Metabolomic profiling of permethrin-treated *Drosophila melanogaster* identifies a role for tryptophan catabolism in insecticide survival

Robert A. Brinzer, Louise Henderson, Alan A. Marchiondo[1], Debra J. Woods[1], Shireen A. Davies and Julian A.T. Dow
Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, Scotland, UK.
[1]Zoetis Inc, Kalamazoo, MI, USA

Abstract

Insecticides and associated synergists are rapidly losing efficacy in target insect pest populations making the discovery of alternatives a priority. To discover novel targets for permethrin synergists, metabolomics was performed on permethrin-treated *Drosophila melanogaster*. Changes were observed in several metabolic pathways including those for amino acids, glycogen, glycolysis, energy, nitrogen, NAD^+, purine, pyrimidine, lipids and carnitine. Markers for acidosis, ammonia stress, oxidative stress and detoxification responses were also observed. Many of these changes had not been previously characterized after permethrin exposure. From the altered pathways, tryptophan catabolism was selected for further investigation. The knockdown of some tryptophan catabolism genes (*vermilion*, *cinnabar* and *CG6950*) in the whole fly and in specific tissues including fat body, midgut and Malpighian tubules using targeted RNAi resulted in altered survival phenotypes against acute topical permethrin exposure. The knockdown of *vermilion*, *cinnabar* and *CG6950* in the whole fly also altered survival phenotypes against chronic oral permethrin, fenvalerate, DDT, chlorpyriphos and hydramethylnon exposure. Thus tryptophan catabolism has a previously uncharacterized role in defence against insecticides, and shows that metabolomics is a powerful tool for target identification in pesticide research.

Keywords

Metabolomics; permethrin; insecticide; RNAi; Drosophila melanogaster; tryptophan catabolism

Introduction

All currently available insecticides are associated with resistant target pest populations, with some being resistant to multiple different modes of action. In addition to this, the occurrence of resistance in previously susceptible populations is becoming more frequent (Hardy, 2014). In order to reduce the magnitude of resistance, increase insecticide efficacy and reduce the cost of active ingredients, synergist compounds can be added to insecticide formulations, typically acting to block insecticide detoxification enzymes, such as cytochrome P450s. However, there are also resistant pest populations that are insensitive to current synergists (Zhang et al., 2008).

Metabolomics can provide a unique view of the impact of insecticide exposure, even if there is no visible phenotype, and so could identify additional modes of action, or metabolic changes associated with attempts to detoxify the xenobiotic. This enables the identification of any metabolic bottlenecks and the compensatory pathways used to alleviate insecticide toxicity, all of which can be potential targets. Metabolomics has already proven successful for target identification 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), for which there are also tissue-specific metabolomes available (Chintapalli et al., 2013).

In this study, metabolomics was used to identify pathways that were altered when the insecticide permethrin, which is an agonist of voltage gated sodium channels (Feng et al., 1992), was topically applied or fed to *Drosophila*. Changes in several pathways, including energy and lipid metabolism, were observed. Some of these pathway alterations can be explained by permethrin interaction with known secondary targets, while many of the pathway perturbations are novel. Amino acid metabolism showed large increases in tryptophan catabolites that are known

to be neuroactive in insects (Cerstiaens et al., 2003). Permethrin challenge down-regulates most enzyme encoding genes of the pathway of tryptophan catabolism. Using a combination of metabolomics, pathway identification, transgenics and comparative toxicology in *Drosophila*, a novel role of tryptophan catabolism pathway genes in permethrin survival is demonstrated.

Materials and Methods

Fly strains and rearing

RNAi expressing effector line for *vermilion* **UAS-*CG2155*-RNAi** (107798), *cinnabar* **UAS-*CG1555*-RNAi** (11322 and 105854) and *CG6950* **UAS-*CG6950*-RNAi** (22322 and 108093) were purchased from the Vienna Drosophila Resource Centre, Vienna, Austria. The KK RNAi effector control line **VDRCKKControl** (p{KC26}VIE) was kindly provided by Dr. Edward Green, University of Leicester, UK. The ubiquitous expressing driver line ***actin*-GAL4/CyO** (4414) and the central nervous system (CNS)-specific driver line ***elav*-GAL4** (8760) were purchased from the Bloomington Stock Centre, Indiana, USA. Another ubiquitous expressing driver line **tub-GAL4:UAS-*DICER*/Tm3Sb** was kindly provided by Dr. Eric Spana, Duke University, USA. The anterior midgut-specific driver line ***tsp42*-GAL4**, the fat body-specific driver line **c564** (Yang et al., 2007), the Malpighian tubule principal cell-specific driver line ***uo*-GAL4** (Terhzaz et al., 2010) and the wild type control **Canton S** (Glasgow Stock) were available in-house. Driver lines were crossed with RNAi effector lines to give F1 experimentals. Driver and expressor lines were each crossed with Canton S resulting in parental controls.

All stocks were reared on standard *Drosophila* medium. Strains used for topical application, feeding survival bioassays and Quantitative (Q)-PCR were kept at 26°C, 50% humidity on a 12:12 light dark cycle (MIR-254, SANYO Incubator).

Metabolomics

All metabolomics was performed using the Canton S wild-type *Drosophila* line. For larval samples, 10ug/ml permethrin was dissolved in molten standard *Drosophila* medium and cooled in 10ml aliquots. 100 third instar larvae were transferred onto this medium for oral exposure. For adult samples, 100 male and female adults were separately exposed to glass vials coated with 1ug of permethrin. The buzz plug was soaked in 5% w/v sucrose solution, to provide food and humidity. Samples were prepared by collecting and flash-freezing all surviving individuals in liquid nitrogen. Samples were collected at 0, 2, 12 and 24 hours after exposure began. Samples were shipped on dry ice to Metabolon Inc. for metabolomic analysis with a proprietary pipeline, and subsequent data processing (Weiner et al., 2012). Experiments were performed in quadruplicate.

Topical application survival bioassays

F1 adult flies between five and nine days old were segregated according to gender and a 69nl volume of permethrin dissolved in acetone applied to the thoracic notum using a microinjector (Nanoject II, Drummond Scientific Company). Doses ranged between 1ng/fly and 25ng/fly covering 0% - 100% mortality. Each dose tested had two technical replicates and three biological replicates with N=25. Treated flies were placed in an inverted food vial to prevent mortality though adherence to the food while in an incapacitated state. Mortality data was recorded 24 hours after application (**Fig. S1**).

Feeding survival bioassays

F1 adult flies between five and nine days old were segregated according to gender, starved for four hours and placed in vials with insecticide containing gels of 3% sucrose and 1% agar. Between 4-6 doses were tested for the following insecticides: permethrin, fenvalerate, DDT, chlopyriphos and hydramethylnon (all Sigma Aldrich). Dose ranges used for each insecticide are listed in **Table S1**. Each dose tested had two technical replicates and three biological replicates with N=30. Treated flies were placed in a horizontal vial as the horizontal orientation had the lowest control mortality. Mortality data was recorded hourly between 8:30-18:30 each day until all flies were dead. All fly strains tested were KK lines driven by an *actin*-GAL4 driver with *actin*-GAL4/VDRCKKControl as the control strain.

RNA extraction and QPCR

For validation of RNAi knockdown, five male and five female seven day old - F1 flies were homogenized and mRNA extracted using a TRIzol:chloroform extraction following standard procedures. The mRNA was DNase treated using an RNase Free DNase Set (50) (QIAGEN) following the off column protocol followed by the PureLink®RNA Minikit (Invitrogen) RNA cleanup protocol. A Superscript II Reverse Transcriptase Kit (Invitrogen) was used to synthesize cDNA from mRNA samples following the manufacturer's instructions. The primers and annealing temperatures used for QPCR are listed in **Table S2**. Taqman Universal PCR 2x Mastermix (Invitrogen) was used for TaqMan probes (Integrated DNA Technologies) and Brilliant III 2x SYBR Green QPCR Master Mix (Agilent) for conventional primers according to manufacturer instructions with 10µl total volume. All QPCR 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). The temperature cycle setup used for TaqMan probes 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 with the quencher set to NFQ-MGB and the passive reference set to ROX. For conventional primers the temperature cycle setup used was 95°C for 10 minutes for one cycle, 95°C for 30 seconds followed by the annealing temperature for 30 seconds and 72°C for one minute for 40 cycles with the passive reference set to none. There were four technical and four biological replicates for each gene tested.

Data Processing

Using Microsoft Excel, survival bioassay data was corrected for control mortality using Abbott's formula (Abbott, 1925) before using Probit analysis (Bliss, 1957; Finney, 1971) to linearize the cumulative Gaussian distribution. Probits from topical application survival assays were plotted against the log of the dose per mg body weight using Graphpad Prism 5 (GraphPad Software Inc., USA) to determine the 50% lethal dose (LD_{50}). Statistical significance between LD_{50}s of different genotypes was determined using both the Litchfield & Wilcoxon method (Litchfield and Wilcoxon, 1949) for the LD_{50} of the whole data set and ANOVA for the LD_{50}s of individual replicates with a Tukey's range test as a post test. Only when both methods showed the two LD_{50}s being compared to be significantly different was the null hypothesis rejected. Probits from feeding survival assays were trimmed to exclude spontaneous mortality and nonresponders (<3% of outer percentiles) before plotting against the log of the time since the assay began using Graphpad Prism 5 (GraphPad Software Inc., USA) to determine the 50% lethal time (LT_{50}). LT_{50}s were then plotted against the log of the dose to make toxicity response graphs. Linear regression analysis of toxicity responses was performed using Graphpad Prism 5 to determine co-linearity.

Results and Discussion

Metabolic changes on permethrin exposure

Metabolites detected from pathways that changed significantly on permethrin exposure are listed in **Supplementary Tables S3-S12** and discussed below.

Amino acid metabolism

Drosophila larvae showed an increase in the majority of free amino acids upon permethrin exposure especially after 12 hours (**Table S3**), as occurs in other species exposed to pyrethroids (Saleem and Shakoori, 1993), which may be due to protein degradation. This is supported by an increase in several free N-Acetyl-amino acids that are only produced post-translationally by protein N-acetylation. The only amino acid to show a decrease was proline, possibly indicating either an inhibition of synthesis from ornithine or increased conversion into glutamate.

A different amino acid profile is observed in permethrin-treated adults (**Table S4**). Only threonine, aspartate, phenylalanine, glutamine, alanine and proline increased on exposure while histidine decreased, indicating that adults are less sensitive to permethrin-induced protein degradation. 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.

It has been assumed that increased free amino acids are used solely for protein synthesis upon permethrin challenge but the metabolomics data for larvae indicate increased amino acid catabolites suggesting that the degradation of amino acids also occurs. As seen in **Fig. 1**, glycine is likely being metabolised via threonine using sarcosine and allo-threonine as sinks. The conversion of glycine to sarcosine occurs via the putative glycine methyltransferase, encoded by *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.

Tryptophan catabolism in larvae (**Table S3**) shows accumulations of the neurotoxic 3-hydroxykynurenine (1.67-fold at 12 hours) and the neuroprotective kynurenic acid (5.52-fold at 12 hours). 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.

The high tyrosine concentrations observed (8.39-fold at 12 hours) potentially indicates the impairment of dopamine metabolism by permethrin (Elwan et al., 2006). L-DOPA is the most significantly increased amino acid catabolite (13.7-fold at 12 hours) in larvae and indicates a potential lesion at DOPA decarboxylase induced by permethrin treatment. Pyrethroid exposure upregulates glutathione metabolism in insects (Lin et al., 2014) and the larval data set suggests an increase in gamma-glutamyl transpeptidase activity as indicated by raised γ-glutamyl amino acids (2-3.2-fold at 4, 12 and 24 h).

Energy Metabolism

The most significant changes in energy metabolites were observed in larvae and not adult flies. The larval data indicate increased amounts of maltotetraose, maltotriose, maltose, mannose, sorbitol and panose, which are products of glycogen utilization, suggesting that mobilization of glycogen energy reserves occurs on permethrin exposure (**Table S5**). In both larvae and adult (**Table S6**) there is increased glycerate with the biggest changes in adults also being observed for glucose-6-phosphate and pyruvate (**Table S6**). Larvae show substantially increased fructose (5.7-fold at 12 hours), indicating stimulation of glycolysis by permethrin. Decreased lactate in both the larvae and adult is consistent with the inhibition of lactate dehydrogenase which also occurs in snails exposed to pyrethroids (Bakry et al., 2011).

The metabolite with the largest increase is trans-aconitate (7.43-fold at 12 hours). This may indicate an impact of permethrin on the Krebs (TCA) cycle (**Fig. S2**) via inhibition of aconitase or acidic reaction conditions. There is also a depletion of succinate and malate but an accumulation of fumarate and α-ketoglutarate. The observed pattern of metabolites can be explained by α-ketoglutarate dehydrogenase complex inhibition or depletion of mitochondrial thiamine pyrophosphate causing a depletion of mitochondrial succinate, fumarate and malate while cytosolic fumarate accumulates due to aspartate being fed into the urea cycle. The high concentrations of trans-aconitate would prevent cytosolic fumarate re-entering the TCA cycle through the inhibition of fumarate hydratase. As fumarate reacts spontaneously with glutathione to form succinated glutathione (Sullivan et al., 2013) the elevated concentrations of fumarate could cause oxidative stress through depleting the pool of available glutathione.

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 utilization of proline for 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 induce energy metabolism perturbations but the increase in free amino acids are specific to pyrethroids.

Nitrogen Metabolism

In larvae there is an accumulation of ornithine and urea (**Table S3**) potentially due to inhibition of ornithine aminotransferase which is a known secondary target of pyrethroids (Bakry et al., 2011). 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. This is consistent with ammonia increases observed in several other species exposed to pyrethroids eg. (Veronica and Collins, 2003). A further indicator of ammonia stress is the elevated concentrations of 2-aminobutyrate in larvae, a compound that accumulates in the presence of high ammonia concentrations.

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 S7** and **Table S8**), 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.

Purine and pyrimidine metabolism

There is increased purine and pyrimidine metabolism in larvae (**Table S9**), most likely driven by RNA degradation. It has been shown that fenvalerate exposure causes a decrease in total RNA in arthropods (Mckee and Knowles, 1986), and 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 occurs in *Drosophila* larvae during permethrin exposure. Only guanine levels are reduced in response to permethrin. Permethrin induces cyclic guanosine monophosphate (cGMP) formation (Bodnaryk, 1982) which may explain the depletion of guanine. 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 S10**).

Pyrimidine metabolism in the larva shows a marginal increase in the nucleic acid thymine indicating increased DNA turnover. The released thymine is catabolised to 3-aminoisobutyrate which accumulates after permethrin exposure. The increased uridine may be utilised to both replenish uracil and form UDP-glucuronate which is involved in the phase II detoxification of permethrin (Shono et al., 1978). The adult data set (**Table S10**) shows a decrease in 2',3'-cUMP suggesting less RNA degradation in adults compared to larvae.

Fatty acid and lipid metabolism

Lipid peroxides produced during pyrethroid exposure (Terhzaz et al., 2015 in press) are degraded by phospholipases releasing free fatty acids, lysolipids and glycerophosphoalcohols. An increase in these compounds and their catabolites is observed in larvae exposed to permethrin (**Table S11**). The accumulation of free fatty acids and dicarboxylate products of ω-oxidation in both larvae and adults (**Table S12**) may indicate a metabolic defect in β-oxidation as occurs in mammals exposed to pyrethroids (Jin et al., 2014) and would explain the synergistic effect of adipokinetic hormone (which mobilises fatty acids) on permethrin exposure (Kodrik et al., 2010).

Lysolipids (monoacylphosphoglycerides) are intermediates in the synthesis and degradation of phospholipids (diacylphosphoglycerides). As pyrethroids cause lipid peroxidation, 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). The acetylcholine that is not reabsorbed is catabolised via betaine feeding into glycine metabolism. This process, shown in **Fig. 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 ethanolamine phosphoglyceride content (both monoacyl and diacyl) of membranes modulates neural excitability (Pavlidis et al., 1994) so the continued degradation of ethanolamine phosphoglycerides caused by permethrin exposure has the potential to make nerves more susceptible to permethrin-induced hyperexcitation under conditions of chronic exposure.

Evidence for oxidative stress

Pyrethroids can induce oxidative stress in insects (Terhzaz et al., 2015 in press) and 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 may be a marker for oxidative stress in invertebrates (Zeitoun-Ghandour et al., 2011). Another indicator of oxidative damage, methionine sulfoxide (Ruan et al., 2002), was also increased (3.5-fold at 24 hours) in larvae. 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. 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 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).

Detoxification responses to permethrin

An elevation in several metabolites related to pathways involved in xenobiotic response was observed in larvae. There is evidence for phase II detoxification mediated by phenol beta-glucosyltransferase and UDP-glucuronyltransferase, indicated by elevated phenylglucopyranoside (a glucosylation product of phenol), and glucarate and xylitol (catabolites of UDP-glucuronate). 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.

Permethrin exposure downregulates tryptophan catabolism

To investigate if the observed changes in tryptophan catabolites could be explained by alterations in gene regulation upon permethrin exposure, the transcripts of genes encoding enzymes involved in the conversion of tryptophan to xanthurenic acid and kynurenic acid (**Fig. 2A**), in addition to two genes for transporter subunits involved in tryptophan and kynurenate transport, were quantified using quantitative (Q)-PCR. The relative expressions of each gene on permethrin exposure compared to an acetone treated control are shown in **Fig. 2B**. The transcription for the *vermilion* gene which encodes the rate limiting enzyme for the pathway is not affected by permethrin exposure. All other genes for enzymes in the pathway show a significant downregulation, potentially indicating a response to reduce flux through the pathway. Interestingly the white gene, which is known to be upregulated in hessian flies fed on insect-resistant wheat (Shukle et al., 2008), was downregulated on permethrin exposure. The gene encoding the dCD98 heavy subunit of several amino acid transporters, which interacts with LAT1 or other small subunits to transport amino acids and amino acid derived metabolites like L-DOPA (Reynolds et al., 2009), is not affected.

Knockdown of tryptophan metabolism enzymes affects survival to permethrin exposure

Conspicuous in the dataset was a range of changes associated with tryptophan metabolism, not previously identified in the context of permethrin intoxication. To investigate whether these changes were incidental, or had bearing on insect survival, the tryptophan catabolism pathway was studied in more depth. As tryptophan catabolites might modulate the neurotoxicity of permethrin, gene knockdowns using RNAi was performed on *vermilion*, *cinnabar* and *CG6950* to determine any changes in permethrin susceptibility (**Fig. 3**). Knockdown efficacy was confirmed using QPCR (**Fig. S3**). 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.

To identify which organ systems play a role in the tryptophan catabolism mediated alterations to surviving permethrin challenge, knockdowns of *vermilion*, *cinnabar* and *CG6950* were performed using relevant tissue-specific drivers. The potential roles of the CNS, 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 LD_{50}s of the tissue-specific knockdown strains relative to controls are shown in **Fig.s 4-7**.

Knockdown of all candidate genes in the CNS had no definitive effect on permethrin survival (**Fig. 4**). Survival data for *vermilion* and *cinnabar* knockdown males were uninformative as the LD_{50} values were an intermediate of the controls; and knockdowns for the three genes did not show significantly different LD_{50} values. Similarly, although the female control Elav>VRDCKK line was more susceptible than the *vermilion* and *cinnabar* knockdowns, none of the gene knockdowns showed differential LD_{50} values compared to the Elav GAL4 parental line. These data indicate that other tissues may be responsible for the changes observed in the ubiquitous gene knock down strains. Alternatively, other tissues are able to supply tryptophan catabolites to the CNS and thus compensate for any loss of neural tryptophan catabolism.

Knockdown of *vermilion* in the fat body of males resulted in increased tolerance to permethrin compared to females, which were more susceptible after *vermilion* knockdown (**Fig. 5**). 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 (**Fig. 3**) 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.

Knockdown of each of *vermillion* and *cinnabar* in female midgut results in increased permethrin susceptibility compared to controls, which is not observed in males (**Fig. 6**). Both genders show increased resistance to permethrin when *CG6950* is knocked down in the midgut. 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 (**Fig. 6**), it is possible that there is a similar system in the *Drosophila* gut.

Malpighian tubule-specific gene knockdowns indicated that *cinnabar* significantly affected permethrin survival, indicating tubule-specific roles for kynurenine or kynurenic acid upon permethrin exposure (**Fig. 7**). In *Drosophila,* kynurenates accumulate in the Malpighian tubule (Tearle, 1991). One 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. Malpighian tubules are key tissues for detoxification and stress responses (Terhzaz et al., 2010; Yang et al., 2007) and these data suggest novel roles for kynurenine metabolism by the tubules for insecticide survival.

Tryptophan catabolism knockdown in each of the CNS, fat body, midgut and Malpighian tubules result 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 that there may be additional tissues and/or pathways involved.

Knockdown of tryptophan metabolism enzymes affects survival against other insecticides

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 (**Fig. 8**). 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 1**). The *cinnabar* knockdown males show opposite trends in survival depending on the route of exposure (**Fig.s 3 and 8**) suggesting the importance of differing routes on the efficacy of permethrin absorption. This also implies that *cinnabar* knockdown may affect the permeability of the cuticle or gut.

There is sexual dimorphism seen in the toxicity responses, with the knockdowns for all genes having increased intercepts without a changed gradient in males. Females 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 implying at least two different mechanisms; perhaps 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.

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 affected survival showing sexually dimorphic alterations to fenvalerate tolerance (**Fig. 9**). 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 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 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 (**Fig. 10**). 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 be insufficient flux through tryptophan catabolism for the *vermilion* and *cinnabar* knockdowns to exert their effect on survival.

Pyrethroids are known to cause the release of the neurotransmitter acetylcholine (Feng et al., 1992) 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 (**Fig. 11**) 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. 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.

The data suggest that permethrin exposure causes perturbations in energy metabolism (**Table S5**). To investigate if interactions in energy metabolism are responsible for the changes in survival observed in tryptophan catabolism knockdowns, hydramethylnon, a suicide inhibitor of mitochondrial complex III, was used in feeding survival assays. 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 kynurenine, 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.

Conclusion

Metabolomics is a powerful tool for identifying the complexity of underlying changes behind superficially simple xenobiotic/organism interactions; and of the “omics” technologies, is the one closest to reporting actual enzymic function. Although the primary target of permethrin is the voltage-gated sodium channel, permethrin poisoning induces alterations in several interlinked metabolic pathways, many of which have the potential to impact survival. The tryptophan catabolism pathway was downregulated by permethrin exposure, and knockdown of genes encoding the tryptophan catabolism enzymes *vermilion*, *cinnabar* and *CG6950* impacted on survival to permethrin. Furthermore, gene knockdown in the whole organism also affects survival against fenvalerate, DDT, chorpyriphos and hydramethylnon, insecticides with a broad range of mode-of-action. The tryptophan pathway could thus be seen as an intrinsic metabolic pathway that could inform in the risk assessment of candidate insecticides. Tissue-specific gene knockdowns demonstrated roles for tryptophan catabolism in fat body, midgut, and Malpighian tubules (tissues associated with xenobiotic detoxification) - but not for the CNS - in permethrin survival. Knockdown of *cinnabar* in Malpighian tubules showed the clearest increase in survival compared to controls and other genes tested, suggesting novel gender- and tissue-specific roles for kynurenine metabolism in response to xenobiotic challenge. Intriguingly, knockdown of at least one of the genes encoding enzymes for tryptophan catabolism increased survival against all of the insecticides tested. This would make *vermilion*, *cinnabar* and *CG6950* potential anti-targets for synergist compounds.

Finally, if tryptophan catabolism were to modulate insecticide tolerance in other insect species there would also be impacts on malaria control strategies due to a trade-off between the reliance on insecticides for vector control

(David et al., 2013) and the possible inhibition of malaria gametogenesis by inhibiting tryptophan catabolism in vectors (Billker et al., 1998).

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Fig. Legends

Fig. 1. Summary of glycine, serine, threonine, methionine, cysteine and glutathione metabolism in larvae. Metabolites detected that were significantly altered on permethrin exposure have an arrow at the upper right, ↑ = p<0.05 - an increase, ↓ = p<0.05 - a decrease. 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.

Fig. 2. Tryptophan catabolism pathway and regulation of associated genes on permethrin challenge. (A) Schematic representation of tryptophan catabolism in *Drosophila* showing all enzymes, substrates and products. Enzymes are in bold and numerals (i) and (ii) represent 4-(2-Aminophenyl)-2, 4-dioxobutanoate and 4-(2-Amino-3-hydroxyphenyl)-2, 4-dioxobutanoate respectively. (B) QPCR showing the expression of genes involved in tryprophan catabolism and the transport of intermediate compounds in adults at 4 hours after being challenged with a sublethal dose of permethrin. Control gene (Black), genes for enzymes listed in order of their occurrence in the pathway where possible (Unshaded) and genes encoding subunits for some transporters involved in tryptophan/kynurenate transport (Grey). v = *vermilion*, kfase = *kynurenine formamidase*, cn = *cinnabar*, cd = *cardinal* and w = *white*. (*) = $P < 0.05$ and (**) = $P < 0.01$.

Fig. 3. Survival of whole fly tryptophan catabolism gene knockdowns of *vermillion*, *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 in blue. N ≥ 1050 per strain. (*) = $P < 0.001$

Fig. 4. 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 in blue. N ≥ 1100 per strain. (*) = $P < 0.05$

Fig. 5. 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 in blue. N ≥ 1050 per strain. (*) = $P < 0.001$

Fig. 6. 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 in blue. N ≥ 1075 per strain. (*) = $P < 0.05$. (**) = $P < 0.001$

Fig. 7. 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 in blue per strain. N ≥ 1060. (*) = $P < 0.001$

Fig. 8. Permethrin toxicity responses of tryptophan catabolism gene knockdowns 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. (*) = $P < 0.005$.

Fig. 9. 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. (*) = $P < 0.005$.

Fig. 10. 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. (*) = $P < 0.005$.

Fig. 11. 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. (*) = $P < 0.005$.

Fig. 12. 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. (*) = $P < 0.005$.