 2013).

The reduction in sterol utilization could be induced either by the activation of negative feedback mechanisms by the endocrine activities of pyrethroids (Kono and Ozeki, 1987) or the planar hydrophobic nature of permethrin allowing competitive binding to the active sites of one or more of the metabolizing cytochrome p450 monooxygenases. An alternative explanation would be upregulation of sterol carrier proteins in the gut improving absorption of dietary sterols. Sterols like cholesterol can form rafts in lipid membranes which interact with both proteins and lipids affecting fluidity, interaction and function. They are known to be able to inhibit the activities of various voltage gated ion channels involved in neuroexcitation, such as the sodium, calcium and chloride channels activated by pyrethroid exposure (Levitan et al., 2010). This protective role of sterols against pyrethroid induced toxicity has been demonstrated by the synergistic effects of sterol carrier protein inhibition, which results in a depletion of sterols, in mosquitoes (Li et al., 2009) and the synergistic effects of sterol metabolism inhibition by some fungicides in honey bees (Pilling and Jepson, 1993).

3.6.4 Carnitine

Carnitine metabolism in the larvae shows an increase in N-6-trimethyllysine indicating increased carnitine synthesis. The increased pool of free carnitine compared to the unaffected pool of acetylcarnitine suggests that there is insufficient acetyl-CoA for fatty acids to form acyl-CoAs. Adult flies do not seem to have this acetyl-CoA bottleneck as females show an increasing concentration of oleoylcarnitine without showing an increase in free fatty acids. There is a depletion of acetyl-carnitine in adults indicating increased activity in the carnitine transport cycle.

3.7 Evidence for acidification, ammonia stress and oxidative stress

3.7.1 Acidification

In both the larval and adult data sets there are metabolic changes that can be interpreted as signs of acidification, ammonia stress and oxidative stress. In larvae there are increases in numerous free fatty acids, especially isovaleric acid (6.3-fold at 12 hours), which would cause the intracellular pH to lower because they are carboxylic acids, as has been well documented in mammalian cases of fatty acid induced acidification (Guo et al., 2006). The administration of adipokinetic hormone has been shown to synergise with permethrin in firebugs where it was assumed to function through accelerating the depletion of energy reserves (Kodrik et al., 2010). As there is evidence for already high concentrations of free fatty acids on permethrin exposure an alternative explanation for the synergism would be the stimulation of lipid metabolism by adipokinetic hormone, resulting in fatty acidosis being even more severe.

When glycerophosphoalcohols are cleaved by phospholipase D or diacylglycerophosphoalcohols by glycerophosphinicocholine diesterase, a cyclic 1, 2-cyclic glycerophosphate intermediate is formed. The cyclic intermediate can then be spontaneously hydrolysed via both acidic and alkaline routes to form either glycerol-3-phosphate or glycerol-2-phosphate (Serdarevich, 1967). As the ratio glyceol-3-phosphate and glycerol-2-phosphate differs depending on the route of hydrolysis this means the intracellular pH has an effect on the ratio of the major (glycerol-3-phosphate) and minor (glycerol-2-phosphate) products, with acidic conditions favouring glycerol-3-phosphate production. The larval data set shows a slight decrease in concentrations of glycerol-2-phosphate on permethrin exposure and a slight but insignificant increase in glycerol-3-phosphate, meaning a reduction in intracellular pH or an increase in glycerol-2-phosphatase activity.

3.7.2 Ammonia stress

It has been observed that pyrethroids exposure causes ammonia increases in several species e.g. (Veronica and Collins, 2003). The urea cycle of larvae showed an accumulation of the known marker for ammonia stress, N-

acetylarginine (Meert et al., 1991). The product of threonine and L-crystathione metabolism, 2-oxobutyrate, is usually rapidly excreted unless high concentrations of ammonia stimulate conversion into 2-aminobutyrate. The increase in 2-aminobutyrate seen in larvae may be indicative of ammonia stress.

3.7.3 Oxidative stress

Pyrethroids are known to cause markers for oxidative stress in several species (**Appendix II**) and cause the generation of peroxide in amphibians (David et al., 2012), C*aenorhabditis elegans* (nematode) (Shashikumar and Rajini, 2010), silkworms (Yamamoto et al., 2011), cotton bollworms (Akbar et al., 2012) and *Drosophila* (Terhzaz et al., 2015). In the dataset, larvae showed markers of such effects strongly. Larvae have a marked accumulation of 2-aminoadipate (4.5-fold at 12 hours), which not only may be a marker for oxidative stress in invertebrates (Zeitoun-Ghandour et al., 2011) but also may cause oxidative stress, as has been seen in mammalian systems. The oxidative stress is caused by the structural similarity to glutamate resulting in the inhibition of glutamate/cysteine transport leading to glutathione depletion (Kato et al., 1993; Schousboe et al., 1977). Another indicator of oxidative damage, methionine sulfoxide (Ruan et al., 2002), was also increased (3.5-fold at 24 hours) in larvae. The reduction in methionine sulfoxide seen in the adult data set could reflect reduced protein oxidation or increased methionine sulfoxide reductase activity. Mannitol, a diet-derived antioxidant, not metabolised by *Drosophila*, is depleted in both larvae and adults on permethrin exposure (0.04-fold in larvae and 0.42-fold in female adults at 12 hours) suggesting increased oxidative stress or changes in absorption by the gut. 5'-methyladenosine is increased in larvae (3.1-fold at 24 hours) and may also be indicative of oxidative stress because the metabolising enzyme has two conserved catalytic cysteine residues of the active site that can be reversibly inactivated by oxidative stress resulting in a lesion of the pathway (Fernandez-Irigoyen et al., 2008). The accumulation of fumarate seen in larval energy metabolism (**Table 3-3**) would also cause oxidative stress by spontaneously reacting with glutathione to form succinated glutathione (Sullivan et al., 2013) depleting the pool of available glutathione. Another potential source for oxidative stress is the inhibition of mitochondrial complex I which is a known target of permethrin (Gassner et al., 1997). Interestingly, it has been shown that *Drosophila* with impaired β-oxidation are more susceptible

to oxidative stress (Strub et al., 2008) suggesting that the lesion seen in the β-oxidation during permethrin exposure (**Section 3.6.1**) might be making exposed flies more prone to oxidative damage.

3.7.4 Ambiguous markers

In mammalian systems it has been shown that the activity of α-ketoglutarate dehydrogenase is sensitive to acidification, ammonia and oxidative stress (Lai and Cooper, 1986; Nulton-Persson and Szweda, 2001). The mammalian and *Drosophila* α-ketoglutarate dehydrogenase share a high degree of homology (Gruntenko et al., 1998) so therefore are likely to suffer from the same inhibitors meaning the lesion observed in larvae could be indicative of all three pathologies. Interestingly it has been found that inhibition of α-ketoglutarate dehydrogenase causes the production of reactive oxygen species (Ambrus et al., 2009) meaning that it could be a pivotal link between the various pathologies observed. Trans-aconitate can be formed by both by the low pH caused by acidification (Ambler and Roberts, 1948) and the inhibition of aconitase by oxidative stress (Gardner, 2002; Nulton-Persson and Szweda, 2001) suggesting that at least one of those two pathologies is occurring during permethrin exposure in *Drosophila*.

3.8 Evidence for xenobiotic detoxification

An elevation in several metabolites related to pathways involved in xenobiotic response were observed in larvae. There is evidence for phase II detoxification mediated by phenol beta-glucosyltransferase and UDP-glucuronyltransferase by elevated concentrations of phenylglucopyranoside (a glucosylation product of phenol) and glucarate and xylitol (catabolites of UDP-glucuronate). The phenol used for phenylglucopyranoside possibly originates from tyrosine degradation via tyrosine phenol lyase which has been found to occur in some insect species (Duffey et al., 1977), but yet to be identified in *Drosophila*, or from the degradation of the phenoxybenzyl moiety of permethrin by bacteria in the gut and standard *Drosophila* food. In larvae the concentrations of xylitol peak around 12 hours after exposure while erythronate peaks around 2 hours indicating the majority of xylitol is not coming from glycolysis or that there is a lesion at sorbitol dehydrogenase, which catabolises both sorbitol and xylitol.

The elevation in N-acetylglucosamine indicates a response to reduce permethrin penetration into the insect or across membranes through the synthesis of both additional chitin, which would reduce permeability of the cuticle, and hyaluronan, an intercellular matrix compound that reduces the permeability of membranes and tissues (Lillywhite and Maderson, 1988). Increased cuticle sclerotization would explain the β-alanine decrease in larvae by high L-Dopa concentrations driving N-β-alanyldopamine synthesis (a catecholamine) (Hopkins and Kramer, 1992). As chitin synthesis is regulated by ecdysones, the perturbations observed in sterol metabolism may be responsible for the flux changes through N-acetylglucosamine.

3.9 Evidence for deregulation of GABA turnover

It is known permethrin and other pyrethroids cause the unregulated release of neurotransmitters (Feng et al., 1992). In larvae the concentrations of GABA are depleted potentially due to permethrin induced neuroexcitation which traffics neurotransmitters for degradation in the astrocytes. An alternative explanation would be a lesion at glutamate decarboxylase which is interesting because, like DOPA decarboxylase and histidine decarboxylase, the enzyme is a group II pyridoxal-5′-phosphate-dependent amino acid decarboxylase (Sandmeier et al., 1994) and the larval data set has evidence for lesions at both other decarboxylases suggesting that permethrin might impair decarboxylases from that group. There is also a depletion of spermidine which potentially is able to be metabolised into GABA via 4-aminobutanal. The synthesis of 4-aminobutanal requires polyamine oxidase however, polyamine oxidase activity has yet to be investigated in *Drosophila* although a candidate gene has been identified *in silico* (Polticelli et al., 2012).

GABA is transaminated forming glutamate and succinate semialdehyde, which can be metabolised into succinate for the TCA cycle, meaning that the pathway can be used to generate energy for metabolising cells (**Figure 3-2**). In mammals it has been shown that a lesion at the α-ketoglutarate dehydrogenase complex stimulates GABA synthesis (Shi et al., 2009) and that up to a third of the ATP of neurons can be supplied by this pathway (Hassel et al., 1998). Interestingly FlyAtlas (http://flyatlas.org) shows that a predicted glutamate decarboxylase CG7811 is highly expressed in the carcass but not in the

thoracicoabdominal ganglion and the putative polyamine oxidase CG8032 has ubiquitous expression while aminobutyrate aminotransferase has 5.4-fold enrichment in the Malpighian tubules, suggesting a role for GABA outside neurotransmission, potentially in energy metabolism, in *Drosophila*.

3.10 Model for permethrin induced pathology

Based on the observed metabolic changes and the literature a model for permethrin poisoning is proposed with interactions shown in **Figure 3-3**. It is known that permethrin causes the hyper excitation of neurons resulting in uncontrolled spasms of muscles. The repetitive firing of neurons is an energy demanding process, so would stimulate glycolysis, cause acidification of the CNS (Rossano et al., 2013) and would result in the dysregulation of neurosecretory cells (Orchard, 1980). The acidification would impair the TCA cycle, cause accumulation of trans-aconitate and start the generation of reactive oxygen species by α-ketoglutarate dehydrogenase inhibition. The reactive oxygen species cause damage to lipids and proteins, sending them down catabolic paths and generating ammonia from free amino acids. The increase in free fatty acids combined with a permethrin induced lesion in β-oxidation contributes to further acidification while the ammonia causes additional inhibition of α-ketoglutarate dehydrogenase. The urea cycle is upregulated to detoxify the ammonia while trans-aconitate causes fumarate to accumulate in the cytosol. The cytosolic fumarate and 2-aminoadipate, from lysine catabolism, deplete glutathione reducing the capacity to prevent oxidative stress. The cycles of acidification, ammonia stress and oxidative stress reinforce each other while pathology spreads to nearby tissues by diffusion and transport.

The secretions of the neurosecretory cells may contribute to pathology by stimulating glycogen and fat utilization. The mobilization of fat reserves and lesion in β-oxidation has the potential to cause acidification and initiate pathology in tissues other than neurons. The inhibition of mitochondrial complex I by permethrin (Gassner et al., 1997) could also act as an initiator if reactive oxygen species scavenging pathways are impaired. As the immobilized organism is unable to drink and losing water at an increased rate (Gerolt, 1976) pathologies from desiccation stress may occur during late exposure.

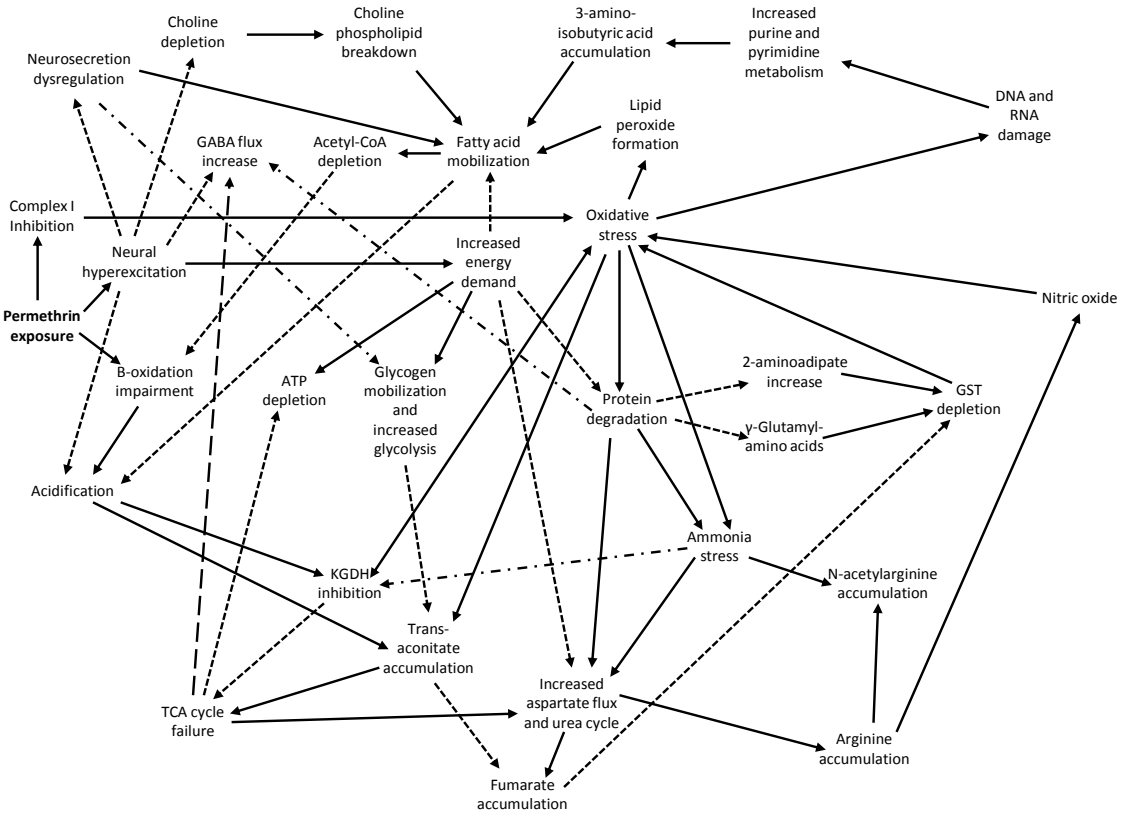

Figure 3-3. Model of permethrin action. Interaction map of permethrin induced physiological changes and pathologies which indicates that permethrin appears to cause pathology only through neuroexcitation and the inhibition of mitochondrial complex I and β-oxidation.

3.11 Candidate genes

Table 3-11. List of candidates

Name	Function	Rationale	Priority
CG8199	**2-oxoisovalerate dehydrogenase E1 component alpha subunit**	**BCOAD subunits, would impair valine, leucine and isoleucine catabolism**	**High**
CG17691	**2-oxoisovalerate dehydrogenase E1 component beta subunit**		**High**
CG6969 (Cd)	**3-hydroxykynurenine transaminase**	**would prevent synthesis of the antioxidant xanthurenic acid, cause an accumulation of neurotoxic 3-hydroxykynurenine and potentially 2-aminoadipate**	**High**
CG7433	**4-aminobutyrate aminotransferase**	**would prevent GABA degradation and use in energy metabolism**	**High**
CG42249	**5'-nucleotidase**	**could interfere with numerous reactions in purine and pyrimidine metabolism and NAD^+ recycling**	**Low**
CG4827	**5'-nucleotidase**		**Low**
CG30104	**5'-nucleotidase**		**Low**
CG32549	**5'-nucleotidase**		**Low**
CG11883	**5'-nucleotidase**		**Low**

CG6465	**Acylase**	**would prevent catabolism of N-acetyl amino acids and encourage excretion**	**Medium**
CG6733	**Acylase**		**Medium**
CG9707 (Acox57D-p)	**Acyl-Coenzyme A oxidase**	**would impair β-oxidation outside mitochondria**	**Medium**
CG9709 (Acox57D-d)	**Acyl-Coenzyme A oxidase**		**Medium**
CG5009	**Acyl-Coenzyme A oxidase**	**would impair β-oxidation outside mitochondria associated with neural survival**	**High**
CG18315 (Aprt)	**Adenine phosphoribosyltransferase**	**would prevent guanine utilization for cGMP, an important signalling compound**	**Medium**
CG5992	**Adenosine deaminase**	**Knockout disrupts insulin signalling in *Drosophila* and causes glucose deregulation and larval death, would increase adenosine causing higher utilization of energy**	**High**
CG9621	**Adenosine deaminase**	**could increase adenosine causing higher utilization of energy**	**Low**
CG11994	**Adenosine deaminase**		**Low**
CG1640	**Alanine transaminase**	**would disrupt the generation of aspartate from alanine and α-ketoglutarate**	**High**
CG9629	**Aldehyde oxidase**	**could prevent spermidine and 4-aminobutanal being used for GABA and impair fatty aldehyde metabolism**	**High**
CG31075	**Aldehyde oxidase**		**High**
CG3752	**Aldehyde oxidase**		**High**
CG3027 (Pyd3)	**Beta-ureidopropionase**	**would impair β-alanine synthesis and deplete 3-amino-isobutyric acid**	**Medium**
CG1673	**Branched-chain amino acid aminotransferase**	**BCOAD subunits, would impair valine, leucine and isoleucine catabolism**	**High**
CG6718	**Calcium-independent phospholipase A2**	**would impair degradation of lipid peroxides and fatty acid mobilization for energy metabolism**	**Low**
CG12891 (whd)	**Carnitine palmitoyltransferase 1**	**would prevent mitochondrial β-oxidation associated with oxidative stress sensitivity**	**High**
CG2107	**Carnitine palmitoyltransferase 2**	**is the bottleneck of mitochondrial β-oxidation mutations are adult lethal involving glial cells**	**High**
CG2791 (dCD98)	**CD98 heavy chain**	**heavy subunit of numerous amino acid transporters involved in tryptophan, BCAA, glutamate and**	**High**

		cysteine transport	
CG2201	**Choline/ethanolamine kinase**	**would disrupt choline and ethanolamine phospholipid synthesis**	**High**
CG8430 (GOT1)	**Cytosolic aspartate transaminase**	**would impair the urea cycle and tyrosine and phenylalanine catabolism**	**Medium**
CG4095	**Cytosolic fumarase**	**would prevent fumarate generated by the urea cycle entering the TCA cycle, associated with maximum energy output**	**High**
CG10160 (ImpL3)	**Cytosolic lactate dehydrogenase**	**lactate acts as an important store during anaerobic metabolism and permethrin already causes a lesion**	**Medium**
CG5362 (MDH1)	**Cytosolic malate dehydrogenase**	**would prevent cytosolic aspartate formation, involved in prevention of oxidative damage, involved in ethanol tolerance**	**Medium**
CG17337	**Cytosolic nonspecific dipeptidase**	**would prevent alternative GABA degradation pathway to homocarnosine**	**Low**
CG31140	**Diacylglycerol kinase**	**would impair phospholipid degradation associated with neurodegeneration**	**Medium**
CG34384	**Diacylglycerol kinase**	**would impair phospholipid degradation**	**Low**
CG8657	**Diacylglycerol kinase**		**Medium**
CG10697	**DOPA-decarboxylase**	**would interfere with dopamine and serotonin synthesis and cause greater increases in L-DOPA, associated with starvation and pathogen stress response**	**High**
CG1543 (Tbh)	**Dopamine beta-monooxygenase**	**would reduce dopamine degradation counteracting the lesion at tyrosinase**	**Medium**
CG3525 (Eas)	**Ethanolamine kinase**	**would cause increase in ethanolamine and depletion of acyl-gelerophosphoethanolamines Involved in seizure prevention**	**High**
CG33116	**Ethanolaminephosphotransferase**	**would increase ethanolamine and deplete acyl-glycerophosphoethanolamines, associated with DDT resistance**	**High**
CG7149	**Ethanolaminephosphotransferase**	**would increase ethanolamine and deplete acyl-glycerophospho-ethanolamines**	**High**
CG6016 (Bbc)	**Ethanolaminephosphotransferase**		**High**

CG14994	**Glutamate decarboxylase**	**would prevent GABA synthesis in CNS, orthologs have been associated with involuntary nerve firing**	**High**
CG7811	**Glutamate decarboxylase**	**would reduce GABA synthesis outside CNS**	**Medium**
CG5320 (GDH)	**Glutamate dehydrogenase**	**would restrict proline, glutamine and glutamate utilization by the TCA cycle**	**High**
CG4625	**Glyceronephosphate O-acyltransferase**	**would prevent lipid synthesis**	**Medium**
CG6188	**Glycine methyltransferase**	**would greatly restrict cysteine synthesis for glutathione**	**High**
CG9310 (HNF4)	**HNF4 receptor**	**required for fat mobilization would identify if permethrin induced changes in fatty acid metabolism are causative or consequential associated with starvation sensitivity**	**High**
CG1555 (Cn)	**Kynurenine 3-monooxygenase**	**would reduce neurotoxic 3-hydroxykynurenine and cause an increase in kynurenine and neuroprotective kynurenic acid**	**High**
CG6950	**Kynurenine transaminase**	**would reduce neuroprotective kynurenic acid and potentially cause an increase in 2-aminoadipate**	**High**
CG13334 (LDH)	**Lactate dehydrogenase**	**lactate acts as an important store during anaerobic metabolism and permethrin already causes a lesion**	**Low**
CG6295	**Lipase**	**would impair fatty acid mobilization**	**Low**
CG8823 (Lip3)	**Lipase**		**Low**
CG32699	**Lysophosphatidylcholine acyltransferase**	**would impair the synthesis of cholinephospholipids**	**Low**
CG18815	**Lysophospholipase II**	**would impair degradation of lipid peroxides and fatty acid mobilization for energy metabolism**	**Medium**
CG31683	**Lysophospholipase III**		**Low**
CG18858	**Lysophospholipase III**		**Low**
CG9655 (Nessy)	**Lysophospholipid acyltransferase**	**involved in recycling lysolipids and lipid synthesis**	**High**
CG2212 (Sws)	**Lysophospholipid hydrolase**	**would prevent degradation of lipid peroxides and fatty acid mobilization for energy metabolism**	**Medium**

		associated with spastic paraplegia involved in neurodegeneration	
CG7266 (MsrA)	**Methionine sulfoxide reductase**	**would prevent breakdown of toxic methionine sulfoxide**	**Medium**
CG4233 (GOT2)	**Mitochondrial aspartate transaminase**	**would impair the urea cycle and tyrosine and phenylalanine catabolism and remove a bypass for part of the TCA cycle**	**High**
CG4094 (Lethal 1)	**Mitochondrial fumarase**	**should have no effect on survival if fumarate is not made in the mitochondria during permethrin exposure**	**Medium**
CG7998 (MDH2)	**Mitochondrial malate dehydrogenase**	**would disrupt functioning part of TCA cycle during permethrin exposure and alter flux to oxaloacetate and trans-aconitate**	**High**
CG3331 (E)	**NBAD-synthase**	**would prevent L-DOPA and β-alanine being used for cuticle tanning**	**Medium**
CG31216 (D-NAAM)	**Nicotinamide amidase**	**bottleneck in NAD^+ recycling associated with sensitivity to oxidative stress in neurons**	**High**
CG7399 (Hn)	**Phenylalanine-4-hydroxylase**	**would prevent potentially toxic phenylalanine being metabolised to tyrosine**	**Medium**
CG11425	**Phosphatidate phosphatase**	**would impair phospholipid synthesis**	**Low**
CG11426	**Phosphatidate phosphatase**		**Medium**
CG11438	**Phosphatidate phosphatase**		**Low**
CG11440	**Phosphatidate phosphatase**		**Low**
CG12746	**Phosphatidate phosphatase**		**Low**
CG8709	**Phosphatidate phosphatase**		**Low**
CG8804	**Phosphatidate phosphatase**		**Low**
CG8805	**Phosphatidate phosphatase**		**Low**
CG8552 (PAPLA2)	**Phosphatidic Acid Phospholipase A1**	**would impair lipid and phospholipid catabolism**	**Medium**
CG5991	**Phosphatidylserine decarboxylase**	**would prevent phosphatidylcholine↔ phosphatidylethanolamine interconversion**	**Medium**
CG4825	**Phosphatidylserine synthase 1**		**Medium**
CG5547	**Phosphoethanolamine cytidylyltransferase**	**would cause depletion of acyl-gelerophospho-ethanolamines, associated with starvation stress resistance**	**High**
CG17191	**Phospholipase A1**	**would impair degradation of lipid peroxides and fatty**	**Low**

CG10133	**Phospholipase A2**	**acid mobilization for energy metabolism**	**Low**
CG12110	**Phospholipase D**	**would prevent choline mobilization from lipids**	**Medium**
CG8032 (DmPAO)	**Polyamine oxidase**	**would prevent spermidine being used for GABA**	**High**
CG16758	**Purine-nucleoside phosphorylase**	**would reduce guanine available for cGMP and impair numerous reactions in purine and pyrimidine metabolism and NAD^+ recycling**	**Medium**
CG1516	**Pyruvate carboxylase**	**would disrupt TCA cycle and aspartate synthesis when there is a lesion at α-ketoglutarate dehydrogenase**	**High**
CG1583	**Secretory phospholipase A2**	**would impair degradation of lipid peroxides and fatty acid mobilization for energy metabolism, involved in reducing Ca^{2+} induced apoptosis, Immune response gene, upregulated under starvation**	**High**
CG11124	**Secretory phospholipase A2**	**would impair degradation of lipid peroxides and fatty acid mobilization for energy metabolism**	**Low**
CG17035	**Secretory phospholipase A2**		**Low**
CG3009	**Secretory phospholipase A2**		**Low**
CG17035	**Secretory phospholipase A2**		**Low**
CG8327 (SpdS)	**Spermidine synthase**	**would prevent flux to GABA and impede methionine recycling pathway**	**High**
CG4300	**Spermine synthase**	**would prevent spermidine going down metabolic pathways other than GABA**	**Medium**
CG6608 (Tpc1)	**Thiamine pyrophosphate carrier protein 1**	**would impair all reactions that rely on thiamine pyrophosphate including acetyl-Coa synthesis, the TCA cycle and BCAA catabolism**	**High**
CG2772	**Triacylglycerol lipase**	**would impair fatty acid mobilization**	**Low**
CG2155 (V)	**Tryptophan 2,3-dioxygenase**	**would reduce tryptophan catabolism and increase the amino acid**	**Medium**
CG42639	**Tyrosinase**	**would increase concentrations of the toxic amino acid tyrosine and reduce flux to cuticle tanning pathways and dopamine**	**High**
CG42640	**Tyrosinase**		**High**

CG10118 (Ple)	**Tyrosine 3-monooxygenase**	**would increase concentrations of the toxic amino acid tyrosine and reduce flux to cuticle tanning pathways and dopamine**	**High**
CG1461	**Tyrosine aminotransferase**	**would impair tyrosine and phenylalanine metabolism and methionine recycling**	**High**

Based on the metabolic changes observed during permethrin exposure a list of proteins involved in the metabolism, transport and signalling of key metabolites which might impact on survival was compiled (**Table 3-11**). The genes of candidate proteins were then prioritized based on the number of reactions or pathways that would be affected by inhibition or knockdown, the redundancy of the protein and the FlyAtlas (http://flyatlas.org) expression profiles preferring genes with high expression in the central nervous system or detoxification tissues (Malpighian tubules, fat body and midgut). Due to the high redundancy of lipases and phosphatidate phosphatises RNAi against a single gene may not cause a permethrin survival phenotype so a chemical genetics approach would be recommended unless siRNAs can be designed to target conserved regions of these enzyme families. From the list, genes involved in the catabolism of tryptophan, valine, leucine and isoleucine were selected for further study primarily because the metabolomics data set showed large increases in downstream catabolites of these amino acids which are neuroactive or able to be utilized for energy but also because they share the same transporters.

Chapter 4 - The role of tryptophan catabolism in surviving permethrin challenge

1 Introduction

1.1 Overview

In the previous chapter it was shown that the metabolites tryptophan, 3-hydroxykynurenine, kynurenic acid, leucine, isoleucine, valine, 3-methyl-2-oxobutanoate, 3-methyl-2-oxopentanoate and 4-methyl-2-oxopentanoate showed large increases on permethrin exposure. 3-hydroxykynurenine and kynurenic acid are both metabolites derived from the tryptophan catabolite kynurenine while 3-methyl-2-oxobutanoate, 3-methyl-2-oxopentanoate and 4-methyl-2-oxopentanoate are all transamination products of the branched chain amino acids valine, leucine and isoleucine respectively. All four amino acids are also known substrates for the L-type amino acid transporter 1(also known as large neutral amino acid transporter) (Kanai et al., 1998).

1.2 Tryptophan catabolism in *Drosophila*

Drosophila are unable to synthesise tryptophan, deriving it from the diet (Sang and King, 1961), and lack Kynurenase so do not form compounds from the anthranilate branch of tryptophan catabolism (Lipke and Fraenkel, 1956). The first and rate-limiting step of tryptophan catabolism in *Drosophila* (**Figure 4-1**) is the opening of the indole ring by Vermilion, a tryptophan 2,3-dioxygenase to form N-formyl-kynurenine. Although N-formyl-kynurenine can spontaneously degrade to kynurenine, the enzyme Kynurenine formamidase is present to increase the rate of kynurenine formation. Kynurenine can then be either transaminated by CG6950, a putative transaminase, to form neuroprotective kynurenic acid, or oxidised by Cinnabar, a kynurenine 3-monooxygenase to neurotoxic 3-hydroxykynurenine. Cardinal then transaminates 3-hydroxykynurenine to form xanthurenic acid which acts as a substrate for the synthesis of ommochromes used to pigment the eyes of *Drosophila*. As mutations that disrupt the conversion of tryptophan to xanthurenic acid result in a loss of brown pigmentation and in some cases an increase in coloured kynurenates, mutant flies have eye colours of various shades of red. Since

mutants for genes in this pathway have obvious phenotypes, *vermilion*, *cinnabar* and *cardinal* were among the first *Drosophila* genes to be discovered (Clausen, 1924; Morgan, 1913; Warren, 1924).

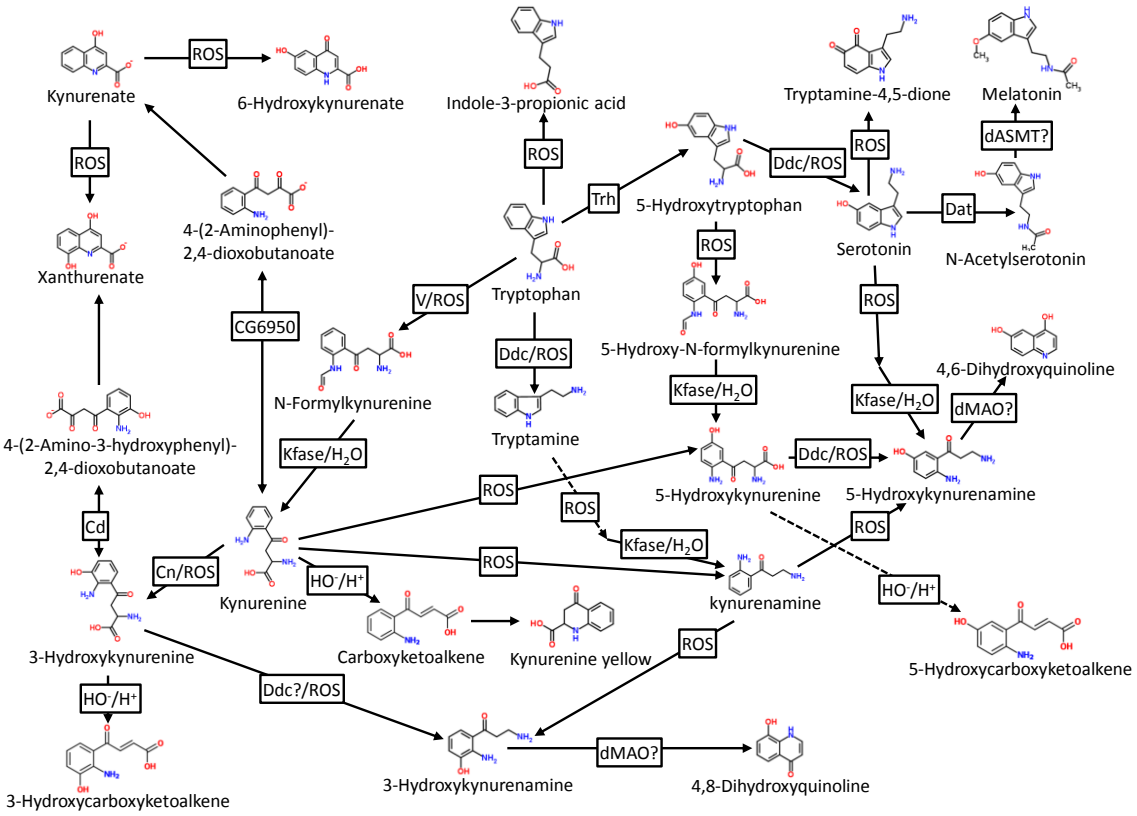

Figure 4-1. Schematic representation of tryptophan metabolism in *Drosophila* showing all enzyme catalysed and nonenzymatic reactions. The boxes are enzymes or chemical species involved in the reactions represented by arrows showing directionality. V = Vermilion, Kfase = Kynurenine formamidase, Cn = Cinnabar, Cd = Cardinal, Ddc = Dopa decarboxylase, Trh = Tryptophan hydroxylase, Dat = Dopamine N-acetyltransferase, dASMT = *Drosophila* Acetylserotonin N-methyltransferase and dMAO = *Drosophila* Monoamine oxidase. Enzymes with names ending in a (?) have known activity in *Drosophila* but no putative gene or have not had the putative substrate tested in *Drosophila*.

1.3 Known properties of the candidate compounds

In addition to roles in protein structure and function the amino acid tryptophan is used to synthesise the aminergic neurotransmitter serotonin, known to be involved in feeding behaviour and desiccation tolerance (Liu et al., 2015), or catabolised down the kynurenate pathway. The kynurenates have numerous physiological properties including the ability to scavenge reactive oxygen species, although 3-hydroxykynuernine can also cause the formation of peroxide (Hiraku et al., 1995; Pineda et al., 2015), impair energy metabolism, with the exception of kynurenine, (Schuck et al., 2007), alter sensitivity to neural pathology (Savvateeva et al., 2000), remodel the neural cytoskeleton in a LIMK-1

dependent manner (Lopatina et al., 2007) and induce paralysis in insects (Cerstiaens et al., 2003). Kynurenic acid is a known antagonist of the NMDA and α7-nicotinic acetylcholine receptors (Pfeiffer-Linn and Glantz, 1991) which has been shown in mammalian neurons to suppress the release of acetylcholine and GABA during neural excitation (Beggiato et al., 2014; Zmarowski et al., 2009). Kynurenine can undergo radical-mediated deamination forming highly reactive carboxyketoalkene, which can perform Michael addition with cysteine, histidine and lysine amino acid residues of proteins potentially modifying their function and stability (Kopylova et al., 2007). 3-hydroxykynurenine can also form adducts, in the presence of transition metal atoms, resulting in damage to the genome (Hiraku et al., 1995). Although primarily formed by enzymatic means, numerous tryptophan metabolites can also be formed by interactions with reactive oxygen species or acid-base chemistry (**Figure 4-1**), usually by cleavage of the indole ring, decarboxylation and deamination, e.g. 5-hydroxykynurenine, 3-hydroxykynurenine, kynurenic acid, N-formylkynurenine, kynurenine, tryptamine, kynurenamine, kynurenine yellow and 6-hydroxykynurenic acid (Hakim and Thiele, 1960; Kopylova et al., 2009; Moenig et al., 1985; Salminen et al., 2008; Stadtman, 1993; Takahashi, 1968; Turjanski et al., 1998).

Metabolites of the branched chain amino acids (valine, leucine and isoleucine) have not been studied in detail in invertebrates with the exception that it is known that branched chain amino acids and their direct transamination products stimulate the TOR (target of rapamycin) signalling pathway (Esslinger et al., 2013). Unlike vertebrates, the branched chained amino acids in arthropods are not used for energy metabolism to any important extent (Öhman et al., 1995). Accumulations of branched chain amino acids and their direct catabolites are known to cause acidosis, inhibition of the TCA cycle and oxidative stress in the branched-chain α-ketoacid dehydrogenase complex mutations that cause maple syrup urine disease in mammals (Sitta et al., 2014). There is also evidence from mammalian studies that 4-methyl-2-oxopentanoate may inhibit kynurenine 3-monooxygenase activity (Botting, 1995). Leucine is known to stimulate protein synthesis while downstream catabolites of leucine reduce protein degradation resulting in a bulking effect in mammals given a surplus of leucine (Tischler et al., 1982). However, none of these symptoms have ever been investigated or validated in invertebrates.

1.4 Related transporters

White is an ABC transporter subunit that together with Scarlet transports 3-hydroxykynurenine (Reed and Nagy, 2005) while interaction with Brown transports GTP (Dreesen et al., 1988), both of which are involved in eye pigmentation in *Drosophila*. As *white* mutants are unable to accumulate pigments in the eye giving it a distinct white-eyed phenotype, it was the first *Drosophila* mutant to be identified (Morgan, 1911). White and other invertebrate orthologs are involved in the transport of cGMP, uric acid, serotonin, histamine, dopamine and riboflavin (Borycz et al., 2008; Evans et al., 2008; Komoto et al., 2009; van Breugel, 1987). The impairment of White functionality has been linked to insensitivity towards anaesthetics (Campbell and Nash, 2001) and *white* has been found to be upregulated in response to insecticidal plant compounds (Shukle et al., 2008) meaning it may have a functional role in xenobiotic response.

The CD98 heavy chain is a subunit for several heterodimeric amino acid transporters. CD98hc (also known as 4F2hc) is known to be involved in the transport of glutamate, cysteine, leucine, isoleucine, valine, tryptophan, methionine, phenyl alanine, tyrosine, histidine, lysine, L-DOPA and 3-hydroxykynurenine making it important for both cellular amino acid uptake and redox, through enabling glutathione metabolism by the XC^- glutamate/cysteine antiporter system (Jin et al., 2003; Kageyama et al., 2000; Kanai et al., 1998; Kaper et al., 2007; Lewerenz et al., 2013; Uchino et al., 2002). It was believed invertebrates lacked a CD98hc ortholog until one was discovered in *Aedes aegypti* (Jin et al., 2003), since then other orthologs have been found in invertebrates including *Drosophila* however, interacting light chains like gender-blind, minidiscs, CG1607, jhl-21 and CG9413 still require characterization (Reynolds et al., 2009).

1.5 The relationship between metabolic profile and gene expression

Any imbalance in metabolic homeostasis is likely to cause the regulation of genes to restore homeostasis. This means pathways that show accumulations of metabolites are likely to have genes upstream of lesions and bottlenecks

downregulated, while those downstream will be upregulated. This trend would not hold true however for pathways with metabolites that have a protective role against stressing factors and pathology as changes in gene regulation that increase concentrations of the metabolite in response to the stimulus would be favoured as is seen with glutathione metabolism (Lin et al., 2014). Metabolic pathways can also show aberrant regulation when cofactors shared with other pathways, like NADH, become limiting (Heux et al., 2006). As metabolomics only detects concentrations of metabolites at a given time point and not the rate of flux through each metabolite, changes in gene expression can occur without changing the concentrations of metabolites in the pathway as the rates of influx compared to efflux can remain balanced. Metabolites with changes in concentrations and associated genes showing a change in regulation on permethrin exposure may have a role in survival but could just be a consequence of general changes in transcriptional regulation and off-target effects by permethrin. This meant survival assays needed to be performed to establish whether the changes are causative or consequential.

1.6 Survival assays

1.6.1 Applications

Survival assays are standard practice in pharmacology and toxicology for determining the relationship between the mortality and exposure ($\propto$concentration and time) for specific compounds. They can be used to compare the sensitivity of different organisms or populations of the same species to specific compounds (Wogram and Liess, 2001; Zhang et al., 2008a) or identify relationships between the structure of related compounds and the toxicity (Sims and Appel, 2007). The interaction between modes of toxicity of one or more compounds (Sprague, 1969) and the synergism of other compounds with a toxic compound (Nash, 1954) can also be investigated. In addition to comparing natural populations, populations with artificially induced genetic lesions or increased expression can be used to validate individual genes responsible for changes in survival when exposed to a toxic compound (Terhzaz et al., 2015).

1.6.2 Factors affecting survival assay data

There are many variables that can affect how organisms are affected by a toxic compound. The route of exposure determines what epithelial tissues the compound initially comes into contact with, affecting the rate of absorption and the proximity to detoxification tissues (Brown, 1978; Rozman et al., 2010); eg. in *Drosophila* the cuticle is in close proximity to the fat body while the gut is closely associated with the Malpighian tubules. Direct injection into the hemolymph bypasses epithelial absorption issues however this method results in higher control mortality due to traumatic injury and infection.

Toxic compounds often have multiple modes of toxicity so the timescale over which the survival assays are performed affects which modes are predominant. During acute exposure (high dose over a single exposure) primary lethal effects are dominant resulting in a brief spike of mortality followed by survivors showing no long term effects (Preisler and Robertson, 1989). With chronic exposure (accumulation of dose over multiple consecutive exposures) mortality results from the cumulative effects of multiple modes of toxicity and the inability of the organism to fully regenerate during iterative damage to tissues (Rozman et al., 2010; Sprague, 1969).

Mortality data usually follows a Gaussian lognormal distribution, assuming a homogenous population, which when plotted as a cumulative function forms a sinusoidal curve. The mortality data can be linearised so the mean becomes the 50% mortality value and the slope the inverse of the standard deviation (Rispin et al., 2002). This can be calculated using various formulae including Probit analysis, Logit analysis and the Spearman-Karber method, with each model having advantages and limitations (Bliss, 1957; Hamilton et al., 1977; Weber, 1991). The main advantage of Probit analysis is that it does not require an equal spacing or number of time points or doses between 16-50% and 50-84% mortality and as it uses percentage mortality it is not necessary for each replicate to have identical numbers.

When mortality data is plotted bias can be introduced by plotting against only dose or time without including the other variable. When plotting acute mortality against dose for a single time point, information is lost about delayed

modes of toxicity that can cause high mortality even if the acute LD_{50} is comparatively low as is seen in honey bees exposed to imidacloprid (Rondeau et al., 2014). With plots of chronic mortality against time it is assumed there are no dose effects (which in the case of detoxification pathways is seldom true), meaning that if the time-dose-mortality relationships of two populations have intercepting gradients between the "no observed adverse effect concentration" (Lethal threshold concentration) and "total lethality" (minimal survival time) then conclusions about changes in survival when exposed to a toxic compound could vary depending on the dose chosen to be used.

Toxicity curves (also known as time-dose-mortality plots and time-to-effect vs. dose plots) are generated by plotting either the $\log(LT_{50})$ (from mortality vs. time plots) vs. log(dose) or the $\log(LD_{50})$ (from mortality vs. dose plots) vs. log(time) giving a comprehensive view of how exposure and mortality interact. At doses approaching the no observed adverse effect concentration and total lethality the plots show a curving trend however, the relationship between LT50 and dose is mostly linear for doses between the two extremes (Sprague, 1969). Deviations from linearity can be caused by mortality data not having a normal distribution and the interaction of primary and secondary modes of toxicity as is seen with cyanic acid and nickel sulphate mixtures (Sprague, 1969). Differences in the gradient of the linear part of toxicity curves can indicate a change in the standard deviation of the population tested, additional modes of toxicity starting to dominate at higher doses, a change in mechanism of toxic action or an increased reliance on concentration gradients (Abel, 1978; Tennekes and Sánchez-Bayo, 2013). Changes in intercept are indicative of changes in absorption and transport (Rozman et al., 2010).

In this chapter the effect of permethrin exposure on the expression of *vermilion*, *kynurenine formamidase*, *cinnabar*, *cardinal*, *CG6950*, *CG1673*, *CG8199* and *CG17691*, which are enzymes involved in the metabolism of tryptophan, 3-hydroxykynurenine, kynurenic acid, leucine, isoleucine, valine, 3-methyl-2-oxobutanoate, 3-methyl-2-oxopentanoate and 4-methyl-2-oxopentanoate, and the *white* and *dCD98* transporter subunits was investigated. The effect of whole fly knockdown of *vermilion*, *cinnabar* and *CG6950* on survival was investigated using topical and oral routes of exposure before looking at tissue specific impacts on survival using tissue specific drivers and a topical route of

exposure. Potential impacts of *CG1673* and *white* on survival were also investigated using a topical route of exposure.

2 Results and discussion

2.1 Regulation of candidate genes by permethrin exposure

To investigate whether observed changes in metabolites could be explained by alterations in gene regulation following permethrin exposure, Canton S flies exposed to an ~LD_{10} dose of permethrin for four hours had transcripts of genes encoding enzymes involved in the conversion of tryptophan to xanthurenic acid and kynurenic acid and the first steps in branched chain amino acid catabolism, in addition to two genes for transporter subunits involved in tryptophan, kynurenate and branched chain amino acid transport quantified using quantitative (Q)-PCR. The relative expressions of each gene on permethrin exposure compared to an acetone treated control are shown in **Figure 4-2**. The transcription for the *vermilion* gene which encodes the rate-limiting enzyme for the pathway was not affected by permethrin exposure. All other genes for enzymes in the tryptophan catabolism pathway showed a significant downregulation, potentially indicating a response to reduce flux through the pathway.

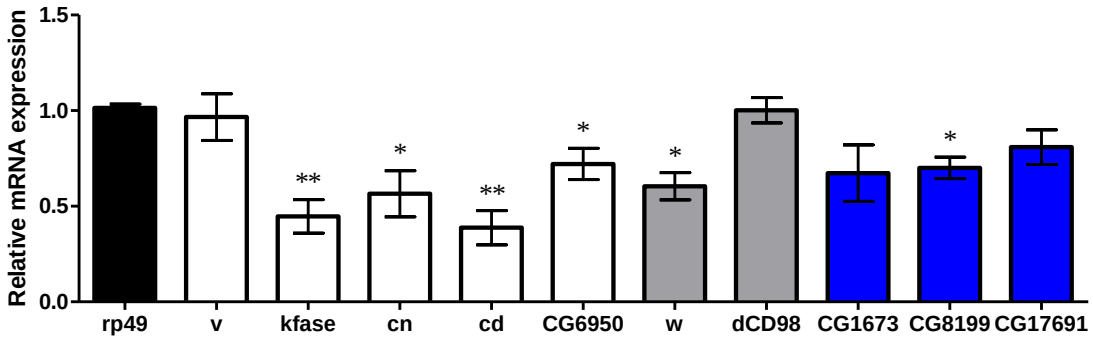

Figure 4-2. Regulation of candidate genes on permethrin challenge. QPCR showing the expression of genes involved in tryptophan and early branched chain amino acid catabolism and the transport of intermediate compounds in Canton S adults at 4 hours after being exposed to an ~LD_{10} dose of permethrin. Control gene (**Black**), genes for tryptophan catabolism enzymes listed in order of their occurrence in the pathway where possible (Unshaded), genes encoding subunits for some transporters involved in tryptophan/kynurenate transport (**Grey**) and genes for branched chain amino acid catabolism enzymes listed in order of their occurrence in the pathway (**Blue**). v = vermilion, kfase = kynurenine formamidase, cn = cinnabar, cd = cardinal and w = white. (*) = $P < 0.05$ and (**) = $P < 0.01$.

Of the genes tested involved in branched chain amino acid catabolism only *CG8199*, the 2-oxoisovalerate-dehydrogenase E1 component alpha subunit, showed downregulation. As the branched-chain α-ketoacid dehydrogenase complex requires multiple proteins to function and 2-oxoisovalerate dehydrogenase E1 component alpha subunit metabolises the first reaction performed by the complex, the downregulation of *CG8199* would reduce irreversible flux down the catabolic pathway. This suggests that *CG1673* is being utilized to regenerate glutamate during times of permethrin induced stress by the transamination of the branched chain amino acids while reducing irreversible catabolism. As the process is reversible, the branched chain amino acids could be regenerated from accumulated branched chain oxoacids when the organism is no longer under stress.

The *white* gene, whose decrease in function has been associated with increasing insensitivity to general anaesthetics in *Drosophila* (Campbell and Nash, 2001), was downregulated. This is interesting because the opposite trend has been observed in hessian flies fed on insect-resistant wheat where an upregulation of *white* occurred (Shukle et al., 2008). The gene encoding the dCD98 heavy chain subunit of several amino acid transporters, which interacts with LAT1 or other light chain subunits to transport amino acids and amino acid derived metabolites like L-DOPA(Reynolds et al., 2009), is not affected. This lack of expression change on permethrin exposure may be because of the vital roles it has with other light chain subunits, like Genderblind which together with dCD98 forms the X_C^- transport system in *Drosophila* that is required for glutathione metabolism (Augustin et al., 2007; Lewerenz et al., 2013).

2.2 Determination of best parental control crosses

To determine whether the manipulation of candidate genes was having an effect on survival, appropriate controls were required. As all KK RNAi lines from the Vienna *Drosophila* Research Centre are isogenic, with the exception of the shRNA insert in the integrated KC26 vector (Green et al., 2014), the use of the VDRCKKControl line, which has an integrated KC26 vector with no RNA hairpin, crossed to the same driver as the RNAi lines, is sufficient. The GD RNAi lines from the Vienna *Drosophila* Research Centre were made by random P-element insertions making them subject to positional effects and insertional mutagenesis

(Green et al., 2014) meaning no single control could be used. This meant parental controls for GD lines had to be made by crossing both the driver line and the GD line to a wild type line to avoid bias caused by gene dosage effects as crosses between driver and GD lines are heterozygous at the P-element insertion sites. Wild types maintained in the Dow/Davies lab were Canton S, Oregon R and CS(w^-), which was derived by backcrossing some white eyed strain with Canton S for over eight generations (P. Cabrero *verbatim*). Using the CS (w^-) line for parental controls was recommended and standard practice in the lab (Dr. S. Terhzaz and Dr. A. Dornan *verbatim*). The LD_{50}s of the wild types are shown in **Figure 4-3**.

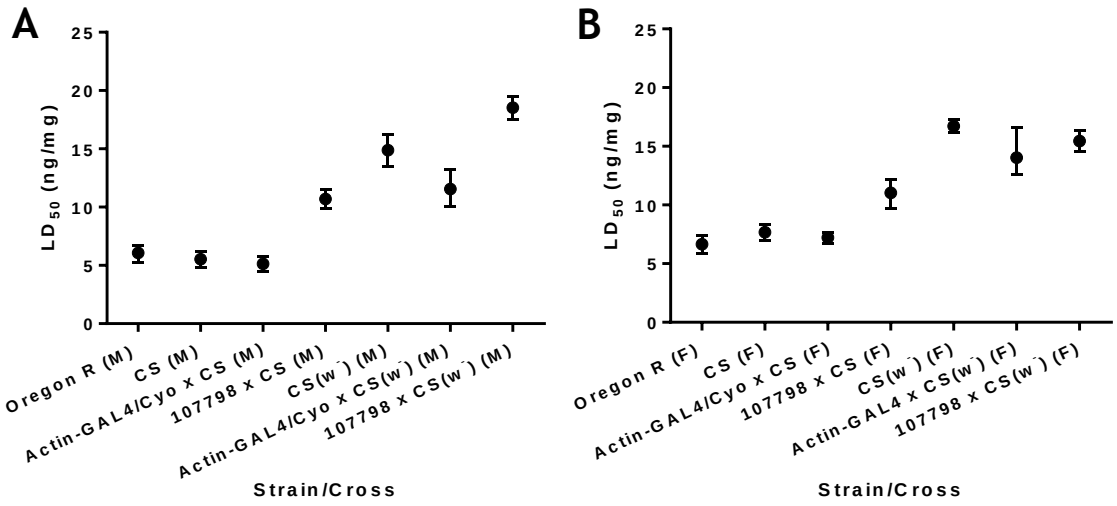

Figure 4-3. Survival of wild type strains and representative crosses when exposed to permethrin. (A) Data shown indicate the LD_{50}s for males of each strain tested and associated 95% confidence intervals. (B) Data shown indicate the LD_{50}s for females of each strain tested and associated 95% confidence intervals. N ≥ 420 per strain.

Oregon R and Canton S had very similar LD_{50}s when topically exposed to permethrin with Oregon R showing no significant difference between genders unlike Canton S where females were slightly less susceptible ($P<0.001$). CS(w^-) however was found to be approximately three times more tolerant than the other wild types. Since DDT was planned to be used in experiments and Oregon R is known to be DDT resistant (King, 1954) it was eliminated as a candidate. Representative parental controls were made using the two Canton S lines crossed to a driver line (Actin-GAL4/CyO) and an RNAi line (107798).

The LD_{50}s of the crosses are shown in **Figure 4-3**. The cross to the driver line using Canton S showed no difference in survival compared to the wild type while the cross to the RNAi line showed some hybrid vigour resulting in an increased

LD_{50}. Crosses to CS(w^-) showed considerably increased tolerance to topical permethrin exposure compared to the other two wild types and to the crosses to Canton S. As insecticide resistance tends to be dominant or co-dominant (Brooke and Koekemoer, 2010) CS(w^-) was determined to be unsuitable for parental controls in survival assays using a topical route of exposure as they would be prone to false positives for detecting increased susceptibility in experimental crosses and false negatives for increased tolerance in experimental crosses. As such crosses to Canton S were used for parental controls of non KK RNAi expressor lines.

2.3 Validation of RNAi lines

2.3.1 QPCR

The knockdown of RNAi lines crossed to appropriate drivers was quantified using QPCR. Tub-GAL4:UAS-Dcr/Tm3Sb was used to drive 22322 while Actin-Gal4/CyO was used for all other crosses except 108365 (*CG2791 RNAi*), which produced no viable adult progeny even when driven with weak drivers. Results of whole fly knockdown are shown in **Figure 4-4A-B**. Crosses using the *vermilion* RNAi line 107798, the *cinnabar* RNAi lines 11322 and 105854, the *CG6950* RNAi lines 22322 and 108093 and the CG1673 RNAi line 110229 had over 50% knockdown (**Figure 4-4A**) so were used for survival assays. The crosses for the branched-chain α-ketoacid dehydrogenase complex RNAi lines 40686 for CG17691 and 24231 and 24232 for CG8199 had less than 40% knockdown (**Figure 4-4B**) so were not used for further experiments.

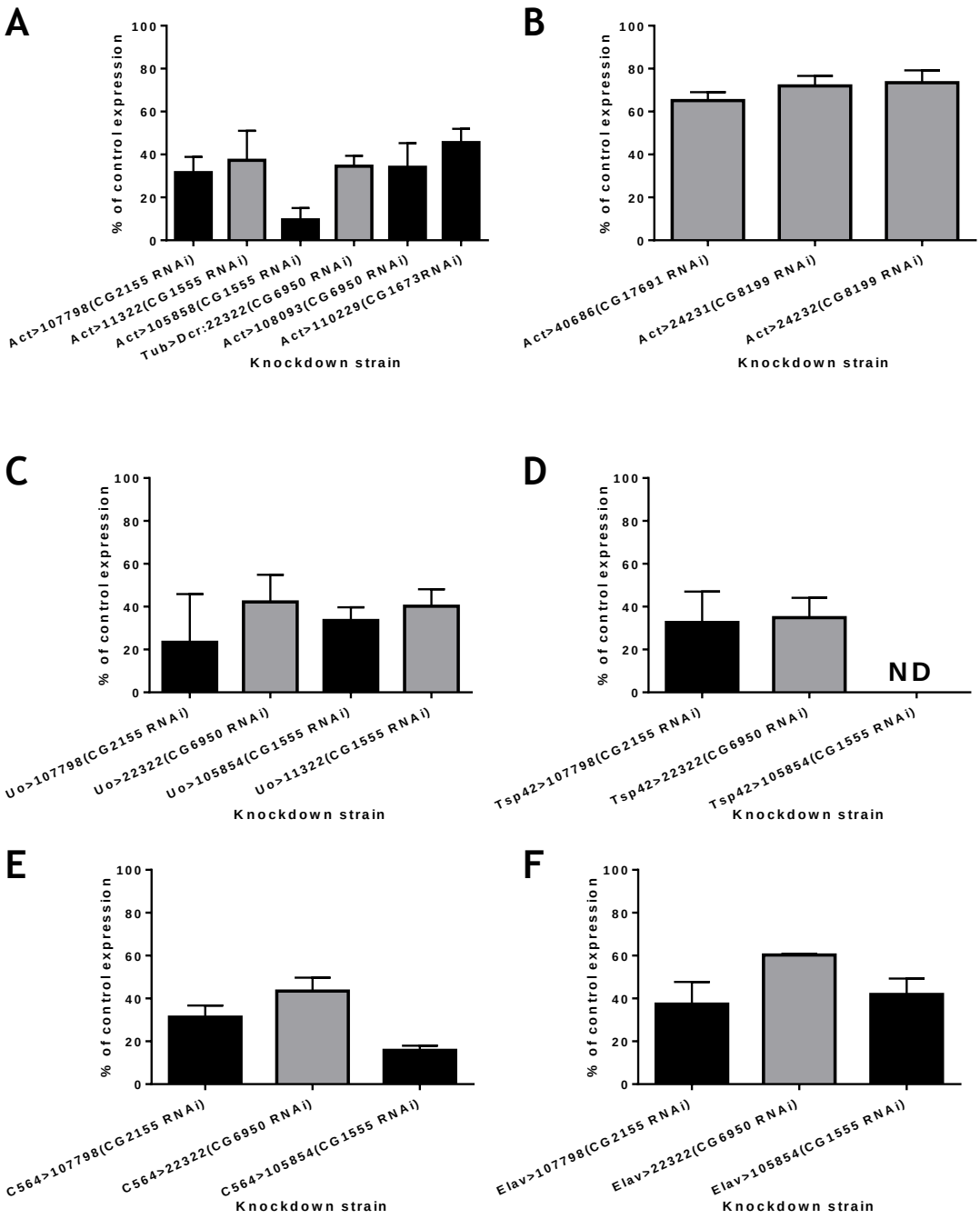

Figure 4-4. QPCR of Knockdown efficacy of RNAi lines used for survival assays. Gene expression levels in GAL4/KK RNAi lines (**Black**) were compared against GAL4>VDRCKKControl and expressed as a % of the control. Gene expression levels GAL4/GD RNAi lines (**Grey**) were compared against parental lines crossed to CS wild-type and expressed as a % of the control with the least expression. Knockdown was validated using primer pairs for the gene of interest (**Ch2., Table 2-6**). (A) Whole fly knockdown in strains used for survival assays. (B) Whole fly knockdown in strains not used for survival assays. (C) Knockdown in Malpighian tubules of Malpighian tubule specific knockdown strains. (D) Knockdown in midgut of midgut specific knockdown strains. ND = Not detected. (E) Knockdown in fat body of fat body specific knockdown strains. (F) Knockdown in the central nervous system of central nervous system specific knockdown strains.

Based on the results of the first round of survival assays (**Section 2.4.1**) the effects of candidate genes from tryptophan catabolism were investigated in specific tissues. To verify that the RNAi constructs were functional in the desired tissues when driven with the tissue specific drivers, the knockdown of 107798, 22322 and 105854 was investigated when crossed with Uo-GAL4 (Malpighian tubules), Tsp42-GAL4 (midgut), c564 (fat body) and Elav-GAL4 (central nervous system) using RNA extracted from the tissue of interest (**Figure 4-4C-F**). The knockdown of 11322 in the Malpighian tubules (**Figure 4-4C**) was also investigated. There was good knockdown in all tissues with the exception of 22322 in the central nervous system (**Figure 4-4F**). In the midgut concentrations of the *cinnabar* transcript were below the detection threshold in both the controls and the knockdown strain (**Figure 4-4D**). This is not unexpected, however, as it is known that cinnabar expression rapidly declines in *Drosophila* after eclosion (Warren et al., 1996) with reserves of 3-hydroxykynurenine (Chintapalli et al., 2013) and other downstream metabolites having formed before then.

2.3.2 Observable phenotypes

2.3.2.1 Eye colour of tryptophan knockdowns compared to mutants and wild type

As tryptophan catabolism mutants are associated with eye colour phenotypes seven day old whole fly KK knockdown strains were compared to mutant stocks for *vermilion*, *cinnabar* and *cardinal* obtained from the Bloomington *Drosophila* Stock Centre and the Canton S wild type (**Figure 4-5**).

Visual inspection under light microscopy could only easily distinguish *cinnabar*$^{-}$, Act>105854(*cn* RNAi) and Act>108093(*CG6950* RNAi) individuals from wild type. Both the mutant and knockdown for *cinnabar* had eyes with a more intense red colour while the *CG6950* knockdown had eyes that appeared to have a pale brown with an orange tint. Photos of fly heads of each genotype and gender had the RGB colour channel intensities of the eyes quantified and expressed as a ratio of Red:Green:Blue (R:G:B) normalized against blue (**Table 4-1**). The R:G:B ratios of the *vermilion* and *cinnabar* mutants are very similar to each other and distinct from the Canton S wild type. With the *vermilion* and *cinnabar* mutants and Canton S both males and females have similar eye colour. With cardinal

mutants females show a R:G:B ratio similar to Canton S while males have a similar profile to *vermilion* and *cinnabar* mutants however both genders show a reduction in the red colour channel.

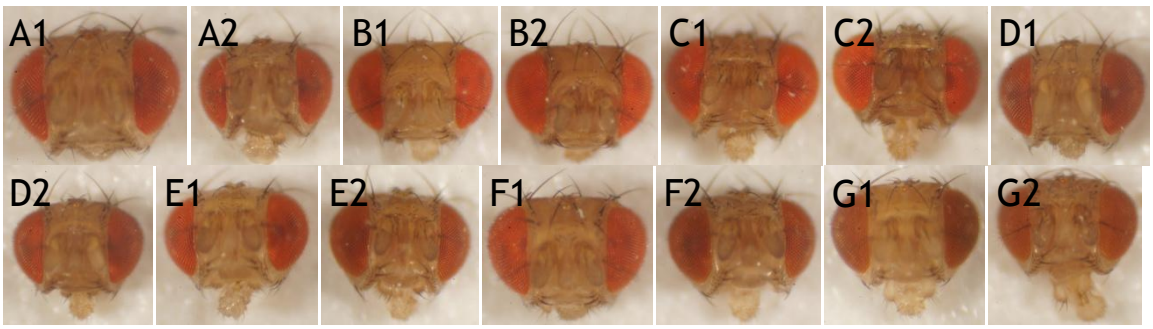

Figure 4-5. *Drosophila* eye colours with impaired tryptophan catabolism. (A1) Male Canton S, (A2) Female Canton S, (B1) Male *vermilion*$^{-}$ mutant, (B2) Female *vermilion*$^{-}$ mutant, (C1) Male *cinnabar*$^{-}$ mutant, (C2) Female *cinnabar*$^{-}$ mutant, (D1) Male *cardinal*$^{-}$ mutant, (D2) Female *cardinal*$^{-}$ mutant, (E1) Male *vermilion* knockdown, (E2) Female *vermilion* knockdown, (F1) Male *cinnabar* knockdown, (F2) Female *cinnabar* knockdown, (G1) Male *CG6950* knockdown and (F2) Female *CG6950* knockdown.

Knockdowns for *vermilion* and *cinnabar* have R:G:B ratios that are intermediates of Canton S and the respective mutants with males having ratios more similar to the mutants while females are more similar to Canton S. This sexual dimorphism in eye colour may indicate differences in the strength of knockdown or gene expression between genders in tissues involved in the eye pigmentation pathway (fat body, Malpighian tubule and eye). Females of the *CG6950* knockdown had a R:G:B ration that was different from all other genotypes while that of the males was similar to the females of the other knockdowns but darker. These results suggest that the knockdown strains exhibit impaired accumulation of downstream metabolites.

Table 4-1. Eye colours of *Drosophila* with impaired tryptophan catabolism

Strain	Gender	Average colour channel intensity			Average Colour	R:G:B ratio
		Red	Green	Blue		
Canton S	Male	150±10	66±9	44±6		3.42:1.5:1
	Female	156±13	69±10	45±6		3.47:1.53:1
Vermilion^{-}	Male	168±17	65±13	38±8		4.41:1.69:1
	Female	174±17	69±13	39±9		4.47:1.77:1
Cinnabar^{-}	Male	170±14	67±9	40±8		4.29:1.7:1
	Female	177±14	72±10	41±7		4.27:1.74:1
Cardinal^{-}	Male	150±12	67±8	40±9		3.8:1.71:1
	Female	147±13	69±8	45±6		3.28:1.54:1
Act>107798	Male	161±13	66±10	39±7		4.12:1.67:1

	Female	**146±15**	**66±11**	**41±9**		**3.58:1.62:1**
Act>105854	**Male**	**163±11**	**67±10**	**38±8**		**4.25:1.75:1**
	Female	**143±13**	**66±10**	**40±7**		**3.58:1.66:1**
Act>108093	**Male**	**135±15**	**66±10**	**37±8**		**3.62:1.76:1**
	Female	**148±15**	**71±13**	**37±9**		**3.96:1.91:1**

Values are Intensity of colour channel per pixel ± SEM

2.3.2.2 Whole fly knockdown of CG2791, CG17691, CG8199 and CG1673 have high mortality at late pupation

RNAi lines were tested for producing viable offspring when crossed with a strong ubiquitous driver, Tub-GAL4:UAS-Dcr/Tm3Sb, the moderate driver Actin-GAL4/CyO and a weak driver, Da-GAL4. The cross was considered to have impaired viability and be unfit for producing the numbers needed for survival assays if six females laying for three days produced less than three adult progeny without a balancer chromosome,. Only 22322 was viable when crossed with Tub-GAL4:UAS-Dcr/Tm3Sb while 107798, 11322, 105858, 108093, 110229, 40686, 24231 and 24232 were viable when crossed to Actin-GAL4/CyO. The RNAi line for *dCD98*, 108365, was not viable even when driven with Da-Gal4.

It was noted that in crosses which knocked down *CG1673*, *CG17691*, *CG8199* and *dCD98* many pupae failed to eclose. According to the Bainbridge scale of pupal development (Bainbridge and Bownes, 1981) mortality occurred at some point between p13 and eclosion (p15). Even in the Actin-GAL4/CyO crosses with 40686, 24231 and 24232 where escapers had a low knockdown there was still high pupal mortality. Individuals that had begun to eclose but failed were adhered to the inside of the pupal case by a clear fibrous substance that was water soluble (Data not shown). As all genes producing this phenotype are involved in the transport and catabolism of the branched chain amino acids there is indication that branched chain amino acid metabolism may be important in late pupation.

2.4 Effect of tryptophan catabolism gene knockdown in the whole organism on surviving permethrin challenge

2.4.1 Topical exposure

Exposure to permethrin was shown to cause a change in both metabolite concentrations (**Ch. 3, Section 3.1.4**) and the expression of enzymes (**Section 2.1**) of tryptophan catabolism. Both of these changes have not been previously identified in the context of permethrin intoxication. To investigate whether these changes were incidental, or had bearing on insect survival, survival assays were performed using RNAi knockdown of *vermilion*, *cinnabar* and *CG6950* to determine any changes in permethrin susceptibility (**Figure 4-6**).

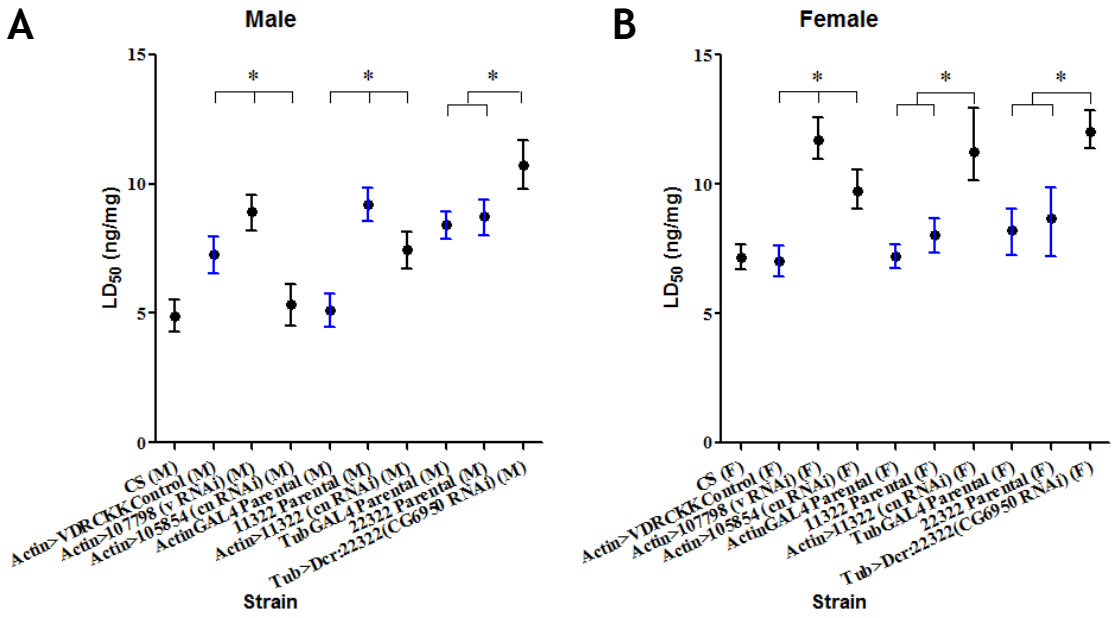

Figure 4-6. Survival of whole fly tryptophan catabolism gene knockdowns of *vermillion*, *cinnabar* and *CG6950* in response to topical permethrin. Data include survival of male and female wild-type (Canton S) and parental control lines. (A) Data shown indicate the LD_{50}s for males of each strain tested and associated 95% confidence intervals. (B) Data shown indicate the LD_{50}s for females of each strain tested and associated 95% confidence intervals. Controls are highlighted (**Blue**). N ≥ 1050 per strain. (*) = P < 0.001. Data published in (Brinzer et al., 2015).

Knockdown of *vermilion* results in *Drosophila* that are more tolerant to permethrin compared to controls, indicating that either the loss of tryptophan or the formation of kynurenates has a negative impact on surviving permethrin intoxication. Male knockdowns of *cinnabar* are more susceptible to permethrin while females are more tolerant, indicating a sexually dimorphic role for the enzyme either by the depletion of downstream catabolites or the accumulation of kynurenine. The putative type 3 kynurenine aminotransferase encoded by

the gene *CG6950*, which is putatively involved in the formation of kynurenic acid, causes an increase in tolerance when knocked down. In mammals kynurenine transaminases have a broad substrate range (Han et al., 2010) and although this is has not yet been demonstrated in insects, it is possible that other transamination products of *CG6950* from lysine, phenylalanine, tyrosine, aspartate, glutamate, cysteine and methionine metabolism may contribute to the observed change in survival against permethrin. Xanthurenic acid is required for malaria gametogenesis (Billker et al., 1998) making the inhibition of the tryptophan catabolism pathway in mosquito vectors a potential target for malaria control strategies however, if inhibition of the pathway in mosquitoes alters permethrin tolerance, like the results show for *Drosophila*, then it would conflict with conventional insecticidal control methods which rely heavily on pyrethroids (David et al., 2013; Wondji et al., 2012).

2.4.2 Oral exposure

As knockdown of tryptophan catabolism affected survival against acute topical permethrin exposure feeding survival assays were performed with permethrin to assess if whole fly knockdown of *vermilion*, *cinnabar* and *CG6950* also affects survival against chronic oral exposure (**Figure 4-7**). The resulting data indicate all three candidate genes affect survival. At 24 hours after initial exposure both the male and female knockdowns show increased permethrin tolerance compared to the control strain (**Table 4-2**). The *cinnabar* knockdown males show opposite trends in survival depending on the route of exposure (**Figures 4-6A** and **4-7A**) suggesting the importance of differing exposure routes on the efficacy of permethrin absorption and metabolism. This also implies that *cinnabar* knockdown may be affecting the permeability of the cuticle or gut. It has been observed that xanthurenic acid causes a thinning of the cuticle in *Bombyx* (Kushida et al., 2012) suggesting that *cinnabar* knockdown *Drosophila* might have a thicker cuticle due to reduced xanthurenic acid formation.

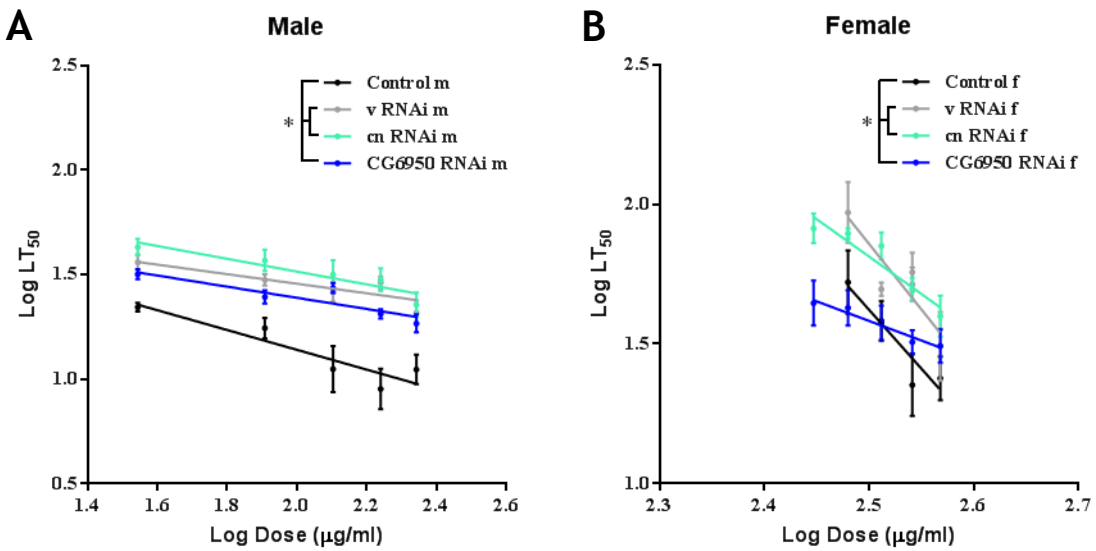

Figure 4-7. Permethrin toxicity responses of tryptophan catabolism gene knockdowns of *vermillion*, *cinnabar* and *CG6950* using an oral route of exposure. Toxicity responses showing the relationship between the dose of permethrin fed and the 50% lethal time (LT_{50}). (A) Toxicity responses for males. (B) Toxicity responses for females. Control strain (**Black**), *vermilion* knockdown strain (**Grey**), *cinnabar* knockdown strain (**Cyan**) and *CG6950* knockdown strain (**Blue**). N ≥ 720 per strain. (*) = P < 0.005. Data published in (Brinzer et al., 2015).

Table 4-2. LD_{50} (µg/ml) at 24 hours when fed permethrin

Strain	Male	Female
Control	31.1±13.8	361.4±21.9
v RNAi	214.8±64	399.9±19.6
cn RNAi	273.5±83.5	458.1±27.8
CG6950 RNAi	107.6±27.3	440.6±43.3

Data are derived from Figure 4-7. Values are LD_{50} (µg/ml) ± SEM

There is sexual dimorphism in the toxicity responses, with the knockdowns for all genes having increased intercepts without a changed gradient in males. Females, however, have increased intercepts in the *vermilion* and *cinnabar* knockdowns, both involved in the xanthurenic acid branch of the pathway, while knockdown of the *cinnabar* and *CG6950* genes, involved in pathways branching from kynurenine, have different gradients. This indicates that tryptophan catabolism is affecting survival in females by at least two different mechanisms. Changes in intercept without changes in gradient are indicative of a changed rate of reaching the threshold concentration without changing the toxicodynamics once reached. This implies that there is either reduced permethrin absorption by the gut or increased permethrin excretion in the *vermilion* and *cinnabar* knockdowns. Both genes are involved in the xanthurenic acid branch of the pathway suggesting that 3-hydroxykynurenine or a downstream metabolite could be responsible.

2.5 The effect of the knockdown of tryptophan catabolism genes in specific tissues on surviving permethrin challenge

As an organism can be considered to be the sum of its parts, with different tissues having different distinct metabolic roles (Chintapalli et al., 2013; Chintapalli et al., 2007), knockdown of *vermilion*, *cinnabar* and *CG6950* in specific tissues was performed to try and identify which tissues are important to survival when tryptophan catabolism is knocked down. The potential roles of the central nervous system, which is the site of the primary target of permethrin and the Malpighian tubules, fat body and midgut, which have a role in xenobiotic detoxification (Perry et al., 2011; Yang et al., 2007) were investigated. The LD50s of the knockdown strains relative to controls for each tissue type are shown in **Figures 4-8** to **4-11**.

2.5.1 Central nervous system

Knockdown of all candidate genes in the central nervous system had no definitive effect on surviving permethrin challenge (**Figure 4-8**). Although significantly different from the controls, vermilion and cinnabar knockdown males were uninformative as the LD50 was an intermediate of the controls and both knockdowns were not significantly different from each other and the CG6950 knockdown that definitely had no effect on survival. The female control for KK lines was more susceptible than the vermilion and cinnabar knockdowns however, none of the strains were significantly different from the CS wild-type and neither were any of the knockdown strains different from each other. This indicates that other tissues are responsible for the changes observed with ubiquitous knock down and the action of the kynurenates on neuroreceptor function are not important for surviving permethrin challenge. Alternatively, other tissues are able to supply tryptophan catabolites to the central nervous system and thus compensate for any loss of neural tryptophan catabolism.

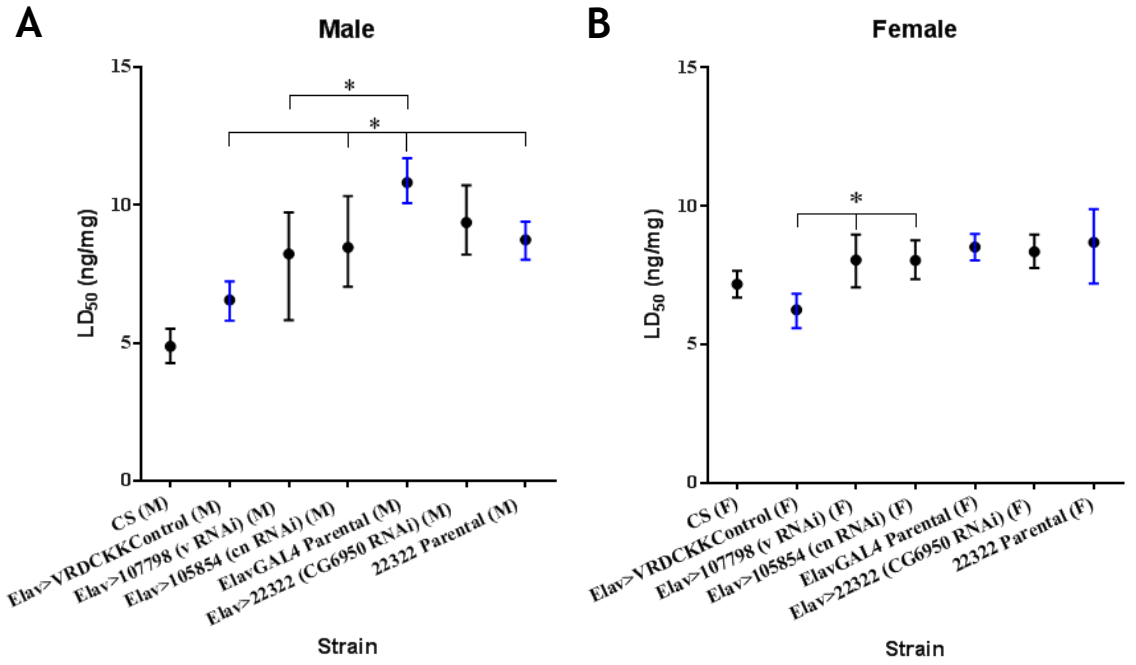

Figure 4-8. Survival of central nervous system-specific tryptophan catabolism gene knockdowns of *vermilion*, *cinnabar* and *CG6950* in response to permethrin. Data include survival of male and female wild-type (Canton S) and parental control lines. (A) Data shown indicate the LD_{50}s for males of each strain tested and associated 95% confidence intervals. (B) Data shown indicate the LD_{50}s for females of each strain tested and associated 95% confidence intervals. Controls are highlighted (**Blue**). N ≥ 1100 per strain. (*) = $P < 0.05$. Data published in (Brinzer et al., 2015).

2.5.2 Fat body

Knockdown of *vermilion* in the fat body of males resulted in increased tolerance to permethrin whereas females were more susceptible after *vermilion* knockdown (**Figure 4-9**). In *Drosophila* the fat body converts tryptophan to kynurenine using vermilion before exporting it to other tissues where it is converted into 3-hydroxykynurenine by *cinnabar* (Tearle, 1991). Therefore impeding kynurenine production in the fat body potentially will have the opposite effect of *cinnabar* knockdown in other tissues. The whole organism *cinnabar* knockdown (**Figure 4-6**) showed contrasting phenotypes to fat body *vermilion* knockdown indicating that, depending on the export pathways and tissues involved, kynurenine produced in the fat body may result in sex-specific survival to permethrin challenge. The knockdown of *CG6950* also showed sex-specific changes to survival with males being more tolerant to permethrin exposure while females showed no change. Although c564 is primarily a fat body driver it is also known to have some off-target expression in the salivary gland, imaginal discs, gut and brain (Harrison et al., 1995). As the central nervous system was found to have no effect on survival it can be eliminated as a potential cause for the observed survival trends.

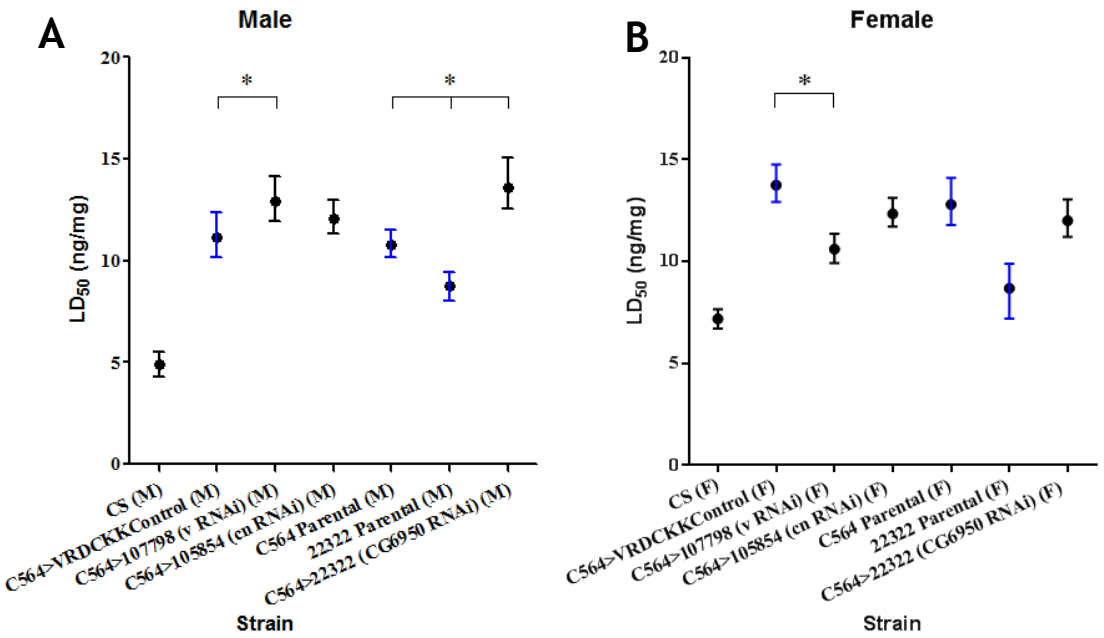

Figure 4-9. Survival of fat body-specific tryptophan catabolism gene knockdowns of *vermilion*, *cinnabar* and *CG6950* in response to permethrin. Data include survival of male and female wild-type (Canton S) and parental control lines. (A) Data shown indicate the LD_{50}s for males of each strain tested and associated 95% confidence intervals. (B) Data shown indicate the LD_{50}s of females for each strain tested and associated 95% confidence intervals. Controls are highlighted (**Blue**). N ≥ 1050 per strain. (*) = P < 0.001. Data published in (Brinzer et al., 2015).

2.5.3 Midgut

Knockdown of either *vermilion* or *cinnabar* in female midgut results in increased permethrin susceptibility compared to controls that is not observed in males (**Figure 4-10**). The step wise increase in susceptibility between *vermilion* and *cinnabar* knockdowns could be explained by the 105854 line having a stronger knockdown than 107798; or that non enzymatic processes (Atherton et al., 1993) can form some kynurenine bypassing vermilion enzymatic activity but not significant amounts of 3-hydroxykynurenine under physiological conditions. Both genders show increased tolerance to permethrin when *CG6950* is knocked down in the midgut indicating products of the enzyme have a negative role in that tissue. Female mosquitoes synthesize and excrete xanthurenic acid into the midgut (Lima et al., 2012). As the xanthurenic acid branch of tryptophan catabolism has been shown to affect permethrin tolerance in the midgut of female *Drosophila* (**Figure 4-10**), it is possible that there is a similar system in the *Drosophila* gut.

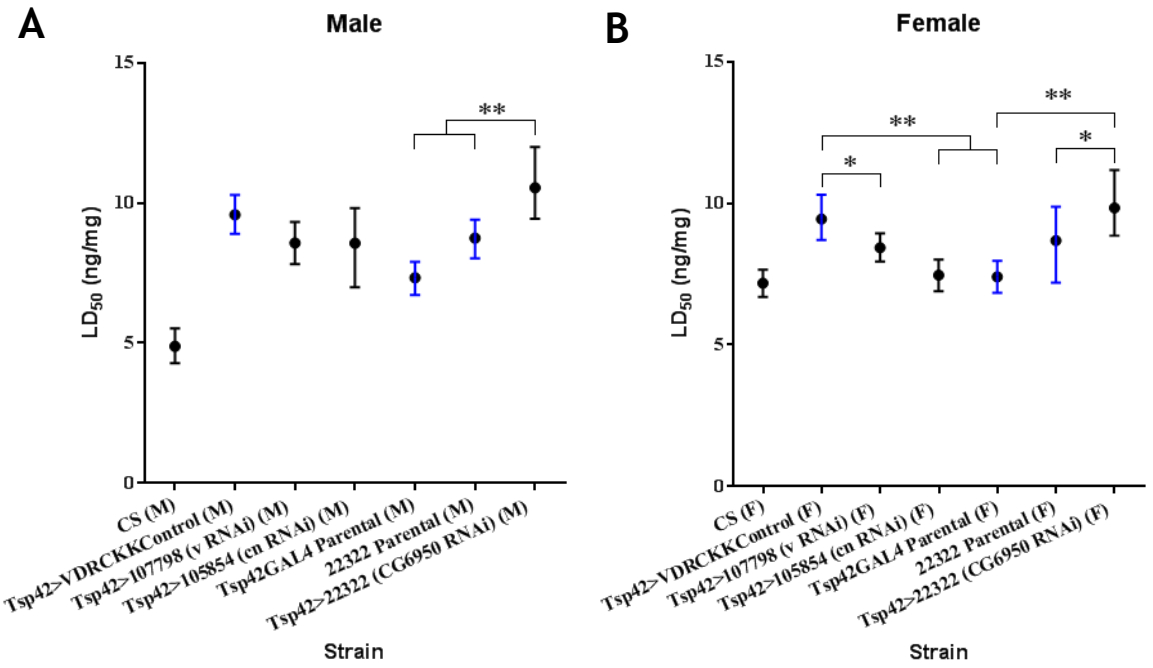

Figure 4-10. Survival of midgut-specific tryptophan catabolism gene knockdowns of *vermilion*, *cinnabar* and *CG6950* in response to permethrin. Data include survival of male and female wild-type (Canton S) and parental control lines. (A) Data indicate the LD_{50}s for males of each strain tested and associated 95% confidence intervals. (B) Data shown indicate the LD_{50}s for females of each strain tested and associated 95% confidence intervals. Controls are highlighted (**Blue**). N ≥ 1075 per strain. (*) = P < 0.05. (**) = P < 0.001. Data published in (Brinzer et al., 2015).

2.5.4 Malpighian tubules

Knockdown of *cinnabar* in the Malpighian tubules significantly increased permethrin tolerance indicating tubule-specific roles for kynurenine or kynurenic acid upon permethrin exposure (**Figure 4-11**). In *Drosophila*, kynurenates accumulate in the Malpighian tubule which acts as a store for ommochrome synthesis during adult eye formation (Tearle, 1991) however, the possibility of other roles have never been investigated. A possible mechanism to explain the observed change in permethrin tolerance is that kynurenine stored in the Malpighian tubules is able to be exported in exchange for metabolites that aid in detoxification. Alternately the prevention of 3-hydroxykynurenine formation in the Malpighian tubules, where release is tightly regulated (Tearle, 1991), allows other tissues to form xanthurenic acid, which is an antioxidant in oxidative stress protection (Pineda et al., 2015). In mammalian kidneys, the sulphate conjugate of xanthurenic acid acts as a natriuretic involved in water homeostasis (Senggunprai et al., 2008), if the same occurs in *Drosophila*, then the *cinnabar* knockdowns might have increased permethrin tolerance due reduced water loss. Malpighian tubules are key tissues for detoxification and stress responses

(Terhzaz et al., 2010; Yang et al., 2007) and these results suggest novel roles for kynurenine metabolism by the Malpighian tubules for insecticide survival.

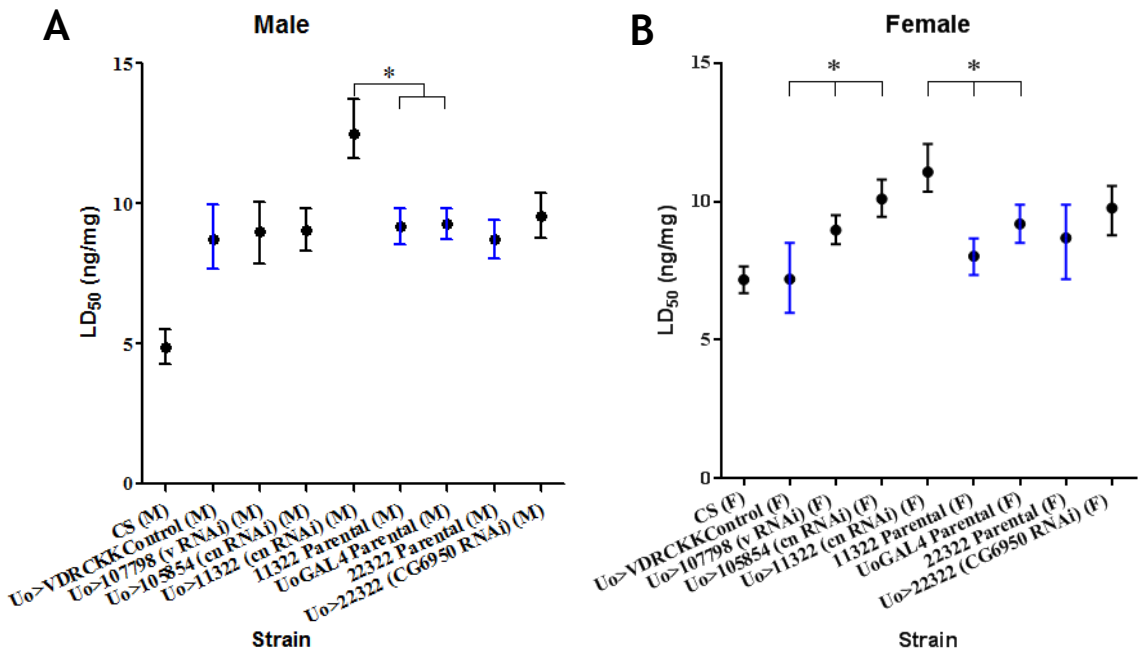

Figure 4-11. Survival of Malpighian tubule-specific tryptophan catabolism gene knockdowns of *vermilion*, *cinnabar* and *CG6950* in response to permethrin. Data include survival of male and female wild-type (Canton S) and parental control lines. (A) Data shown indicate the LD_{50}s for males of each strain tested and associated 95% confidence intervals. (B) Data shown indicate the LD_{50}s for females of each strain tested and associated 95% confidence intervals. Controls are highlighted (**Blue**). N ≥ 1060. (*) = P < 0.001. Data published in (Brinzer et al., 2015).

2.5.5 Potential for compensation by other tissues

Tryptophan catabolism knockdown in each of the CNS fat body, midgut and Malpighian tubules results in different effects on survival against permethrin exposure. However, none of these alterations in survival can fully explain some of the data from the whole organism knockdowns, indicating there are additional tissues involved. Although transcripts for *vermilion* are primarily enriched in the fat body (http://flyatlas.org/), while *cinnabar* transcripts are mostly found in the Malpighian tubule (Warren et al., 1996) and *CG6950* is enriched in both Malpighian tubules and fat body (http://flyatlas.org/), there is still some expression in other tissues. As tissue types tested consist of a comparatively small number of cells compared to the whole organism it is possible that the soluble kynurenates are able to be supplied by other tissues exporting them into the hemolymph.

2.6 Effect of the knockdown of *CG1673* on survival

In addition to tryptophan catabolites the metabolites of the branched chain amino acids also showed large increases on permethrin exposure. Due to associated mortality (**Section 2.3.2.2**) and low knockdown in escapers (**Section 2.3.1**) only *CG1673* could be investigated for causing changes to permethrin susceptibility (**Figure 4-12**). Although females were significantly more tolerant than the control, they were not significantly different from the wild type suggesting that *CG1673* only has a minor role, if any, during permethrin exposure.

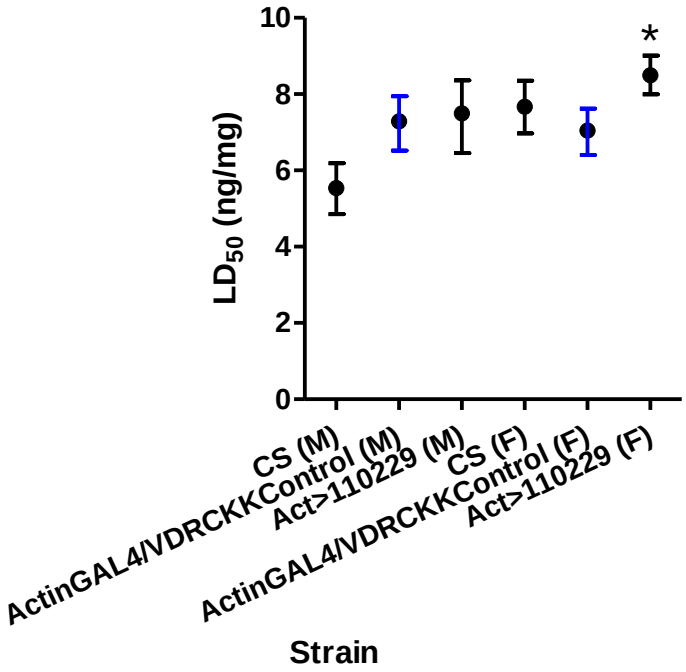

Figure 4-12. Survival of whole fly *CG1673* knockdowns in response to topical permethrin. Data include survival of male and female wild-type (Canton S) and parental control lines. Data shown indicate the LD_{50}s for males and females of each strain tested and associated 95% confidence intervals. Controls are highlighted (**Blue**). N ≥ 1075. (*) = P < 0.001.

2.7 Effect of *white* overexpression on survival

There was evidence from the QPCR of gene expression during permethrin exposure (**Section 2.1**) and from the comparison of Canton S and CS(w^-) (**Section 2.2**) that *white* may have a role in surviving permethrin challenge. The data to date indicated that a loss of white function caused increased permethrin tolerance. As all driver lines present in the lab used *white* as a marker gene with no suitably strong RNAi line that could counteract the over-expression of the driver lines, an over-expression strategy was used. The B28 stock which was

kept in-house contained a construct of white fused to YFP under the control of a UAS promoter. Expression of the construct was confirmed in a qualitative manner by observing YFP expression using a Universal visualizing light source for fluorescent stereo microscopy attached to a LEICA MZ6 microscope. The over-expression of white made females more tolerant to permethrin (**Figure 4-13**) but males were unaffected. This and previous data suggests that *white* may affect survival when exposed to permethrin both by a reduction in expression and over-expression. This suggests at least two mechanisms affecting survival.

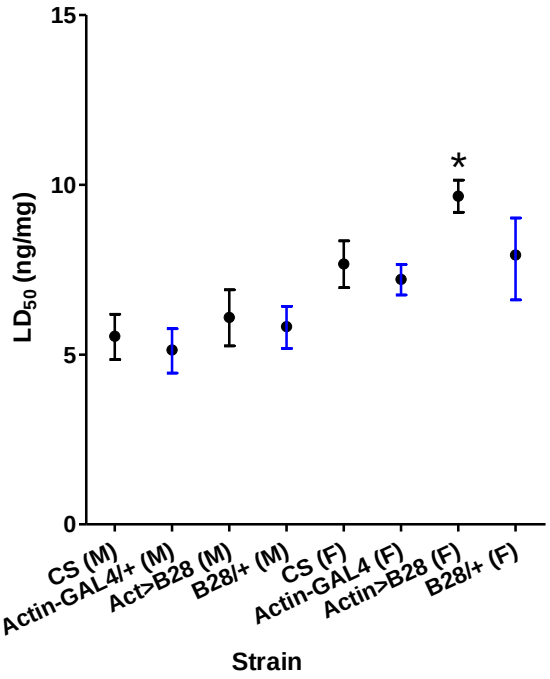

Figure 4-13. Survival of whole fly White over-expression in response to topical permethrin. Data include survival of male and female wild-type (Canton S) and parental control lines. Data shown indicate the LD_{50}s for males and females of each strain tested and associated 95% confidence intervals. Controls are highlighted (**Blue**). N ≥ 1180. (*) = P < 0.01.

In this chapter there is evidence that the RNAi lines chosen are affecting tryptophan metabolism as intended and tryptophan catabolism is not only downregulated by permethrin exposure but also has an impact on survival when exposed to permethrin by different routes of exposure. Furthermore tryptophan catabolism in the midgut, fat body and Malpighian tubules each have distinctive roles in surviving permethrin challenge however, tryptophan catabolism has no role in the central nervous system which is the tissue with the primary target of permethrin. This raises the question about the capacity for tryptophan catabolism to affect survival against insecticide exposure for insecticides other than permethrin.

Chapter 5 - The role of tryptophan catabolism in surviving challenge by other insecticides

1 Introduction

1.1 Overview

Tryptophan catabolism was shown in the previous chapter to have an impact on the survival of *Drosophila* when exposed to the pyrethroid insecticide permethrin. Depending on how specific the unknown mechanism is through which tryptophan catabolism affects survival will determine if it has an effect on survival against other insecticides (**Figure 5-1**). Furthermore differences in secondary targets and the modes of actions of insecticides tested might give further insight into how tryptophan catabolism is affecting survival.

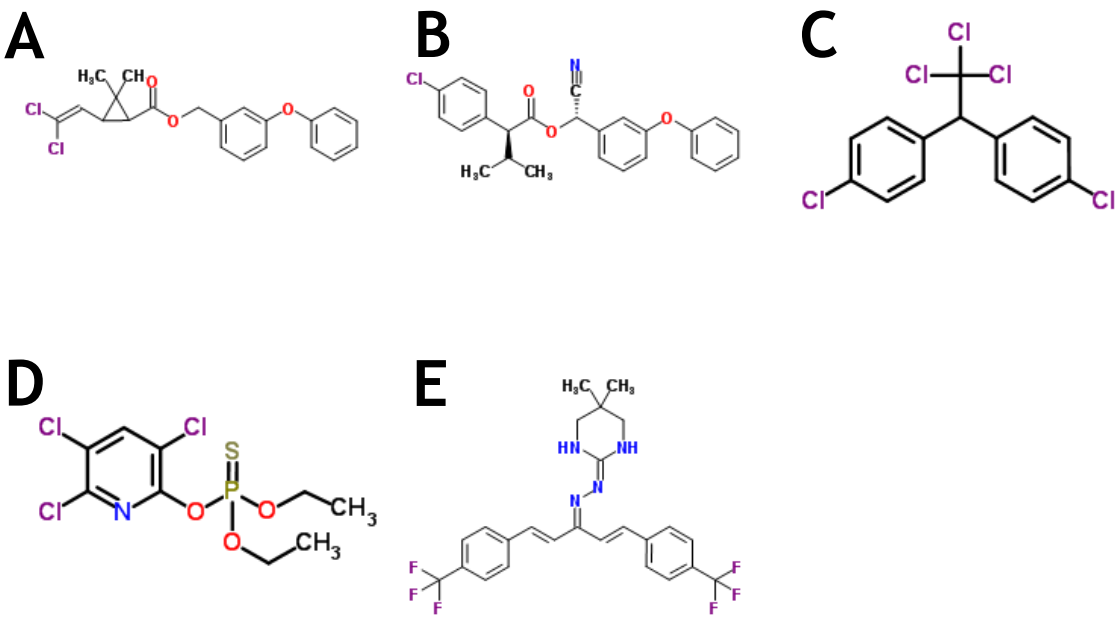

Figure 5-1. Chemical structures of insecticides tested. (A) Permethrin. (B) Fenvalerate. (C) DDT. (D) Chlorpyriphos. (E) Hydramethylnon.

1.2 The pyrethroid insecticide fenvalerate

The atypical third generation type-II pyrethroid fenvalerate (**Figure 5-1B**) was developed in 1974 by the Sumitoma company (Yoshioka, 1978) and is structurally very different from permethrin (**Figure 5-1A**). Unlike most pyrethroids which use a derivatised chrysanthemate group, fenvalerate has a chlorinated

phenylisovalerate group and unlike the type-I pyrethroid permethrin, there is a degradation-inhibiting α-cyano group (Zerba, 1988) on the 3-phenoxybenzyl alcohol motif. Fenvalerate also stimulates the opening of voltage gated calcium channel and chloride channel secondary targets to a differing extent compared to permethrin (Breckenridge et al., 2009; Cao et al., 2011). Like permethrin (Armstrong and Bonner, 1985; He et al., 1998; Luo and Bodnaryk, 1988), fenvalerate also has an antifeedant effect (Reynaldi et al., 2006) and inhibits Na^+, Ca^{2+} and Mg^{2+} ATPases (Kumari et al., 2014). The reduction in juvenile hormone esterase activity on exposure to fenvalerate (Wei et al., 2010) is the only known off-target effect of fenvalerate that has not been validated using permethrin.

1.3 The organochlorine insecticide DDT

DDT (**Figure 5-1C**) was first synthesised in 1874 (Weiler, 1874) however, its insecticidal properties were only identified several decades later (Läuger et al., 1944) for which Paul Müller got the Nobel prize in 1948. Like the pyrethroids, DDT primarily functions through being a voltage gated sodium channel agonist except that it has a binding site higher up in the binding pocket of the channels compared to the pyrethroids (O'Reilly et al., 2006). DDT and associated catabolites are highly lipophilic and thermodynamically stable which is why they are prone to environmental persistence and biomagnification (Huq, 2006).

As far as known secondary targets go, DDT is a known inhibitor of ATPases (Koch et al., 1969), like pyrethroids, but also inhibits DNA replication (Bahçeci, 1985), cytochrome oxidase (mitochondrial complex IV) and succinate dehydrogenase (Barsa and Ludwig, 1959). The major catabolite of DDT, p,p′-dichlorodiphenyldichloroethylene, is known to cause oxidative stress (Dowling et al., 2006). From mammalian studies there is evidence that dopamine transport may also be impaired (Hatcher et al., 2008).

1.4 The organophosphate insecticide chlorpyriphos

Chlorpyriphos (chlorpyrifos in the US) (**Figure 5-1D**) was developed by the DOW Chemical Company in 1965 (U.S. Patent 3244586)(Kenaga et al., 1965) and primarily functions by being a suicide inhibitor of acetylcholine esterase (Casida

and Quistad, 2004; Solomon et al., 2014). Like all organophosphates, chlorpyriphos requires activation by cytochrome p450s to form an oxono intermediate that can then reversibly covalently bind with the catalytic serine residue of serine hydrolases (Solomon et al., 2014). Cleavage of the 3,5,6-trichloropyridinol leaving group by the serine hydrolase then irreversibly phosphorylates the enzyme preventing catalysis of natural substrates (Solomon et al., 2014). Spontaneous hydrolysis of the organophosphate-enzyme complex can hydrolyze the serine-O-P bond restoring enzyme activity or cleave one of the two ethylester groups stabilising the serine-O-P bond permanently preventing resoration of enzymatic activity (Solomon et al., 2014).

Organophosphates are known to be non-specific serine hydrolase inhibitors with secondary targets including phospholipases, N-acetylpeptide hydrolases and kynurenineformamidase (Casida and Quistad, 2004). Unfortunately very few of these secondary targets have been validated in invertebrates with the exception of the *swiss cheese* (*sws*) gene product in *Drosophila* which is known to be mipafox sensitive (da Cruz et al., 2008). Organophosphates also inhibit succinate and malate dehydrogenase activity (Nath et al., 1997). Chlorpyriphos and other organophosphates, with the exception of malathion, have been shown to cause a decrease in cellular protein along with increased free amino acids, ammonia and urea, while the activities of aspartate transaminase, alanine transaminase and glutamate dehydrogenase are elevated in exposed invertebrates (Narra et al., 2013; Nath et al., 1997). There is evidence that chlorpyriphos and other organophosphates also cause oxidative stress (Gupta et al., 2010; Khalil, 2015).

1.5 The amidinohydrazone insecticide hydramethylnon

Hyramethylnon (**Figure 5-1E**) was developed in 1978 as a slow acting poison for the control of red imported fire ant infestations (Williams et al., 1980). It is an irreversible suicide inhibitor of site-II of the mitochondrial complex III (Hollingshaus, 1987) causing disruption of respiration and death through an ATP depletion/starvation cycle. Hydramethylnon has poor photostability (Mallipudi et al., 1986) which prevents agricultural use with primary applications being in poisoned baits for the control of species that inhabit dark spaces and colony

forming species where returning individuals distribute the bait. The possibility of secondary targets has never been investigated for this insecticide.

2 Results and discussion

Significantly observable changes in survival can be determined between LT_{16} to LT_{84}. Doses tested are listed in **Ch. 2, Table 2-7**.

2.1 Symptoms of insecticide poisoning in *Drosophila*

As insecticides with different modes of actions were tested different symptoms of poisoning were to be expected however, many of the insecticides tested have never had their symptoms described in a *Drosophila* model or even in any Dipteran. Since the time before symptom onset was experimentally found to decrease with increased insecticide dose, symptoms are divided into early, middle and late intoxication in a sequential order with no specific time scale given. Pictures of flies showing symptoms described are presented in **Figures 5-2** to **5-6**. The Act>KK RNAi, Act>roGFP and CS strains were tested to identify any strain-specific symptoms.

2.1.1 Permethrin and fenvalerate

The differences in symptoms between type-I and type-II pyrethroids are well documented in mammals (Ray and Forshaw, 2000) and have been documented in cockroaches (Gammon et al., 1981). In *Drosophila* symptoms for poisoning with permethrin (type-I) (**Figure 5-2**) began within <6 hours of starting the experiment depending on dose. The first symptom to manifest is ataxia in the form of loss of coordination when walking resulting in frequent stumbling and falling over which progresses to immobilization. Flies then show clambering actions of legs against each other as the knees start to bend in on themselves. This is accompanied by a hunching of the abdomen known as prostration (**Figure 5-2A**). The loss of coordination and prostration has been observed in cockroaches and *Musca domestica* (house fly) exposed to type-I pyrethroids (Adams and Miller, 1980; Gammon et al., 1981) however, the flaccid paralysis of individual legs while still mobile known to occur in *Musca domestica* (Adams and Miller, 1980) was not observed. The mouth, legs and feet continue to be moved

but with the knees rigid in a completely bent position while the abdomen ceases movement (**Figure 5-2B-D**) marking the end of early intoxication at around 6-8 hours after beginning the experiment.

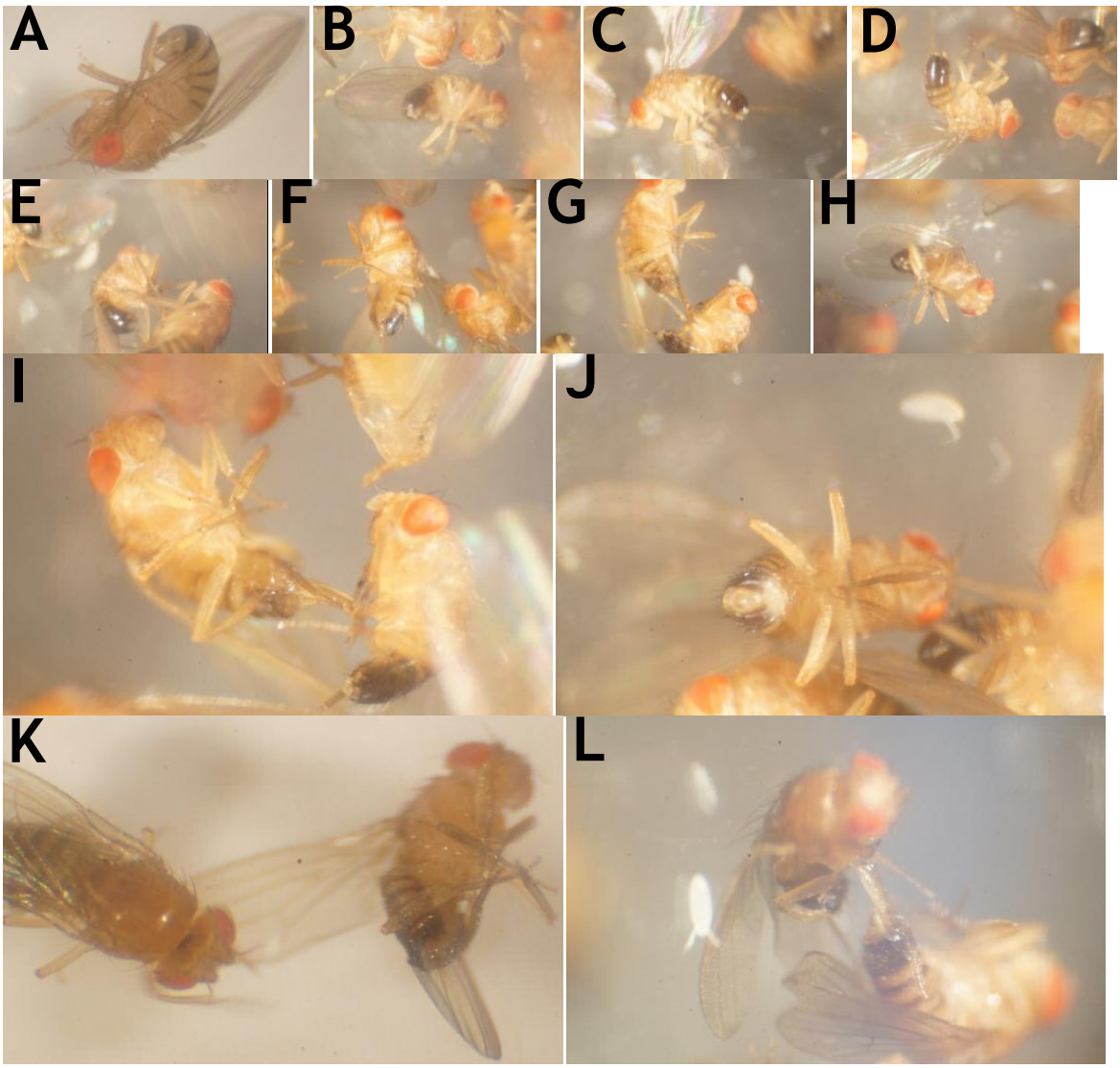

Figure 5-2. Symptoms of permethrin poisoning. All flies were still alive when images were taken. (A) Hunching of abdomen and clambering during early poisoning. (B-D) Positions of legs and abdomen during mid intoxication. (E) Size of abdomen during late poisoning. (F-L) Flies with visible moisture on the abdomen caused by fluid released from the posterior.

During mid intoxication there is then a gradual loss of mouth and leg movement. The abdomen shrinks in size, probably due to dehydration, showing muscle movement at the anus with occasional visible accumulations of fluid at the mouth and the slow excretion of a clear sticky fluid from the abdomen (**Figure 5-2F-L**). The fluid loss through the mouth and rear on insecticide exposure has also been observed in *Musca domestica* (Gerolt, 1976). Late permethrin intoxication is defined as the period when all movement has ceased except for the occasional twitch of the feet, usually >16 hours after starting the

experiment, until death. During this period the abdomen looks almost completely desiccated (**Figure 5-2E**).

Exposure to fenvalerate (type-II) (**Figure 5-3**) begins showing loss of coordination and prostration like permethrin but within two hours of symptoms starting the abdomen goes rigid and is extended away from the body (**Figure 5-3A-C**), potentially representing the convulsion symptom seen in cockroaches (Gammon et al., 1981). Many individuals show an inflation of the abdomen (**Figure 5-3D-F**), especially at higher doses with males being more prone, within one hour of symptoms starting, potentially being the *Drosophila* equivalent of the choreoathetosis symptom seen in mammals exposed to type-II pyrethroids (Ray and Forshaw, 2000). The loss of fluid from the rear also starts sooner and is more profuse (**Figure 5-3G-J**) than with permethrin. All other symptoms are the same as permethrin with the abdomen undergoing prostration as intoxication progresses (**Figure 5-3C** and **I-J**) as has been seen in cockroaches (Gammon et al., 1981).

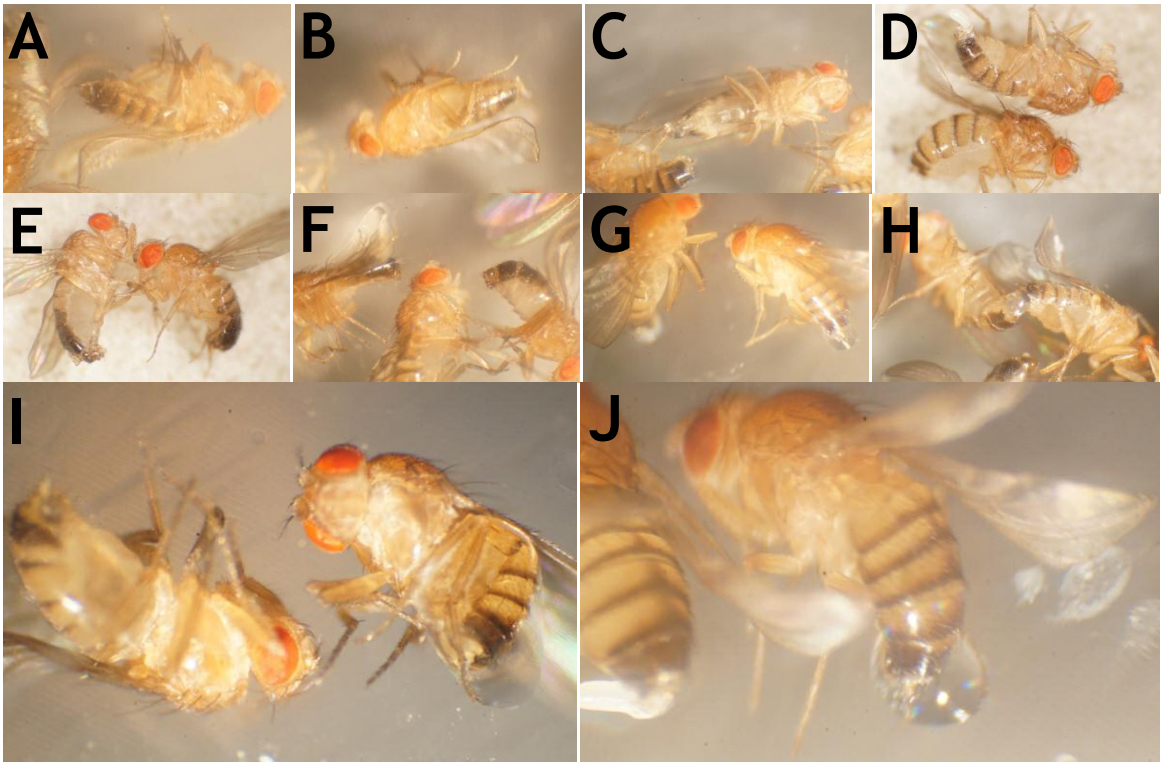

Figure 5-3. Symptoms of fenvalerate poisoning. All flies were still alive when images were taken. (A-C) Typical positions of legs and abdomen during mid intoxication. Fly in lower left of (C) shows prostration that occurs during mid intoxication. (D) Inflation in females. Top control fed, bottom fenvalerate fed. Wing closest to viewing was removed from both individuals to prevent obstruction. (E) Inflation in males. Right control fed, left fenvalerate fed. (F) Inflation in male on the right. (G-J) Fluid release from the posterior.

2.1.2 DDT

DDT exposure showed hyperactivity shortly after experiments began, as has been seen in *Musca domestica* (Adams and Miller, 1980), with flies being in constant movement and no individuals at rest or standing. Within 3 hours of starting experiments legs showed uncontrolled walking actions that led to ataxia and immobilization. All other symptoms (**Figure 5-4**) were identical to permethrin except the volume of liquid lost through the rear was greater, a symptom also observed in *Musca domestica* (Gerolt, 1976).

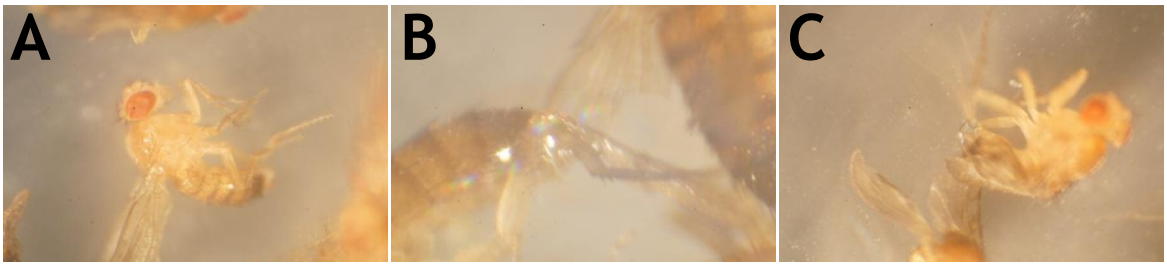

Figure 5-4. Symptoms of DDT poisoning. Flies in (A) and (B) were still alive when images were taken. (A) Typical position of legs and abdomen during mid intoxication. (B) Fluid release from the posterior. (C) Dead individual adhered to side of vial by drying fluid.

2.1.3 Chlorpyriphos

Chlorpyriphos poisoning (**Figure 5-5**) had symptoms very similar to permethrin with the following exceptions. Unlike the voltage gated sodium channel targeting insecticides there was no bending of the knees during early poisoning with legs having a tendency to be kept extended (**Figure 5-5A-C**) until near the end of mid intoxication (**Figure 5-5G**). There would be regular bouts of uncontrolled flight attempts with extended wings during early intoxication that reduced to the vibration of the wings in their resting position during mid intoxication. By late intoxication all wing movements had stopped. Similar to fenvalerate and DDT there was a comparatively larger volume of fluid (**Figure 5-5D-G**) lost through the rear compared to permethrin although release occurred later than the voltage gated sodium channel targeting insecticides and persisted until just before death (**Figure 5-5G**). This fluid loss symptom has also been observed during organophosphate poisoning in *Musca domestica* (Gerolt, 1976) indicating that all neuroexcitatory insecticides may cause increased fluid loss through the posterior of the Diptera.

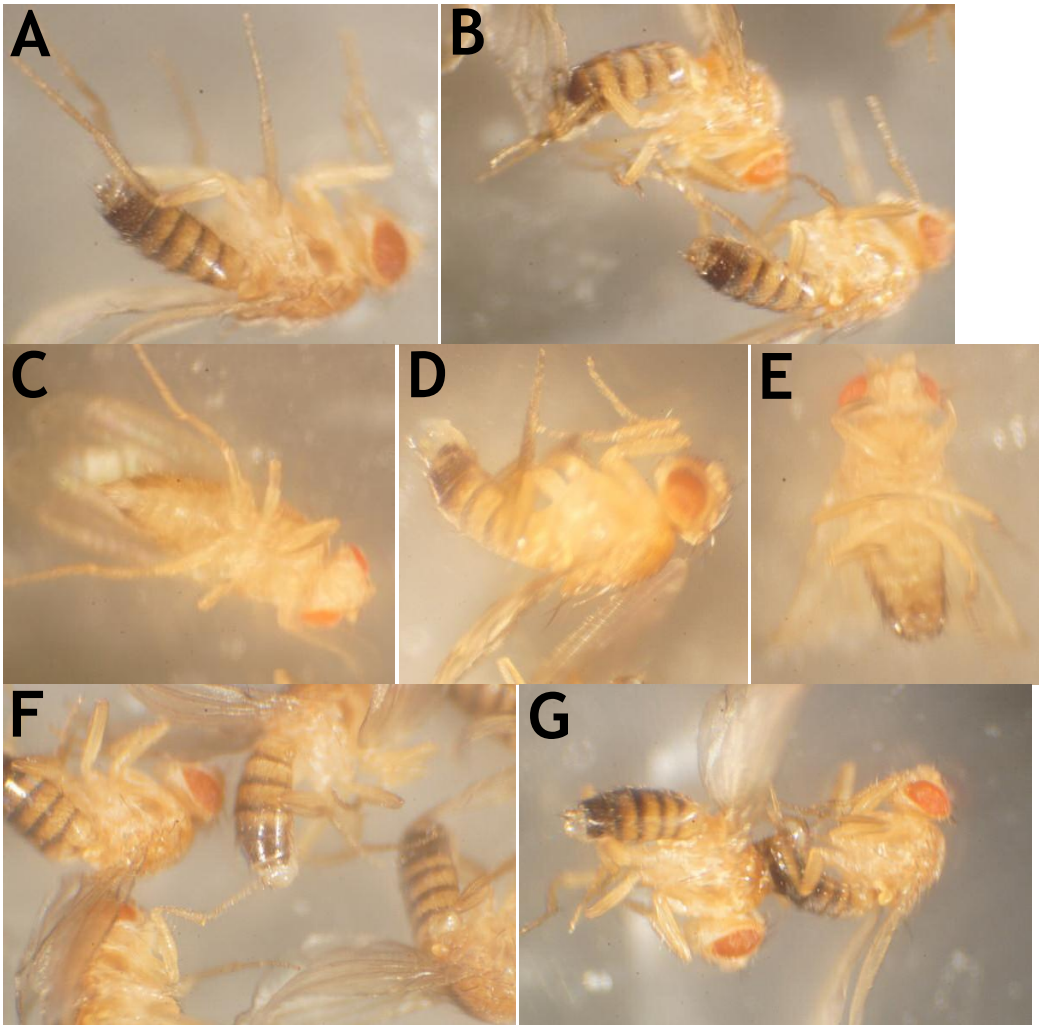

Figure 5-5. Symptoms of chlorpyriphos poisoning. All flies were still alive when images were taken. (A-C) Typical position of legs and abdomen during early poisoning. (D-G) Flies with visible moisture on the abdomen caused by fluid released from the posterior. (G) Position of legs and abdomen during late poisoning with two flies adhered to each other by fluid released from the posterior of the fly on the right.

2.1.4 Hydramethylnon

Hydramethylnon was a slow acting poison with mortality only beginning on the third day of experiments. Symptoms (**Figure 5-6**) only began after 24 hours of exposure with an inability to climb the food vials leading to a splaying of the legs (**Figure 5-6A-F**) and inability to fly by the beginning of the second day of exposure. By the end of the second day of exposure individuals are unable to right themselves if knocked over. Between the inability to right themselves and death a starvation like cavitation of the abdomen occurs (**Figure 5-6G-H**), potentially caused by a utilization of fat reserves and tissue for energy metabolism.

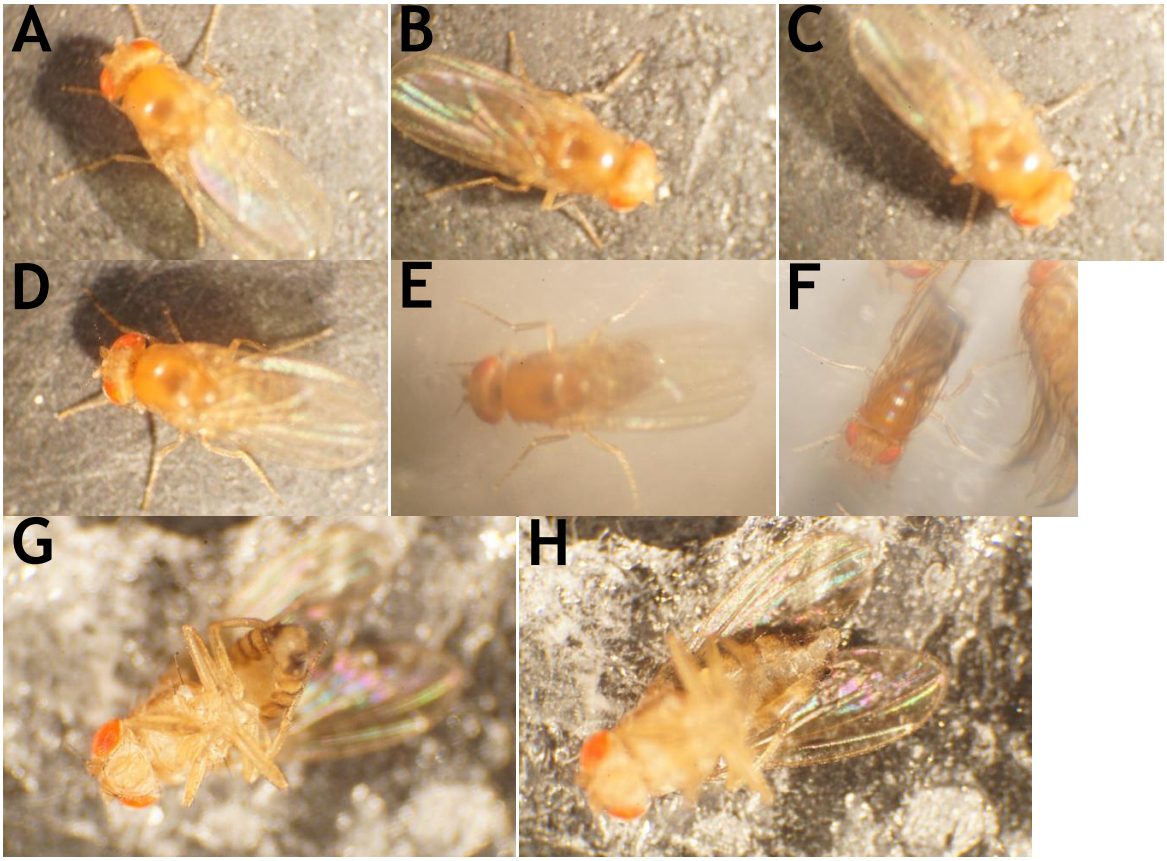

Figure 5-6. Symptoms of hydramethylnon poisoning. All flies were still alive when images were taken. (A-D) Splaying of legs on a rough surface. (E-F) Splaying of legs on a smooth surface. (G) Cavitation of the abdomen in the resting position. (H) View of cavitation of the abdomen when straightened using double sided tape.

Despite the neurotoxic insecticides sharing many symptoms and therefore potentially having mechanisms of action in common, all insecticides tested produced some symptoms unique each insecticide. As symptoms were shown in Act>KK RNAi, Act>roGFP and CS strains it can be concluded that the symptoms caused are not due to genotype specific effects. The mix of shared and unique symptoms combined with the differing modes of action and known targets offered the opportunity to compare trends in survival when tryptophan catabolism is knocked down with specific pathways.

2.2 The effect of tryptophan catabolism knockdown on survival against different insecticides

2.2.1 Fenvalerate

To investigate if the knockdown of tryptophan catabolism affects tolerance to pyrethroids other than permethrin, the atypical third generation pyrethroid fenvalerate was used in feeding survival assays. As with permethrin the knockdown of all three candidate genes (*vermilion*, *cinnabar* and *CG6950*)

affected survival showing sexually dimorphic alterations to fenvalerate tolerance (**Figure 5-7**).

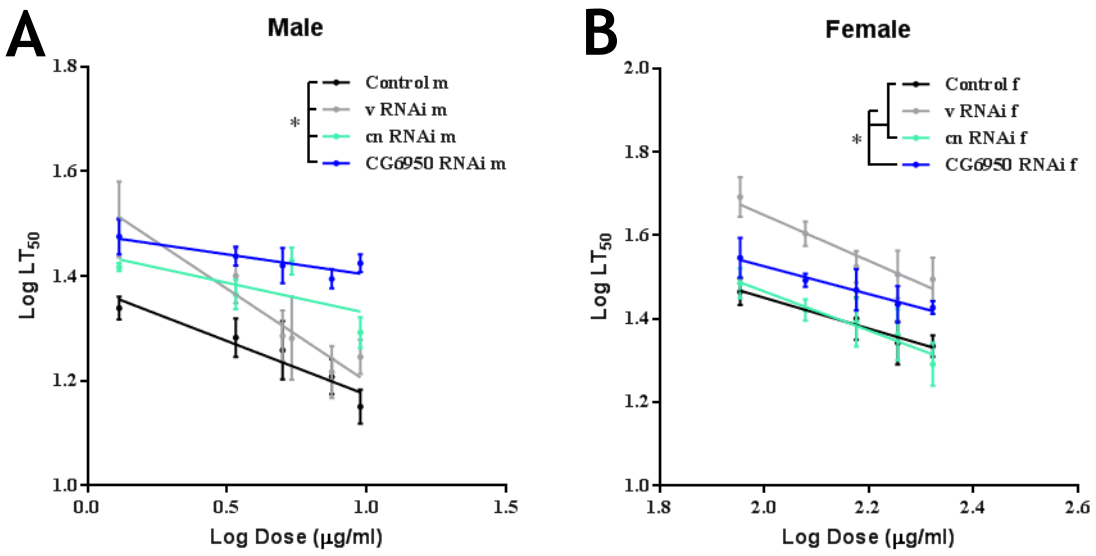

Figure 5-7. Fenvalerate toxicity responses of tryptophan catabolism gene knockdowns using an oral route of exposure. Toxicity responses showing the relationship between the dose of fenvalerate fed and the 50% lethal time (LT_{50}). (A) Toxicity responses for males. (B) Toxicity responses for females. Control strain (**Black**), *vermilion* knockdown strain (**Grey**), *cinnabar* knockdown strain (**Cyan**) and *CG6950* knockdown strain (**Blue**). N ≥ 900 per strain. (*) = P < 0.005. Data published in (Brinzer et al., 2015).

In males, knockdown caused increased tolerance as was observed with permethrin while in females *vermilion* and *CG6950* knockdown increased tolerance but *cinnabar* knockdown had no effect. The toxicity response of *vermilion* knockdown males had a different slope from the *cinnabar* and *CG6950* knockdowns indicating at least two different mechanisms affect survival. The sequential clustering of the toxicity responses for fenvalerate exposure are different compared to permethrin exposure suggesting the involvement of pyrethroid secondary targets in the changes in survival caused by tryptophan catabolism gene knockdown. As with permethrin there was sexual dimorphism with *cinnabar* and *CG6950* knockdowns except a change in intercept instead of a change in gradient was observed. Only one example of a type-I and type-II pyrethroid was tested so it would be of interest to investigate the responses of tryptophan catabolism knockdown in *Drosophila* to other compounds in the two pyrethroid groups.

2.2.2 DDT

As pyrethroids share many secondary targets, an organochlorine voltage gated sodium agonist, DDT, was used in feeding survival assays to investigate if the voltage gated sodium channel is involved in the changes in tolerance observed with the pyrethroid insecticides. The knockdown of *vermilion* and *cinnabar* had no effect on survival while *CG6950* knockdown caused increased tolerance in both genders (**Figure 5-8**).

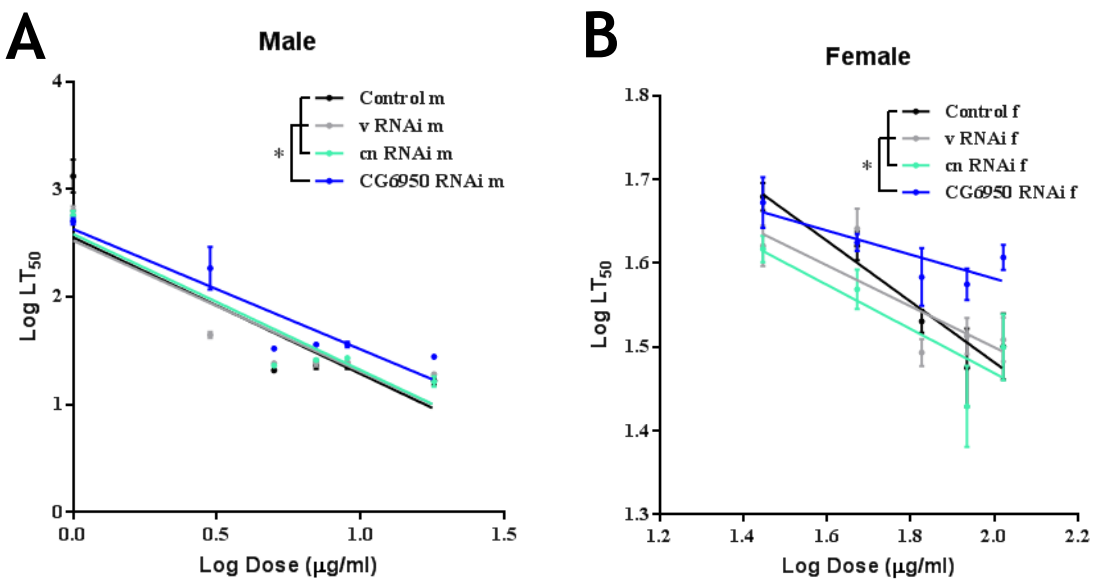

Figure 5-8. DDT toxicity responses of tryptophan catabolism gene knockdowns using an oral route of exposure. Toxicity responses showing the relationship between the dose of DDT fed and the 50% lethal time (LT_{50}). (A) Toxicity responses for males. (B) Toxicity responses for females. Control strain (**Black**), *vermilion* knockdown strain (**Grey**), *cinnabar* knockdown strain (**Cyan**) and *CG6950* knockdown strain (**Blue**). N ≥ 900 per strain. (*) = P < 0.005. Data published in (Brinzer et al., 2015).

These results suggest that tryptophan catabolism affects survival through mechanisms that do not involve the voltage gated sodium channel. Alternatively since DDT exposure does not affect free amino acid profiles (Mansingh, 1965), there may not be enough flux through tryptophan catabolism for the vermilion and cinnabar knockdowns to exert their effect on survival. As CG6950 is a putative transaminase, a class of enzymes which are known to be highly promiscuous in mammals (Han et al., 2010), it is possible that some of the effects seen in survival trends when exposed to voltage gated sodium channel agonists are caused by off-target transamination reactions and might explain why it was the only candidate gene tested to affect DDT survival when knocked down. Since DDT exposure also causes oxidative stress and may impair dopamine transport (Dowling et al., 2006; Hatcher et al., 2008), which also

occurs during permethrin exposure (Elwan et al., 2006; Terhzaz et al., 2015), it can be deduced that both oxidative stress and dopamine transport are not responsible for the changes seen in survival with the knockdown of *vermilion* and *cinnabar*.

2.2.3 Chlorpyriphos

Pyrethroids are known to cause the release of the neurotransmitter acetylcholine (Feng et al., 1992; Yan et al., 2011) which is likely to impact on cholinergic neurotransmission. To investigate if cholinergic neurotransmission is responsible for the survival phenotypes observed with tryptophan catabolism knockdown, the organophosphate chlorpyriphos, an acetylcholine esterase inhibitor, was used in feeding survival assays.

As with the pyrethroids, *vermilion*, *cinnabar* and *CG6950* affected survival when knocked down (**Figure 5-9**) causing increased tolerance in females while males were only more tolerant at higher doses. A potential mechanism for the change in chlorpyriphos tolerance seen in the knockdown strains is the ability of kynurenates to modulate acetylcholine release, receptor function and trafficking (Zmarowski et al., 2009) which would reduce cholinergic neurotransmission counteracting the increase caused by the acetylcholinesterase inhibition.

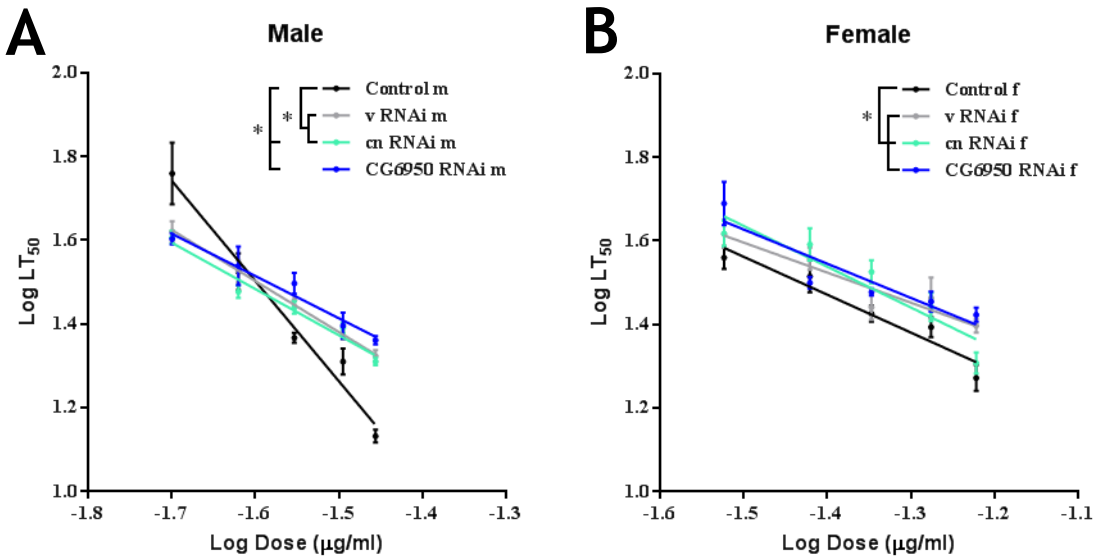

Figure 5-9. Chlorpyriphos toxicity responses of tryptophan catabolism gene knockdowns using an oral route of exposure. Toxicity responses showing the relationship between the dose of chlorpyriphos fed and the 50% lethal time (LT_{50}). (A) Toxicity responses for males. (B) Toxicity responses for females. Control strain (**Black**), *vermilion* knockdown strain (**Grey**), *cinnabar* knockdown strain (**Cyan**) and *CG6950* knockdown strain (**Blue**). N ≥ 900 per strain. (*) = P < 0.005. Data published in (Brinzer et al., 2015).

The toxicity responses of the knockdown males show a change in gradient compared to the control while females have a change in the x-axis intercept indicating a sexually dimorphic effect of cholinergic neurotransmission on survival. As the toxicity responses for permethrin and fenvalerate lacked the features seen in the chlorpyriphos toxicity responses it can be concluded that cholinergic neurotransmission is unlikely to play a major role in pyrethroid toxicity. Interestingly some organophosphates cause changes in lipid peroxidation, free amino acid concentrations and urea cycle metabolism (Gupta et al., 2010; Narra et al., 2013; Nath et al., 1997) which is similar to those known to occur during pyrethroid exposure (**Appendix II**) e.g. (Reddy and Bhagyalakshmi, 1994; Terhzaz et al., 2015; Veronica and Collins, 2003). Organophosphates inhibit lipases impairing the metabolism of lipid peroxides, which degrade into aldehydes (Turnwald et al., 1998) that form mono and dicarboxylic acids (Raghavamenon et al., 2009). Unlike the organophosphates and pyrethroids DDT does not impair fatty acid metabolism and does not show an increase in free amino acids. It can therefore be hypothesised that neural hyperexcitation must be combined with impaired fatty acid/lipid metabolism, to trigger an increase in free amino acids and associated changes in the urea cycle when under oxidative stress.

2.2.4 Hydramethylnon

The data suggests that permethrin exposure causes perturbations in energy metabolism (**Ch. 3, Section 3.2**). To investigate if interactions in energy metabolism are responsible for the changes in survival observed in tryptophan catabolism knockdowns, hydramethylnon, a suicide inhibitor of site-II of the mitochondrial complex III, was used in feeding survival assays (**Figure 5-10**).

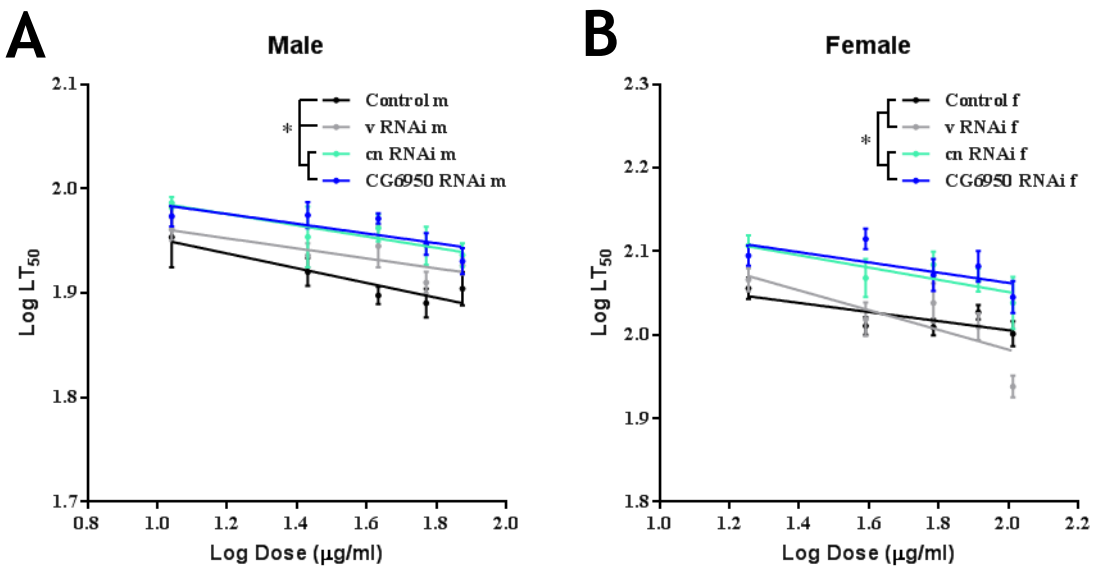

Figure 5-10. Hydramethylnon toxicity responses of tryptophan catabolism gene knockdowns using an oral route of exposure. Toxicity responses showing the relationship between the dose of hydramethylnon fed and the 50% lethal time (LT_{50}). Toxicity responses for males are on the left and the toxicity responses for females are on the right. Control strain (**Black**), vermilion knockdown strain (**Grey**), cinnabar knockdown strain (**Cyan**) and CG6950 knockdown strain (**Blue**). N ≥ 900 per strain. (*) = P < 0.005. Data published in (Brinzer et al., 2015).

Both genders of *cinnabar* and *CG6950* knockdowns show increased tolerance while only male *vermilion* knockdowns had increased tolerance. The clustering of the *cinnabar* and *CG6950* knockdowns suggest that both are acting via the same mechanism. These data indicate that tryptophan catabolism has an important link to energy metabolism or energy utilization. The inhibition of mitochondrial complex III is likely to cause oxidative stress (Sanz et al., 2010) which would impact on survival in addition to the loss of ATP generation. Glycolysis and the Krebs cycle have been found to be impaired by 3-hydroxykynurenine and kynurenic acid in rats (Schuck et al., 2007). If the same happens in insects, it would explain the changes in survival seen with hydramethylnon exposure as the accumulation of tryptophan catabolites in the *cinnabar* and *CG6950* knockdowns would reduce the flux of electrons into impaired ROS generating electron transport chains. Type-II pyrethroids are known to undergo hydrolysis on degradation releasing cyanide (Matsuya et al., 2012), an inhibitor of the mitochondrial complex IV. Since tryptophan catabolism knockdown appears to have some beneficial role during the impairment of energy metabolism and oxidative respiration there is the potential to explain some of the characteristics seen in the toxicity response graphs of flies exposed to fenvalerate (**Figure 5-7**).

Survival against all of the insecticides tested was affected by at least one of the genes from tryptophan catabolism tested, usually causing increased tolerance. This would make *vermilion*, *cinnabar* and *CG6950* anti-targets and interaction with them should be avoided in designing and screening for new insecticidal or synergist compounds. Also if tryptophan catabolism were to similarly affect insecticide tolerance in other insect species there would also be impacts on malaria control strategies as there would be a trade off between the reliance on insecticides for vector control (David et al., 2013) and the possibility to inhibit malaria gametogenesis by inhibiting tryptophan catabolism in vectors (Billker et al., 1998).

The *white* (Klemenz et al., 1987) and *vermilion* (Fridell and Searles, 1991) genes, both involved in tryptophan catabolism, are used as markers for the majority of transgenic flies including commercial RNAi lines, such as the KK and GD, Nig-Fly and the TRIP libraries (Green et al., 2014); http://www.shigen.nig.ac.jp/fly/nigfly/about/pdf/mating_scheme.pdf; http://www.flyrnai.org/supplement/VALIUM20_map_seq.pdf). As there is evidence that tryptophan metabolism affects insecticide survival (**Section 2.2**), the strength of marker gene expression caused by positional effects, especially in P-element generated lines, has the potential to interact with genes of interest during insecticide exposure which could add bias to results. There is evidence from the results that tryptophan catabolism affects survival against insecticide exposure with more than one mechanism however, there is no link between the known interactions of tryptophan and its catabolites and those of permethrin meaning that further investigation is required to identify causative interactions.

Chapter 6 - Functional characterization of candidate genes using metabolomics

1 Introduction

In the previous chapters it was demonstrated that knocking down genes involved in tryptophan catabolism affects survival against insecticide exposure (**Ch. 4** and **5**). Unfortunately, based on what is known about the candidate genes, their associated metabolites and the insecticides was not enough to explain the observed changes in survival for many of the insecticides. Untargeted metabolomics offers a potential linking stratagem in that it can identify downstream or pleiotropic metabolic changes induced outside established lesions (Kamleh et al., 2008).

The one-enzyme-one-reaction hypothesis has been disproven with some enzymes being highly promiscuous (Han et al., 2010) leading to the concept of an "underground metabolism" formed from side-reactions that is able to contribute greatly to metabolic flexibility (Guzman et al., 2015; Patrick et al., 2007). Surprisingly, the Vermilion and Cinnabar enzymes have never been tested for activity on substrates that are not indoleamines or other tryptophan catabolites while CG6950 is completely uncharacterised. The impacts of increased concentrations of tryptophan and the kynurenates (anthranilate branch excluded) outside of tryptophan metabolism has also been limited to very few studies (Nagaraj et al., 2010; Schuck et al., 2007). In this chapter untargeted metabolomics was applied to Act>KK RNAi strains and the control to verify the efficacy of the RNAi at inducing the desired metabolic lesions and to identify any pleiotropic or off-target changes in metabolism.

2 Results and discussion

Unpoisoned larvae were selected for study because the tryptophan catabolic pathway is more active in larvae than in adults (Sullivan et al., 1973). Due to the size and format of the metabolomic data tables, **Tables 6-4** to **6-12** are listed at the end of **Chapter 6** (**Pages 172-212**).

2.1 Categorization of metabolites and pathways affected by RNAi knockdown

Compared to the control strain the *vermilion* knockdown strain showed significant changes in the concentration of 136 metabolites and both the *cinnabar* knockdown and the *CG6950* knockdown showed changes in 103 metabolites respectively. In total 274 unique metabolites (16 identified, 258 putative) were affected in the tryptophan catabolism knockdown strains with an additional 157 related metabolites (16 identified, 141 putative) having a probability of $0.05<p<0.01$. The majority of metabolites belonged to eight metabolic pathways (**Table 6-1**). The number of metabolites that are shared between two or more RNAi knockdowns is shown in **Figure 6-1**.

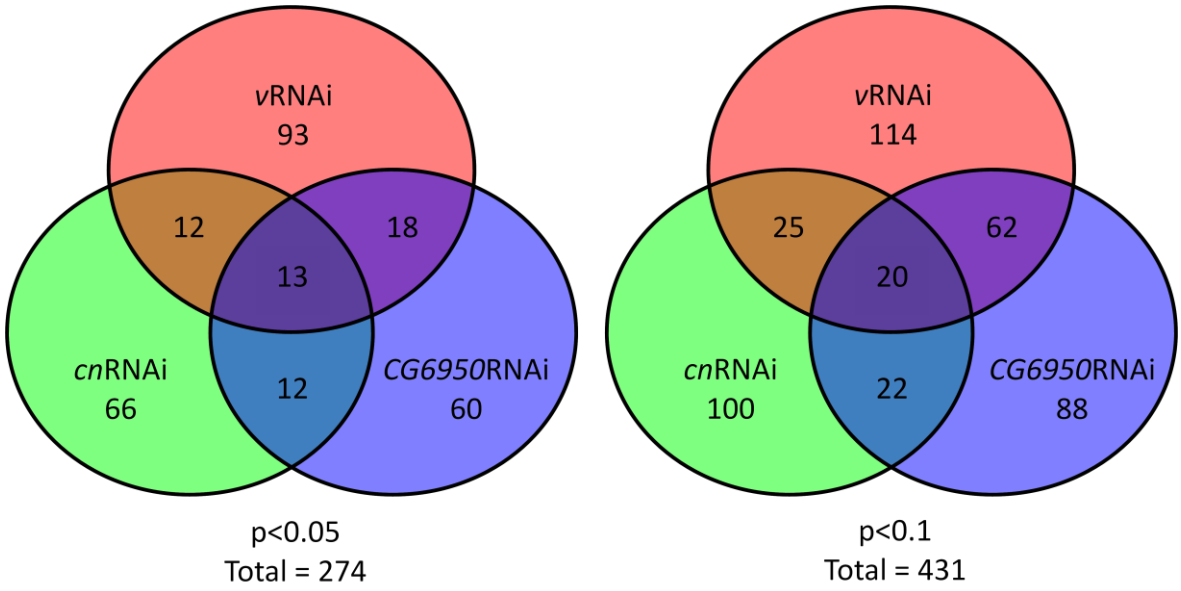

Figure 6-1. Venn diagrams of metabolite numbers that had significant concentration changes in the RNAi strains compared to the control and numbers at a more relaxed significance value. *v*RNAi = *vermilion* knockdown strain, *cn*RNAi = *cinnabar* knockdown strain and *CG6950*RNAi = *CG6950* knockdown strain. Area sizes are not proportional.

The automated annotation process used by by the Glasgow in-house analysis pipeline IDEOM uses a scoring system based on similarity of retention time, then mass before allocating isomers according to peak height using lists that prioritize central metabolism followed by the lowest unused database entry number (Creek et al., 2012). This means that two different compounds with the same score will be given the same annotation and unlikely isomer annotations with a lower data base entry number will be given priority over more logical choices which have a higher database entry number.

Of the putative metabolites identified, 26 at $p<0.05$ (46 at $p<0.1$) were highly unlikely to be found either as an endometabolite or exometabolite in *Drosophila* indicating probable misannotation. These putative compounds were annotated as compounds that are man-made xenobiotics which are not common environmental contaminants eg. 2,4-dinitrophenylhydrazine or as compounds that only occur in select species of plants or bacteria that inhabit niches not associated with *Drosophila* or any of the dietary components eg. L-hypoglycin. These putative compounds are more likely to be one of the alternative isomers.

Table 6-1. Metabolic pathways significantly altered in tryptophan catabolism knockdown strains.

***vermilion* knockdown**	***cinnabar* knockdown**	***CG6950* knockdown**
Unsaturated fatty acid metabolism		
	Hydroxy-epoxide fatty acid metabolism	**Hydroxy-epoxide fatty acid metabolism**
Acyl-glycerophosphoalcohol metabolism		**Acyl-glycerophosphoalcohol metabolism**
	Energy metabolism	**Energy metabolism**
Amino acid metabolism	**Amino acid metabolism**	**Amino acid metabolism**
Purine metabolism		**Purine metabolism**
Pyrimidine metabolism	**Pyrimidine metabolism**	
Vitamin B6 metabolism		**Vitamin B6 metabolism**

Among all the metabolites detected, 26 at $p<0.05$ (42 at $p<0.1$) had metabolites with a duplicate annotation in the data set of which 6 at $p<0.05$ (three pairs) (7 pairs at $p<0.1$) had putative duplicates that also showed significant concentration changes in the knockdown strains. Of the metabolites with duplicate annotation 6 at $p<0.05$ (8 at $p<0.1$) were short peptides. Since short peptides made of the same amino acids but arranged in a different sequence usually have the same retention time and are among the possible isomers, it is probable that duplicate short peptide annotations are just one of the rearrangements. If metabolites with annotation duplicates are isomers within the same pathway, then the trends in concentration relative to each other and related metabolites could be used for identification. Where possible a likely alternative annotation for duplicates is given (**Tables 6-4** to **6-12**).

2.2 Changes in tryptophan metabolism

Expected changes in tryptophan catabolites caused by lesions in the pathway have been reviewed (Savvateeva et al., 2000). As the desired targets for the RNAi knockdown strains used were in tryptophan catabolism, it was to be expected that there would be changes in metabolites involved in tryptophan catabolism and pathways that use tryptophan (**Table 6-4**).

The *vermilion* gene product metabolises the first and rate limiting step of tryptophan catabolism so it was expected that there would be a decrease in all downstream metabolites and potentially increased flux towards serotonin in the RNAi knockdown strain however, the only significantly altered tryptophan catabolite was kynurenate which is the minor product of the pathway. This indicates that despite a 64% knockdown in adults at the transcriptional level (**Ch. 4, Section 2.3.1**) the loss in enzymatic activity is not enough to impact significantly on metabolites in the xanthurenic acid branch of the pathway under non stress-inducing conditions in larvae.

The *cinnabar* gene knockdown strain (90% knockdown in adults) had an expected increase in kynurenine concentrations and a marginal increase in the upstream metabolite N-formylkynurenine however, there were no significant changes to 3-hydroxykynurenine and downstream metabolites. This indicates that the cinnabar knockdown is not producing a bottleneck that would impair the accumulation and storage of metabolites downstream of 3-hydroxykynurenine before adulthood.

CG6950 is a putative kynurenine transaminase, a family of enzymes known to be highly promiscuous in mammals (Han et al., 2010), so changes in off target transaminations could be expected, however, the RNAi knockdown strain (66% knockdown in adults) shows significant decreases in most of tryptophan metabolism with only marginal changes to the transamination products kynurenate and xanthurenic acid ($0.05<p<0.1$). The *CG6950* RNAi knockdown strain also showed a marginal decrease in the pool of free tryptophan ($0.05<p<0.1$) and a significant decrease in 5-hydroxy-L-tryptophan suggesting that the observed changes in tryptophan metabolism may be due to a lack available tryptophan to act as a substrate. As tryptophan is an essential amino

acid of dietary origin, the deficiency could be caused by reduced absorption across the gut or by the synthesis of tryptophan-containing peptides like Ans-Phe-Trp-Cys (12.7 fold increase in the *CG6950* RNAi knockdown strain) depleting the available pool (**Table 6-6**). These results imply that the *v*RNAi and *cn*RNAi strains have some reduction in flux through the pathway at the desired lesions but the function of the *CG6950* RNAi as the knockdown of the kynurenate synthesizing enzyme is put into doubt.

2.3 Changes in other amino acid metabolic pathways

There were 25 metabolites belonging to the metabolism of other amino acids that were significantly changed in the knockdown strains (**Table 6-5**). In *cinnabar* knockdowns and to a lesser extent in *CG6950* knockdowns there is an increase in L-arginine phosphate suggesting increased arginine kinase activity in those two strains. The *vermilion* and *cinnabar* knockdown strains show decreases in the spermidine metabolites deoxyhypusine and N1,N8-diacetylspermidine and an increase in the spermine degradation product 1-5-diazabicyclononane indicating either increased spermine synthesis or increased polyamine oxidase activity. The changes in phosphotyrosine, N6-acetyl-L-lysine and N2-acetyl-L-lysine seen in the *cinnabar* knockdowns suggest increases in protein degradation or post-translational modification. There is also an increase in the glutathione precursor gamma-L-glutamyl-L-cysteine in *cinnabar* knockdowns which is potentially linked to the depletion in glycine suggesting restricted availability in the correct cellular compartments. The *vermilion* and *cinnabar* knockdowns showed opposite trends for N-acetylcysteamine, a decarboxylation product of N-acetylated cysteine residues, which might be linked to glutathione metabolism since vermilion knockdowns showed a decrease in glutathione and an increase in N-acetylcysteamine.

In *vermilion* knockdowns an accumulation of 4-Hydroxyphenylacetaldehyde and depletion of 4-Hydroxyphenylacetate, both tyrosine derived metabolites, is observed suggesting impaired aldehyde dehydrogenase activity. Tyrosine metabolism also showed many changes in metabolites derived from L-Dopa including the eumelanin precursor dopaquinone which was depleted (>74% decrease) in all three strains (2-carboxy-2,3-dihydro-5,6-dihydroxyindole and L-dopachrome also showed marginal change in *vermilion* and *cinnabar* knockdowns

(0.05<*p*<0.1) indicating increased flux to cuticle tanning pathways (Sugumaran, 2002) which might impact on the strength and permeability of the cuticle and peritrophic membrane. Changes in cuticle structure would also affect water loss (Noh et al., 2015). Interestingly xanthurenic acid has been linked to changes in cuticle thickness in *Bombyx mori* (Kushida et al., 2012) so the results suggest that other tryptophan catabolites may also influence chitin synthesis. The decreases in 3,4-dihydroxymandelaldehyde in *CG6950* knockdowns and epinephrinesulfate in *vermilion* knockdowns indicate that adrenergic signalling may also be perturbed.

2.4 Changes in short peptides

Of all the significantly changed metabolites 44 were short peptides of two to four amino acids in length (**Table 6-6**). This is probably caused by altered protein degradation that occurs as a consequence of RNAi by co-translational degradation and ribosomal drop-off producing truncated peptides which are degraded (Eulalio et al., 2008). Tryptophan catabolism has been implicated in the efficacy of protein glycosylation (Nagaraj et al., 2010), which has an effect on protein stability, and the modulation of the RNAi pathway in general by interaction with lysosomal sorting (Harris et al., 2011) making it possible that the peptide fragments are from proteins other than the targets of the knockdown strains. It is known that the *C. elegans vermilion* ortholog acts as a regulator of protein homeostasis with depletions being associated with decreased proteotoxicity (van der Goot et al., 2012), so the observed decreases in basic, hydrophopbic and polar peptides indicate the same is happening in *Drosophila*. The peptides changed in the *vermilion* and *CG6950* knockdown strains were similar to each other showing the same decreased trend but very different from those found in the *cinnabar* knockdown strain which had no obvious trend. Interestingly the Asn-Phe-Trp-Cys peptide, which was the only peptide to be greatly increased in all three knockdown strains, has a high degree of similarity to part of a peptide that was able to inhibit substrate binding by human β-adrenergic receptors (Kim et al., 1994) suggesting it might be a fragment of a regulatory peptide and could explain the changes in adrenaline catabolites observed (**Table 6-5**).

2.5 Changes in fatty acid and lipid metabolism

The knockdown strains also showed alteration in fatty acid metabolism unique to each strain with the exception of two metabolites (**Table 6-7**). The essential fatty acid linolenic acid, which comes from dietary sources, was increased in the *vermilion* RNAi knockdown strain while the *cinnabar* RNAi knockdown strain showed a decrease. This pattern was also seen in all fatty acid metabolites derived from linolenic acid. The depletion of several short chain acyl-carnitines, short chain fatty acid derivatives and malonate in the *vermilion* RNAi knockdown strain suggests the increase in long chain fatty acids is the result of increased fatty acid elongation. The *cinnabar* RNAi knockdown strain shows an increase in unsaturated fatty acids with a C6-9 length carbon backbone but there is a decrease in all fatty acid metabolites derived from unsaturated fatty acids with a longer backbone indicating a lesion between medium and long chain fatty acid metabolism.

Fatty aldehydes are formed from the cleavage of diols that are a consequence of lipid peroxidation (Turnwald et al., 1998), so the reduced concentrations of several fatty aldehydes in the *vermilion* RNAi knockdown strain can be interpreted as either a reduction in oxidative stress, a reduction in cyclooxygenase or epoxide hydrolase activity or increased medium chain aldehyde dehydrogenase activity. There is an accumulation of choline and ethanolamine derived phospholipids in the *vermilion* RNAi knockdown strain, the only exception being N-(11R,15S-dihydroxy-9-oxo-5Z,13E-prostadienoyl)-ethanolamine which was depleted in all three knockdown strains. The depletion of glyceraldehyde, an oxidation product of glycerol phosphate, and orthophosphate in the *vermilion* RNAi knockdown strain also suggests increased phospholipid synthesis. The *CG6950*RNAi strain showed changes in fatty acid metabolism that paralleled the *v*RNAi strain however the differences were not large enough to be significant.

Lipoyllysine, the post translationally modified catalytic oleoyl-lysine residues of several dehydrogenases, showed accumulations in the *cinnabar* and *CG6950* RNAi knockdown strains (>2 fold) while the substrate bound intermediates S-acetyldihydrolipoamide, from pyruvate dehydrogenase, and S-succinyldihydrolipoamide, from oxoglutarate dehydrogenase show depletion in

the *CG6950* RNAi knockdown strain. This indicates either reduced flux through pyruvate and oxoglutarate dehydrogenase or increased expression of those enzymes.

2.6 Changes in nucleotide metabolism

There were several changes in the metabolism of nucleotides (**Table 6-8**). The pentose phosphate pathway has a decrease in 2-Deoxy-D-ribose 5-phosphate indicating a decrease in DNA degradation in the *vermilion* RNAi and *CG6950* RNAi knockdown strains. This is supported by the decreases in deoxyadenosine ($0.05<p<0.1$), deoxyguanosine, thymidine and thymine. D-Ribose 5-phosphate was depleted in *vermilion* RNAi knockdowns while showing an increase in *cinnabar* RNAi knockdowns indicating that kynurenine and downstream metabolites might stimulate D-ribose 5-phosphate synthesis. All three knockdown strains showed a weak trend ($0.05<p<0.1$) of having depleted mono and diphosphates of purine metabolism indicating either increased RNA and DNA synthesis or a bottleneck in purine synthesis. The *vermilion* RNAi knockdown strain showed an increase in 5-hydroxyisourate from the uric acid pathway suggesting that the more severe depletion of purine metabolites seen in that strain may be due to increased flux through the uric acid pathway.

In the *vermilion* RNAi knockdown strain there are large decreases in modified nucleotides associated with tRNA and rRNA, especially cytidine derived nucleotides, indicating that the knockdown of *vermilion* causes impairment of post transcriptional modification. There is a weak depletion of pseudouridine 5'-phosphate ($0.05<p<0.1$) in the *vermilion* and *CG6950* RNAi knockdown strains implying either a reduction in the conversion of uridine to pseudouridine or a decrease in the degradation of pseudouridine containing RNAs, eg. rRNAs. There is also a large but insignificant increase in 2',3'-Cyclic CMP, 2',3'-Cyclic UMP and CMP (whether 3’ or 5’ was not able to be determined), which are RNA degradation products, in the *vermilion* RNAi knockdown, probably from tRNA and rRNA residues that should have been modified. The *cinnabar* RNAi knockdown strain had depletions of 2',3'-Cyclic CMP and CMP and an increase in 5-methylcytidine indicating increased RNA methylation of cytidine.

As pseudouridine is recycled into uracil, the weak decrease in 5,6-dihydrouracil ($0.05<p<0.1$) and decrease in malonate which are uracil catabolites seen in the *vermilion* RNAi knockdown strain might be the result of a reduction in flux through uracil. The *CG6950* RNAi knockdown strain mimics the trends seen in the *vermilion* RNAi knockdown strain but again the differences were not large enough to be significant. Strangely, there is a depletion of guanosine, 3-methylguanine and deoxyguanosine in the *CG6950* RNAi knockdown strain, potentially due to decreased DNA cycling or a lesion in the pathway.

2.7 Changes in energy metabolism

It is known that kynurenate and 3-hydroxykynurenine act as inhibitors of energy metabolism in rats (Schuck et al., 2007), so changes in energy metabolism (**Table 6-9**) were to be expected. The *cinnabar* RNAi knockdown strain showed the most changes to energy metabolism with increases in sucrose and phosphoenolpyruvate and decreases in sedoheptulose, arbutin 6-phosphate and 6-acetyl-D-glucose, sugars of dietary origin. There was also a marginal decrease in D-glucose and the starch catabolite maltotriose ($0.05<p<0.1$) suggesting an increase in glycolysis but a bottleneck at entering the Kreb's cycle, possibly at pyruvate dehydrogenase. The neuroactive compound (-)-salsolinol forms spontaneously from L-Dopa and either pyruvate or acetaldehyde by Pictet-Spengler condensation with the rate of formation being dependent on pyruvate and acetaldehyde availability (Dostert et al., 1990; Xie et al., 2012). By the *cinnabar* RNAi knockdown strain having reduced concentrations of (-)-salsolinol (**Table 6-5**) it can be concluded that there is a greater utilization of pyruvate and acetaldehyde in tissues where L-Dopa is present.

There was evidence for a stimulated Kreb's cycle in the *vermilion* RNAi knockdown strain with a depletion of malate and 2-oxoglutaramate, a glutamine catabolite that feeds into the Kreb's cycle. Since the *vermilion* RNAi knockdown strain showed a depletion of kynurenate (**Table 6-4**), it is possible the increase in energy metabolism is caused by a loss of kynurenate induced inhibition. The only energy metabolite to show any change in the *CG6950* RNAi knockdown strain was D-erythrose, a potential mass spectroscopy artefact of D-erythrose 4-phosphate, suggesting increased utilization of ß-D-fructose 6-phosphate for D-glyceraldehyde 3-phosphate synthesis.

2.8 Changes in glycosylation and sugar conjugates

From mammalian studies there is evidence that tryptophan catabolism can modulate the glycosylation process (Nagaraj et al., 2010) and therefore potentially other processes that form sugar conjugates. Several metabolites associated with oligosaccharide formation were found to have altered concentrations in the knockdown strains (**Table 6-10**). N-acetyl-D-glucosamine, the monomers of chitin, are decreased in the *cinnabar* and *CG6950* RNAi knockdown strains giving further evidence for potential differences in cuticle and peritrophic membrane composition in the knockdown strains. The oligosaccharide intermediate 2-(acetylamino)-1-5-anhydro-2-deoxy-3-O-b-D-galactopyranosyl-D-arabino-Hex-1-enitol shows large increases in the *vermilion* and *cinnabar* RNAi knockdown strains (6.8 fold in the *cinnabar* knockdown). Interestingly, this sugar has only previously been identified in one other published study investigating the oligosaccharides of human milk (Zhang et al., 2005) (http://www.hmdb.ca/metabolites/HMDB02278), without investigating the origins of the compound. Inspection of the chemical structure revealed galactose conjugated onto 1,5-anhydro-D-arabino-hex-1-enitol, a tautomeric intermediate of glucose found during the catalytic mechanism of glycosyltrasferase (Díaz et al., 2012). The increase of this compound not only confirms the influence that tryptophan catabolism has on glycosylation in *Drosophila* but also gives insight as to what step in the catalytic mechanism is affected.

The data set showed changes in two metabolites originating from glycoproteins. N4-(Acetyl-beta-D-glucosaminyl)asparagine was depleted in *cinnabar* knockdowns while procollagen 5-(D-galactosyloxy)-L-lysine was depleted in *CG6950* knockdowns. Two glycosides and a glucuronide of diet derived compounds of plant origin (1-Salicylateglucuronide, Chrysophanol 8-O-beta-D-glucoside and 4'-Hydroxychalcone 4'-glucoside) were also found to be depleted across the three knockdown strains suggesting that sugar conjugation involved in phase II detoxification might also be affected by tryptophan catabolism.

2.9 Changes in metabolites of environmental origin

Of the 274 metabolites that showed significant changes in concentration across the three knockdown strains 72 (26.3%) were exometabolites (**Table 6-11**) originating either from the standard *Drosophila* medium (**Ch. 2, Section 2.3, Table 2-5**) or from bacterial species known to colonize the *Drosophila* gut indicating either a large change in the composition of the gut flora, changes in absorption across the gut or changes in excretion from the Malpighian tubules. The possibility of differing degrees of food contamination between samples being responsible for the concentration changes of exometabolites can be excluded as none of the strains showed any unidirectional trend in exometabolite concentrations. There is further evidence of cuticle and peritrophic membrane modification by the depletion of dietary derived compounds used for cuticle tanning pathways like p-benzenediol (Dennell, 1958) and the marginal decrease of chlorogenate ($0.05<p<0.1$) (Sugumaran et al., 1987) in the *vermilion* and *CG6950* RNAi knockdown strains. Surprisingly the *vermilion* RNAi knockdown strain showed accumulations of the carbamate insecticide aldicarb, traces of which probably contaminate the ingredients used for the *Drosophila* food. Based on this observation it could be predicted that the *vermilion* RNAi knockdown strain might be more susceptible to that class of insecticide.

2.10 Miscellaneous metabolic changes

Other metabolic changes observed in the data set are listed in **Table 6-12**. As occurred with the changes in short peptides, there is a similarity between *vermilion* and *CG6950* knockdowns in the changes in miscellaneous metabolites, while the *cinnabar* knockdown shows a different trend. The *vermilion* and *CG6950* RNAi knockdown strains had a reduction in vitamin B6 metabolites. It is known that vitamin B6 is required as a cofactor by the CG6950 and cardinal enzymes in the formation of kynurenic acid and xanthurenic acid respectively and that vitamin B6 deficiency causes an accumulation of tryptophan catabolites (Yess et al., 1964) however, the inverse relationship of the effect of tryptophan catabolites on vitamin B6 regulation has never been investigated. As vitamin B6 is used as a cofactor by many enzymes it is possible that some of the metabolic changes observed in the *vermilion* and *CG6950* knockdowns are being caused by

a loss in activity of vitamin B6 dependent enzymes. It is known that supplementation with vitamin E reduces pyrethroid toxicity in mammals (Yousef et al., 2006) but vitamin B6 supplementation has never been investigated.

2.10.1 Probable misannotions

There were 23 metabolites with significant concentration changes which were not duplicates in the data set that were putatively annotated as metabolites unlikely to be found in *Drosophila* or as an exometabolite in the food, gut flora or environment (**Tables 6-11** and **6-12**). Where possible these metabolites were given an additional manual annotation, listed under the notes for that metabolite, suggesting a compound that had a lower percent retention time error and also fitted the mass spectroscopy data which was more likely to be present in *Drosophila* or as an exometabolite. The majority of misannotated compounds were annotated as alkaloids, flavonoids or man-made chemicals and pharmaceuticals often having possible alternative isomers that were fatty acids, short peptides, other alkaloids and flavonoids or sugars. An example of this was methyl 2-diazoacetamidohexonate ($0.05<p<0.1$), a compound with no literature indicating natural occurrence. Of the six potential alternative annotations listed, which were all xenobiotics, 2-Dimethylamino-5,6-dimethylpyrimidin-4-ol was a catabolite of aldicarb, and aldicarb was present in the data set so it is more probable that it is the alternative annotation.

2.10.2 Changes in ROS associated metabolites

Evidence for changes in oxidative stress in the data set is inconclusive, however, it is a known problem with metabolomics that some potential markers of oxidative stress can also be oxidation artefacts (Winnik and Kitchin, 2008). Methionine sulfoxide (**Table 6-5**), an oxidation product of methionine only formed by reactive oxygen species (Weissbach et al., 2002), is decreased in the *CG6950* RNAi knockdown strain and marginally in the *vermilion* RNAi knockdown strain suggesting a decrease in oxidative stress. The *cinnabar* and *CG6950* RNAi knockdown strains show a marginal decreasing trend in the antioxidant hypotaurine ($0.05<p<0.1$) (Aruoma et al., 1988) which potentially suggests a slight increase in oxidative stress. The biotin oxidation product biotinsulfone (**Table 6-12**), the short peptide oxidation product glycylprolylhydroxyproline

(**Table 6-6**) (also showed marginal depletion in *vermilion* knockdowns (0.05<p<0.1)), and the ascorbate and N-acetylglucosamine oxidation product threonate (**Table 6-10**) were depleted in *CG6950* knockdowns which potentially indicates reduced oxidative stress.

In *vermilion* knockdowns N-acetylcysteamine (**Table 6-5**), a deamination product of N-acetylcysteine potentially formed by oxidative stress, was increased however, lipid peroxidation products (**Table 6-7**) and glyceraldehyde (**Table 6-7**), the oxidation product of glycerol, were depleted. There is a marginal decrease in the oxidative stressor aminoacetone (0.05<p<0.1) (Dutra and Bechara, 2004) in *vermilion* and *CG6950* knockdowns which would reduce the production of reactive oxygen species slightly. Of all the knockdown strains the *vermilion* RNAi knockdown strain alone showed a decrease in glutathione (**Table 6-5**) potentially indicating increased oxidative stress. There was an increase in the dietary isoflavone antioxidant glycitein (**Table 6-11**) seen in *vermilion* knockdowns which would reduce oxidative stress while the *cinnabar* RNAi knockdown strain shows a depletion which would reduce tolerance to oxidative stress.

2.11 Correlation of metabolites altered to the changes in survival observed in the feeding assays

The feeding survival assay results (**Ch. 4** and **5**) showed that some tryptophan catabolism knockdown strains had extremely similar trends (**Figures 4-7** and **5-7** to **5-10**) depending on the insecticides used. Any significantly changed metabolites that were shared between these knockdowns that showed the same concentration change trends compared to the control were investigated to possibly identify candidate pathways responsible for the changes in survival (**Table 6-2**).

Table 6-2. metabolites with trends shared between knockdown strains

Putative metabolites shared			
***v* and *cn* RNAi**	***cn* and *CG6950* RNAi**	***v* and *CG6950* RNAi**	**All three strains**
1-5-diazabicyclononane	**Asp-Leu-Ser-His**	**S-Acetyldihydrolipoamide**	**Glu-Arg**
Leu-Asp-Cys	**Glycine**	**2-Oxopentanoic acid**	**2-C-Methyl-D-erythritol 4-phosphate**
2-(acetylamino)-1-	**3-oxo-hexadecanoic**	**2-heptenal**	**N-(11R,15S-dihydroxy-**

5-anhydro-2-deoxy-3-O-b-D-galactopyranosyl-D-arabino-Hex-1-enitol	**acid**		**9-oxo-5Z,13E-prostadienoyl)-ethanolamine**
Deoxyhypusine	**4'-Hydroxychalcone 4'-glucoside**	**Ovalitenin C**	**4-Amino-4-deoxychorismate**
Tiglylcarnitine	**N-Acetyl-D-glucosamine**	**Asn-Leu-Gln-Pro**	**Asn-Phe-Trp-Cys**
	Ser-Tyr	**Asp-Val-Pro-Ser**	**Glu-Glu-Ile-Thr**
	9S-hydroxy-10E,12Z-octadecadienoic acid	**Val-Asn-Pro**	**Dopaquinone**
	N2-Acetyl-L-aminoadipate	**L-Hypoglycin**	**Isoquinoline**
	1-Aminocyclopropane-1-carboxylate	**N-Methyl-L-glutamate**	**Norerythrostachaldine**
	1-4-beta-D-Glucan	**2-Deoxy-D-ribose 5-phosphate**	**Vinylacetylglycine**
	2-Aminomuconate semialdehyde	**Cys-Pro-Ser**	
	Lipoyllysine	**Deoxyguanosine**	
	Ricinine	**4-Pyridoxolactone**	
		Isopyridoxal	
		1-Salicylateglucuronide	
		3-Methylguanine	
		Diethylene glycol	
		Spiro[benzofuran-2(3H),1'-[2]cyclohexene]-7-chloro-4,6-dimethoxy-6'-methyl-2'-(methylthio)-3,4'-dione	

Data derived from Tables 6-4 to 6-12.

In the feeding survival assays using pyrethroids (**Ch. 4, Section 2.4.2** and **Ch. 5, Section 2.2.1**) the *vermilion* knockdown showed differences in the mechanisms affecting survival compared to the *cinnabar* and *CG6950* knockdowns. Interestingly, *vermilion* knockdowns showed increased accumulations of glyciein, a compound able to attenuate some of the lipid changes caused by cypermethrin exposure in mammals (Yousef et al., 2003). There were also great differences in the fatty acid and phospholipid profiles of *vermilion* knockdowns compared to the other two strains.

Many octadecenoic acid derivatives and catabolites are known to be chemically and pharmacologically active being capable of radical mediated reactions, rearrangements, cyclizations and polymerizations (Spiteller, 1998), while having properties ranging from neuroprotective (Kim et al., 2012) to cytotoxic (Schneider et al., 2001) as well as modulating the function of ion channels (Seebungkert and Lynch, 2002). Unfortunately, as this group of metabolites has such diverse effects on physiology, with many members lacking characterization of their physiological properties, it is possible that the perturbation in concentration of any individual member could cause the observed changes in survival against insecticide exposure in the tryptophan catabolism knockdown strains. As the composition of lipid membranes is known to affect the excitability of neurons (Pavlidis et al., 1994), the difference in lipid profiles may explain the differences seen between the *vermilion* RNAi knockdown strain and the other knockdown strains when exposed to different insecticides.

The *cinnabar* and *CG6950* knockdown strains both showed similarities to each other in survival trends when exposed to pyrethroids and hydramethylnon (**Ch.5, Section 2.2.4**). N-Acetyl-D-glucosamine and lipoyllysine are the most probable candidates for these similarities since modification of the chitinous peritrophic membrane might impair absorption of the insecticides by the gut while lipoyllysine is involved in the catalysis of many energy metabolism and amino acid catabolic reactions. For the changes in survival seen with the pyrethroid insecticides L-arginine phosphate is also a candidate because it could act as a sink for arginine which might reduce the formation of toxic guanido compounds caused by arginine accumulation (Meert et al., 1991) during pyrethroid induced ammonia stress (Veronica and Collins, 2003). However, despite being a polysaccharide which is depleted in both the *cinnabar* and *CG6950* knockdowns, 1-4-beta-D-glucan is a cellulose catabolite unable to be metabolised by *Drosophila* and as such is an unlikely candidate for explaining the observed changes in survival against hydramethylnon exposure.

From the metabolites with the same trends across all three knockdown strains those that stand out most as potentially being able to explain the changes in survival seen in the feeding survival assays are the phospholipid N-(11R,15S-

dihydroxy-9-oxo-5Z,13E-prostadienoyl)-ethanolamine, the cuticle tanning compound dopaquinone and isoquinoline which is a potential ABC transporter and cytochrome p450 inhibitor (Wink, 2012). N-(11R,15S-dihydroxy-9-oxo-5Z,13E-prostadienoyl)-ethanolamine depletion could possibly cause a change in membrane fluidity which would result in a reduction in the sensitivity of neurons towards hyperexcitation when fed neuroexcitatory insecticides. Dopaquinone can be used for sclerotization in insects (Hopkins and Kramer, 1992; Sugumaran et al., 1987) and there was some evidence for increased flux through the eumelanin pathway (**Section 2.3**) that, when combined with trends in N-acetyl-D-glucosamine and other tanning compounds, suggests increased chitin synthesis which would reduce the permeability of the cuticle and peritrophic membrane.

There were no obvious metabolic changes that could explain the differences in survival of the knockdown strains when fed DDT (**Ch. 5, Section 2.2.2**). Surprisingly, the neurotransmitter acetylcholine showed accumulation in the *CG6950* RNAi knockdown strain (**Table 6-7**). This would have a negative impact during organophosphate poisoning however, since the feeding survival assays (**Ch. 5, Section 2.2.3**) did not show any differences in survival for *CG6950* knockdowns compared to the other knockdown strains when fed the organophosphate chlorpyriphos it can be concluded that the accumulation of acetylcholine may be limited to the larval stages of development.

2.12 Candidate substrates and products for CG6950

The *CG6950* gene product is a putative Type III kynurenine transaminase (Yu et al., 2006), however the substrate specificity and functionality of the enzyme has never been investigated. Like many transaminases it could be highly promiscuous (Han et al., 2010), utilizing a broad range of 2-amino acids and 2-oxoacids as substrates and products. As transamination reactions are reversible, compounds able to undergo transamination with increased concentrations were considered candidate substrates while those showing depletion were considered candidate products. Despite tryptophan catabolism in the *CG6950* RNAi knockdown strain not being very informative as to whether CG6950 is able to transaminate kynurenine, kynurenine and two other transamination substrates from tryptophan catabolism were included in the list of candidates. Candidates are listed in **Table 6-3**. Interestingly, the

transamination product of tryptophan, indolepyruvate, was the only transaminated tryptophan catabolite to be significantly depleted in the knockdown strain suggesting CG6950 might have tryptophan aminotransferase activity, previously only known to occur in plants and fungi.

Table 6-3. Candidate substrates and potential products of CG6950

Potential substrates	Potential products
Imidazol-5-yl-pyruvate	Glycine
L-Kynurenine	2-Amino-9,10-epoxy-8-oxodecanoic acid
3-Hydroxy-L-kynurenine	2-Oxopentanoic acid
Tryptophan	Indolepyruvate
	3-Methyl-2-oxobutanoic

Data derived from Tables 6-4 to 6-12.

In this chapter metabolic differences occurring in the knockdown strains were identified along with evaluating the efficacy of the RNAi disruption of desired steps in tryptophan catabolism. Many known metabolic perturbations caused by tryptophan catabolites were identified along with several potentially novel metabolic alterations. The most changes occurred in metabolites of dietary origin indicating differences in absorption and metabolites able to be used for cuticle synthesis, both of which might affect survival against insecticides. Several candidate compounds for explaining the differences in feeding survival assay results (**Ch. 4** and **5**) were identified although the function of CG6950 as a kynurenine transaminase has been put into question and requires further investigation.

Table 6-4. Tryptophan metabolites altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
224.08	9.78	C10H12N2O4	C10H12N2O4 (2) C10H9NO4 (2) C8H9NO4 (5) C20H24N4O8 (1)	3-Hydroxy-L-kynurenine	21.3	-0.7	Tryptophan metabolism	0.63	0.108	0.87	0.382	0.53	0.008	Tryptophan catabolite; decrease expected in the *cinnabar* knockdown
161.05	7.54	C9H7NO2	C9H7NO2 (6) C9H9NO3 (6) C10H18N4O6S (2)	4,6-Dihydroxyquinoline	18.1	-0.1	Tryptophan metabolism	1.25	0.624	0.88	0.364	0.59	0.025	5-hydroxykynuramine metabolite; *Drosophila* has enzymatic activity
220.08	8.53	C11H12N2O3	C11H12N2O3 (2) C11H9NO3 (1) C9H9NO3 (6) C11H14N2O4 (1)	5-Hydroxy-L-tryptophan	10.1	-0.5	Tryptophan metabolism	0.77	0.413	0.84	0.232	0.55	0.012	Serotonin precursor present in *Drosophila*
203.06	4.95	C11H9NO3	C10H7NO (1) C11H6O3 (1) C9H6O3 (1)	Indole-pyruvate	-39.1	-0.9	Tryptophan metabolism	1.05	0.953	0.57	0.137	0.33	0.044	Tryptophan→ indolpyruvate; transamination reaction; no known gene in *Drosophila*; part of IAA synthesis in plants; potentially of dietary origin
189.04	6.67	C10H7NO3	C10H7NO3 (5) C10H9NO4 (6)	Kynurenate	-9.3	0.5	Tryptophan metabolism	0.02	0.007	1.03	0.925	0.61	0.070	Transamination product of kynurenine; neuroactive; expected to decrease in the *CG6950* knockdown
236.08	8.05	C11H12N2O4	C11H12N2O4 (1) C11H9NO4 (2)	L-Formyl-kynurenine	7.8	-1.8	Tryptophan metabolism	0.86	0.641	1.27	0.059	0.66	0.248	trp→kyn intermediate; expected to decrease in the *vermilion* knockdown
208.08	8.67	C10H12N2O3	C10H12N2O3 (1) C10H9NO3 (3) C9H10N2O (1) C8H9NO3 (7) C9H8N2O2 (1) C10H14N2O4 (2) C20H24N4O6 (1)	L-Kynurenine	0.3	-0.6	Tryptophan metabolism	1.20	0.626	1.81	0.010	1.07	0.800	Trp→XA intermediate; neuroactive; can form protein adducts; had standard

			C30H36N6O9 (1)											
189.04	4.85	C10H7NO3		N-Acetylisatin	-34.7	-0.4	Tryptophan metabolism	0.43	0.032	1.11	0.617	0.56	0.054	Enzyme present in maize; formed from inoxyl that forms indigo; plant seed peroxygenase makes precursor; probably of dietary origins
205.04	8.40	C10H7NO4	C10H7NO4 (1) C10H9NO5 (6)	Xanthurenic acid	12.8	0	Tryptophan metabolism	0.72	0.352	0.72	0.194	0.57	0.073	Antioxidant; ommochrome precursor; product of *cardinal* gene in *Drosophila*
141.04	9.09	C6H7NO3	C6H7NO3 (2) C5H5NO (1) C4H4O3 (1) C6H9NO4 (3)	2-Amino-muconate semialdehyde	10.9	0.3	Tryptophan metabolism	0.57	0.077	0.62	0.034	0.63	0.029	Tryptophan catabolite produced by some bacteria; probably of dietary origin
141.04	7.79	C6H7NO3	C6H7NO3 (3) C6H4O3 (1) C5H5NO (3) C4H4O3 (1) C6H9NO4 (2)	2-Amino-muconate semialdehyde	-3.9	0.2	Tryptophan metabolism	0.53	0.007	1.41	0.251	1.04	0.856	Had duplicate annotation in the data set; probably N-Methyl-2-oxoglutaramate (-0.7, 0.2), a methylamine and oxoglutarate transamination product produced by bacteria that is common in the environment from the degradation of proteins, herbicides and insecticides; probably of dietary origin
204.09	9.16	C11H12N2O2	C11H12N2O2 (2) C9H9NO2 (1) C11H14N2O3 (1) C22H24N4O4 (1) C33H36N6O6 (1)	L-Tryptophan	-0.2	-0.1	Tryptophan metabolism	0.88	0.707	0.92	0.587	0.70	0.097	

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm error)

Table 6-5. Other amino acid metabolites altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
254.08	11.02	C6H15N4O5P	C8H16N4O3 (2)	L-Arginine phosphate	-19.8	-1.1	Arginine and proline metabolism	1.09	0.847	2.34	0.005	2.34	0.055	Product of arginine kinase
202.14	14.54	C8H18N4O2		NG,NG-Dimethyl-L-arginine	26.5	0	Arginine and proline metabolism	0.72	0.284	0.95	0.636	0.69	0.034	Product of protein methylation associated with histones; *Drosophila* has enzyme to degrade into citrulline and dimethylamine; inhibits nitric oxide synthase
104.02	8.96	C2H4N2O3		Urea-1-carboxylate	1	0	Arginine and proline metabolism	0.73	0.344	1.12	0.257	0.67	0.020	Urea catabolite
126.12	11.67	C7H14N2	C7H14N2 (1)	1-5-diazabicyclononane	22.7	0.1	Arginine and proline metabolism	1.32	0.040	1.22	0.047	1.14	0.437	Oxidation product of spermine
229.18	11.61	C11H23N3O2	C9H20N2O2 (1)	N1,N8-diacetyl-spermidine	34.1	-0.4	Arginine and proline metabolism	0.19	0.000	0.79	0.353	0.57	0.121	Diacetylated spermidine known to occur in mammals; never investigated in insects
119.04	4.61	C4H9NOS		N-acetyl-cysteamine	-42.7	-0.1	Cysteine metabolism	2.13	0.015	0.38	0.015	0.95	0.895	Wrongly annotated man made compound or ROS decarboxylation product of N-acetylcysteine
145.04	7.14	C5H7NO4	C5H7NO4 (1) C4H5NO2 (2) C3H4O4 (2) C4H3NO3 (1) C5H9NO5 (1)	2-Oxo-glutaramate	-11.7	0.1	Glutamate metabolism	0.36	0.027	1.04	0.844	0.63	0.187	Glutamine transamination product; catabolite forms oxoglutarate in yeast; *CG8132* catabolises
307.08	9.64	C10H17N3O6S	C10H17N3O6S (2) C31H54N7O17P3S (1)	Glutathione	0.1	-1	Glutathione metabolism	0.35	0.006	1.04	0.836	1.05	0.877	Had standard

250.06	9.61	C8H14N2O5S	C8H14N2O5S (1) C8H11NO5S (3) C8H16N2O6S (1)	gamma-L-Glutamyl-L-cysteine	-6.6	-1.4	Glutathione metabolism	1.13	0.725	1.79	0.043	1.08	0.811	γ-Glutamyl amino acid used to synthesise glutathione
75.03	10.84	C2H5NO2	C2H2O2 (1) CH3N (1) H2O2 (1) CHNO (1)	Glycine	-0.1	0.2	Glycine, serine and threonine metabolism	0.77	0.407	0.72	0.038	0.70	0.034	Had standard
188.12	10.37	C8H16N2O3	C8H16N2O3 (4) C6H13NO3 (1)	N2-Acetyl-L-lysine	-0.3	-0.2	Lysine metabolism	1.42	0.369	1.38	0.034	1.02	0.910	Acetylated lysine involved in protein stability and degradation; had standard
160.04	9.89	C6H8O5	C6H8O5 (4) C5H6O3 (5) C6H10O6 (23)	2-Oxoadipate	9.7	0	Lysine metabolism	0.52	0.013	1.11	0.494	0.66	0.149	2-Aminoadipate transamination product; formed by KATs; can replace oxoglutarate for some reactions; transported by Slc25a21 into mitochondria
188.12	9.36	C8H16N2O3	C8H16N2O3 (6) C8H13NO3 (1) C6H13NO3 (2)	N6-Acetyl-L-lysine	12.5	-0.5	Lysine metabolism	0.93	0.809	1.38	0.014	1.08	0.665	Acetylated lysine associated with histones
217.18	19.79	C10H23N3O2	C9H19N3O (1)	Deoxy-hypusine	32.9	-0.2	Lysine metabolism	0.37	0.010	0.61	0.034	1.02	0.903	Spermidine conjugate with lysine; formed post translationally on eIF5A precursor; gene found in *Drosophila; had duplicate annotation in the data set*
165.05	9.44	C5H11NO3S	C5H11NO3S (3) C3H8O3S (1) C4H7NO2S (1) C5H13NO4S (1)	L-Methionine S-oxide	1.3	-0.4	Methionine metabolism	0.60	0.067	0.98	0.831	0.66	0.025	Oxidation product of methionine caused by ROS
168.04	4.85	C8H8O4	C7H6O2 (2)	3,4-Dihydroxy-mandel-aldehyde	-31.9	0.2	Tyrosine metabolism	0.99	0.987	0.64	0.061	0.57	0.034	Noradrenaline and adrenaline catabolite; cytotoxic; formed by monoamine oxidase; generates radicals

195.05	7.74	C9H9NO4	C9H9NO4 (7) C9H6O4 (5) C8H7NO2 (8) C7H6O4 (10) C9H11NO5 (2) C18H18N2O8 (1)	Dopaquinone	-3.5	0.6	Tyrosine metabolism	0.20	0.006	0.38	0.014	0.26	0.011	L-Dopa→melanin intermediate found in *Drosophila*
195.0532	10.95308	C9H9NO4	C9H9NO4 (1) C18H18N2O8 (1)	2-carboxy-2,3-dihydro-5,6-dihydroxyindole	28.9	0.2	Tyrosine metabolism	7.57	0.10989	0.55	0.06158	1.29	0.544381	Intermediate in L-DOPA→Eumelanin synthesis found in *Drosophila*
193.0375	10.96771	C9H7NO4	C9H7NO4 (1) C9H9NO5 (1)	L-Dopachrome	24.3	0.2	Tyrosine metabolism	10.96	0.094889	0.49	0.079411	1.32	0.553406	Part of eumelanin synthesis found in *Drosophila*; phenol oxidase of from the melanization defence pathway uses as substrate
261.04	10.70	C9H12NO6P	C9H12NO6P (1) C10H11NO5 (2)	Phospho-tyrosine	-1.1	-0.8	Tyrosine metabolism	0.88	0.524	1.48	0.016	1.18	0.329	Product of protein phosphorylation; *Drosophila* has enzymes; protein degradation product
136.05	4.71	C8H8O2	C8H8O2 (5) C16H16O4 (9)	4-Hydroxy-phenyl-acetaldehyde	-28.3	0.3	Tyrosine metabolism	2.71	0.036	1.06	0.618	9.89	0.209	Tyrosine→ 4-hydroxyphenylacetate intermediate; enzyme activity found in *Drosophila*; probably produced via tyramine; fenton oxidation product of tyrosol; pyruvate decarboxylase and monoamine oxidase can make; found in plants and yeast
152.05	6.69	C8H8O3	C8H8O3 (24) C7H6O (1) C8H10O4 (6) C16H16O6 (6)	4-Hydroxy-phenyl-acetate	-8.2	0.1	Tyrosine metabolism	0.45	0.024	1.04	0.757	0.76	0.297	Intermediate of tyrosine catabolism before conjugation and excretion
168.04	7.53	C8H8O4	C8H8O4 (16) C7H6O2 (5) C16H16O8 (2)	Homo-gentisate	2	0	Tyrosine metabolism	0.43	0.060	0.56	0.102	0.37	0.041	Formed from 2-Hydroxyphenylacetate by some unknown, potentially radical mediated, mechanism;

														tyrosine catabolite
179.09	4.84	C10H13NO2	C10H13NO2 (2) C10H10O2 (7) C8H10O2 (3)	(-)-Salsolinol	-38.7	-0.3	Tyrosine metabolism	3.32	0.149	0.36	0.001	0.60	0.103	Alkaloid derived from dopamine and pyruvate; S-enantiomer found in food and when peripheral aromatic L-amino acid decarboxylase is inhibited; inhibits tryptophan hydrolase and tyrosine hydrolase; forms spontaneously by Pictet-Spengler condensation; is neuroactive
263.05	11.50	C9H13NO6S	C9H13NO6S (1) C9H10O6S (1)	Epinephrine-sulfate	33.5	-0.6	Tyrosine metabolism	0.46	0.032	0.96	0.706	0.82	0.407	Sulfate conjugate of epinephrine; produced by phenol sulfotransferase; *Drosophila* has enzyme and precursor; inactivated epinephrine
116.05	7.43	C5H8O3	C5H8O3 (8) C4H6O (3) C4H4O2 (2) C5H10O4 (8)	3-Methyl-2-oxobutanoic acid	2.5	-0.3	Valine, leucine and isoleucine metabolism	0.77	0.440	1.00	0.997	0.60	0.043	Closest metabolite to valine in valine synthesis and degradation
143.06	7.38	C6H9NO3	C6H6O3 (8) C4H6O3 (7) C5H5NO2 (7) C6H11NO4 (5)	Vinylacetyl-glycine	-4.8	0	Valine, leucine and isoleucine metabolism	0.50	0.022	0.75	0.008	0.57	0.015	Glycine conjugate of vinylacetic acid which forms from crotonic acid on heating

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm error)

Table 6-6. Short peptides altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
303.15	11.60	C11H21N5O5	C11H18N4O5 (2)	Glu-Arg	8.5	-0.7	Basic peptide	0.46	0.027	0.38	0.003	0.31	0.001	Had duplicate annotation in data set
244.07	11.00	C17H24N6O9S		Asp-Asp-Cys-His	-45.4	3	Basic peptide	0.78	0.294	0.32	0.018	0.60	0.075	
311.17	10.92	C12H21N7O3		Arg-His	10.2	-1	Basic peptide	1.39	0.445	0.56	0.020	1.09	0.749	
259.19	13.45	C12H25N3O3		Ile-Lys	40.2	-0.7	Basic peptide	0.53	0.064	0.45	0.022	1.19	0.500	
275.15	11.01	C11H21N3O5	C11H21N3O5 (1) C9H18N2O5 (1) C10H17N3O4 (2)	L-a-glutamyl-L-Lysine	6.1	-0.2	Basic peptide	1.13	0.739	1.45	0.030	1.44	0.241	
389.12	9.11	C14H23N5O4S2	C12H20N4O4S2 (1)	Met-Cys-His	23.3	-1.7	Basic peptide	0.82	0.468	0.62	0.034	0.58	0.084	
470.21	12.77	C19H30N6O8		Asp-Leu-Ser-His	-25.3	-2.7	Basic peptide	0.52	0.061	0.54	0.035	0.47	0.023	
309.17	7.84	C15H23N3O4	C15H23N3O4 (1) C15H20N2O4 (1)	Lys-Tyr	-10.5	-0.8	Basic peptide	1.18	0.703	0.51	0.043	0.89	0.727	
151.10	15.67	C12H26N6O3		Lys-Arg	26.9	0.3	Basic peptide	1.59	0.372	0.83	0.050	0.94	0.784	
568.21	7.94	C27H32N6O6S		Asn-Phe-Trp-Cys	-25.9	1.7	Hydro-phobic peptide	34.03	0.003	28.06	0.000	12.70	0.006	Similar to human β-adrenergic receptor peptide
488.22	15.72	C19H32N6O9		Asn-Leu-Asp-Gln	36.4	-2.2	Hydro-phobic peptide	0.61	0.173	1.79	0.003	0.98	0.891	
342.19	6.86	C15H26N4O5	C15H26N4O5 (5) C13H23N3O5 (2) C15H28N4O6 (8)	Leu-Asn-Pro	-12.1	-0.7	Hydro-phobic peptide	0.60	0.145	1.58	0.005	0.79	0.354	
349.13	8.45	C13H23N3O6S	C13H23N3O6S (3) C21H19NO4 (2)	Leu-Asp-Cys	-2.5	0.3	Hydro-phobic	0.10	0.000	0.63	0.009	0.54	0.086	

			C12H21N3O4S (1)				peptide							
232.14	11.08	C10H20N2O4	C10H20N2O4 (1)	Leu-Thr	32.7	-0.6	Hydrophobic peptide	0.86	0.731	2.54	0.015	2.65	0.127	Had duplicate annotation in the data set
451.17	8.49	C19H25N5O8	C19H22N4O8 (1) C18H23N5O6 (2) C17H22N4O8 (2) C19H27N5O9 (1)	Asn-Phe-Asp-Gly	-17.8	0.4	Hydrophobic peptide	2.10	0.228	0.18	0.016	1.04	0.927	
386.18	8.74	C16H26N4O7	C16H26N4O7 (2) C16H23N3O7 (1) C15H24N4O5 (2) C14H23N3O7 (1) C16H28N4O8 (10)	Asp-Val-Gly-Pro	-10	-1.3	Hydrophobic peptide	1.58	0.684	4.84	0.031	7.02	0.086	
389.18	7.62	C16H27N3O8	C15H25N3O6 (3)	Glu-Glu-Ile	-30.8	-1	Hydrophobic peptide	0.76	0.332	0.59	0.042	0.92	0.684	
526.24	15.75	C27H34N4O7	C18H31N9O5S (1)	Asp-Phe-Phe-Val	36.5	2.8	Hydrophobic peptide	0.80	0.488	1.19	0.042	0.92	0.524	
490.23	7.66	C20H34N4-O10		Glu-Glu-Ile-Thr	-30.6	-0.7	Hydrophobic peptide	0.50	0.042	0.67	0.044	0.50	0.009	
309.17	8.86	C15H23N3O4	C15H23N3O4 (1)	Pro-Pro-Pro	-10.7	-0.9	Nonpolar peptide	1.11	0.785	0.53	0.023	0.99	0.977	
321.08	10.42	C11H19N3O4S2		Cys-Cys-Pro	24.7	2.7	Nonpolar peptide	1.61	0.320	0.46	0.040	1.29	0.626	Similar to the rat neuropeptide pGlu-Asn-Cys(Cys)-Pro-Arg-Gly-NH2
321.15	7.84	C12H23N3O7	C11H21N3O5 (4) C11H19N3O6 (1)	Thr-Thr-Thr	-10.5	0.2	Polar peptide	1.22	0.675	0.47	0.048	0.55	0.264	
204.07	10.13	C7H12N2O5	C7H12N2O5 (3) C7H9NO5 (1) C6H10N2O3 (2) C5H9NO5 (4) C6H8N2O4 (2)	Ala-Asp	-4.3	-0.8	Acidic peptide	0.74	0.333	0.89	0.201	0.64	0.048	
305.12	8.00	C11H19N3O7	C10H17N3O5 (2)	Glu-Ala-Ser	-28.5	-0.7	Acidic peptide	0.54	0.083	0.87	0.407	0.63	0.037	
262.08	9.54	C9H14N2O7	C8H12N2O5 (1)	Glu-Asp	-35.3	-0.2	Acidic	0.57	0.062	0.92	0.501	0.70	0.049	

			C19H32N4O9S2 (1)				peptide							
317.17	10.82	C12H23N5O5	C12H23N5O5 (1) C12H20N4O5 (3) C11H21N5O3 (1)	Lys-Asn-Gly	-4.1	0	Basic peptide	0.97	0.907	0.77	0.196	0.85	0.031	
380.21	7.87	C18H28N4O5	C18H28N4O5 (2) C18H25N3O5 (1) C16H25N3O5 (1) C18H30N4O6 (1)	Lys-Ala-Tyr	-10.2	-1	Basic peptide	0.26	0.001	1.18	0.197	0.58	0.104	
442.25	11.44	C19H34N6O6	C19H31N5O6 (1) C17H31N5O6 (6)	Ala-Lys-Gln-Pro	-39.9	-0.9	Basic peptide	0.60	0.198	1.32	0.238	0.36	0.046	
394.20	7.83	C17H26N6O5	C17H26N6O5 (1)	Gly-Tyr-Arg	-1	-2.8	Basic peptide	0.40	0.006	1.06	0.729	0.65	0.093	
257.66	12.61	C21H41N9O6		Arg-Lys-Val-Asn	-26.9	-0.8	Basic peptide	0.55	0.122	1.04	0.894	0.38	0.047	
278.09	8.64	C10H18N2O5S		Glu-Met	-0.8	0	Hydro-phobic peptide	0.79	0.447	0.85	0.329	0.36	0.011	
357.19	6.75	C16H27N3O6	C15H25N3O4 (1)	Glu-Ile-Pro	-24.7	-1	Hydro-phobic peptide	0.31	0.016	0.83	0.366	0.58	0.075	
534.28	7.71	C25H38N6O7		Gln-Leu-Phe-Gln	-29.8	0.2	Hydro-phobic peptide	0.15	0.004	0.85	0.414	0.63	0.150	
470.25	7.32	C20H34N6O7		Asn-Leu-Gln-Pro	-36.7	-0.8	Hydro-phobic peptide	0.24	0.034	1.28	0.472	0.26	0.033	
505.29	6.76	C25H39N5O6		Asn-Leu-Leu-Phe	-48	0	Hydro-phobic peptide	0.14	0.000	1.09	0.564	0.53	0.085	
328.17	7.46	C14H24N4O5	C14H24N4O5 (1) C12H21N3O5 (2) C14H26N4O6 (11)	Val-Asn-Pro	-9.2	-0.7	Hydro-phobic peptide	0.45	0.038	1.04	0.798	0.46	0.012	
406.17	7.84	C15H26N4O9	C14H24N4O7 (10)	Asp-Val-Ser-Ser	-27.6	-0.3	Hydro-phobic peptide	0.23	0.005	1.01	0.944	1.03	0.846	
416.19	7.77	C17H28N4O8	C16H26N4O6 (1)	Asp-Val-Pro-Ser	-28.7	-1	Hydro-phobic	0.51	0.041	1.01	0.974	0.65	0.046	

							peptide							
268.11	11.02	C12H16N2O5	C12H13NO5 (1) C10H13NO5 (1) C24H32N4O10 (1)	Ser-Tyr	31.7	-1	Hydro-phobic peptide	72.93	0.057	0.00	NA	0.00	NA	
376.14	6.84	C14H24N4O6S	C14H24N4O6S (1) C12H26O7P2 (1) C14H26N4O7S (4)	Ala-Cys-Pro-Ser	-23.6	-1.5	Polar peptide	0.44	0.029	0.87	0.403	0.93	0.648	
305.10	6.87	C11H19N3O5S	C13H12ClN5 (1) C11H21N3O6S (2)	Cys-Pro-Ser	-23.5	-1.7	Polar peptide	0.33	0.005	0.88	0.403	0.57	0.023	
216.11	7.24	C9H16N2O4	C9H16N2O4 (2) C8H14N2O2 (1) C7H13NO4 (1) C8H12N2O3 (1)	Thr-Pro	-18.3	0	Polar peptide	0.44	0.049	0.87	0.589	0.53	0.080	
285.13	6.75	C12H19N3O5	C12H16N2O5 (1) C10H16N2O5 (1) C11H15N3O4 (1)	Glycylprolylhydroxyproline	-42.1	0.4		0.54	0.087	0.87	0.265	0.64	0.020	Tripeptide with modified proline; can be caused by ROS
449.25	4.57	C23H35N3O6	C23H41NO5 (1) C19H37O7P (1) C21H42NO8P (3)	N-Acetyl-leu-leu-tyr	12.6	2.8		3.27	0.023	0.94	0.937	0.56	NA	Acetylated short peptide

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm error)

Table 6-7. Fatty acids and lipids altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
249.09	7.06	C10H19NO2-S2	C9H9N5O (1) C8H16O2S2 (1) C10H13N5O4 (3)	S-Acetyl-dihydro-lipoamide	25.9	1	Alanine and aspartate metabolism	0.52	0.026	1.10	0.241	0.56	0.001	Pyruvate→acetyl-CoA intermediate; concentration probably linked to lipoyllysine and S-succinyl-dihydrolipoamide
243.22	6.73	C14H29NO2	C14H29NO2 (1) C14H26O2 (1)	[FA amino(14:0)] 2-amino-tetradecanoic acid	21.8	-0.5	Amino fatty acids	2.61	0.003	1.07	0.828	2.03	0.289	Transamination product of 2-oxotetradecanoic acid, an α-oxidation intermediate of tetradecanoic acid
130.03	11.62	C5H6O4	C5H6O4 (4) C5H8O5 (9)	2,5-Dioxopentanoate	34.3	0.1	Ascorbate and aldarate metabolism	0.52	0.049	0.98	0.917	0.62	0.093	Probably a xylose catabolite or 5'-unsaturated 2-oxofatty acid peroxidation catabolite
308.27	3.47	C20H36O2	C20H36O2 (11) C20H38O3 (8) C40H72O4 (2) C60H108O6 (28)	Icosadienoic acid	-22.3	1.5	Un-saturated fatty acid Bio-synthesis	1.64	0.075	0.48	0.037	1.26	0.770	Fatty acid
160.07	6.94	C7H12O4	C7H12O4 (2) C6H10O2 (21) C6H8O3 (9) C7H14O5 (2) C14H24O8 (2)	[FA (7:0/2:0)] Heptanedioic acid	-19	-0.2	Biotin metabolism	0.14	NA	0.68	0.086	0.46	0.032	Dicarboxylic acid from ω-oxidation of lipid peroxide catabolites; had duplicate annotation in the data set
104.05	7.77	C4H8O3	C4H8O3 (12) C3H4O2 (4) C4H10O4 (2)	(R)-3-Hydroxy-butanoate	3.8	-0.2	Butanoate metabolism	0.63	0.122	1.04	0.761	0.64	0.050	Metabolite made from acetoacetate or beta oxidation of butanoate; found in *Drosophila*; ethyl ester used in insect olfaction and pheromone synthesis; had standard
307.09	7.80	C12H21NO4-	C11H17NO9 (1)	S-Succinyl-	25.3	-1.2	Citrate	0.68	0.352	1.17	0.229	0.46	0.033	Product of ODO1 (EC

		S2	C10H15NO7 (2) C10H13NO8 (1) C11H19NO10 (1)	dihydro-lipoamide			cycle (TCA cycle)							1.2.4.2) from lipoamide found in lipolylpeptides
337.24	3.81	C20H33O4		6,7-dihydro-12-epi-LTB4	-24.9	-3	Eico-sanoids	0.56	0.017	0.87	0.264	0.76	0.126	β-Oxidation product of leukotriene B4; present in *Drosophila*
395.27	3.72	C22H37NO5	C22H34O5 (1) C20H34O5 (3)	[FA hydroxy,oxo(2:0)] N-(11R,15S-dihydroxy-9-oxo-5Z,13E-prostadienoyl)-ethanolamine	-25.8	0.9	Eicos-anoids	0.34	0.006	0.61	0.026	0.53	0.016	Arachdionic acid derived phospholipid
144.12	4.16	C8H16O2	C16H32O4 (6)	[FA (8:0)] octanoic acid	-44.1	0.1	Fatty acid bio-synthesis	1.28	0.164	1.52	0.019	1.62	0.118	Fatty acid produced by *Drosophila*; toxic to *Drosophila*
104.01	10.40	C3H4O4	C3H4O4 (2) C2H2O2 (1)	Malonate	-0.3	-0.1	Fatty acid bio-synthesis	0.59	0.040	0.73	0.128	0.73	0.122	Plants and *Drosophila* have CoA bound form; bacteria can produce directly; used for fatty acid synthesis; inhibits succinate dehydrogenase; had standard
228.21	3.59	C14H28O2	C13H24O (1)	Tetra-decanoic acid	-32.8	0.4	Fatty acid bio-synthesis	2.00	0.018	0.70	0.077	1.73	0.384	Fatty acid produced by *Drosophila*
254.22	3.55	C16H30O2	C15H26O (4) C16H32O3 (1)	(9Z)-Hexa-decenoic acid	-29.7	0.4	Fatty acid bio-synthesis	1.47	0.064	0.66	0.027	1.22	0.726	(Palmitoleic acid) Product of desaturases on palmitate; *desat1* gene involved; part of cuticle; involved in Wnt signalling
282.26	3.49	C18H34O2	C18H34O2 (27) C17H30O (1) C18H36O3 (1) C54H102O6 (5)	[FA (18:1)] 9Z-octadecenoic acid	-24.8	-0.3	Fatty acid metabolism	1.70	0.031	0.62	0.042	1.55	0.568	(Oleic acid) Fatty acid produced by *Drosophila*
130.10	4.25	C7H14O2		[FA (7:0)] heptanoic	-48	0.4	Fatty acids and	1.12	0.418	1.44	0.024	1.50	0.273	Produced by plants and lipid peroxidation;

				acid			conjugates							*Drosophila* attractant
246.15	4.84	C12H22O5	C11H20O3 (9)	3-Hydroxy-dodecane-dioicacid	-39.4	-0.1	Fatty acids and conjugates	0.78	0.530	1.16	0.278	0.71	0.029	β-Oxidation product of dodecanedioic acid (an ω-oxidation product); had duplicate annotation in data set; probably [FA oxo(11:0)] 2-oxo-undecanoic acid (-11.1, 0), an α-oxidation intermediate of undecanoic acid
158.13	3.88	C9H18O2	C18H36O4 (19)	Nonanoic acid	-47.7	0.1	Fatty acids and conjugates	1.27	0.235	1.16	0.017	1.12	0.575	Linolenic acid peroxidation product; undergoes ω-oxidation to azelaic acid followed by β-oxidation to pimelic
238.19	3.58	C15H26O2	C14H22O (1) C15H28O3 (1)	[FA (15:0)] 3-penta-decynoic acid	-33.5	0.3	Fatty acids and conjugates	3.89	0.005	1.07	0.907	1.88	0.402	Unknown
116.05	4.82	C5H8O3		2-Oxo-pentanoic acid	-47.3	-0.1	Fatty acids and conjugates	0.24	0.009	0.85	0.472	0.40	0.021	α-Oxidation intermediate of pentanoate; potentially synthesised from glycoxylate; found in plants and animals; substrate for LDH; ROS scavenger
118.06	6.79	C5H10O3	C5H10O3 (11) C4H8O (4) C4H6O2 (7) C5H12O4 (2)	5-Hydroxy-pentanoate	-10.2	0.4	Fatty acids and conjugates	0.13	0.003	0.82	0.333	0.81	0.243	Formed from 1,5-pentanediol or ω-oxidation intermediate of pentanoate
270.22	3.56	C16H30O3	C16H30O3 (1) C15H28O (1) C16H32O4 (1)	[FA oxo(16:0)] 3-oxo-hexa-decanoic acid	-29.4	0.7	Fatty acids and conjugates	0.94	0.780	0.77	0.040	0.65	0.042	Intermediate in fatty acid biosynthesis and β-oxidation
240.21	3.61	C15H28O2	C15H30O3 (1)	[FA dimethyl(13:0)] 2,5-dimethyl-2E-tridecenoic acid	-34.7	0.5	Fatty acids and conjugates	1.64	0.008	0.76	0.259	1.61	0.439	Unknown branched chain fatty acid
300.27	3.46	C18H36O3	C18H36O3 (1)	[FA	-26.8	0.4	Fatty acids	1.07	0.852	0.73	0.029	0.74	0.110	α-Oxidation product

			C17H34O (4)	hydroxy(18:0)] 2S-hydroxy-octadecanoic acid			and conjugates							
226.19	3.61	C14H26O2		(9Z)-Tetra-decenoic acid	-36.2	0.6	Fatty acids and conjugates	1.99	0.030	0.69	0.204	1.83	0.364	Product of Desat2 from myristate in *Drosophila*; used for ω-5 fatty acid synthesis
314.25	3.73	C18H34O4	C17H32O2 (17) C17H30O3 (1)	[FA hydroxy(18:1)] 9,10-dihydroxy-12Z-octadecenoic acid	-27.4	1.5	Fatty acids and conjugates	1.01	0.987	0.57	0.022	0.61	0.027	Oxidised linoleneic acid derivative; lipid peroxide catabolite
200.10	7.75	C10H16O4	C10H16O4 (5) C9H14O2 (11) C9H12O3 (2) C10H18O5 (2)	[FA (10:1/2:0)] 2E-Decenedioic acid	8.8	0.2	Fatty acids and conjugates	2.34	0.168	0.55	0.026	1.26	0.520	(Decenedioate) ω-Oxidation product of oleic acid; commonly found in acid urea associated diseases; found in royal jelly; synthesised from stearic acid
202.12	7.52	C10H18O4	C9H16O2 (10) C9H14O3 (1)	[FA (10:0/2:0)] Decanedioic acid	8.3	0.5	Fatty acids and conjugates	1.71	0.309	0.43	0.034	0.87	0.654	(Sebacic acid) Dicarboxylic acid formed by ω-oxidation; predominant dicarboxylic acid in bees
230.19	3.97	C13H26O3	C12H24O (6)	[FA hydroxy(13:0)] 2-hydroxy-tridecanoic acid	-29.3	-0.1	Fatty acids and conjugates	3.00	0.033	0.22	NA	2.28	0.287	α-Oxidation product
243.15	7.04	C12H21NO4	C12H18O4 (4) C10H18O4 (1) C11H17NO3 (5)	Tiglyl-carnitine	-7.2	-0.5	Fatty acyl carnitines	32.34	0.066	7.49	NA	0.00	NA	Carnitine conjugate of tiglic acid found in *Drosophila* part of fatty acid catabolism
259.18	5.05	C13H25NO4	C13H22O4 (1) C12H21NO3 (1)	Hexanoyl-carnitine	-36	-0.9	Fatty acyl carnitines	1.55	0.429	1.36	0.013	0.82	0.481	Carnitine conjugate of hexanoic acid
231.15	7.57	C11H21NO4	C11H21NO4 (2) C10H17NO3 (4)	O-Butanoyl-carnitine	-1.3	0.7	Fatty acyl carnitines	0.40	0.006	1.05	0.687	0.94	0.705	Acyl-carnitine of butanoyl-CoA; part of

														fatty acid synthesis
191.63	3.80	C21H37NO5		3-Hydroxy-5,8-tetradecadien carnitine	-45.1	0.6	Fatty acyl carnitines	0.20	0.035	0.95	0.865	0.41	0.091	Carnitine conjugate of a fatty acid; had duplicate annotation in data set
210.20	3.90	C14H26O	C14H26O (5) C14H28O2 (4)	[FA methyl,methyl,ethyl(10:2)] 3-methyl-6-(1-methyl-ethyl)-3,9-decadien-1-ol	-21.4	0.3	Fatty alcohols	0.34	0.019	1.12	0.583	0.63	0.223	Unknown
196.18	4.00	C13H24O	C13H26O2 (6) C26H48O2 (3) C39H72O3 (2)	[FA (13:1)] 2-tridecenal	-12.2	-0.4	Fatty aldehydes	0.33	0.042	1.31	0.322	0.68	0.360	Insecticidal essential oil from plants and pork; β-scission product of a lipid peroxide; potentially formed from 4Z,7Z-octadecadienoic acid
208.18	3.96	C14H24O	C14H24O (1) C14H26O2 (10) C28H48O2 (9)	[FA (14:2)] 5,8-tetra-decadienal	-14.6	0.3	Fatty aldehydes	0.30	0.034	1.21	0.449	0.56	0.177	Lipid peroxidation product; related compounds used as pheromones in moths
236.21	3.79	C16H28O	C16H28O (2) C15H24 (12) C16H30O2 (18)	[FA (16:2)] 6,11-hexa-decadienal	-13.4	0.2	Fatty aldehydes	0.15	0.028	1.03	0.923	0.41	0.140	Common Lepidopteran pheromone
112.09	4.79	C7H12O	C7H12O (4) C7H14O2 (1) C14H24O2 (1) C21H36O3 (3)	[FA (7:1)] 2-heptenal	-15.4	-0.2	Fatty aldehydes	0.40	0.022	0.69	0.085	0.57	0.031	Compound found in scent glands of several insects; lipid peroxidation product
353.33	3.79	C22H43NO2	C22H40O2 (5) C20H40O2 (11)	[FA (20:0)] N-(11Z-eicosaenoyl)-ethanolamine	-3	0.4	Fatty amides	1.59	0.395	1.86	0.035	2.89	0.186	(Anandamide (20:I, n-9)) Endocannabinoid; more saturated form of Anandamide; formed by lipid cleavage
341.29	3.48	C20H39NO3	C18H36O3 (1)	[FA (18:0)] N-octadecanoyl-glycine	-24	0.8	Fatty amides	1.94	0.045	0.63	0.151	0.72	0.329	Glycine conjugate of stearic acid
339.28	3.49	C20H37NO3	C20H34O3 (1) C18H34O3 (4)	[FA (18:0)] N-(9Z-	-14.5	1.7	Fatty amides	1.39	0.130	0.56	0.044	0.51	0.142	Glycine conjugate of oleic acid; neuroactive

			C19H33NO2 (1)	octadecenoyl)-glycine										
327.28	3.49	C19H37NO3	C18H35NO (1) C17H34O3 (2)	Margaroyl-glycine	-27	0.1	Fatty amides	1.23	0.614	0.55	0.034	0.54	0.208	Glycine conjugate of margaric acid
254.10	9.80	C9H18O8	C8H14O7 (2)	3-beta-D-Galactosyl-sn-glycerol	23.8	0.1	Galactose metabolism /Glycero-lipid metabolism	1.33	0.482	0.79	0.135	0.80	0.025	Galactose conjugate of glycerol; found in *Drosophila* (*CG7997*); used for glycolipid synthesis
90.03	10.88	C3H6O3	C3H6O3 (2)	Glycer-aldehyde	34.7	-2.2	Glycero-lipid metabolism	0.45	0.036	1.27	0.227	1.04	0.841	Glyceraldehyde 3-phosphate with phosphate group lost during MS; formed from glycerol and peroxide or glycolaldehyde and formaldehyde; forms alanine with ammonia; had duplicate annotation in the data set
410.24	4.53	C19H39O7P	C19H39O7P (2) C24H36O4 (1) C21H40O5 (1)	[GP (16:0)] 1-hexadecanoyl-2-sn-glycero-3-phosphate	11.7	-0.1	Glycero-phosphates	3.93	0.036	1.25	0.443	1.41	0.454	(1-Palmitoylglycerol 3-phosphate) Glycerophosphate of plamitic acid
397.22	4.82	C17H36NO7P	C17H36NO7P (4) C19H37NO5 (1) C20H31O5 (2) C20H29O6 (1) C42H66O14 (2)	[PC (9:0)] 1-nonanoyl-sn-glycero-3-phospho-choline	16.9	-1.2	Glycero-phospho-cholines	4.14	0.012	1.76	0.354	0.52	0.546	Acylglycerophospho-choline
675.48	3.94	C36H70NO8P	C36H70NO8P (7)	PC(14:0/14:1-(9Z))	-1.6	-0.6	Glycero-phospho-cholines	1.40	0.049	1.13	0.285	1.27	0.164	Acylglycerophospho-choline
467.30	4.68	C22H46NO7P	C22H46NO7P (2) C27H43NO4 (1)	[PC (14:0)] 1-tetradecanoyl-sn-glycero-3-phospho-choline	14.6	-0.6	Glycero-phospho-cholines	2.06	0.034	1.06	0.829	0.84	0.559	Myristic acid acylglycerophospho-choline
495.33	4.52	C24H50NO7P	C24H50NO7P (4) C29H47NO4 (2) C27H41F3O2 (1)	[PC (16:0)] 1-hexadecanoyl-sn-glycero-3-phospho-choline	11.6	-0.1	Glycero-phospho-cholines	2.34	0.050	1.01	0.980	0.97	0.911	Glycerophosphate of plamitic acid

507.33	4.50	C25H50NO7P	C25H50NO7P (2) C28H41F3O2 (1)	[PC (17:0)] 1-(10Z-heptadecenoyl)-sn-glycero-3-phospho-choline	11.1	-0.1	Glycero-phospho-cholines	0.98	0.903	0.84	0.172	0.65	0.016	Unsaturated acylglycerophospho-choline
647.45	3.97	C34H66NO8P	C34H66NO8P (1)	PE(14:1(9Z)/15:0)	-0.8	-0.1	Glycero-phosphoethanolamines	1.56	0.008	1.25	0.110	1.08	0.298	Acylglycerophospho-ethanolamine
491.30	4.63	C24H46NO7P	C24H48NO8P (6)	1-18:2-lyso-phosphatidyl ethanolamine	13.5	-0.6	Glycero-phosphoethanolamines	2.37	0.021	0.94	0.801	0.81	0.487	Lysolipid with an acyl group of linolic acid
451.27	4.53	C21H42NO7P	C21H42NO7P (2) C23H43NO5 (1) C21H39O7P (3) C19H39O7P (3)	1-16:1-lyso-phosphatidyl ethanolamine	11.8	0.3	Glycero-phosphoethanolamines	2.53	0.029	0.88	0.532	0.87	0.653	Lysolipid with a monounsaturated acyl group 16 carbons long
475.27	4.46	C23H42NO7P		1-18:3-lyso-phosphatidyl ethanolamine	10.4	-2.6	Glycero-phosphoethanolamines	1.82	0.035	0.83	0.450	0.63	0.143	Lysolipid with an acyl group of linolenic acid
477.29	4.42	C23H44NO7P	C23H44NO7P (1) C21H41O7P (5) C23H46NO8P (3)	LysoPE(0:0/18:2(9Z,12Z))	9.6	-0.6	Glycero-phosphoethanolamines	1.79	0.044	0.68	0.299	0.57	0.185	Acylglycerophospho-ethanolamine
145.11	13.43	C7H15NO2		Acetylcholine	0.1	-0.2	Glycerophospholipid metabolism	3.68	0.126	1.22	0.644	2.43	0.048	Neurotransmitter; KYNA known to affect release and extracellular concentrations; had standard
334.14	7.44	C14H26N2O3S2	C13H22N2O8 (1) C12H18N2O7 (1) C14H20N6O5 (1)	Lipoyllysine	16.1	-0.8	Glycine, serine and threonine metabolism	1.10	0.685	2.66	0.017	2.01	0.001	Post-translationally modified lysine found in acyltransferase, pyruvate dehydrogenase, ketoglutarate dehydrogenase and branched chain ketoacid dehydrogenase; associated with GSH and oxidative stress levels
242.02	7.04	C6H11O8P	C11H8O5 (3)	D-myo-Inositol 1,2-cyclic	-42.3	-0.7	Inositol phosphate metabolism	0.49	0.042	1.03	0.910	0.64	0.082	Product of phospholipid cleavage found in *Drosophila*; had

				phosphate										duplicate annotation in data set; could be purpurogallin (-14.6,-0.6)
296.24	3.63	C18H32O3	C18H32O3 (6) C17H30O (1)	[FA hydroxy(18:2)] 9S-hydroxy-10E,12Z-octa-decadienoic acid	-28.7	0.2	Linoleic acid metabolism	0.89	0.313	0.64	0.017	0.71	0.049	Linolenic acid derivative found in animals
312.23	3.78	C18H32O4	C18H32O4 (17) C17H30O2 (5) C17H28O3 (1) C18H34O5 (1)	[FA (18:2)] 9S-hydroperoxy-10E,12Z-octa-decadienoic acid	-19.9	1.6	Linoleic acid metabolism	1.25	0.272	0.62	0.041	0.72	0.069	Lipohydroperoxide derived from linolenic acid as a result of lipoxygenases; soya has allene oxide synthases that catabolize
280.24	3.54	C18H32O2	C18H32O2 (22) C17H28O (1) C18H34O3 (8) C36H64O4 (1) C54H96O6 (10)	Linoleate	-26.1	-0.2	Linoleic acid metabolism	1.64	0.038	0.54	0.034	1.35	0.661	Deprotonated form of linoleic acid; has long term affects on dopamine and serotonin neurotransmission; incorperation into phospholipids affects drug symptom severity; modulates sodium, potassium and calcium channels
330.24	4.13	C18H34O5	C18H34O5 (9) C17H32O3 (7) C18H36O6 (1)	[FA trihydroxy(18:1)] 9S,12S,13S-trihydroxy-10E-octadecenoic acid	-20.1	-0.6	Octa-decanoids	1.08	0.851	0.78	0.037	0.91	0.649	Lipid peroxidation product; found in wheat; produced from 9(S)-hydroperoxy-10(E),12(Z)-octadecadienoic acid (9-HPODE) by epoxy alcohol synthase or peroxygenase followed by epoxide hydrolase; effect in insects unknown
217.13	8.03	C10H19NO4	C10H16O4 (6) C9H17NO2 (1) C8H16O4 (2) C9H15NO3 (5)	O-Propanoyl-carnitine	-1.2	-0.1	Oxidation of branched fatty acids	0.32	0.002	1.09	0.560	0.73	0.439	Carnitine with propanoic acid attached; would be formed from propanoly-CoA from isoleucine, beta alanine and propanoate metabolism of *Drosophila*

116.08	4.56	C6H12O2	C6H12O2 (2) C5H10 (1) C18H36O6 (1)	Hexanoic acid	-45	0	Oxidation of very long chain fatty acids	1.37	0.296	1.32	0.042	1.22	0.061	Intermediate in fatty acid chain elongation
97.98	9.90	H3O4P		Ortho-phosphate	1	-1.2	Oxidative phos-phorylation	0.61	0.043	1.08	0.541	0.76	0.179	Free phosphate; can reflect ATP turnover with a decrease reflecting reduced ATP turnover; could be linked to glyceraldehyde concentrations; had standard
241.20	4.13	C14H27NO2	C14H24O2 (19) C12H24O2 (2)	[SP (14:0/2:0)] tetradecasphinga-4E,6E-dienine	-37.9	0.7	Sphingoid bases	0.25	0.021	1.33	0.381	0.57	0.176	Had duplicate annotation in the data set; probably [FA methyl(11:0)] 3-methyl-undecanoic acid (3.2, 0.9) or [FA (14:2)] 2E,4E-tetradecadienoic acid (-21, 0.8) which are both fatty acids
241.20	6.72	C14H27NO2	C14H24O2 (8) C12H24O2 (9)	[SP (14:0/2:0)] tetradecasphinga-4E,6E-dienine	15.1	-0.7	Sphingoid bases	1.61	0.007	0.86	0.488	1.58	0.436	Found in manduca; may have role in metamorphasis; 14 carbon long doubly unsaturated sphingolipid; causes flight muscle degeneration in *Drosophila*; catabolised by Sply; causes apoptosis; unknown how it is formed
228.14	4.85	C12H20O4	C12H20O4 (1) C11H18O2 (11)	Traumatic acid	-31.7	0.6	α-Linolenic acid metabolism	2.16	0.228	0.43	0.013	0.97	0.928	α-Linolenic acid catabolite
278.22	3.55	C18H30O2	C18H30O2 (1) C18H32O3 (1)	[FA (18:3)] 9Z,12Z,15Z-octa-decatrienoic acid	-28.9	-0.1	α-Linolenic acid metabolism	1.83	0.047	0.56	0.035	1.26	0.709	α-Linolenic acid→arachidonic acid intermediate
178.63	3.91	C19H35NO5		2,3-dioctanoyl-	-5.8	-0.2		0.33	0.026	1.13	0.584	0.67	0.319	Formed from glycerate, ammonia and octanoic

				glyceramide										acids; had duplicate annotation in the data set
160.12	12.86	C7H16N2O2	C7H16N2O2 (3) C7H13NO2 (1) C5H13NO2 (1)	L-Carnitin-amide	12.1	-0.3		1.19	0.631	1.38	0.021	1.53	0.143	Ammonia salt of carnitine; unknown if naturally occuring

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm error)

Table 6-8. Nucleotide metabolites altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
427.03	9.90	C10H15N5O10P2	C10H15N5O10P2 (5) C12H16N5O8P (1)	ADP	0	-0.7	Purine metabolism	0.60	0.067	0.69	0.127	0.53	0.040	Diphosphate of adenosine
214.02	8.47	C5H11O7P	C5H11O7P (5) C10H8O4 (5) C7H12O5 (7) C6H10O6 (26) C4H9O5P (2) C5H13O8P (6)	2-Deoxy-D-ribose 5-phosphate	-11.5	0.5	Pentose phosphate pathway	0.29	0.020	0.68	0.180	0.41	0.039	DNA backbone degradation product
290.04	9.15	C7H15O10P	C7H15O10P (5) C6H13O8P (4) C6H11O9P (4)	D-Sedo-heptulose 7-phosphate	-7	-0.7	Pentose phosphate pathway	1.34	0.518	0.66	0.045	0.78	0.232	Intermediate of the pentose phosphate pathway; used for ribose synthesis; had duplicate annotation in data set
230.02	10.15	C5H11O8P	C5H11O8P (15) C7H12O6 (5) C6H10O7 (16) C4H9O6P (1) C4H7O7P (2)	D-Ribose 5-phosphate	0.2	-0.2	Pentose phosphate pathway	0.47	0.023	1.51	0.046	0.93	0.891	Used for nucleotide synthesis in all life and histidine synthesis in plants
184.02	9.13	C5H4N4O4	C4H2N4O2 (1) C5H6N4O5 (2)	5-Hydro-xyisourate	15.8	-0.6	Purine metabolism	1.60	0.040	1.01	0.978	0.88	0.746	Product of urate catabolism in *Drosophila*
267.10	8.89	C10H13N5O4	C10H13N5O4 (2) C9H11N5O2 (3) C8H10N4O4 (1) C9H9N5O3 (1)	Deoxy-guanosine	19.2	0.1	Purine metabolism	0.61	0.001	1.03	0.787	0.75	0.031	DNA precursor found in *Drosophila*
251.1014	7.268088	C10H13N5O3	C10H13N5O3 (3) C8H10N4O3 (1) C10H15N5O4 (3)	Deoxyadenosine	-0.8	-1.7	Purine metabolism	0.71	0.09801	0.97	0.873412	0.93	0.827765	DNA precursor found in *Drosophila*; had standard
443.02	11.19	C10H15N5O11P2		GDP	0	0.3	Purine metabolism	0.43	0.046	0.76	0.295	0.57	0.094	Used for GTP synthesis in *Drosophila*
283.09	9.65	C10H13N5O5	C10H13N5O5 (3) C10H10N4O5 (3)	Guanosine	0.4	-1.2	Purine metabolism	0.95	0.860	1.13	0.488	0.73	0.010	Nucleotide found in *Drosophila*; had

			C9H11N5O3 (4)											standard
281.11	6.99	C11H15N5O4	C11H15N5O4 (5)	1-Methyl-adenosine	-4.9	-1.2	Purine metabolism	0.54	0.038	1.29	0.175	0.65	0.116	Modified nucleotide from RNA; commonly associated with tRNA; associated with adenosine deaminase activity
165.07	9.82	C6H7N5O	C6H7N5O (1)	3-Methyl-guanine	25.3	0.1	Purine metabolism	0.49	0.039	1.00	0.972	0.74	0.001	DNA methylation product formed by alkylating agents; impairs DNA replication
311.12	7.54	C12H17N5O5	C11H15N5O3 (1) C10H14N4O5 (1) C11H13N5O4 (1) C12H19N5O6 (1) C32H38N4O7S (1)	N2-N2-Dimethyl-guanosine	4	-0.7	Purine metabolism	0.45	0.017	1.15	0.496	0.82	0.340	tRNA modification found in yeast and *Drosophila*
231.54	11.06	C14H18N5-O11P		N6-(1,2-Dicarboxy-ethyl)-AMP	0	0.2	Purine metabolism	0.35	0.036	0.94	0.798	0.48	0.068	(Adenylosuccinate) Conjugate of AMP and succinate; IMP→AMP pathway; requires GTP to form and consumes aspartate; broken down to form fumarate
305.04	8.93	C9H12N3O7P	C9H12N3O7P (2) C11H13N3O5 (1) C9H14N3O8P (3)	2',3'-Cyclic CMP	1.6	-0.7	Pyrimidine metabolism	3.00	0.180	0.15	0.006	0.94	0.873	RNA degradation catabolite found in *Drosophila*
306.03	8.16	C9H11N2O8P	C9H11N2O8P (1) C6H12O9S (2) C9H13N2O9P (3)	2',3'-Cyclic UMP	-4.8	0.7	Pyrimidine metabolism	2.83	0.176	0.25	0.010	0.90	0.753	RNA degradation catabolite found in *Drosophila*
323.05	8.15	C9H14N3O8P	C11H15N3O6 (1) C9H11N2O8P (2)	CMP	-25.7	-0.5	Pyrimidine metabolism	2.99	0.156	0.21	0.019	0.85	0.667	Nucleotide monophosphate found in *Drosophila*; can be product of cCMP
242.09	6.81	C10H14N2O5	C9H10N2O4 (1)	Thymidine	2.1	-0.6	Pyrimidine metabolism	0.18	0.010	1.23	0.440	0.53	0.216	Had standard
126.04	6.81	C5H6N2O2	C5H6N2O2 (1) C4H4N2 (1) C10H12N4O4 (2)	Thymine	-1.6	-0.3	Pyrimidine metabolism	0.20	0.007	1.10	0.726	0.57	0.144	Thymidine catabolite; had standard
241.11	7.93	C10H15N3O4	C10H12N2O4 (4) C8H12N2O4 (1)	5-Methyl-2'-deoxy-	11.1	-1.1	Pyrimidine metabolism	0.45	0.066	0.82	0.513	0.37	0.038	DNA methylation product; produced by

			C9H11N3O3 (1) C10H17N3O5 (2)	cytidine										DNA methylases
303.09	6.83	C11H17N3O5S		5-methyl-aminomethyl-2-thiouridine	-22.1	0.5	Pyrimidine metabolism	0.36	0.039	0.79	0.393	0.46	0.066	Modified nucleotide from RNA; commonly associated with tRNA; enzyme activity present in *Drosophila*
257.10	7.87	C10H15N3O5	C10H12N2O5 (1) C9H13N3O3 (1) C8H12N2O5 (1) C10H17N3O6 (1)	5-Methyl-cytidine	5.1	-0.7	Pyrimidine metabolism	0.32	0.002	1.34	0.037	0.65	0.193	Modified nucleotide from RNA; commonly associated with rRNA and tRNA; associated with methyltransferase activity present in *Drosophila*
285.10	7.37	C11H15N3O6	C9H12N2O6 (2)	N4-Acetyl-cytidine	-1.9	-0.9	Pyrimidine metabolism	0.17	0.004	0.99	0.957	0.38	0.073	tRNA and rRNA modification known to occur in eukaryotes including *Drosophila*
363.058	10.65482	C10H14N5O8P	C10H14N5O8P (4) C9H12N5O6P (1) C10H16N5O9P (1)	GMP	4.5	0.1	Purine metabolism	0.51	0.141962	0.42	0.091554	0.37	0.07584	Nucleotide monophosphate found in *Drosophila* can be product of cGMP
347.0628	9.223854	C10H14N5O7P	C10H14N5O7P (6) C10H11N4O7P (2) C10H12ClN5O4 (1)	AMP	4.7	-0.9	Purine metabolism	0.71	0.366445	0.47	0.07932	0.50	0.108291	Nucleotide monophosphate found in *Drosophila* can be product of cAMP; had standard
324.036	9.953244	C9H13N2O9P	C9H13N2O9P (2) C14H10N2O6 (1) C10H12N2O8 (1)	Pseudouridine 5'-phosphate	-5.6	0.4	Pyrimidine metabolism	0.38	0.096993	0.83	0.674572	0.23	0.0609	Pseudouradine→uracil intermediate found in *Drosophila*
114.0429	7.083334	C4H6N2O2	C4H6N2O2 (5) C4H3NO2 (1) C3H4N2 (1) C2H3NO2 (1) C4H8N2O3 (3)	5,6-Dihydrouracil	-3.3	0	Pyrimidine metabolism	0.49	0.059818	0.88	0.541024	0.77	0.266866	Uracil→β-alanine catabolite found in *Drosophila*; formed from cytosine by ionizing radiation; indicitive of DNA damage; uses same enzyme as thymine also forms from DNA under oxidising conditions

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm error)

Table 6-9. Energy metabolites altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
134.02	10.52	C4H6O5	C4H6O5 (3) C3H4O3 (2)	(S)-Malate	0.2	-0.3	Citrate cycle (TCA cycle)	0.70	0.031	1.08	0.404	0.80	0.105	Had standard
120.04	8.26	C4H8O4	C4H8O4 (8) C3H6O2 (7) C3H4O3 (3)	D-Erythrose	9.5	-0.3	Pentose phosphate pathway	0.64	0.188	0.88	0.387	0.64	0.048	Could be a MS artefact from D-Erythrose 4-phosphate which is present in *Drosophila*
342.12	10.84	C12H22O11	C12H22O11 (31)	Sucrose	2.8	-0.5	Galactose metabolism /Starch and sucrose metabolism	0.58	0.073	1.27	0.039	0.90	0.455	Had standard
504.1692	11.04777	C18H32O16	C18H32O16 (21) C18H34N4O7S2 (1)	Maltotriose	0	0.4	glycogen degradation I	0.93	0.84098	0.57	0.068032	0.66	0.119416	Starch catabolite; forms glucose; found in diet
167.98	11.04	C3H5O6P	C3H5O6P (2) C5H6O4 (4) C4H4O5 (3) C3H7O7P (3)	Phosphoenol pyruvate	0.2	0	Glycolysis /Citrate cycle (TCA cycle)	1.12	0.709	1.93	0.007	1.81	0.277	Pyruvate precursor; can be formed from oxaloacetate; had standard

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm error)

Table 6-10. Glycosylation metabolites altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
416.11	6.81	C21H20O9	C21H20O9 (1) C20H15F3N4O3 (1) C17H22N4O6 (1) C20H18O7 (2) C20H16O8 (3)	Chrysophanol 8-O-beta-D-glucoside	41.3	-1	Aromatic polyketides	0.35	0.003	0.99	0.911	0.80	0.339	Glucose conjugate of chrysophanol
136.04	9.06	C4H8O5	C4H8O5 (2) C3H6O3 (5) C3H4O4 (3)	[FA trihydroxy(4:0)] 2,3,4-trihydroxy-butanoic acid	1.6	0.3	Ascorbate and aldarate metabolism	0.63	0.163	0.83	0.222	0.68	0.040	(Threonate) Ascorbate degradation and GlcNAc oxidation product
386.14	9.45	C21H22O7	C16H22N4O5 (3)	[Fv Hydrox] 4'-Hydroxychalcone 4'-glucoside	41.7	-1.3	Flavonoids	0.55	0.081	0.63	0.007	0.49	0.005	Glycoside conjugate of a soya polyphenol
221.09	9.10	C8H15NO6	C8H15NO6 (6) C7H13NO4 (3) C6H12O6 (57) C7H11NO5 (4)	N-Acetyl-D-glucosamine	-0.2	-0.4	Glutamate metabolism	0.59	0.104	0.54	0.010	0.54	0.018	Chitin monomer; had standard
314.06	8.35	C13H14O9	C13H14O9 (1) C13H16O10 (2)	1-Salicylate-glucuronide	-18.5	1		0.42	0.047	0.86	0.370	0.53	0.011	Glucuronic acid conjugate of salicylic acid
365.13	7.97	C14H23NO10	C14H23NO10 (1) C12H20O10 (4) C14H25NO11 (8)	2-(acetylamino)-1-5-anhydro-2-deoxy-3-O-b-D-galacto-pyranosyl-D-arabino-Hex-1-enitol	-2.7	-0.5		7.65	0.007	6.80	0.002	2.58	0.209	Glycosaminoglycan formed by glycosylation; galactose conjugated onto a UDP-glucose that is in an intermediate state found during glycosyltransferase activity
335.13	10.33	C12H21N3O8	C12H21N3O8 (2) C11H19N3O6 (4) C10H18N2O8 (1)	N4-(Acetyl-beta-D-glucosaminyl)asparagine	4.9	0.6		1.18	0.624	0.43	0.018	1.06	0.858	Derived from asparagine linked glycans; *Drosophila* has pathways

324.15	**9.10**	**C12H24N2O8**		**Procollagen 5-(D-galactosyl-oxy)-L-lysine**	**-8.3**	**-0.6**		**0.70**	**0.224**	**0.59**	**0.106**	**0.52**	**0.027**	**Peptogalactan of lysine; *Drosophila* has enzymes; protein degradation product; had duplicate annotation in the data set**

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm error)

Table 6-11. Metabolites of dietary origin altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
151.06	9.75	C8H9NO2	C8H9NO2 (1) C8H11NO3 (1)	N-Methyl-anthranilate	28.3	-0.4	Acridone alkaloid metabolism	0.68	0.186	0.83	0.215	0.60	0.026	Formed from kynurenine→anthranilate but *Drosophila* does not have pathway; probably from diet
216.04	9.96	C5H13O7P	C10H10O4 (2) C6H12O6 (52) C4H11O5P (1) C4H9O6P (1)	2-C-Methyl-D-erythritol 4-phosphate	5.9	1.4	Metabolism of steroids	0.24	0.012	0.35	0.019	0.39	0.020	Plant metabolite of dietary origin
158.07	7.40	C6H10N2O3	C6H7NO3 (4) C4H7NO3 (5) C5H6N2O2 (2) C6H12N2O4 (3) C12H20N4O6 (1)	4-Methylene-L-glutamine	-18.6	0.1	C5-Branched dibasic acid metabolism	0.35	0.047	0.83	0.530	0.61	0.216	Plant metabolite of dietary origin; found in soya; used for nitrogen transport
290.04	9.15	C7H15O10P	C7H15O10P (5) C6H13O8P (4) C6H11O9P (4)	Sedo-heptulose	-7	-0.7	Carbon fixation	1.02	0.950	0.72	0.043	0.80	0.134	Sugar of plant origin
254.06	5.38	C15H10O4	C15H10O4 (13) C14H8O2 (3) C15H12O5 (23)	[PK] Chrysophanol	-0.5	-1.2	Chrysophanol metabolism	3.28	0.000	0.44	0.017	0.99	0.983	Plant derived pigment of dietary origin; catabolites are genotoxic; produced by some endosymbionts in leaf beetles; had duplicate annotation in the data set
254.06	6.76	C15H10O4	C15H10O4 (14) C14H8O2 (3) C15H12O5 (23)	[PK] Chrysophanol	20	0.5	Chrysophanol metabolism	0.33	0.011	0.90	0.713	0.58	0.075	Had duplicate annotation in the data set; probably 3,9-dihydroxypterocarpan (12.6, 0.5), a soya alkaloid; probably of dietary origin
200.08	7.66	C8H12N2O4	C8H9NO4 (6) C6H9NO4 (2)	Dihydro-clavaminic	-28.1	0.3	Clavulanic acid	0.99	0.989	0.86	0.135	0.77	0.026	Arginine→clavaminic acid pathway

			C7H8N2O3 (1)	acid			metabolism							intermediate; beta lactamase inhibitor; synthesis pathway not in *Drosophila* but found in some bacteria; probably of dietary origin
121.02	10.12	C3H7NO2S	C3H7NO2S (1) C3H9NO3S (1)	D-Cysteine	14.9	-0.1	Cysteine metabolism	0.29	0.050	0.81	0.452	1.20	0.680	L-cysteine catabolite; *Drosophila* lacks enzymes for metabolism; toxic; had duplicate annotation in data set; probably of dietary origin
169.99	11.32	C3H6O6S	C3H6O6S (5)	3-Sulfolactate	9.6	-0.7	Cysteine metabolism	1.24	0.540	1.66	0.012	1.26	0.519	Cysteine catabolite found in bacteria; probably of dietary origin
216.14	7.11	C11H20O4	C10H18O2 (21) C10H16O3 (9)	[FA (11:0/2:0)] Undecane-dioic acid	7.6	-0.3	Fatty acids and conjugates	1.09	0.766	0.62	0.010	0.97	0.902	Plant lipid of dietary origin; had duplicate annotation in data set
215.12	4.68	C10H17NO4	C8H14O4 (1)	2-Amino-9,10-epoxy-8-oxodecanoic acid	-48.2	0	Fatty acids and conjugates	0.91	0.815	1.36	0.538	0.48	0.019	Rare amino acid produced by microbes and fungi; used for antimicrobial peptides and HC toxin; transamination product; probably of dietary origin
213.14	3.90	C11H19NO3	C33H57N3O9 (1)	N-Heptanoyl-homoserine lactone	-40.9	0.9	Fatty amides	0.43	0.022	1.06	0.805	0.74	0.501	An acyl-homoserine lactone used by gram negative bacteria for quorum sensing; probly from gut bacteria
229.12	3.73	C30H34O4	C30H34O4 (2)	[Fv] Sophoradochromene	-7.1	-0.9	Flavonoids	2.02	0.242	1.84	0.019	1.98	0.415	Found in some legumes; probably of dietary origin
225.06	7.89	C10H11NO5	C10H8O5 (1) C9H9NO3 (6) C8H8O5 (4) C9H7NO4 (4)	4-Amino-4-deoxy-chorismate	-31.9	0.3	Folate metabolism	0.29	0.003	0.56	0.030	0.30	0.005	THF precursor from phyenylalanine synthesis present in yeast and gut bacteria; probably of dietary origin
356.13	10.39	C13H24O11		galactopinitol A	19.1	0.7	galactosylcyclitol	0.63	0.127	0.91	0.458	0.70	0.048	Stachyose synthase from legumes produces;

							metabolism							found in soya; probably of dietary origin
169.05	9.49	C4H12NO4P	C6H13NO2 (11) C16H18O8 (3)	Phospho-dimethyl-ethanolamine	15.6	-0.7	Glycerophospholipid metabolism	1.07	0.792	1.40	0.032	0.98	0.925	Phosphoethanolamine→ phosphocholine synthesis pathway; not present in *Drosophila*; present in plants including soya; probably of dietary origin
352.06	11.02	C12H17O10P		Arbutin 6-phosphate	22	-2.4	Glycolysis	1.00	0.993	1.80	0.043	1.69	0.142	Phosphorylated form of gluconated hydroquinone; produced by *E.coli* and plants; found in bran; is a tyrosinase inhibitor; probably of dietary origin
156.05	7.04	C6H8N2O3	C6H8N2O3 (3) C6H5NO3 (5) C5H6N2O (2) C4H5NO3 (1) C5H4N2O2 (1) C6H10N2O4 (3)	4-Imidazolone-5-propanoate	-16.5	0.7	Histidine metabolism	0.36	0.017	0.73	0.072	0.77	0.296	Histidine→4-Imidazolone-5-propanoate via urocanate; enzyme not in *Drosophila*; probably of dietary origin
154.04	8.11	C6H6N2O3	C6H6N2O3 (1) C4H3NO3 (1) C6H8N2O4 (2)	Imidazol-5-yl-pyruvate	5.2	-0.1	Histidine metabolism	1.61	0.308	1.32	0.109	1.60	0.002	Precursor of imidazol lactate; histidine derivative produced by bacteria; found in human urine
221.06	9.78	C6H12N3O4P	C8H13N3O2 (1)	L-Histidinol phosphate	19.8	-1.1	Histidine metabolism	0.74	0.159	0.94	0.545	0.79	0.011	Histidine synthesis precursor found in soya and yeast; *Drosophila* can utilize using alkaline phosphatase but cannot utilize histadinol which is toxic
194.08	8.22	C7H14O6	C7H14O6 (17) C6H12O4 (14) C6H10O5 (24) C7H16O7 (2)	1-O-Methyl-myo-inositol	2.1	0.2	Inositol phosphate metabolism	0.70	0.335	0.70	0.212	0.41	0.041	Myoinositol derivative found in legumes; cyclitols affect osmotic stress in plants; probably of dietary origin
284.07	4.83	C16H12O5	C16H12O5 (15) C15H10O3 (5) C15H8O4 (1)	Glycitein	-21.5	-1.3	Iso-flavonoid metabolism	3.79	0.029	0.27	0.029	1.30	0.622	Isoflavone found in soya; metabolized by gut flora; protects against

			C16H14O6 (20)											oxidative stress; may be insecticidal; protects rabbits against cypermethrin
228.12	4.34	C15H16O2	C15H16O2 (4) C14H14 (1) C14H12O (2)	[PR] Cacalol	7.7	0.6	Isoprenoids	0.94	0.908	1.77	0.137	2.75	0.013	(Sesquiterpene) Insecticidal compound of plant origin; probably derived from diet
203.08	9.10	C8H13NO5	C8H13NO5 (1) C8H10O5 (2) C7H11NO3 (2) C6H10O5 (24) C7H9NO4 (3) C8H15NO6 (7) C25H35N7O11 (1)	N2-Acetyl-L-aminoadipate	-2.6	0.4	Lysine metabolism	0.59	0.105	0.54	0.011	0.56	0.025	Lysine synthesis intermediate used by some bacterial species; probably of dietary origin
206.04	11.03	C7H10O7	C7H10O7 (4) C6H8O5 (4) C6H6O6 (3)	2-Hydroxy-butane-1,2,4-tricarboxylate	-11.6	-0.1	Lysine metabolism	1.00	0.996	2.04	0.035	2.01	0.137	(Homocitrate) Lysine precursor formed from acetly coa and ketoglutarate; present in yeast; probably of dietary origin
117.08	6.73	C5H11NO2	C5H11NO2 (4) C5H8O2 (16) C4H9N (1) C3H8O2 (5) C4H7NO (2)	5-Amino-pentanoate	-29.1	0.3	Lysine degradation	0.61	0.106	0.64	0.106	0.47	0.028	Lysine catabolite produced by bacteria; probably of dietary origin
161.07	10.00	C6H11NO4	C6H11NO4 (8) C6H8O4 (11) C5H9NO2 (2) C4H8O4 (5) C5H7NO3 (5) C6H13NO5 (10)	N-Methyl-L-glutamate	-10.8	0.2	Methane metabolism	0.39	0.004	0.89	0.283	0.58	0.039	Produced by bacteria known to be present in aquatic molluscs; made by N-Methylglutamate synthetase; probably of dietary origin
101.05	9.14	C4H7NO2	C4H7NO2 (2) C4H4O2 (1) C2H4O2 (2) C4H9NO3 (11) C8H14N2O4 (4) C12H21N3O6 (5)	1-Amino-cyclopropane-1-carboxylate	-2.1	0.2	Methionine metabolism	0.55	0.064	0.60	0.021	0.60	0.034	SAM→MTA catabolite found in soya and maize; common in plants; used for ethylene production; NMDA associated glycine receptor agonist; bacteria and fungi can form from 2-oxobutanoate; had standard; probably of dietary origin

136.02	7.78	C4H8O3S	C3H6OS (3) C4H10O4S (1)	S-Methyl-1-thio-D-glycerate	16.3	0	Methionine metabolism	1.39	0.443	1.11	0.602	2.06	0.007	Methionine catabolite; methylthiol conjugated with G3P; present in plants and bacteria; probably of dietary origin
131.02	8.00	C4H5NO4	C4H5NO4 (1) C4H2O4 (1)	Imino-aspartate	-19.9	-0.6	Nicotinate and nicotin-amide metabolism	0.29	0.032	0.84	0.489	0.53	0.105	Asp→quinolate; present in plants and many bacteria species; probably of dietary origin
294.22	3.57	C18H30O3	C18H30O3 (3) C17H28O (1) C18H32O4 (1)	[FA oxo(5:1/5:0/8:0)] (1S,2S)-3-oxo-2-(2'Z-pentenyl)-cyclopentane octanoic acid ((9S,13S)-10,11-dihydro-12-oxo-15-phytoenoic acid)((9S,13S)-12-Oxo-phytodienoic acid)(OPC8)	-37.7	-0.5	Octa-decanoids	0.67	0.041	0.78	0.132	0.84	0.637	Jasmonate precursor present in plants; probably of dietary origin
240.17	3.91	C14H24O3	C13H20O2 (1) C14H26O4 (2) C28H48O6 (4)	[FA oxo(5:2/5:0/4:0)] (1S,2S)-3-oxo-2-pentyl-cyclopentane butanoic acid	-41.2	-7	Octa-decanoids	1.97	0.001	0.87	0.507	1.31	0.468	Jasmonate precursor analog made from linoleic acid; present in plants; probably of dietary origin
150.05	10.54	C5H10O5	C5H10O5 (1) C4H8O3 (7) C4H6O4 (4)	L-Arabinose	26.1	-0.4	Pentose and glucuronate interconversions	1.29	0.576	0.37	0.038	1.02	0.952	Sugar present in plants; *Drosophila* can convert to arabitol but not utilize; probably of dietary origin
166.06	4.74	C9H10O3	C9H10O3 (2) C8H8O (5) C9H12O4 (1) C18H20O6 (4)	3-(3-Hydroxy-phenyl)-propanoic acid (m-hydroxy-Hydro-cinnamic	-44.8	-0.2	Phenyl-alanine metabolism	1.98	0.407	0.59	0.020	2.05	0.206	Flavonoid and hydroxycinnamate degradation product produced by gut bacteria; precursor compound found in soya and maize; product of

				acid, Dihydro-3-coumaric acid)										chlorogenic acid catabolism; probably of dietary origin
114.03	10.59	C5H6O3	C5H6O3 (1) C4H4O (1) C5H8O4 (7)	2-Hydroxy-2,4-penta-dienoate	28.3	0.3	Phenyl-alanine metabolism	1.48	0.416	0.41	0.043	1.15	0.727	Phenylpropanoate catabolite produced by bacteria; phenylpyruvate tautomerases catabolise and are found in plants but not *Drosophila*; probably of dietary origin
210.09	7.27	C11H14O4	C11H14O4 (3) C10H12O2 (14) C10H10O3 (11) C11H16O5 (1)	Sinapyl alcohol	20.5	0.5	Phenyl-propanoid metabolism	0.47	0.055	0.99	0.962	0.52	0.050	Precursor in lignin synthesis found in plants; syringin is degraded by gut bacteria to this compound; probably of dietary origin
369.21	4.34	C19H31NO6	C18H29NO4 (1)	[SP] Pramanicin	-12	-0.8	Sphingoid bases	0.40	0.033	0.93	0.667	0.64	0.371	Antibiotic formed by a fungal grass pathogen from 2,4-tetradecadienoic acid; probably of dietary origin
536.16	10.82	C18H32O18		1-4-beta-D-Glucan	0	1	Starch and sucrose metabolism	0.80	0.498	0.56	0.041	0.51	0.029	Cellulose oligomer from plants; probably of dietary origin
828.28	11.42	C30H52O26	C30H52O26 (3)	Cello-pentaose	0	1.3	Starch and sucrose metabolism	0.74	0.347	0.32	0.025	0.53	0.059	Cellulose oligomer; probably of dietary origin
222.07	10.94	C8H14O7	C8H14O7 (2) C7H12O5 (7) C7H10O6 (4) C24H42O21 (14)	6-Acetyl-D-glucose	33.1	-0.8	Starch and sucrose metabolism	1.13	0.760	0.40	0.028	0.67	0.132	Acetylation product of the linear form of glucose; found in soya; could also be one of many other isomeric plant derived sugars
203.06	4.95	C11H9NO3	C10H7NO (1) C11H6O3 (1) C9H6O3 (1)	Indole-pyruvate	-39.1	-0.9	Tryptophan metabolism	1.05	0.953	0.57	0.137	0.33	0.044	Tryptophan→indolpyruvate; transamination reaction; no known gene in *Drosophila*; part of IAA synthesis in plants; potentially of dietary origin

189.04	4.85	C10H7NO3		N-Acetylisatin	-34.7	-0.4	Tryptophan metabolism	0.43	0.032	1.11	0.617	0.56	0.054	Enzyme present in maize; formed from inoxyl that forms indigo; plant seed peroxygenase makes precursor; probably of dietary origins
141.04	9.09	C6H7NO3	C6H7NO3 (2) C5H5NO (1) C4H4O3 (1) C6H9NO4 (3)	2-Amino-muconate semialdehyde	10.9	0.3	Tryptophan metabolism	0.57	0.077	0.62	0.034	0.63	0.029	Tryptophan catabolite produced by some bacteria; probably of dietary origin
141.04	7.79	C6H7NO3	C6H7NO3 (3) C6H4O3 (1) C5H5NO (3) C4H4O3 (1) C6H9NO4 (2)	2-Amino-muconate semialdehyde	-3.9	0.2	Tryptophan metabolism	0.53	0.007	1.41	0.251	1.04	0.856	Had duplicate annotation in the data set; probably N-Methyl-2-oxoglutaramate (-0.7, 0.2), a methylamine and oxoglutarate transamination product produced by bacteria common in the environment; made from degradation of proteins, herbicides and insecticides; probably of dietary origin
190.05	7.21	C7H10O6	C6H8O4 (7) C6H6O5 (2)	[FA hydroxy(7:1/2:0)] 2,4-dihydroxy-2-heptenedioc acid	-34.8	0.1	Tyrosine metabolism	0.53	0.036	0.82	0.308	0.65	0.064	Homoprotocatechuate→ succinate degradation pathway of tyrosine and phenylalanine found in bacteria; probably of dietary origin
110.04	7.52	C6H6O2	C6H6O2 (4) C6H8O3 (9) C12H12O4 (2) C18H18O6 (4)	p-Benzenediol	17.4	-0.5	Tyrosine metabolism /Riboflavin metabolism	0.43	0.055	0.58	0.139	0.40	0.047	(Hydroquinone) Found in plants and yeast; used for riboflavin synthesis; *Drosophila* has enzyme to recycle into quinone; able to be formed from lignin hydrolysates used for yeast fermentation; prophenoloxidase and laccases metabolise in *Drosophila*; inhibits apoptosis; used for cuticle tanning in the

														bluebottle fly; can form spontaneously from catechol and orthobenzoquinone
176.07	8.80	C7H12O5	C7H12O5 (6) C6H10O3 (12) C6H8O4 (12) C7H14O6 (18)	(2S)-2-Isopropyl-malate	-2.6	0.3	Valine, leucine and isoleucine metabolism	0.86	0.682	0.88	0.570	1.48	0.022	Leucine precursor found in soya, maize and yeast; had duplicate annotation in the data set; probably of dietary origin
165.04	4.65	C8H7NO3		4-Pyridoxo-lactone	-39.2	0.7	Vitamin B6 metabolism	0.38	0.008	0.75	0.072	0.68	0.045	Vitamin B6 catabolite found in rhizome associated bacteria and some other bacteria; probably of dietary origin
331.13	4.93	C14H21NO8		5'-O-beta-D-Glucosyl-pyridoxine	-43.8	-0.6	Vitamin B6 metabolism	0.24	0.003	0.94	0.784	0.52	0.078	Glucose conjugate of pyridoxine found in pea like legumes, like soya; probably of dietary origin
188.03	6.44	C7H8O6	C8H8NO3 (1) C6H6O4 (3)	(E)-3-(Methoxycarbonyl)pent-2-enedioate	-43.7	0.4		0.30	0.036	0.90	0.732	0.37	0.054	*E.coli* metabolite formed from trans-aconitate and SAM; probably of dietary origin
170.13	3.78	C10H18O2	C10H18O2 (2) C10H20O3 (1) C20H36O4 (2)	(E)-3,7-Dimethylocta-1,6-diene-3,8-diol	-48.2	0.6		2.81	0.006	1.28	0.586	1.63	0.316	Linalool catabolite found in maize; probably of dietary origin
168.05	6.81	C7H8N2O3	C7H5NO3 (4) C6H6N2O (4) C5H5NO3 (1)	2,3-Diaminosalicylic acid	-10.8	0		0.40	0.050	0.73	0.273	0.82	0.457	Putative compound of dietary origin; unknown
138.00	7.22	C3H6O4S	C3H6O4S (1) C2H4O2S (1)	3-sulfopropanal	-7.5	0		2.86	0.213	1.25	0.523	3.06	0.017	Homotaurine catabolite produced by bacteria; probably of dietary origin
190.08	4.75	C7H14N2O2S		Aldicarb	-21	-0.4		2.27	0.029	1.13	0.603	1.62	0.328	Carbamate insecticide; common food and groundwater contaminant; probably of dietary origin
564.15	8.91	C26H28O14	C26H28O14 (4) C22H30N4O11 (2) C22H32N4O7S2 (1) C25H24O13 (1)	Apigenin 7-O-[beta-D-apiosyl-(1->2)-beta-D-	0	-0.9		0.62	0.217	0.86	0.401	0.53	0.030	Formed from apigenin; found in some plants; present in lentils; probably of dietary

			C26H30O15 (1)	glucoside]										origin; could be schaftoside (0, -0.9), a flavonoid found in wheat
155.09	8.43	C8H13NO2	C8H13NO2 (3) C8H10O2 (1) C7H11N (1) C6H10O2 (16) C8H15NO3 (5)	Arecoline	24.1	0.5		0.61	0.167	0.81	0.112	0.61	0.017	Alkaloid found in some nuts; probable misannotation; could be heliotridine (14.3, 0.5) or scopoline (13.4, 0.5) both of which are from common wheat contaminants; probably of dietary origin
162.09	7.78	C7H14O4	C7H14O4 (1) C6H12O2 (13) C6H10O3 (15)	beta-Cymaro-pyranose	13.6	-0.1		0.68	0.274	0.81	0.333	0.62	0.049	Steroidal glycoside found in some plants; probably of dietary origin
178.08	7.68	C7H14O5	C7H14O5 (1) C6H12O3 (13) C6H10O4 (16)	beta-D-Digitalo-pyranose	7.6	0.1		0.27	0.041	0.85	0.549	0.50	0.105	Steroidal glycoside found in some plants; probably of dietary origin
175.08	9.21	C7H13NO4	C7H13NO4 (2) C7H10O4 (4) C6H11NO2 (8) C5H10O4 (7) C6H9NO3 (1)	Calystegin B2	10.5	0		1.01	0.990	2.99	0.013	2.85	0.194	Alkaloid found in mulberry and some other plants; found in potato; inhibits beta glucosidase and alpha glactosidase; probably of dietary origin
275.10	6.90	C11H17NO7	C11H17NO7 (2)	Cardio-spermin	-3.1	-0.9		0.74	0.348	1.16	0.237	2.08	0.001	Cyanogenic compound of plant origin; only found in a few families of plants; possible misannotation; could be 1,6-anhydro-N-acetylmuramate (-19.4, -0.9), a product of anhydromuropeptide recycling found in gram positive bacteria; probably of dietary origin
218.09	9.68	C8H14N2O5	C8H14N2O5 (2) C9H16N3O2 (1) C7H12N2O3 (2) C6H11NO5 (1) C7H10N2O4 (1)	gamma-L-Glutamyl-D-alanine	-11.1	-0.3		0.85	0.614	0.84	0.353	0.64	0.017	γ-Glutamyl amino acid of D-alanine; found in legumes
321.10	9.21	C11H19N3O6	C11H19N3O6S (3)	gamma-L-	-8.5	-0.8		0.24	0.000	0.77	0.064	0.80	0.422	(Homoglutathione)

		S	C10H17N3O4S (1) C34H60N7O17P3S (1)	Glutamyl-L-cysteinyl-beta-alanine										Found in plants; present in legumes; could be tridecanoyl-CoA (0.3, 0), a fatty acid-CoA conjugate
313.19	4.25	C16H27NO5		Heliotrine	-35.1	1.2		0.41	0.010	1.19	0.341	0.80	0.328	Mutagenic plant alkaloid; can be from *Heliotropium popovii* seeds contaminating grain; probably of dietary origin; had duplicate annotation in the data set
429.32	3.39	C27H43NO3	C27H40O3 (13)	Imperialine	-18.1	0.5		1.63	0.016	0.78	0.392	0.58	0.060	Exotic plant alkaloid; probable misannotation; probably a secosteroid isomer (same ppm and rt error); probably of dietary origin
250.08	4.74	C12H14N2O2S	C11H12N2S (1) C11H10N2OS (1) C12H16N2O3S (1) C23H24N4O9 (2)	indol-3-ylmethyl-cysteine	-45.8	-1		14.29	0.009	0.46	0.267	0.74	0.566	Myrosinase product found in *Brassicaceae* plants; misannotation or food contaminant; had duplicate annotation in the data set; probably of dietary origin
245.16	7.13	C12H23NO4	C12H23NO4 (3) C12H20O4 (3) C11H21NO2 (1) C11H19NO3 (1) C12H25NO5 (1)	N-(octanoyl)-L-homoserine	15.4	-1		0.50	0.006	1.03	0.701	0.76	0.159	Quorum sensing molecule produced by gram negative bacteria; probably of dietary origin
219.07	7.64	C8H13NO6	C8H13NO6 (1) C7H11NO4 (2) C6H10O6 (26) C8H15NO7 (3)	N-Acetyl-D-mannos-aminolactone	-2	-0.2		0.62	0.170	0.77	0.048	0.83	0.289	Oxidation product of ManNac; parent compound found in bacteria and yeast; enzyme known to be present in *Flavobacterium*; probably of dietary origin
183.10	7.47	C8H13N3O2	C7H11N3 (1)	Nalpha,Nalpha-Dimethyl-L-histidine	-14.7	0.6		0.61	0.006	0.78	0.105	0.67	0.177	Methylation product of histidine formed by the fumigation of grain; probably of dietary origin

399.13	9.41	C21H21NO7		Narcotoline	40.3	-1.6		0.74	0.360	1.53	0.041	1.03	0.933	Poppy alkaloid limited to the genus *papaver*; potential food contaminant; probably of dietary origin
206.06	6.80	C11H10O4	C11H10O4 (5) C10H8O2 (5) C10H6O3 (2) C11H12O5 (1)	Scoparone	12.6	-0.2		0.34	0.019	1.05	0.790	0.58	0.221	Coumarin derivative found in citrus plants; probably misannotated; probably Sinapate (-0.2, -0.2), a lignin precursor found in all plants including maize and soya
424.19	4.84	C25H28O6	C25H28O6 (37) C24H26O4 (1) C24H24O5 (1) C25H30O7 (2)	Sophora-flavanone G	17.3	-1.5		0.55	0.082	0.85	0.248	0.61	0.043	Antibacterial compound of *Sophora flavescens*; probable misannotation; probably one of the many flavonoid isomers with the same %RT error; probably of dietary origin
468.00	9.46	C16H12N4O9S2		tartrazine	0	-1.1		0.37	0.042	0.82	0.466	0.41	0.051	Man made food colourant; additive in ingredients used for *Drosophila* food or misannotation
354.0954	7.71581	C16H18O9	C16H18O9 (1) C15H16O7 (1) C15H14O8 (1)	Chlorogenate	3.4	1	Phenylpropanoid biosynthesis__Flavonoid biosynthesis	0.47	0.088815	1.20	0.549796	0.79	0.509119	Chlorogenic acid found in plants; used to make antibacterial compounds by some insects; used for cuticle tanning

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm error)

Table 6-12. Miscellaneous metabolites altered in the knockdown strains

Mass	RT	Predicted formula	No. of alternative formulas from common adducts and isomers with <2 ppm and <25% rt error	Metabolite	%RT error	ppm error	Pathway	*v*		*cn*		*CG6950*		Notes
								Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
276.08	7.63	C10H16N2O5S	C9H12N2O4S (1)	Biotinsulfone	-3.8	0.8	Biotin metabolism	0.72	0.389	0.75	0.112	0.46	0.038	Oxidation product of biotin; found in animals; may reflect ROS
129.06	6.85	C9H7N	C9H7N (2) C9H9NO (3)	Isoquinoline	10.9	0.2		0.18	0.011	0.26	0.020	0.20	0.015	Probably formed from benzylisoquinoline
407.27	3.75	C23H37NO5	C23H37NO5 (1) C23H34O5 (1) C22H35NO3 (2) C21H34O5 (1) C23H39NO6 (2)	Norerythro-stachaldine	-6.6	0.4		0.21	0.001	0.65	0.031	0.38	0.009	Toxic plant alkaloid found in a few related legumes; probable misannotation; probably [FA hydroxy,hydroxy,oxo(3:0/2:0)] N-(1,3-dihydroxypropan-2-yl)-9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoyl amine (-6.6, 0.4), an amide of PGD2 which insects have and serinol formed from transamination of glycerone phosphate; had duplicate annotation in the data set
182.17	4.13	C12H22O	C12H22O (5) C11H18 (1) C12H24O2 (2) C24H44O2 (1)	Geosmin	-36.8	0.6	geosmin metabolism	0.23	0.038	1.35	0.375	0.59	0.247	Repellant to *Drosophila*; produced by toxic microbes probable misannotation; probably 2-dodecenal (-11.9, 0.6), a lipid peroxidation product
130.06	4.45	C6H10O3	C6H10O3 (1) C6H12O4 (4)	6-Hydroxyhexan-6-olide	-47.7	0.3		0.27	0.047	0.75	0.392	0.53	0.177	Formed from 2-hydroxycyclohexan-1-one; cyclohexanol

														catabolite able to be producted by some bacteria; probable misannotation; probably paratose (0, 0.3)
322.08	8.49	C19H14O5	C10H16N2O5S (1)	[Fv] Ovalitenin C	39.1	-1.9	Flavonoids	0.37	0.019	0.91	0.580	0.33	0.027	Flavonoid only found in the legume *Millettia ovalifolia*; probable misannotation; could be biotinsulfone (6.8, 0.1)
368.05	9.46	C17H17O5ClS		Spiro[benzofuran-2(3H),1'-[2]cyclohexene]-7-chloro-4,6-dimethoxy-6'-methyl-2'-(methylthio)-3,4'-dione	42.9	-1.7		0.41	0.024	0.87	0.551	0.34	0.011	Compound found in coal tar; probable misannotation
106.06	7.81	C4H10O3	C3H6O2 (7)	Diethylene glycol	15.5	-0.6		0.45	0.020	0.86	0.348	0.50	0.011	Man made compound; probable misannotation; could be propanoate (0,-0.9)
167.06	4.85	C8H9NO3	C8H9NO3 (1)	Isopyridoxal	-37.3	1	Vitamin B6 metabolism	0.47	0.011	0.71	0.116	0.68	0.030	Vitamin B6 catabolite found in bacteria and humans
176.06	9.79	C9H8N2O2	C9H10N2O3 (3)	4-Hydro-xyamino-quinoline N-oxide	33.3	0.1		0.54	0.057	0.89	0.422	0.56	0.003	Carcinogen; man made compound; probable misannotation; probably 4-Aminohippuricacid (19.9, 0.1), an amino acid glycine conjugate of PABA from folate synthesis in yeast and probably of dietary origin
266.09	8.02	C17H14O3	C8H16N2O3S (2)	Benzarone	33.3	-2.9		0.58	0.141	0.90	0.466	0.36	0.011	Catabolite of a man made compound; probable misannotation; probably Met-Ala (7, -0.5) or Val-Cys (6, -0.5)
168.09	5.72	C8H12N2O2	C8H12N2O2 (1) C8H9NO2 (16)	Pyridoxamine	-49	-0.2	Vitamin B6 metabolism	0.67	0.028	0.70	0.063	0.70	0.127	Vitamin B6 metabolite found in *Drosophila*;

			C7H10N2 (1) C6H9NO2 (1) C7H8N2O (1)											inactive form required for proline synthesis
141.08	8.65	C7H11NO2	C7H11NO2 (1) C5H8O2 (12) C6H7NO (3) C7H13NO3 (8)	L-Hypoglycin	4.4	0.1	Hypoglycin metabolism	0.70	0.039	0.84	0.111	0.75	0.033	Found in ackee tree and close relatives; highly toxic; inhibits beta oxidation; probable misannotation; had duplicate annotation in the data set; probably 3-dehydrocarnitine (6.5, -0.7)
204.10	8.72	C9H16O5	C8H14O3 (3) C8H12O4 (4) C9H18O6 (1)	Diethyl (2R,3R)-2-methyl-3-hydroxy-succinate	35.2	0.6		0.74	0.230	0.67	0.069	0.55	0.031	Unknown; could be 4-Hydroxycyclohexylacetic acid (14, 0.8), a product of tyrosine epoxidation followed by reduction; associated with defects in 4-hydroxyphenyl-pyruvate dioxygenase
152.05	7.75	C5H12O3S	C4H10OS (1) C4H8O2S (2) C10H16N4O7 (1)	Pentane-sulfonate	15.4	-0.3		0.77	0.408	1.31	0.142	0.69	0.027	Compound used in the mobile phase of HPLC; probable artefact; could also be methionol (21, -0.5) or 3-(Methylthio)propionic acid (6.4, -0.4), both methionine derivatives made by yeast and numerous vegetable plants or Strecker degradation of methionine; could be of dietary origin
197.05	8.65	C5H12NO5P	C5H12NO5P (1) C10H9NO2 (4) C7H13NO3 (10) C6H11NO4 (8) C3H9O5P (2) C5H8NO3Cl (1) C5H14NO6P (1)	2-Amino-5-phosphopentanoic acid	-25.1	0		0.81	0.593	0.62	0.234	0.27	0.049	NMDA receptor antagonist; man made compound; probable misannotation; probably sn-glycero-3-Phosphoethanolamine (10, 0)
213.11	11.50	C9H15N3O3	C7H12N2O3 (2)	Methyl 2-diazoacetamidohexanoate	43.8	0		0.87	0.634	1.32	0.005	1.19	0.522	Compound of unknown origin; probably cyanogenic from plants;

														unknown; possible misannotation; could be L-prolyl-L-glycine (15.7, 0), a dipeptide
231.15	4.73	C11H21NO4	C10H17NO3 (1)	N(alpha)-t-Butoxy-carbonyl-L-leucine	-31.9	-0.6		0.97	0.937	0.62	0.219	0.46	0.042	Derivative of leucine using a man made BOC group; probable misannotation; probably N-hexanoyl-D,L-homoserine lactone (-21.3, -0.7), a quorum sensing molecule produced by gram negative bacteria in the gut or diet and probably of dietary origin
284.97	9.93	C6H8N3O4ClS2		Chloramino-phenamide	30.8	2.9		1.14	0.772	1.14	0.660	1.39	0.031	Man made compound; probable misannotation
213.11	7.25	C8H13N3O	C9H15N3O3 (1) C8H13N3O (1) C7H12N2O3 (1) C9H17N3O4 (3)	Methyl 2-diazoacetamidohexonate	8.9	0		1.17	0.687	0.55	0.070	1.91	0.179	Compound of unknown origin; probably cyanogenic from plants; unknown; possible misannotation; could be 2-Dimethylamino-5,6-dimethylpyrimidin-4-ol (8.7,0), a catabolite of some carbamate insecticides
84.02	10.60	C4H4O2	C4H6O3 (2) C8H8O4 (2)	3-Butynoate	23.6	-1.9	Butanoate metabolism	1.24	0.605	0.47	0.015	1.02	0.933	3-Butyn-1-ol product; suicide inhibitor of alcohol dehydrogenase; not known to be naturally occuring so probable misannotation; had duplicate annotation in data set; probably 4-Hydroxycrotonicacid (24, -1.5), a GHB neurotransmitter catabolite found in mammals able to be used for GABA synthesis but never investigated in insects

130.11	15.85	C6H14N2O		ε-amino-caproamide	49.7	0.3		1.54	0.272	1.28	0.030	1.04	0.824	Man made compound; probable misannotation
214.19	3.65	C13H26O2	C13H26O2 (2)	CAI-1	-25.4	0.7		1.71	0.027	0.64	0.066	1.90	0.321	Part of *V.cholerae* quorum sensing; probable misannotation; probably [FA methyl(12:0)] 4-methyl-dodecanoic acid (-9.6, 0.7), a branched chain fatty acid found in plants and animals
198.04	7.56	C6H6N4O4	C6H6N4O4 (1) C5H4N4O2 (3)	2,4-Dinitrophenyl hydrazine	13.2	0		2.05	0.012	1.97	0.517	1.63	0.369	Man made compound; probable misannotation; probably 6-8-Dihydroxypurine (-6.7, 0.1), a hypoxanthine→ uric acid intermediate
207.11	13.48	C8H17NO5	C7H13NO4 (1)	N-Ethyl-glycocyamine	48.2	0		6.33	0.095	1.85	0.558	5.82	0.006	Potential transethylation product of glycocyamine or glucamine; literature contradicting and confused about structure and origin; unknown
164.06	10.98	C8H8N2O2		Ricinine	38.9	0.2		67.21	0.070	0.00	NA	0.00	NA	Toxic alkaloid of castor beans; probable misannotation
109.0198	10.55779	C2H7NO2S	C2H4O2S (1)	Hypotaurine	18.8	0.2	Taurine and hypotaurine metabolism	0.62	0.127393	0.63	0.069961	0.49	0.051408	Cysteine→taurine intermediate found in *Drosophila*; antioxidant and free radical scavenger; marker for ROS; competes with gaba and β-alanine for transport into brain; concentrated in insect CNS
73.05274	5.243888	C3H7NO	C3H4O (2) CH4O (1)	Aminoacetone	-33.6	-0.3	Glycine, serine and threonine metabolism	0.69	0.074617	0.76	0.16684	0.62	0.05921	Glycine and threonine catabolite found in *Drosophila*; causes oxidative stress though auto-oxidation with copper releasing

														peroxide; forms methylglyoxal which is cytotoxic

RT = retention time; numbers in parenthesis next to chemical formulae are the number of candidate isomers for that formula; ppm = parts per million; possible alternative annotations are listed with (% retention time error, ppm erro

Chapter 7 - Role of peroxide and other oxidative stress in permethrin toxicity

1 Introduction

There was evidence from both the metabolomics data (**Ch. 3, Section 3.7.3**) and the literature e.g. (Akbar et al., 2012; Yamamoto et al., 2011) that pyrethroid exposure induces oxidative stress. There is however no information as to when during pyrethroid induced pathology oxidative stress begins and how significantly it contributes to mortality. There is also the issue as to whether it is the insecticide itself or, as in the case of mammals, the catabolites that are responsible for the increase in oxidative stress (Vadhana et al., 2011).

1.1 Types of reactive oxygen species and the cellular defence mechanisms against oxidative stress

1.1.1 Problems caused by oxidative stress

Reactive oxygen species (ROS) are highly reactive radicals or oxidising compounds capable of modifying proteins and metabolites. The reaction of proteins with reactive oxygen species can result in altered protein stability (Davies, 2005) and reactivity (Fernandez-Irigoyen et al., 2008; Nulton-Persson and Szweda, 2001). The reaction of reactive oxygen species with metabolites can result in the formation of adducts or oxidation products, that unless corrected by metabolic proofreading (Van Schaftingen et al., 2013; Weissbach et al., 2002), are sent down dead-end pathways, with potentially toxic results (Napolitano et al., 1999; Wrona and Dryhurst, 2001) or form substrate analogs that act as competitive inhibitors of unmodified substrates (Doorn et al., 2006).

The reaction of hydroxyl radicals with compounds followed by dehydration results in the formation of radicals of the compounds (Hela et al., 1999). These radicals of compounds can then undergo deamination, decarboxylation, fragmentation, rearrangement, isomerisation, cyclization, polymerization and adduct formation (Eberhardt, 1981; Moenig et al., 1985; Stadtman, 1993; Turnwald et al., 1998; Yurkova, 2012) producing further chemical complexity and potentially pharmoactive deadend products. Products of oxidative stress can then also impact on protein function (Crabb et al., 2002) and stability

(Davies, 2005). Oxidative stress can cause the direct cross-linking of compounds which can be detrimental as in the case of DNA dimers (Cao and Wang, 2007) or the formation of reactive compounds like quinones (Bremner et al., 2006) that can also cause cross-linking however, in insects, this particular chemical process has been harnessed to the organisms advantage for cuticle tanning (Hopkins and Kramer, 1992). As invading pathogens can cause oxidative stress, some components of the *Drosophila* immune system respond to oxidative stress in a NF-κB signalling pathway dependent manner (Nappi et al., 2000).

1.1.2 Common oxidative species

Nitric oxide can be formed enzymatically from arginine or non-enzymatically from both nitrite or nitrate using transition metal (Fe, Cu or Zn) catalysts (Ischiropoulos, 1998; Lundberg et al., 2008). Nitric oxide can nitroslyate radicals or react with superoxide forming peroxynitrite which is capable of nitration, oxidation and hydroxylation of lysine, histidine, arginine, cysteine and tryptophan amino acid residues and other compounds with aromatic groups adding on to both carbon and nitrogen atoms (Ischiropoulos, 1998). Nitrosylating species can also be formed by the interaction of peroxide and hydroxyl radicals with nitrate (Ischiropoulos, 1998). The nitration of tyrosine residues inhibits and sometimes prevents phosphorylation which can impact the regulation of proteins (Gow et al., 1996).

Superoxide radicals are primarily produced by the mitochondrial complexes I and III as a result of molecular oxygen interacting with electrons leaked from the electron transport chain (Sanz et al., 2010) but can also be produced as a product of some enzymes like xanthine oxidase and the autoxidation of catechols (Bindoli et al., 1992). Superoxide radicals can act as an electron donor for transition metal ions stimulating the formation of hydroxyl radicals from peroxide (Sutton and Winterbourn, 1989) and the release of iron from iron-sulphur clusters (Gardner, 2002). Hydroxy radicals and hydrogen peroxide are generated from water molecules by Fenton chemistry reactions involving Fe, Cu, Mn and Zn transition metal ions, even if these elements are complexed to protein ligands (Stadtman, 1993). Hydroxy radicals are highly reactive and react with most unsaturated bonds that are not sterically hindered leading to hydroxylation and even peroxidation, through radical mediated addition of

molecular oxygen. Hydrogen peroxide can be formed from two hydroxyl radicals but is also produced by many oxidoreductase enzymes like urate oxidase.

1.1.3 Mechanisms for reducing oxidative stress

Due to the toxicity and structural damage able to be caused by oxidative stress, cells have numerous pathways for scavenging reactive oxygen species and their products. Nitric oxide is degraded via peroxynitrite which is metabolised by the peroxynitrite reductase activity of glutathione peroxidise (Ischiropoulos, 1998). Superoxide radicals are converted into hydrogen peroxide by superoxide dismutase (Felton and Summers, 1995) which is then converted into water by either catalase (Mockett et al., 2003), glutathione peroxidase (Simmons et al., 1989) or ascorbate peroxidase (Mathews et al., 1997). Insects also accumulate and recycle many compounds that can act as antioxidants (Felton and Summers, 1995). There are also enzymes to reverse the products of oxidative damage or enable repair for example methionine sulfoxide reductase which corrects oxidised methionine residues (Weissbach et al., 2002) or phospholipases which remove oxidised fatty acid chains from lipids to allow replacement (Grossmann and Wendel, 1983).

1.2 The model Lepidopteran *Bombyx mori*

The silkworm *Bombyx mori* (able to grow up to 9-11cm) is a model oligophagous Lepidopteran that was domesticated approximately 5000 years ago (Li et al., 2005) and has primarily been used for food and textile production. Although usually reared on a diet of white mulberry (*Morus alba*), silkworms can also be reared on foliage of several other plant species from the families *Asteraceae*, *Rosaceae*, *Moraceae*, *Campanulaceae*, *Ulmaceae* and *Salicaceae* (Clarke, 1839; Kenrick, 1839; Legay, 1958; Nasreen et al., 1999; Tazima, 1978) however, the quality of silk produced when reared on alternative food plants is inferior to that from a white mulberry diet so is not used commercially. As *Bombyx* is the focus of an entire industry the physiology, nutritional requirements and heritable characteristics have been studied in great detail e.g. (Hamamura, 2001; Tazima, 1978) (http://www.shigen.nig.ac.jp/silkwormbase) with even chemically defined diets being available (Hamamura, 2001). In addition to a rich

background literature, *Bombyx* has several parameters that make it an attractive organism for physiological studies, biotechnology and transgenesis.

1.2.1 Lifecycle and germplasm

Details of the lifecycle of *Bombyx mori* have been detailed in numerous publications e.g. (Hamamura, 2001; Tazima, 1978). Eggs hatch within 9-13 days of laying for non-diapausing eggs while diapausing eggs hatch within 9-13 days after exposure to temperatures greater than 16°C after having spent at least twenty days at temperatures between 4-10°C. The larval developmental parameters of *Bombyx* are more flexible than those of *Drosophila* depending on the strain (race) used. The number of generations per year can vary between one (univoltine) to as many as eight (multivoltine), while the number of larval instars before pupation can range from as few as three (bimolter) to as many as seven (hexamolter)(Daimon et al., 2012; Kosegawa et al., 1995; Morohoshi, 1975). There are also genes like *late maturing* that affect the duration of each instar, although they do interact with alleles of moltinism associated genes like *moltinism* or *dimolting* (Morohoshi, 1975). This means the duration of the larval, and therefore maintenance intensive, stage of development can range from approximately 9 to >26 days with most commercial strains taking approximately 21 days before cocoon spinning and pupation (http://www.shigen.nig.ac.jp/silkwormbase).

Cocoon spinning starts approximately three days before pupation which lasts just over a week. Adults are non-feeding and flightless, mating within hours of eclosing. At least three hours of mating is required to transfer enough sperm to fertilize the 280-700 eggs that the female lays. Whether diapausing or non-diapausing eggs are laid depends on the interaction of female genotype with the rearing photoperiod and temperature (Grenier et al., 2004). Non-diapausing eggs remain pale in colour and do not have accumulated carotenoids while diapausing eggs have carotenoid accumulations (progeny of *yellow blood* mutant females excepted) and start ommochrome synthesis on the third day after having been laid. Diapause in diapausing eggs can be prevented by thermal or acid treatment within twenty hours of laying (Saheb et al., 1990). By the fourth day of being laid, diapausing eggs have begun to diapause.

The presence of polyphagy in some stocks (Asaoka, 2000) allows for the investigation of xenobiotics and use of cheaper dietary ingredients which would normally not be consumed. As the majority of *Bombyx* germplasm is able to undergo diapause during the egg stage of the development cycle, unlike *Manduca sexta* and other Sphingids which diapause during the pupal stage of development, stocks can be maintained at a high density for up to two years between maintenance cycles (Iizuka et al., 2008). Like for *Drosophila* genetic resources there are several stock centres around the world maintaining a high diversity of *Bombyx* genotypes (Jingade et al., 2011). Cryopreservation techniques are also being investigated which may allow for the low cost and long term preservation of transgenic germplasm (Relina and Gulevsky, 2013).

1.2.2 Transgenics and biotechnology

Like *Drosophila* transgenics is possible in *Bombyx*. There is a large variety of transformation methods available for *Bombyx* including embryo injection, bioballistic delivery, electroporation, gonad injection (has several variations), attenuated baculovirus vectors and sperm mediated gene transfer (Guo et al., 2004; Li et al., 2014)(reviewed in (Xue et al., 2012)). Interestingly a primitive form of the larval variant of the gonad injection method allegedly was used to repair a lesion at kynurenine 3-monooxygenase by the injection of genomic DNA from a strain with the wild type allele (Nawa et al., 1971), several decades before the first official *Bombyx* transformation (Tamura et al., 2000). There are several marker genes that can be used for *Bombyx* transgenics including *green-fluorescent protein* (Tamura et al., 2000), *red egg* (Osanai-Futahashi et al., 2012), *neomycin-resistance* (Zhang et al., 2012a), *white egg-1* (*cinnabar* ortholog) (Kobayashi et al., 2007) and *white egg-3*^{oily} (translucent larvae allele of *white* ortholog) (Kobayashi et al., 2011). The genetic toolbox available for *Bombyx* is also similar to *Drosophila* with transposons (Tamura et al., 2000), site specific recombinases (Long et al., 2013; Long et al., 2012), the UAS/GAL4 system (Zhang et al., 2012a), conditional promoters (Tan et al., 2013) and the CRISPR/Cas9 system (Wei et al., 2014) being available for use.

One advantage *Bombyx* has over *Drosophila* is the ability to rapidly generate large clonal populations by thermal induced parthenogenesis (Grenier et al., 2004). The application of axenic rearing conditions not only enables study of

host pathogen interactions in *Bombyx* but also allows for the larval stage of the lifecycle to be completed without maintenance (Matsubara et al., 1967). The silk glands of larval *Bombyx* are very efficient at protein expression enabling the production of transgenic proteins in large quantities (Xue et al., 2012).

2 Results and discussion

2.1 Acute permethrin exposure has no effect on oxidative stress in *Drosophila*

If oxidative stress plays an important role in pathology, then an increase in reactive oxygen species would be expected in parallel with increased mortality. As acute exposure means mortality occurs from primary toxic action and before catabolites are able to accumulate it offers the opportunity to determine if the permethrin compound itself is responsible for oxidative stress. Oxidative stress levels were therefore determined in Canton S *Drosophila* using samples taken at several time points over the period during which mortality occurred after acute exposure to three different doses, close to the LD_{16}, LD_{50} and LD_{84} values, of topically applied permethrin (**Figure 7-1**).

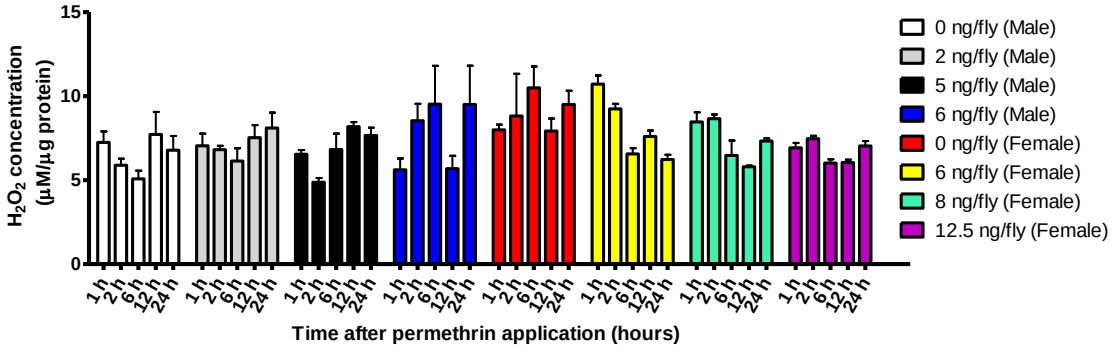

Figure 7-1. Oxidative stress in whole *Drosophila* after acute topical permethrin exposure. Treatment groups are clustered together in order of increasing time since exposure. Treatment groups are listed in order of increasing dose. Male treatment groups are on the left and female treatment groups are on the right. N = 30.

There was no significant difference detected between the different treatment groups at any time point. Using the Bradford assay kit protocol on whole fly homogenates, the viscosity of the sample potentially prevented the plate reader from ensuring proper mixing which might result in underestimations and variations in the readouts for sample protein concentration. This issue was avoided in future experiments by performing a 10^2-10^3 dilution of samples used

for the Bradford assay and adding the larger volume of Bradford's reagent to the smaller volume of sample as opposed to adding samples to the reagent as per kit instructions.

2.2 Effect of acute permethrin exposure on oxidative stress in *Bombyx mori*

As whole *Drosophila* samples did not demonstrate changes in oxidative stress on acute permethrin exposure, the tissue specificity of permethrin induced oxidative stress was investigated using *Bombyx*, a larger organism known to show oxidative stress in the fat body when acutely exposed to high doses of permethrin (Yamamoto et al., 2011). The time point of 48 hours was chosen because other studies had been successful in Lepidoptera using that time point.

2.2.1 Characterization of the *Bombyx* strain (race) used

The supplier had no information as to the origins of the silkworm strain (race) sold so a characterization of the stock was required. Visual inspection and rearing over several generations identified it as bivoltine (can lay diapausing eggs) with a yellow chorion and haemolymph that spins white cocoons with a mix of wild type and peanut shapes. The egg serosa colour was wild type while the larval population had a mix of normal and plain markings (alleles of the *plain* gene). Most individuals were tetramolters however 4.9% were pentamolters (having six instars). The occurrence of pentamolters did not show Mendelian inheritance excluding the M^5 allele of the *moltinism* gene as a cause. The wild wing spot phenotype, common in strains (races) of the "Chinese" breed, was observed in some adults. These phenotypic markers are classically associated with silkworms descended from the "Chinese" breed while the peanut cocoon shape is associated with the "Japanese" breed indicating the strain (race) used is of mixed genetic origins. The voltinism excludes strains (races) from the "European" and "Tropical" breeds from being candidates in the ancestry.

Average fecundity was 385 eggs with an average hatchability of 67%. Larval mortality before the pupation was 3-10%. The molting of the strain (race) was delayed at each instar compared to the average wild type (**Figure 7-2**). The longer development time combined with the occurrence of pentamolters

indicates the presence of the *Lm* allele of *late maturing*. The strain (race) used had difficulty pupating, producing a high proportion of larval-pupal intermediates with a mortality rate of 22.5% among those that did manage to pupate. A gender bias was observed among the pupae (dead pupae included) and adults with there being twice as many females suggesting male associated mortality at either the egg or prepupal stage.

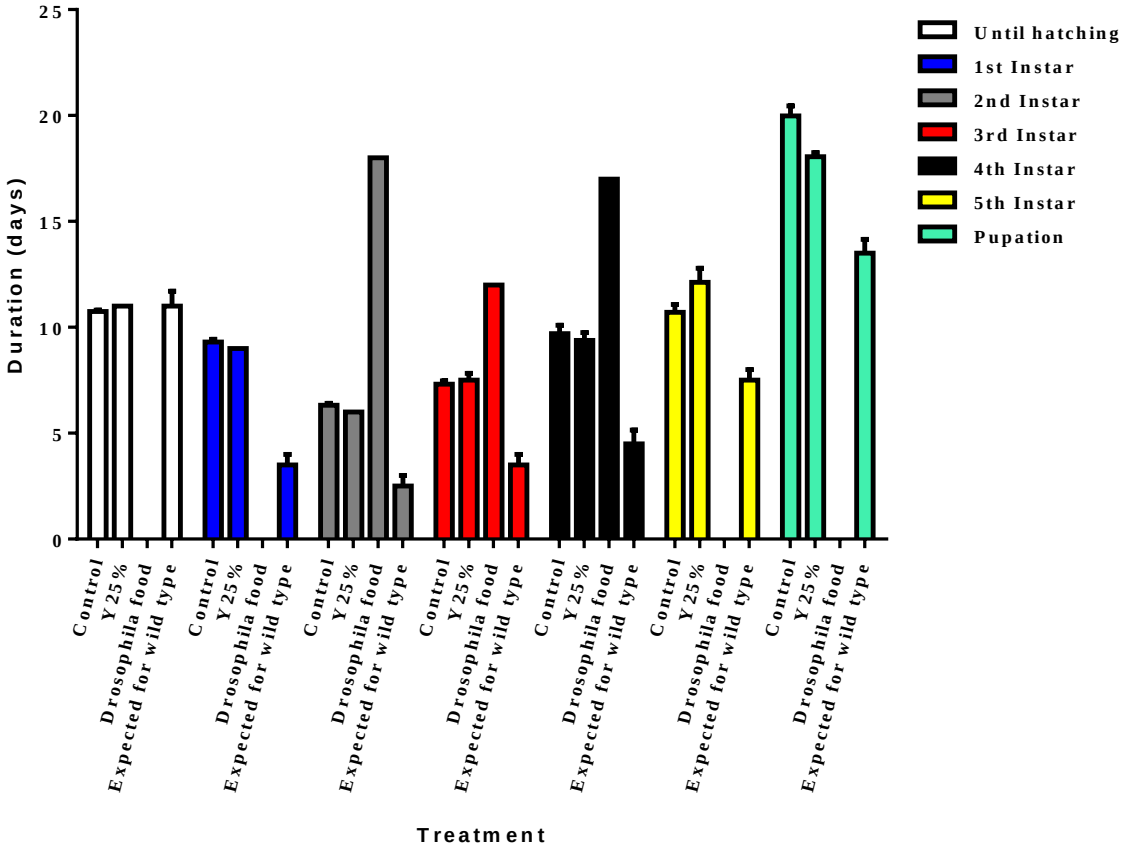

Figure 7-2. Duration of stages in the *Bombyx* pre-adult lifecycle. Control = growth data for the *Bombyx* strain (race) used in lab. Y25% = growth when fed on a 25% yeast diet. Expected growth data for wild types was obtained from http://www.shigen.nig.ac.jp/silkwormbase.

The presence of polyphagy was investigated using yeast, a known phagorepellant of non-polyphagous *Bombyx* strains (races) (Yanagawa et al., 1989). Larvae from the majority of clutches would not eat a diet where 25% of the mulberry powder had been replaced with yeast (Y25%) however one batch of eggs produced larvae that accepted the modified diet. The growth rate of larvae reared on the Y25% diet was not affected, implying that the slower development observed in the strain (race) used is not due to lack of vitamin B or protein. Interestingly, there was a reduced pupal mortality of 5.4% however, it is possible that genotypes which would have shown pupal lethality were removed by the selective pressure imposed by the change in diet. Progeny of the polyphagous

larvae had approximately 75% hatchability and two thirds acceptance of the Y25% diet indicating the presence of a dominant homozygous deleterious allele conferring polyphagy. The gene causing the phenotype could not be determined because there are several in *Bombyx* that have alleles which confer polyphagy and are dominant but homozygous deleterious like *non-preference* and *beet feeder*. To eliminate influences of diet in response to permethrin, attempts were made to rear second instar polyphagous larvae on standard *Drosophila* medium (**Figure 7-2**). Larvae ate the diet but failed to survive past the fourth instar having delayed development and often dying of constipation which suggests both nutritional insufficiency and insufficient roughage in the diet.

2.2.2 Determination of permethrin toxicity in Bombyx

In order to determine what doses of permethrin could be considered low, medium and high for *Bombyx*, topical application survival assays were performed on larvae (**Figure 7-3**). Surprisingly, the LD_{50} was found to be 2.68ng/g (95%CI = 2.3-3.17) which is very low even compared to the 38-43ng/g cited in the literature (Yamanoi, 1986) suggesting that compared to *Drosophila* which have an LD_{50} of 5.54-7.67 ng/mg (**Ch. 4, Section 2.4.1**), *Bombyx* is more susceptible to permethrin and the stock purchased is especially sensitive.

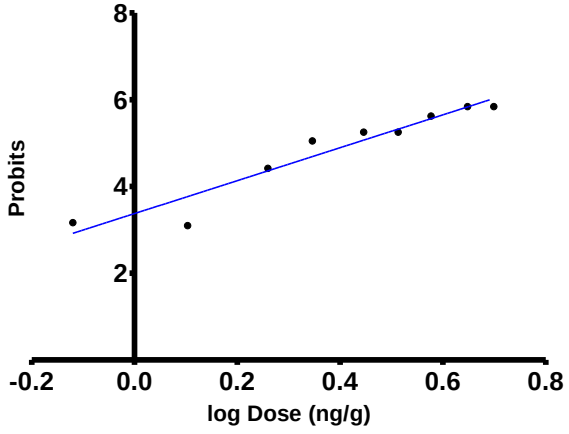

Figure 7-3. Probit analysis of mortality data at 48 hours for *Bombyx* larvae exposed to permethrin. The dose where the linear regression line intercepts 5 probits is the LD_{50}. N = 208.

2.2.3 Identification of a functional dose

Bombyx Malpighian tubules were used as a representative tissue to test for oxidative stress at 48 hours after exposure to permethrin (**Figure 7-4**), with two doses of permethrin, one above and one below the LD_{50}, being tested. The 2 ng/g dose caused a significant increase in peroxide concentrations while the 3.5 ng/g dose did not. This suggests that although permethrin exposure causes oxidative stress, the oxidative stress scavenging pathways are probably able to suppress increases in oxidative stress when upregulated by doses above a certain threshold until the oxidative stress scavenging pathways become overwhelmed by extremely high doses. Alternatively higher doses could result in earlier oxidative stress but are already detoxified at the point of observation.

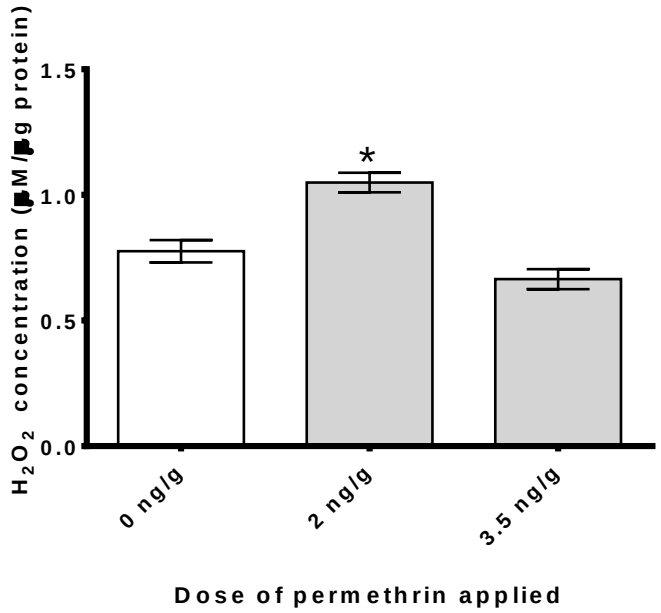

Figure 7-4. Oxidative stress in *Bombyx* at different permethrin doses after 48 hours. White = control, **Gray** = permethrin exposed. N = 30. (*) = P < 0.001.

2.2.4 *Bombyx* tissues involved in permethrin induced peroxide

Based on the results of **Section 2.2.3**, a dose of 2 ng/g was selected for determining when and where oxidative stress occurs in *Bombyx* (**Figure 7-5**). Samples for Malpighian tubules were taken at twelve hour intervals up 72 hours after permethrin exposure to try and determine when oxidative stress increases begins and when it ends for acute exposure. As the cuticle, which contains the fat body, a detoxification tissue, showed the lowest concentrations of hydrogen peroxide of all the tissues tested, it was sampled at 12 and 48 hours after exposure. The whole organism, head, midgut and silk gland were only sampled

at 48 hours after exposure. As occurred with *Drosophila* exposed to an acute dose of permethrin, the whole organism did not show any significant changes in oxidative stress. Surprisingly, only two of the tissues tested, the Malpighian tubules and the silk glands showed changes in oxidative stress in response to permethrin.

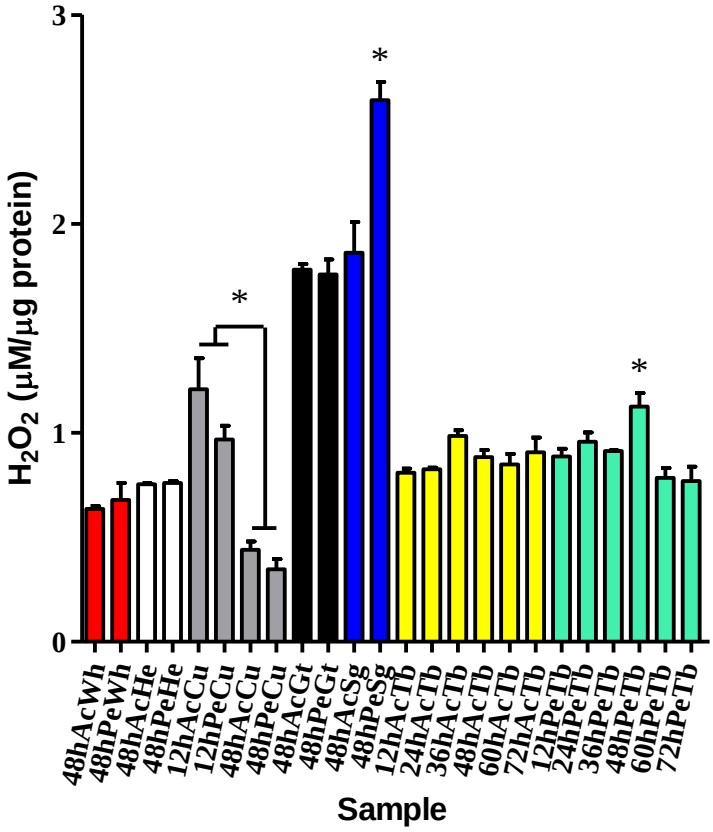

Figure 7-5. Peroxide concentrations in different *Bombyx* tissues at different time points after permethrin exposure. Ac = acetone, Pe = permethrin. **Red** = whole larvae, **White** = head, **Grey** = cuticle, **Black** = midgut, **Blue** = silk gland, **Yellow** = Malpighian tubules from acetone treated controls and **Cyan** = Malpighian tubules from permethrin treated larvae. N = 30. (*) = P < 0.05.

2.2.4.1 The Malpighian tubules

Permethrin exposure increases hydrogen peroxide concentrations in the Malpighian tubules however, this only occurs within a narrow time window after exposure. The Malpighian tubules only show a significant increase at 48 hours after exposure indicating that hydrogen peroxide is only produced at a distinct phase during acute exposure. By oxidative stress not beginning immediately on exposure suggests that it is the detoxification products of permethrin that induce the oxidative stress as has been observed in mammals (Vadhana et al., 2011).

2.2.4.2 The Silk gland

The largest increase in hydrogen peroxide during permethrin exposure occurred in the silk glands. It is well known that exposure to insecticides reduces the quality of silk produced (Kuribayashi, 1988) and these results offer a potential mechanism because the silk protein sericin functions as a potent antioxidant (Fan et al., 2009). Next to the midgut the silk gland had the highest control levels of hydrogen peroxide suggesting high metabolic activity in those tissues.

2.2.4.3 Relation of cuticle H_2O_2 and time since molting

The cuticle of the *Bombyx* control shows a decrease in peroxide content dependent on time since the start of the experiment that is not related to permethrin exposure. No increase in oxidative stress was caused by permethrin exposure suggesting that survivable doses of permethrin may not cause significant oxidative stress in the fat body unlike 1µg/g permethrin which is several times the LD_{50} (Yamanoi, 1986) and known to cause increased oxidative stress in the fat body (Yamamoto et al., 2011). As fifth instar larvae weighing 1g were used for experimentation, a weight obtained within three days since molting, it is possible that continuing cuticle tanning processes which produce oxidative species (Bindoli et al., 1992; Hopkins and Kramer, 1992) are responsible for the differences in oxidative stress between the 12 and 48 hour cuticle samples. Alternatively the number of hours since last having eaten could also explain the decrease since it would give the fat body within the cuticle samples more time to detoxify compounds of dietary origin.

2.3 Effect of chronic permethrin exposure on ROS production in *Drosophila*

2.3.1 Contact exposure

As acute topical exposure had no impact on whole fly oxidative stress levels, contact survival assays were used to determine if chronic exposure for 24 hours would cause oxidative stress (**Figure 7-6**). As with the topical application survival assays no difference in oxidative stress could be detected. These results suggest that permethrin exposure, entering via the cuticle, does not

produce significant amounts of oxidative stress in *Drosophila* or that oxidative stress generation is limited to very few tissue types.

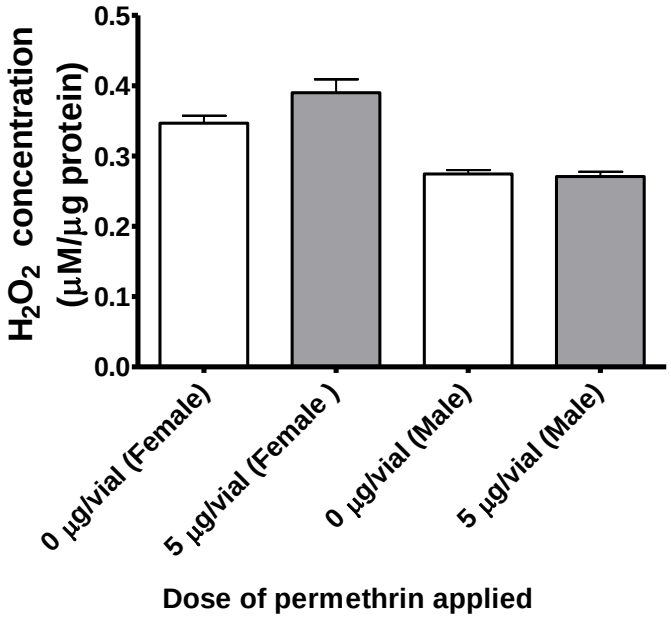

Figure 7-6. Oxidative stress in whole *Drosophila* after chronic contact permethrin exposure. **White** = control, **Gray** = permethrin exposed. N = 30.

2.3.2 Feeding exposure using a roGFP reporter

The results from **Section 2.2.3** and **2.2.4** indicated that the detection of oxidative stress generation by permethrin exposure is highly sensitive to both the dose and the time points used. This means a high throughput screen capable of investigating the dose and time relationship of permethrin induced oxidative stress in *Drosophila* was required. As the Amplex®Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) lacked the capacity for sufficient throughput, UAS-*roGFP* reporter containing transgenic fly lines were generated (similar to strains used by Albrecht *et al.*, 2011 (Albrecht et al., 2011) but lacking the domain that gave peroxide selectivity) for measuring oxidative stress and trialled in the whole organism using a ubiquitous *actin*-GAL4 driver (**Figure 7-7**).

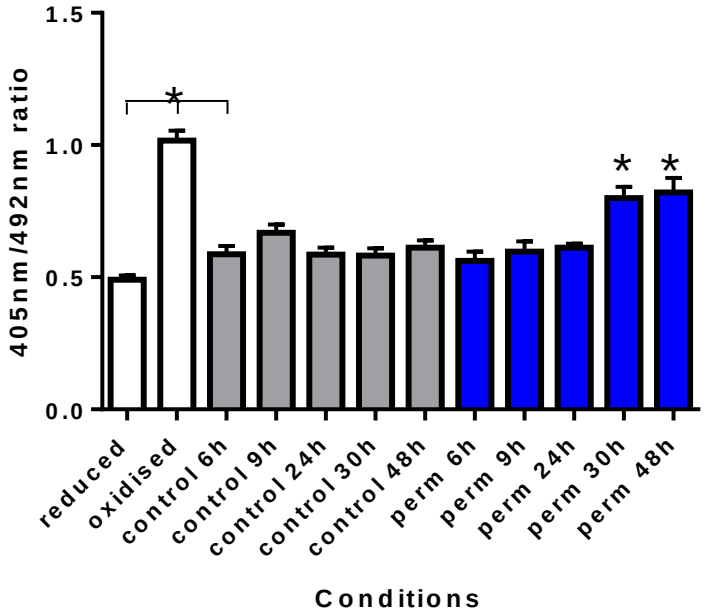

Figure 7-7. Readouts of the roGFP reporter in *Drosophila* larvae under different conditions. Samples were excited at 405nm and 492nm and fluorescence detected at 530nm. The degree of probe oxidisation is expressed as the ratio of the 405nm and 492nm readouts. Permethrin exposed samples were compared against controls for the same time point using a t-test. **White** = controls for the probe, **Grey** = Control fed, **Blue** = Permethrin fed (127µg/ml). N = 160. (*) = P < 0.01.

2.3.2.1 Validation of the roGFP reporter lines

Stable lines were generated for the roGFP probe driven by *actin*-GAL4 (whole fly), *c564* (fat body), *tsp42*-GAL4 (midgut), *elav*-GAL4 (CNS) and *uo*-GAL4 (Malpighian tubules) while a stable *actin*-GAL4 driven mitochondrially targeted roGFP probe line was also generated. The sensitivity of the emissions of probes from whole fly homogenates to full reduction and oxidation was tested using 10mM DTT and 25µM H_2O_2 and compared to untreated control homogenates. It was experimentally determined that the pigments found in the eyes and cuticle of adult flies interfered with the reading so larvae were used for further experiments (although decapitated adult also could be used but with reduced sensitivity). The Actin-GAL4, UAS-mito-roGFP/CyO line showed sufficient difference between the control and the fully oxidised and reduced states of the probe (**Figure 7-7**) so was utilized for feeding assays. Preliminary data (not shown) suggested that whole larvae homogenates could also be used for the fat body specific roGFP line while all other lines lacked sufficient sensitivity meaning dissection (purification in the case of mitochondria) of the expressing tissues would be required for utilization. Due to time constraints the other lines were not investigated further.

2.3.2.2 Time of ROS production relative to exposure and onset of pathology

Larvae fed on 127µg/ml permethrin (a moderate dose used for males in the feeding survival assays) showed no change in oxidative stress levels within the first 24 hours of exposure (**Figure 7-7**) by which time ~6.2% mortality had occurred. Between 24 and 30 hours (~10.2% mortality) of exposure there was a sudden increase in oxidative stress that showed no further increase by the 48 hour time point (~18.6% mortality). This result of delayed oxidative stress indicates either that catabolites of permethrin are the causative agents of oxidative stress, as in mammals (Vadhana et al., 2011), or that it takes several hours after symptoms and mortality have started for permethrin to reach a high enough concentration within the survivors to induce oxidative stress. The results of the assays using acute exposure however, suggest the second hypothesis is unlikely. The oxidative stress levels do not show a strong correlation with mortality implying that oxidative stress is not an indicator for how close the individual is to death. All changes in oxidative stress appear to occur within a 6 hour window so further investigation is required.

2.4 Effect of Catalase overexpression in *Drosophila* on surviving permethrin challenge

Since the results showing the increase in hydrogen peroxide in the Malpighian tubules in *Bombyx* after permethrin exposure, it has been discovered that chronic permethrin exposure also causes increased hydrogen peroxide in the Malpighian tubules of *Drosophila* (Terhzaz et al., 2015). To investigate if increases in hydrogen peroxide are having an influence on survival, the hydrogen peroxide decomposing enzyme Catalase was overexpressed (**Figure 7-8**). Catalase overexpression had no effect on survival in males while ubiquitous overexpression in females conferred increased tolerance to acute permethrin exposure. The overexpression of catalase in the Malpighian tubules had no effect on survival when exposed to permethrin suggesting that it is oxidative stress in other tissues that are responsible for the change in female survival when catalase is ubiquitously overexpressed. It would also be interesting to see the impact of catalase targeted to the mitochontria on survival to permethrin exposure because it has been shown that expression of catalase in the

mitochondria can confer resistance to stresses that cytosolic catalase cannot (Mockett et al., 2003).

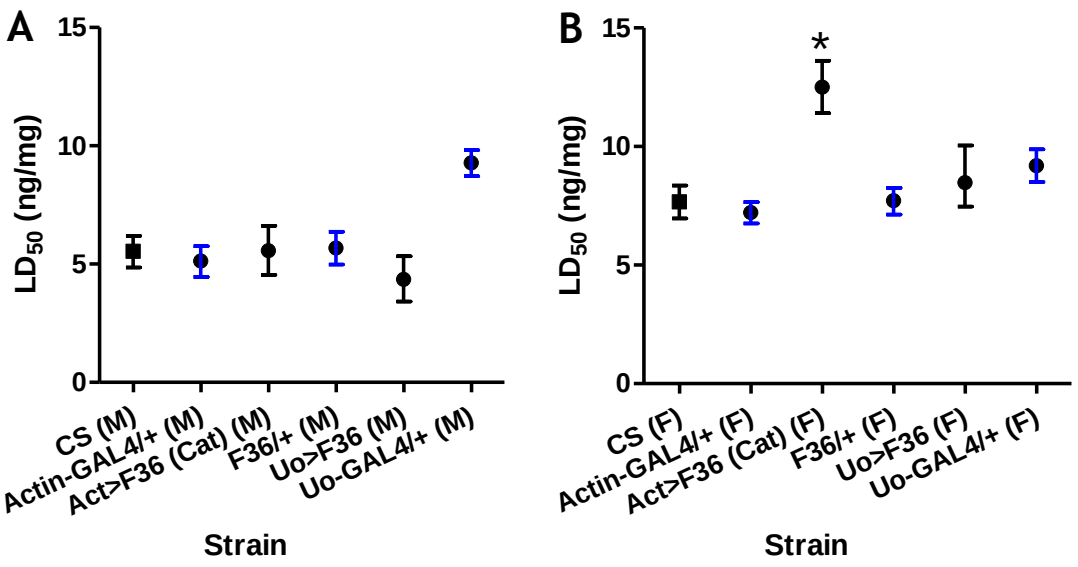

Figure 7-8. Survival of whole fly Catalase overexpression in response to topical permethrin. Data include survival of male and female wild-type (Canton S) and parental control lines. (A) Data shown indicate the LD_{50}s for males of each strain tested and associated 95% confidence intervals. (B) Data shown indicate the LD_{50}s for females of each strain tested and associated 95% confidence intervals. Controls are highlighted in **Blue**. N ≥ 420 per strain. (*) = $P < 0.001$.

In this chapter there is evidence that oxidative stress caused by permethrin exposure is unlikely to be caused by the compound itself but more likely from downstream catabolites and that oxidative stress is not necessary for mortality. Progress has been made to establishing a framework for the investigation of permethrin induced oxidative stress with the quicker roGFP based screening method, stable reporter lines that can be directly crossed to expresser lines of interest and identification of a timeframe when the changes in oxidative stress occurs. A mechanism has also been found that could explain the reduction in silk quality found when *Bombyx* is exposed to insecticide contaminants.

Chapter 8 - Conclusions

1 Summary of project research achievements

Identified metabolic pathways in amino acid, glycogen, glycolysis, energy, nitrogen, NAD^+, purine, pyrimidine, lipid and carnitine metabolism that are disrupted by permethrin exposure.

Found evidence for lesions in β-oxidation and an aspartate shunt which had previously only been observed in vertebrates.

Found evidence for acidosis, ammonia stress and oxidative stress.

Proposed a model for permethrin induced pathology.

Identified 99 genes that might impact on survival when exposed to permethrin.

Validated three candidate genes from tryptophan catabolism.

Demonstrated genes involved in tryptophan catabolism affect survival against permethrin exposure both in the whole organism and in specific tissues.

Showed the candidate genes also affect survival against other insecticides.

Evaluated the efficacy of the knockdowns at disrupting tryptophan metabolism using metabolomics.

Found evidence for disrupted energy metabolism and glycosylation by tryptophan catabolism which had only previously been demonstrated in mammals.

Found candidate substrates and products of the enzyme CG6950 which is functionally uncharacterized.

Found evidence for altered gut absorption in tryptophan catabolism knockdowns

Found evidence for changes in cuticle tanning in the tryptophan catabolism knockdowns

Identified the tissues producing ROS in *Bombyx mori* after acute permethrin exposure which is able to explain the deterioration of silk quality caused by insecticides in that species

Found a timeframe when oxidative stress begins in *Drosophila* when given chronic exposure to permethrin

In terms of proving or disproving the hypothesis stated in **Ch. 1, Section 7**, the **Achievements 1-3** showed that metabolomics can be used in insects to find metabolic changes that could impact on survival. **Achievements 6-8** showed that impairing some of these pathways with RNAi does impact on survival and, in this case demonstrated an increase in tolerance towards permethrin and other insecticides with the tryptophan catabolism genes chosen for validation. The use of metabolomics on the RNAi knockdown strains for hypothesis generation (**Achievements 9-13**) identified several potential mechanisms that could explain the observed differences in surviving insecticide exposure but this would require future validation.

2 Evaluation of techniques

Currently with insecticide development and pharmacology the limiting step is target identification (Cong et al., 2012). This thesis has demonstrated that metabolomics can offer a glut of candidate targets shifting the progress-limiting step to the comparative toxicology used to validate candidates. One solution to this new bottleneck in the insecticide discovery pipeline would be automation however an accurate high-throughput method of determining mortality would be required.

The metabolomics study revealed many pathways being perturbed by what is assumed to be a single insecticidal compound. This occurrence is probably not limited to xenobiotics with endometabolites probably having an equally large number of uncharacterized interactions. Manipulations in one enzyme could therefore potentially cause unpredictably complex changes in metabolism outside the pathway the enzyme is from. The lack of information about the non-metabolic interactions of most metabolites is therefore a main factor limiting the prediction of changes in physiology based on biochemical data.

The low dose of permethrin that was used to generate the provided adult metabolomics data set probably was too low to cause many of the physiological changes reported in the literature and seen in the larval data set and this experiment should be repeated using dose values obtained from the survival assays (7 µg/vial for males and 20.2 µg/vial for females). Although the use of several timepoints after permethrin exposure began gave some insights into metabolic flux though pathways, by the accumulation and peaking of some metabolites, it would require heavy isotope labelling to be able to quantify the changes in metabolic activity (Rabinowitz et al., 2011). Future research should also compare data sets for *Drosophila* exposed to other insecticides or specific catabolites of permethrin over a timecourse which would enable the dissection of metabolic changes caused by the mode of action and as a consequence of insecticide degradation.

With the untargeted metabolomics studies there were several putatively annotated compounds of potential interest, for example dopaquinone, however there is always uncertainty in the automated annotation procedures for metabolites (Dunn et al., 2013), especially those that are not part of core metabolism. To validate the metabolimics data would require additional runs using standards, heavy isotope labelling or derivatization. Alternatively direct quantification of the metabolite could be performed using traditional methods for biochemistry e.g. colorimetric assays or titrations however, these experiments are low throughput. From the perspective of insecticide researchers interested in the role the metabolite has in survival, a direct chemical genetics approach is probably more informative.

3 Future work

Although the candidate genes chosen from tryptophan catabolism that were investigated during this study tended to cause increased tolerance to insecticide exposure, there are still the 95 other candidates identified during this study which will require screening with comparative toxicology. It was shown that *vermilion*, *cinnabar* and *CG6950* knockdown affects survival, however the mechanisms causing the changes are still unknown and require further investigation. A chemical-genetics approach of feeding wild type *Drosophila* on a tryptophan catabolite rich diet and comparing their survival to insecticide exposure would not only verify the data obtained from the survival assays on the knockdown strains but also allow dissection of the effects the tryptophan catabolites from off-target effects of the associated enzymes, like protein-protein interactions. This approach could also be applied to investigating some of the metabolic changes seen in the tryptophan catabolism knockdowns.

It is known that both the catabolites of permethrin and the kynurenates can form adducts with proteins (Kopylova et al., 2007; Noort et al., 2008) so development of an antibody against permethin catabolite adducts could be used to identify proteins modified by adducts derived from those compounds. This could highlight proteins inhibited by permethrin catabolites and could contribute to understanding the basis for some of the metabolic changes observed during permethrin exposure.

The work using the roGFP reporter was left unfinished due to the time constrains of the submission of this document. Once a timepoint showing a transition in the oxidative state was found a dose-time response of oxidative stress induced by permethrin could be investigated. The tissue specificity of oxidative stress during this timepoint would also be informative. By using ladders of chemicals that produce specific ROS species, like hydrogen peroxide, paraquat and nitroprusside, the roGFP reporter could be calibrated and used in combination with peroxide specific roGFP reporters to determine what proportion of the total oxidative stress caused by permethrin is peroxide related.

Appendix I

Seuence of plasmid pra305. **Red** = start codon for mitochondrially targeted roGFP; **Blue** = start codon for roGFP; **Orange** = Stop codon

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGT
TACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACG
TCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGT
GGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGC
CCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTA
TGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCG
GTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCC
ACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGT
CGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATA
TAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTCGAG
CTCGAATTCGGGCTGAAGGAGACTTGGGGGCACCCGCGTCGTGCCTCCTGGGTTGTGAGG
AGTCGCCGCTGCCGCCACTGCCTGTGCTTC**ATG**AGGAAGATGCTCGCCGCCGTCTCCCGCG
TGCTGTCTGGCGCTTCTCAGAAGCCGGCAAGCAGAGTGCTGGTAGCATCCCGTAATTTTGC
AAATGATGCTACATTTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACC
ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGAC
GGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTAC
GGCAAGCTGACCCTGAAGTTCATCTCCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCC
TCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCA
GCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTC
AAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTG
AACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAG
CTGGAGTACAACTACAACTGCCACAAACGTCTATATCATGGCCGACAAGCAGAAGAACGGC
ATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGAC
CACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTAC
CTGAGCACCTGCTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTG
CTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG**TAA**AGC
GGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAA
AACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTT
GTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAG
CATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGGCGTAAA
TTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAA
CCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTG
AGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAG
GGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTT
TTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGA
GCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGC
GGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGC
GCTTAATGCGCCGCTACAGGGCGCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAAC
CCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGA
TAAATGCTTCAATAATATTGAAAAAGGAAGAGTCCTGAGGCGGAAAGAACCAGCTGTGGAA
TGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGC
ATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAA
GTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATC
CCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATT
TATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTT
TTGGAGGCCTAGGCTTTTGCAAAGATCGATCAAGAGACAGGATGAGGATCGTTTCGCATGA
TTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTA
TGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAG
GGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAAGACG
AGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACG
TTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCC
TGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTG
CATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAG
CACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGG
GCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGAGCATGCCCGACGGCGAGGATCT

**CGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTG
GATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTAC
CCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGT
ATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGC
GGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCG
ATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTG
GATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCTAGGGGGAGGCTA
ACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGGCAATAAAAAGA
CAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCT
GGCACTCTGTCGATACCCCACCGAGACCCCATTGGGGCCAATACGCCCGCGTTTCTTCCTTT
TCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCGGGGCG
GCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTT
TAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGT
GAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCC
TTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTT
GTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCA
GATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAG
CACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAG
TCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCT
GAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGAT
ACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGT
ATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAAC
GCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGA
TGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCC
TGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATA
ACCGTATTACCGCCATGCAT**

Appendix II

Note that this is not a comprehensive list but a sample of the variety of taxa certain common pyrethroid induced changes are found in. As mammal and fish models dominate the literature only one example from each group is given. Examples from plant models have not been included. Changes in compounds were directly quantified in the referenced papers while changes in enzymes include references for changes in activity, transcription and protein concentration.

Table S2-1. List of commonly found metabolic changes caused by pyrethroids.

Parameter	Examples of animals reported in	References
Protein content decrease	**Frog, Crab, Chicken, Rat, Fish, Red flour beetle, Ostracod, Snail, Earthworm, Water flea**	**(Anwar et al., 2004; David et al., 2012; Liang et al., 2013; Mckee and Knowles, 1986; Reddy and Bhagyalakshmi, 1994; Saleem and Shakoori, 1987; Shakoori et al., 1996; Tripathi et al., 2010; Tripathi and Singh, 2004; Zeba and Khan, 1995)**
Free amino acid increase	**Frog, Crab, Chicken, Rat, Fish, Red flour beetle, Snail**	**(Anwar et al., 2004; David et al., 2012; Liang et al., 2013; Reddy and Bhagyalakshmi, 1994; Saleem and Shakoori, 1993; Shakoori et al., 1996; Tripathi and Singh, 2004)**
Alanine aminotransferase activity increase	**Crab, Fish, Rat, Ostracod, Snail, Toad, Red flour beetle**	**(Anwar et al., 2004; Kumar et al., 2011; Prashanth and Neelagund, 2008; Reddy and Bhagyalakshmi, 1994; Sakr and Hanafy, 2002; Saleem et al., 1998; Tripathi and Singh, 2004; Yousef et al., 2006; Zeba and Khan, 1995)**
Aspartate transaminase activity increase	**Crab, Fish, Rat, Lesser grain borer, Ostracod, Snail, Toad**	**(Ali et al., 2014; Kumar et al., 2011; Prashanth and Neelagund, 2008; Reddy and Bhagyalakshmi, 1994; Sakr and Hanafy, 2002; Tripathi and Singh, 2004; Yousef et al., 2006; Zeba and Khan, 1995)**
Glutamate dehydrogenase activity increase	**Crab, Fish, Cotton bollworm, Ostracod**	**(Konus et al., 2013; Kumar et al., 2011; Prashanth and Neelagund, 2008; Reddy and Bhagyalakshmi, 1994; Zeba and Khan, 1995)**
Lactate dehydrogenase activity increase	**Cotton bollworm, Red flour beetle, Rat, Snail**	**(Akbar et al., 2012; Saleem and Shakoori, 1987; Tripathi and Singh, 2004; Yousef et**

		al., 2006)
Lactate dehydrogenase activity decrease	**Snail, Chicken, Fish, Earthworm, Mosquito**	**(Anwar et al., 2004; Azmi et al., 2002; Bakry et al., 2011; Shakoori et al., 1996; Singh and Agarwal, 1987; Tripathi et al., 2010)**
Increased glycolysis	**Snail, Fish, Rat, Red flour beetle**	**(Bakry et al., 2011; Liang et al., 2013; Saleem et al., 1998; Shakoori et al., 1996; Yousef et al., 2006)**
Glycogen depletion	**Red flour beetle, Fish, Chicken, Locust, Snail, Water flea**	**(Anwar et al., 2004; Mckee and Knowles, 1986; Saleem et al., 1998; Shakoori et al., 1996; Singh, 1986; Tripathi and Singh, 2004)**
DNA decrease	**Fish, Lesser grain borer, Snail, Water flea**	**(Ali et al., 2014; Mckee and Knowles, 1986; Shakoori et al., 1996; Tripathi and Singh, 2004)**
RNA decrease	**Fish, Red flour beetle, Chicken, Snail, Water flea**	**(Anwar et al., 2004; Mckee and Knowles, 1986; Saleem and Shakoori, 1987; Shakoori et al., 1996; Tripathi and Singh, 2004)**
Increased free fatty acids or lipids	**Water flea, Fish, Rat, Chicken, Red flour beetle, Locust**	**(Anwar et al., 2004; Saleem et al., 1998; Shakoori et al., 1996; Singh, 1986; Taylor et al., 2010; Yousef et al., 2006)**
Increased lipid peroxides	**Fruit fly, Rat, Cotton bollworm, Prawn, Brown planthopper, Frog, Fish**	**(Akbar et al., 2012; David et al., 2012; Dinu et al., 2010; Hu et al., 2010; Terhzaz et al., 2015; Vijayavel and Balasubramanian, 2009; Vontas et al., 2001)**

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