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Abstract

Insecticides are a key tool in the management of many insect pests of agriculture, including soybean aphids. The selection imposed by insecticide use has often lead to the evolution of resistance by the target pest through enhanced detoxification mechanisms. We hypothesised that exposure of insecticide-susceptible aphids to sublethal doses of insecticides would result in the up-regulation of genes involved in detoxification of insecticides, revealing the genes upon which selection might act in the field. We used the soybean aphid biotype 1 reference genome, version 6.0 as a reference to analyze RNA-Seq data. We identified multiple genes with potential detoxification roles that were up-regulated 12 h after sublethal exposure to

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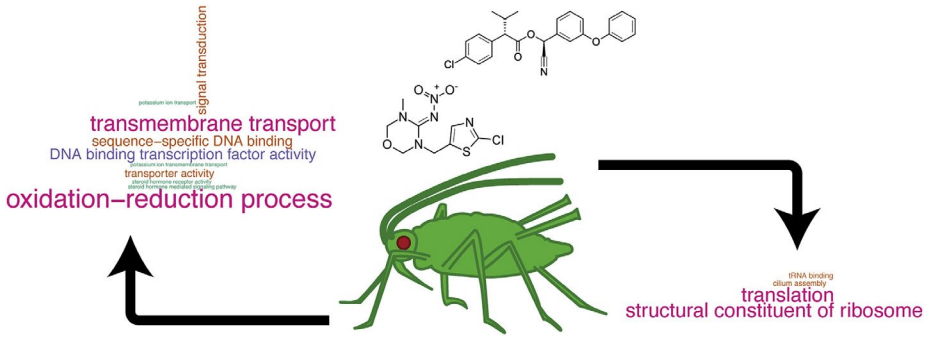
esfenvalerate or thiamethoxam. However, these genes were part of a dramatic burst of differential gene expression in which thousands of genes were up- or down-regulated, rather than a defined response to insecticides. Interestingly, the transcriptional burst observed at 12 h s declined dramatically by 24-hrs post-exposure, suggesting a general stress response that may become fine-tuned over time.

Keywords: esfenvalerate, RNA-seq, Gene expression, Aphis glycines, Thiamethoxam



1. Introduction

Management of aphid pests within agro-ecosystems relies extensively on the use of insecticides. Organophosphates, carbamates, pyrethroids and neonicotinoids are the most common insecticidal groups used to control aphids (Koch et al., 2018). These toxins generally target critical functions, such as transmission of nerve impulses, cellular respiration and lipid biosynthesis (Nauen et al., 2011; Yu, 2011). However, intensive use of insecticides worldwide has lead to the evolution of resistance in many aphid pests, most notably *Myzus persicae* and *Aphis gossypii* (Bass et al., 2014; Carletto et al., 2010). At least 20 aphid species, including the invasive soybean aphid (*Aphis glycines*), have developed resistance to multiple groups of insecticides (Foster et al., 2007; Hanson et al., 2017).

Several mechanisms exist in aphids that render individuals insensitive to chemical insecticides, including mutations of targeted receptor sites, reduced cuticular penetration and heightened activity of detoxification enzymes (Bass et al., 2014; Foster et al., 2007). Activation of genes involved in various phases of xenobiotic detoxification appears to be a core mechanism by which aphids counteract exposure to insecticidal compounds (Enders and Miller, 2016). Transcriptional responses of aphids exposed to several insecticides show increased expression of many detoxifying enzymes, including cytochrome P450 microsomal monooxygenases, esterases, and

glutathione-S-transferases (Cabrera-Brandt et al., 2014; Pan et al., 2015; Silva et al., 2012; Wu et al., 2018; Xi et al., 2015). For example, among 183 genes up-regulated in a pirimicarb-sensitive *M. persicae* genotype, 60 were involved in xenobiotic detoxification (Silva et al., 2012).

Although detoxification is a primary coping mechanism in aphids, transcriptomic studies have revealed that a greater level of complexity underlies response to insecticides than previously believed (Enders and Miller, 2016). A significant portion of the aphid transcriptome is altered under selective pressure from insecticide stress. Hundreds of genes involved in a broad spectrum of pathways are differentially expressed in resistant genotypes (Pan et al., 2015; Xi et al., 2015). For example, general stress responsive genes associated with restoration of homeostasis are differentially expressed in insecticide stressed aphids, such as heat shock proteins and peptidases that interact with damaged proteins (Cabrera-Brandt et al., 2014; Silva et al., 2012; Xi et al., 2015). Increased expression of cuticular proteins also likely functions as a first line of defense against insecticides, by reducing permeability through the aphid exoskeleton (Silva et al., 2012). Finally, comparison of transcriptional changes among genotypes with various resistance mechanisms demonstrates aphids exhibit considerable plasticity in gene expression when exposed to insecticides (Silva et al., 2012).

Despite considerable advances in our understanding of aphid molecular responses to insecticides, the underlying mechanisms responsible for resistance remain uncharacterized in several species, including the soybean aphid. Approximately 15 years after being introduced into North America, pyrethroid resistant populations of *A. glycines* were documented in Minnesota and Iowa (Hanson et al., 2017). A recent study found increased esterase and cytochrome P450 (CYP) activity in laboratory populations of *A. glycines* selected for pyrethroid resistance (Xi et al., 2015). However, it is unclear to what extent general detoxification mechanisms are involved in response to multiple pyrethroids or additional insecticide classes used to control *A. glycines*. We therefore aimed to characterize the transcriptional response of *A. glycines* exposed to sublethal doses of two insecticides: thiamethoxam (neonicotinoid) and esfenvalerate (pyrethroid). We hypothesised that differential gene expression would reveal insecticide responsive detoxification genes that may respond to selection and lead to resistance evolution.

2. Material and methods

2.1. Insect rearing and stress treatments

The colony of soybean aphids (*A. glycines*) used in this experiment was established in 2011 from a single viviparous parthenogenetic female collected in Madison, Wisconsin. Microsatellite markers (Kim et al., 2010; Michel et al., 2009) confirmed this colony consisted of a single genotype (Enders et al., 2014). In addition, the colony was unable to successfully colonize a panel of soybean varieties containing various resistance genes (*Rag*: Resistance to *Aphis glycines*) and was therefore considered a biotype 1 clonal line (Enders et al., 2014). Aphids were continuously maintained in a growth chamber (24 ± 1 °C, 16:8 photoperiod) on a single soybean plant (variety KS4202) grown in a plastic Cone-tainer (Ray Leach Cone-tainer, Hummert International, Earth City, MO) and covered by a custom fitted cylindrical plastic cage (30.5 cm × 4.4 cm). Soybean variety KS4202 was used for aphid colony maintenance because it does not adversely affect aphid survival or development and is tolerant of large populations (Enders et al., 2014; Pierson et al., 2010). All soybean plants used for aphid colony maintenance and experimental treatments were grown in a greenhouse (16L:8D photoperiod), using a potting medium comprised of peat moss, perlite, pine bark, and vermiculite (Fafard 3B Mix).

Plants of a standard aphid susceptible soybean variety SD76R (Chiozza et al., 2010) were grown under greenhouse conditions to the V2 vegetative stage for use in age-synchronizing adult aphids and experimental treatments. Groups of aphids were age-synchronized prior to exposure to insecticide stress and control treatments by allowing adults from the aphid colony to produce offspring for 48 h in a growth chamber (24 ± 1 °C, 16L:8D hours photoperiod). When age-synchronized offspring reached reproductive age (~7 days old) they were exposed to the following treatments: **1)** control conditions (no insecticide) **2)** Esfenvalerate stress (LC_{50} = 10 ng/μl) and **3)** Thiamethoxam stress (LC_{50} = 10 ng/μl). We used a modified aphid-dip bioassay technique (Chandrasena et al., 2011) to expose groups of age-synchronized adult aphids to each insecticide or control solution. Preliminary experiments determined the LC_{50} for each insecticide that resulted in approximately 50% mortality 48 hs post-exposure was 10 ng/μl.

Insecticide solutions were prepared by dissolving in acetone and then diluting with distilled water to reach the desired concentration.

Groups of 40 adult aphids were dipped for 10 s in each of the 3 treatment solutions: Control (0.05% acetone in distilled water), Esfenvalerate (10 ng/μl), and Thiamethoxam (10 ng/μl). The aphids were then transferred with a paint brush to a single V1 trifoliate of a susceptible soybean plant and covered with a custom-built plastic Petridish cage (8.9 cm × 2.5 cm). Nine replicate cages were set up for each treatment. Aphids were harvested at 12 and 24 h post exposure from independent cages, flash-frozen in liquid nitrogen and stored at −80 °C for further transcriptomic processing. Survival and nymph production were also measured at each time point.

2.2. Transcriptomic methods and analysis

Total RNA was isolated and purified from groups of 30 whole apterous adults using a Qiagen RNeasy extraction kit according to manufacturer protocols. Four replicate RNA samples were prepared for each of the 3 experimental treatments (2 insecticides and control) at each time point (12 h and 24 hr) by randomly pooling aphids from across 4–5 replicate cages (24 total biological replicates). RNA integrity was confirmed using an Agilent 2100 Bioanalyzer. RNA-Seq libraries were prepared, pooled and sequenced on two lanes of an Illumina HiSeq 2000 platform at the University of Nebraska Medical Center Genomics Core facility. Sequencing resulted in between 6,596,272 and 14,656,030 (mean 11,253,320) pairs of 100 nucleotide reads per biological replicate. Read data were deposited with the NCBI Sequence Read Archive under BioProject PRJNA515901.

Technical sequences (e.g. sequencing adapters) and regions of low-quality sequence were removed from reads using Trimmomatic (Bolger et al., 2014). Trimmed reads were aligned to the *Aphis glycines* biotype 1 reference genome, version 6, using HISAT2 (Kim et al., 2015). The number of sequenced fragments mapping to each gene in the *Aphis glycines* official gene set (OGS) version 6.0 was computed for each RNA-Seq library using featureCounts (Liao et al., 2014). Tests for differential gene expression between each insecticide/time point treatment and the corresponding control were performed with DESeq2 (Love et al., 2014). Genes were declared significantly differentially-expressed at a false discovery rate (Benjamini and Hochberg, 1995) of 0.1.

Putative functions of select differentially-expressed genes were inferred from the results of InterProScan and Blast2GO analysis of the OGS 6.0 gene models prepared by the Bioinformatics Platform for Agrosystem Arthropods (BIPPA) and an additional Blast2GO analysis provided by Ravi Kiran Donthu, Puerto Rico Science, Technology & Research Trust, San Juan, Puerto Rico. Gene Ontology-term enrichment analyses for sets of differentially-expressed genes were performed using GOSeq (Young et al., 2010) using a consolidated, non-redundant set of gene to GO term mappings prepared from the sources given above. GO terms were declared significantly overrepresented at a false discovery rate of 0.1. Ancestor charts of overrepresented GO terms were generated using the online tools provided by the EBI QuickGo site (www.ebi.ac.uk/QuickGO). Genes with possible detoxification roles were identified by searching the "description" field of the InterProScan results for case-insensitive matches to the strings "Cytochrome P450", "ABC transporter", "glutathione S-transferase" and "carboxylesterase".

3. Results

3.1. Overall transcriptional response to insecticide treatments

The number of sequenced fragments that were mapped to gene models in the official gene set version 6.0 ranged from 2,279,371 to 9,411,020 (mean 6,400,514) per sample.

The number of genes that were significantly differentially expressed changed rapidly over time for both insecticides (**Table 1, Fig. 1**). At 12 h post-exposure, thousands of genes were differentially expressed (Table 1, Fig. 1). At 24 h post-exposure, the number of differentially expressed genes had declined to a few hundred for

Table 1. Total numbers of differentially expressed genes.

	Esfenvalerate		*Thiamethoxam*	
	12 h	*24 h*	*12 h*	*24 h*
Up-Regulated	4163	131	2177	1
Down-Regulated	4169	72	1982	6

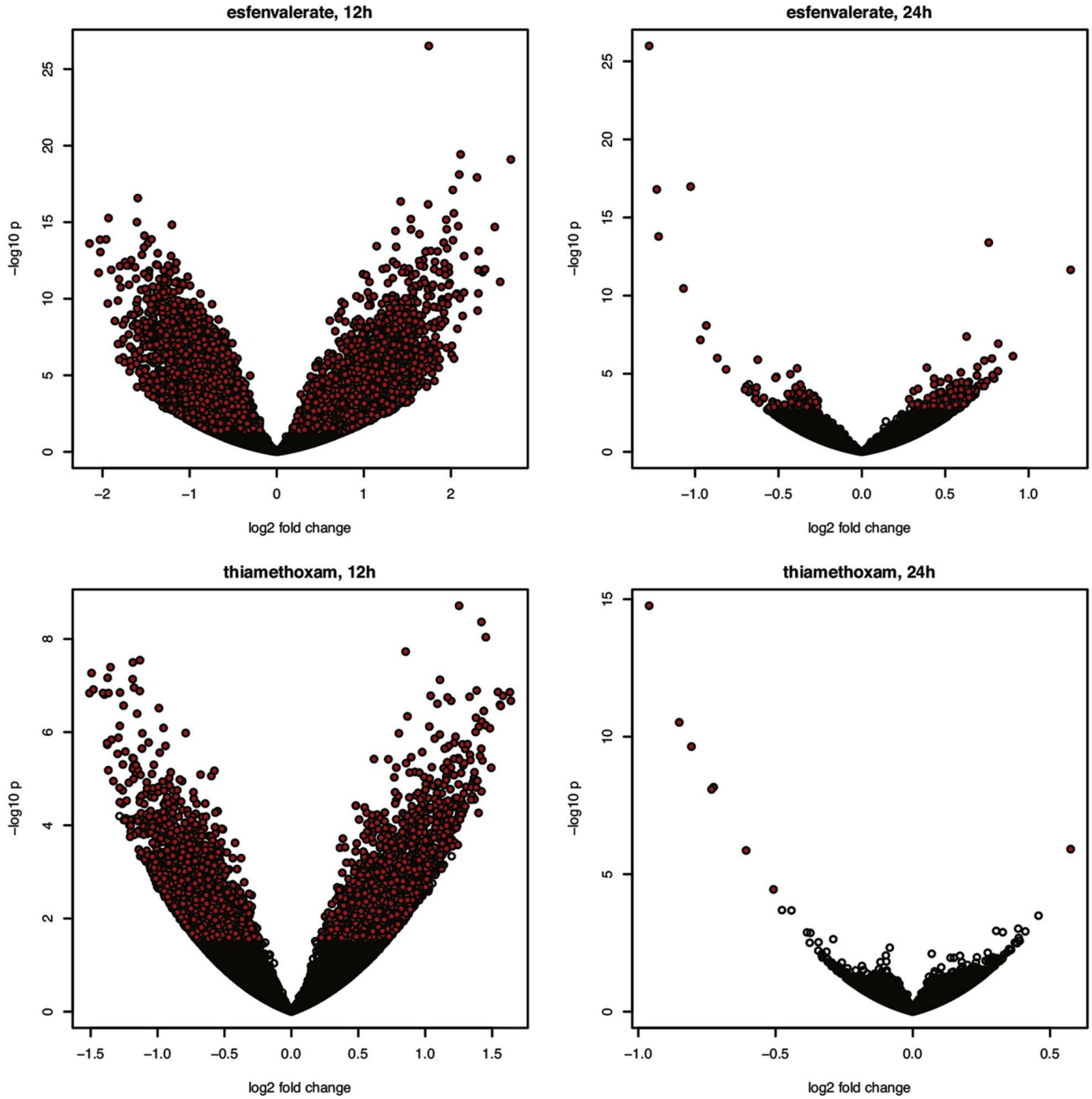


Fig. 1. Volcano plots showing changes in gene expression, relative to control conditions, 12 and 24 h after exposure to esfenvalerate or thiamethoxam. Red circles indicate genes that were significantly differentially-expressed at a false discovery rate of 0.1.

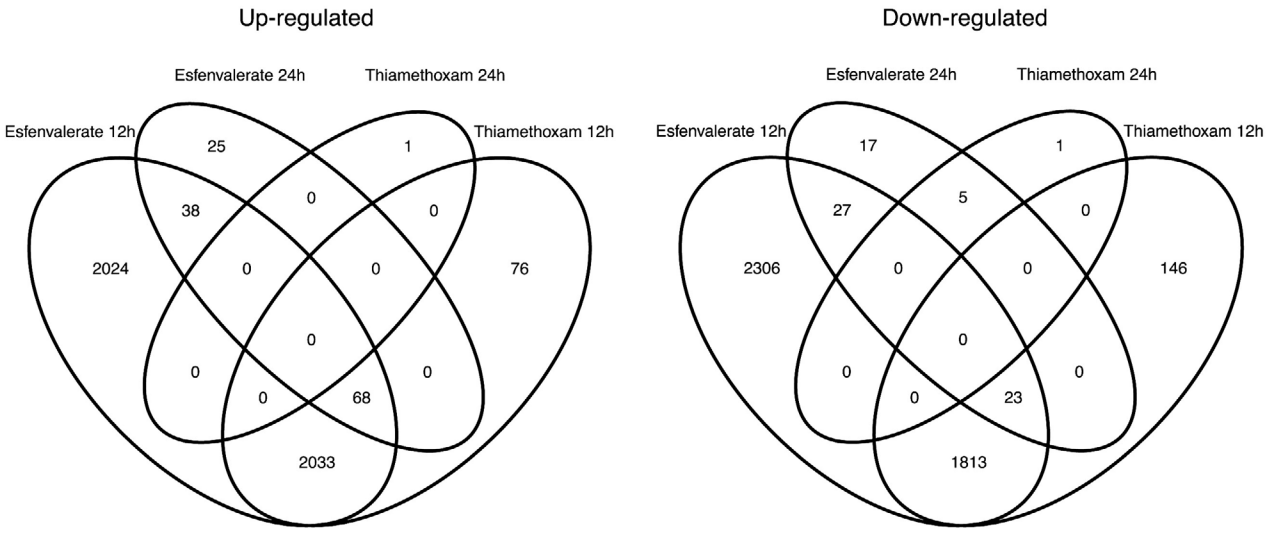


Fig. 2. Venn diagrams showing the numbers of up- and down-regulated transcripts after exposure to two insecticides.

esfenvalerate and less than ten for thiamethoxam (Table 1, Fig. 1). There was considerable overlap between the two insecticides in the genes that were significantly up- or down-regulated in response to exposure after 12 h (**Fig. 2**). To a lesser extent, there was also some overlap between genes that were up- or down-regulated 12 and 24 h after exposure to esfenvalerate. Similarly, some genes that were up- and down-regulated following exposure to esfenvalerate were shared with those up- or down-regulated 12 h after exposure to thiamethoxam (Fig. 2).

GO-term enrichment analyses of the genes that were differentially regulated in response to each insecticide or to both insecticides, 12 h after exposure, revealed some consistent patterns. Overrepresented GO terms associated with genes that were upregulated were mainly indicative of signaling, regulation of transcription and oxidation/reduction processes (**Tables 2–4**). Overrepresented GO terms associated with genes that were downregulated were mostly indicative of translation and protein synthesis (**Tables 5–7**). Ancestor charts of overrepresented GO terms are given in **Supplementary Figs. S1–S6**.

Table 2. Overrepresented GO terms associated with genes up-regulated 12 h after exposure to esfenvalerate. CC: cellular component, MF: molecular function, BP: biological process, *q*: false discovery rate *q*-value, *N*: number of genes in category.

GO term	*Ontology*	*Description*	*q*	*N*
GO:0043565	MF	sequence-specific DNA binding	<0.01	115
GO:0003700	MF	DNA binding transcription factor activity	<0.01	116
GO:0005215	MF	transporter activity	<0.01	68
GO:0005509	MF	calcium ion binding	<0.01	97
GO:0003707	MF	steroid hormone receptor activity	0.062	18
GO:0004930	MF	G-protein coupled receptor activity	0.064	51
GO:0102336	MF	3-oxo-arachidoyl-CoA synthase activity	0.083	8
GO:0102337	MF	3-oxo-cerotoyl-CoA synthase activity	0.083	8
GO:0102338	MF	3-oxo-lignoceronyl-CoA synthase activity	0.083	8
GO:0102756	MF	very-long-chain 3-ketoacyl-CoA synthase activity	0.083	8
GO:0055114	BP	oxidation-reduction process	<0.01	227
GO:0055085	BP	transmembrane transport	<0.01	159
GO:0006355	BP	regulation of transcription, DNAtemplated	<0.01	199
GO:0007186	BP	G-protein coupled receptor signaling pathway	<0.01	62
GO:0007165	BP	signal transduction	0.01	89
GO:0006813	BP	potassium ion transport	0.038	15
GO:0006633	BP	fatty acid biosynthetic process	0.039	21
GO:0043401	BP	steroid hormone mediated signaling pathway	0.049	19
GO:0007155	BP	cell adhesion	0.049	33
GO:0008152	BP	metabolic process	0.055	118
GO:0016310	BP	phosphorylation	0.065	38
GO:0005975	BP	carbohydrate metabolic process	0.083	72
GO:0016020	CC	membrane	<0.01	272
GO:0016021	CC	integral component of membrane	<0.01	1162

Table 3. Overrepresented GO terms associated with genes up-regulated 12 h after exposure to thiamethoxam. CC: cellular component, MF: molecular function, BP: biological process, *q*: false discovery rate *q*-value, *N*: number of genes in category.

GO term	*Ontology*	*Description*	*q*	*N*
GO:0043565	MF	sequence-specific DNA binding	<0.01	63
GO:0005215	MF	transporter activity	<0.01	47
GO:0003700	MF	DNA binding transcription factor activity	<0.01	66
GO:0003707	MF	steroid hormone receptor activity	<0.01	15
GO:0055085	BP	transmembrane transport	<0.01	107
GO:0006813	BP	potassium ion transport	<0.01	14
GO:0055114	BP	oxidation-reduction process	<0.01	130
GO:0071805	BP	potassium ion transmembrane transport	0.014	15
GO:0043401	BP	steroid hormone mediated signaling pathway	0.016	15
GO:0007165	BP	signal transduction	0.035	58
GO:0016021	CC	integral component of membrane	<0.01	670
GO:0016020	CC	membrane	0.082	163

Table 4. Overrepresented GO terms associated with genes up-regulated 12 h after exposure to esfenvalerate and 12 h after exposure to thiamethoxam. CC: cellular component, MF: molecular function, BP: biological process, *q*: false discovery rate *q*-value, *N*: number of genes in category.

GO term	*Ontology*	*Description*	*q*	*N*
GO:0043565	MF	sequence-specific DNA binding	<0.01	61
GO:0005215	MF	transporter activity	<0.01	46
GO:0003700	MF	DNA binding transcription factor activity	<0.01	65
GO:0003707	MF	steroid hormone receptor activity	<0.01	15
GO:0055085	BP	transmembrane transport	<0.01	105
GO:0006813	BP	potassium ion transport	<0.01	14
GO:0055114	BP	oxidation-reduction process	<0.01	126
GO:0071805	BP	potassium ion transmembrane transport	<0.01	15
GO:0043401	BP	steroid hormone mediated signaling pathway	0.01	15
GO:0007165	BP	signal transduction	0.049	56
GO:0016021	CC	integral component of membrane	<0.01	646

Table 5. Overrepresented GO terms associated with genes down-regulated 12 h after exposure to esfenvalerate. CC: cellular component, MF: molecular function, BP: biological process, *q*: false discovery rate *q*-value, *N*: number of genes in category.

GO term	*Ontology*	*Description*	*q*	*N*
GO:0003735	MF	structural constituent of ribosome	<0.01	94
GO:0003723	MF	RNA binding	<0.01	121
GO:0005524	MF	ATP binding	<0.0	1 357
GO:0008080	MF	N-acetyltransferase activity	0.017	18
GO:0019843	MF	rRNA binding	0.043	10
GO:0016887	MF	ATPase activity	0.043	56
GO:0006412	BP	translation	<0.01	93
GO:0006396	BP	RNA processing	<0.01	46
GO:0002181	BP	cytoplasmic translation	<0.01	11
GO:0006886	BP	intracellular protein transport	0.024	53
GO:0032259	BP	methylation	0.028	29
GO:0045859	BP	regulation of protein kinase activity	0.039	8
GO:0090305	BP	nucleic acid phosphodiester bond hydrolysis	0.039	25
GO:0002098	BP	tRNA wobble uridine modification	0.052	9
GO:0006367	BP	transcription initiation from RNA polymerase II promoter	0.062	9
GO:0005840	CC	ribosome	<0.01	91
GO:0005622	CC	intracellular	<0.01	193
GO:0022625	CC	cytosolic large ribosomal subunit	<0.01	13
GO:0022627	CC	cytosolic small ribosomal subunit	<0.01	8
GO:0031011	CC	Ino80 complex	<0.01	14
GO:0005759	CC	mitochondrial matrix	0.012	14
GO:0019013	CC	viral nucleocapsid	0.039	14
GO:0030529	CC	intracellular ribonucleoprotein complex	0.052	22
GO:0005815	CC	microtubule organizing center	0.09	14

Table 6. Overrepresented GO terms associated with genes down-regulated 12 h after exposure to thiamethoxam. CC: cellular component, MF: molecular function, BP: biological process, *q*: false discovery rate *q*-value, *N*: number of genes in category.

GO term	*Ontology*	*Description*	*q*	*N*
GO:0003735	MF	structural constituent of ribosome	<0.01	27
GO:0000049	MF	tRNA binding	0.028	8
GO:0006412	BP	translation	<0.01	27
GO:0060271	BP	cilium assembly	<0.01	8
GO:0005840	CC	ribosome	<0.01	26

Table 7. Overrepresented GO terms associated with genes down-regulated 12 h after exposure to esfenvalerate and 12 h after exposure to thiamethoxam. CC: cellular component, MF: molecular function, BP: biological process, *q*: false discovery rate *q*-value, *N*: number of genes in category.

GO term	*Ontology*	*Description*	*q*	*N*
GO:0003735	MF	structural constituent of ribosome	0.014	26
GO:0000049	MF	tRNA binding	0.021	8
GO:0060271	BP	cilium assembly	0.014	8
GO:0006412	BP	translation	0.014	26
GO:0005840	CC	ribosome	0.018	24

3.2. Putative functions of highly differentially-expressed genes

The InterProScan and Blast2GO results for the 20 most strongly up and down-regulated genes, relative to controls, for each condition were inspected to infer putative gene function. In the case of thiamethoxam 24 h after treatment, fewer genes were inspected as only one gene was significantly up-regulated and six genes significantly down-regulated. Details of the inferred functions of strongly differentially expressed genes are given in **Supplemental Tables S1–S6**.

Two genes were strongly upregulated, relative to controls, 12 h after treatment with either esfenvalerate or thiamethoxam. Gene AG6014660 was a putative hexokinase. GO terms associated with AG6014660 indicated possible roles in glycolysis or glucose homeostasis. The upregulation of AG6014660 may represent part of a general stress response, as changes in energy metabolism are a common feature of stress responses in aphids (Enders and Miller, 2016). Gene AG6027850 was annotated as a putative tropomyosin gene. Blast2GO results for AG6027850 also indicated nucleic acid binding and alaninine-tRNA ligase functions, which are not characteristic of

tropomyosin. A blastx search of the NCBI non-redundant protein database revealed strong similarity between the first 81 amino acid residues encoded by AG6027850 (essentially, exon 1) and multiple aphid tropomyosins. Investigation of nearby gene models on the BIPPA genome browser identified two other gene models with blastx hits to insect tropomyosins. It is likely that AG6027850 represents part of a tropomyosin gene that is fragmented into multiple gene models.

A putative α-tocopherol transporter gene was highly-expressed, relative to controls, 12 h after exposure to esfenvalerate. This may also represent part of a general stress response as α-tocopherol is known to protect against oxidative stress in *Drosophila* (Bahadorani et al., 2008). Similar to the tropomyosin gene, blastx searches suggested that the α-tocopherol transporter gene was fragmented into at least two adjacent gene models (AG6026920, AG6026921), possibly with one or more undetected exons in-between. Two probable membrane-bound Ras protein genes (AG6034507, AG6032677), likely to be involved in G-protein mediated signaling were highly expressed 12 h after exposure to thiamethoxam.

The most strongly down-regulated genes, relative to controls, 12 h after exposure to either esfenvalerate or thiamethoxam were overwhelmingly genes with no identifiable function or unidentified membrane proteins (Supplemental Tables S3 and S4). Of the handful of genes that were strongly differentially expressed 24 h after exposure to thiamethoxam, none had an identifiable function.

Genes showing strong differential expression 24 h after exposure to esfenvalerate (Supplemental Tables S5 and S6) frequently had no known function or were unidentified membrane proteins. For those genes for which function could be inferred, no obvious common pathways or processes were apparent. One notable highly-expressed gene, AG6020067, had a putative role in glucuronidation in phase II metabolism of xenobiotic compounds. Conversely, a putative cytochrome P450 monooxygenase gene (AG6003000) was strongly down-regulated, relative to controls.

3.3. Transcriptional response of detoxification-related genes

The total number of cytochrome P450 monooxygenase, ABC transporter, glutathione-S-transferase and carboxylesterase genes identified in the OGS were 60, 70, 15 and 35, respectively. Appreciable

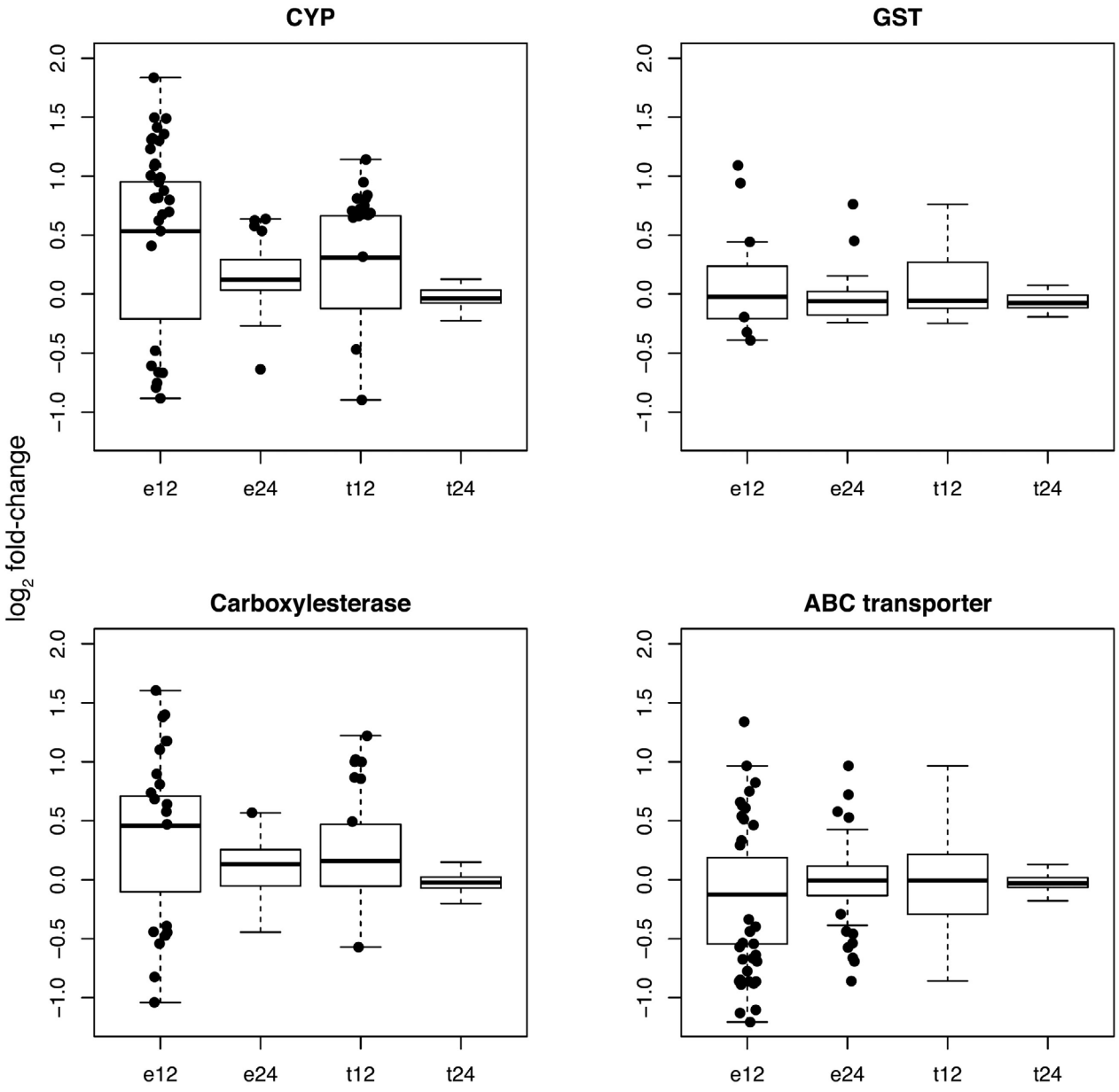


Fig. 3. Distributions of changes in expression (log_2 fold-change, relative to controls) of gene families commonly associated with detoxification of insecticides. Dots denote genes with significant changes in expression at a false discovery rate of 0.1. CYP: cytochrome P450 monooxygenases, GST: glutathione-S-transferases, e12: 12 h after exposure to esfenvalerate, e24: 24 h after exposure to esfenvalerate, t12: 12 h after exposure to thiamethoxam, t24: 24 h after exposure to thiamethoxam.

numbers of these genes were among those that were differentially expressed in response to insecticide treatments, especially 12 h post-exposure (**Fig. 3, Supplemental Tables S7–S10**). The magnitude of

up- or downregulation of the potential detoxification-related genes, as estimated by log2 fold-change relative to control conditions, was not significantly different from that of other differentially-expressed genes (Wilcox test p-values > 0.15).

4. Discussion

When exposed to insecticides the prevailing response across many aphid species is activation of xenobiotic detoxification. For example, resistance to neonicotinoids is associated with overexpression of CYPs in *M. persicae* (Bass et al., 2014), *A. gossypii* (Pan et al., 2015; Wu et al., 2018), *Aphis craccivora* (Abdallah et al., 2016) and *Rhopalosiphum padi* (Wang et al., 2018). Enhanced detoxification is also reportedly involved in the ability of Chinese *A. glycines* populations to overcome pyrethroid insecticides (Xi et al., 2015). However, it remains unclear whether similar mechanisms underlie resistance observed in *A. glycines* populations invasive to North America (Hanson et al., 2017). We therefore exposed soybean aphids to sublethal doses of two commonly used insecticides (esfenvalerate and thiamethoxam) and measured transcriptional changes at 12 h s and 24 h s post-exposure in order to identify genetic mechanisms that could potentially contribute to resistance evolution. Overall, we identified multiple genes with potential detoxification roles that were up-regulated in response to both insecticides, but these genes were part of a dramatic transcriptional burst involving thousands of differentially expressed genes that occurred 12 h s postexposure and largely dissipated by 24 h s.

In contrast to previous research, soybean aphids exposed to sublethal doses of insecticide in the current study did not exhibit a strong detoxification response, relative to overall transcriptional changes. differentially expressed following insecticide exposure, they were neither significantly overrepresented nor significantly strongly up- or downregulated, compared to other differentially expressed genes. Three main factors could explain this lack of a distinctive detoxification response in the current study: 1) route or method of insecticide exposure 2) differences in prior history of exposure to insecticides across laboratory populations and 3) method used to measure gene expression (RNA-Seq vs qRT-PCR).

In the current study aphids were dipped directly into insecticide solution (i.e. aphid dip bioassay), therefore absorption through the cuticle is likely the primary route of exposure. However, many studies utilize either leaf-dip or detached-leaf bioassays (e.g. Magalhaes et al., 2008), where insecticides are coated onto leaves or taken up systemically by the plant prior to aphid infestation and exposure therefore involves direct ingestion. A number of studies reporting enhanced detoxification in response to insecticides have used leaf-dip methods (Abdallah et al., 2016; Pan et al., 2015; Wu et al., 2018), including recent work in *A. glycines* (Xi et al., 2015). Our results suggest molecular responses vary depending on the route of insecticide exposure. Aphids may mount a stronger xenobiotic detoxification response when insecticides are ingested as opposed to absorbed through the cuticle.

In laboratory populations of *A. glycines* selected for pyrethroid resistance over multiple generations (Xi et al., 2015) increased expression of esterases and CYPs has been observed. In contrast, we measured transcriptional responses using a susceptible *A. glycines* clone with no prior history of exposure to insecticides. Although we found several CYPs differentially expressed in aphids exposed to insecticides (Fig. 3, Supplemental Table S7), detoxification was not a dominant feature of the overall transcriptional profile. It is possible that susceptible individuals exhibit a less pronounced detoxification response relative to insecticide resistant individuals or populations with a history of insecticide exposure, which may explain in part why our results differ from previous studies. For example, thiamethoxam resistant *A. gossypii* show heightened detoxification responses compared to susceptible individuals (Pan et al., 2015; Wu et al., 2018).

Finally, it is worthwhile to note that previous work reporting enhanced detoxification reponses in *A. glycines* used a targeted approach that measured expression of seven pre-selected genes with qRT-PCR (Xi et al., 2015), whereas the current study employed a global transcriptional approach using RNA-Seq. qRT-PCR based methods provide insight into differential expression of specific genes, which can be informative but biological relevance should be interpreted with caution. When viewed in the context of global transcriptional responses, our results demonstrate xenobiotic detoxification is part of a complex response involving many genes. RNA-Seq is useful for

uncovering broader gene expression patterns that can be investigated in depth using focused or targeted studies that employ qRT-PCR based methods.

Interestingly, we observed a dramatic burst of differential gene expression 12 h after exposure to insecticides that had largely subsided by 24 h post-exposure. Transcriptional spikes or "impulse responses" are a common phenomenon observed in many organisms in response to environmental stress or perturbations (Lopez-Maury et al., 2008; Yosef and Regev, 2011). For example, RNA-Seq studies have reported thousands of differentially expressed genes within a few hours of exposure to abiotic stressors in insects (Liu et al., 2017), plants (Lou et al., 2018) and fungi (Wang et al., 2017). Several studies also suggest stress-responsive genes are structured into distinct stages (e.g. early vs. late) and that transcriptional responses to various stressors become less pronounced over time (Bendjilali et al., 2017; Kawasaki et al., 2001; Sorensen et al., 2005). Widespread changes in gene expression that occur directly following exposure to stress are often transient and over time fine-tuned responses specific to the stressor are observed (Lopez- Maury et al., 2008). Our results suggest that the transcriptional stress response of *A. glycines* also potentially becomes more precise over time, which may help to balance physiological trade-offs with growth or reproduction, as prolonged stress responses can be energetically costly.

In conclusion, although our original hypothesis regarding enhanced detoxification under low-dose insecticide stress was not supported, our results suggest a complex process involving various metabolic pathways may be involved in *A. glycines* response to insecticides. Further research is needed to determine whether constitutive over-expression and/or induction of detoxification genes are the primary driver of observed insecticide resistance in North American populations of *A. glycines*.

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Appendix A. Supplementary data — Supplementary data to this article follows:

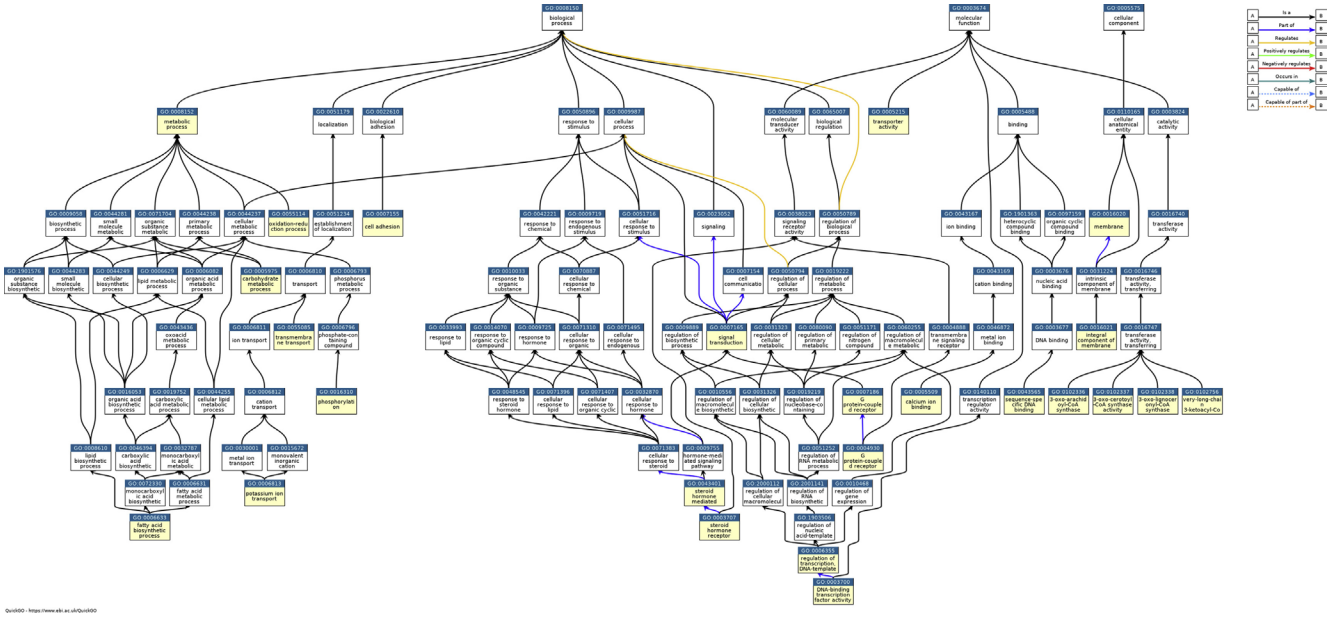

Fig S1. Ancestor chart of overrepresented GO terms associated with genes up-regulated 12 hours after exposure to esfenvalerate.

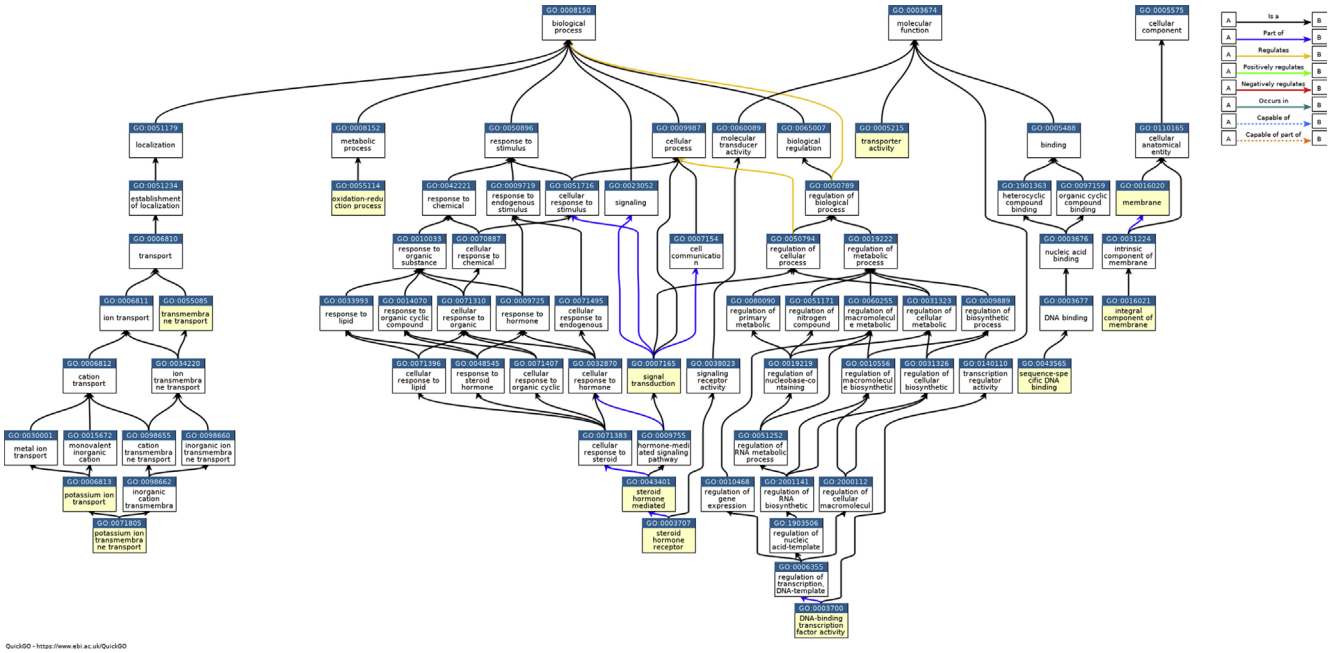

Fig S2. Ancestor chart of overrepresented GO terms associated with genes up-regulated 12 hours after exposure to thiamethoxam.

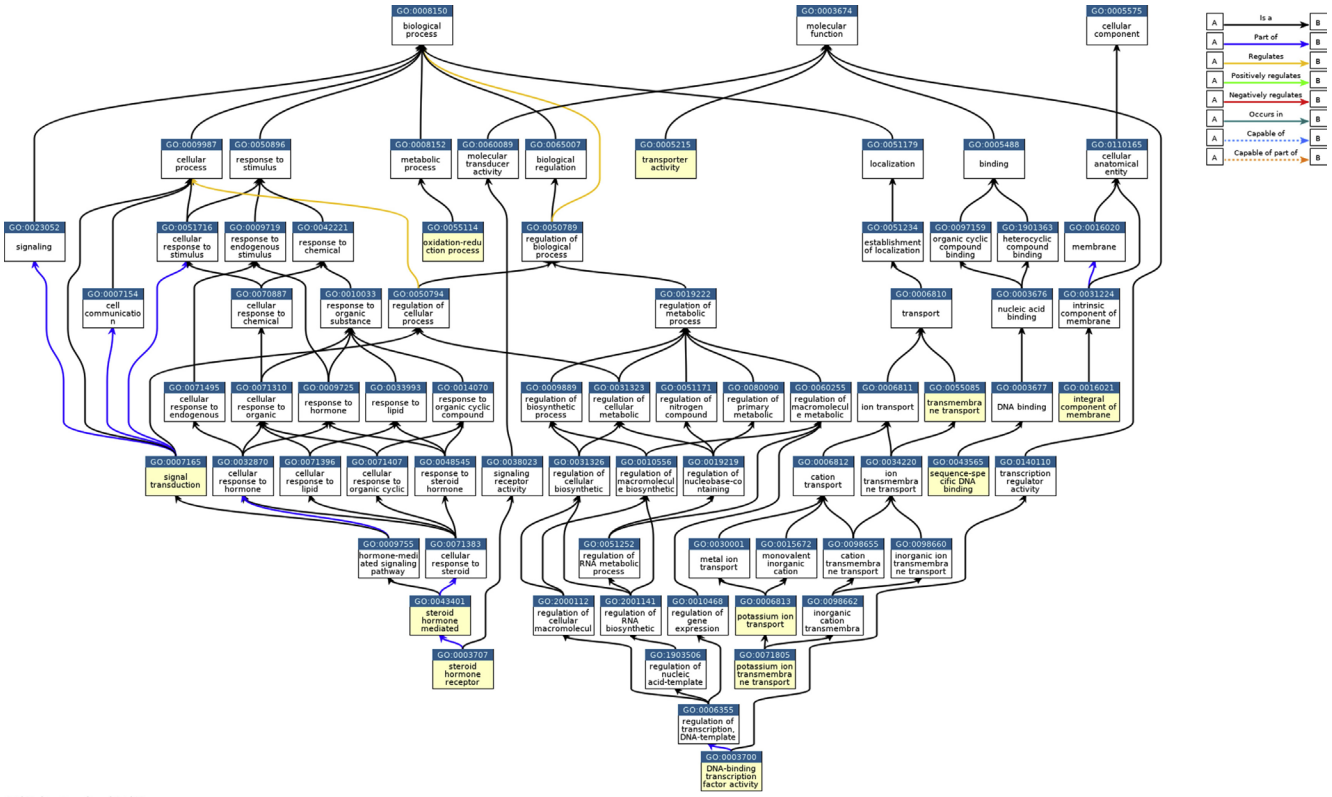

Fig S3. Ancestor chart of overrepresented GO terms associated with genes up-regulated 12 hours after exposure to esfenvalerate and 12 hours after exposure to thiamethoxam.

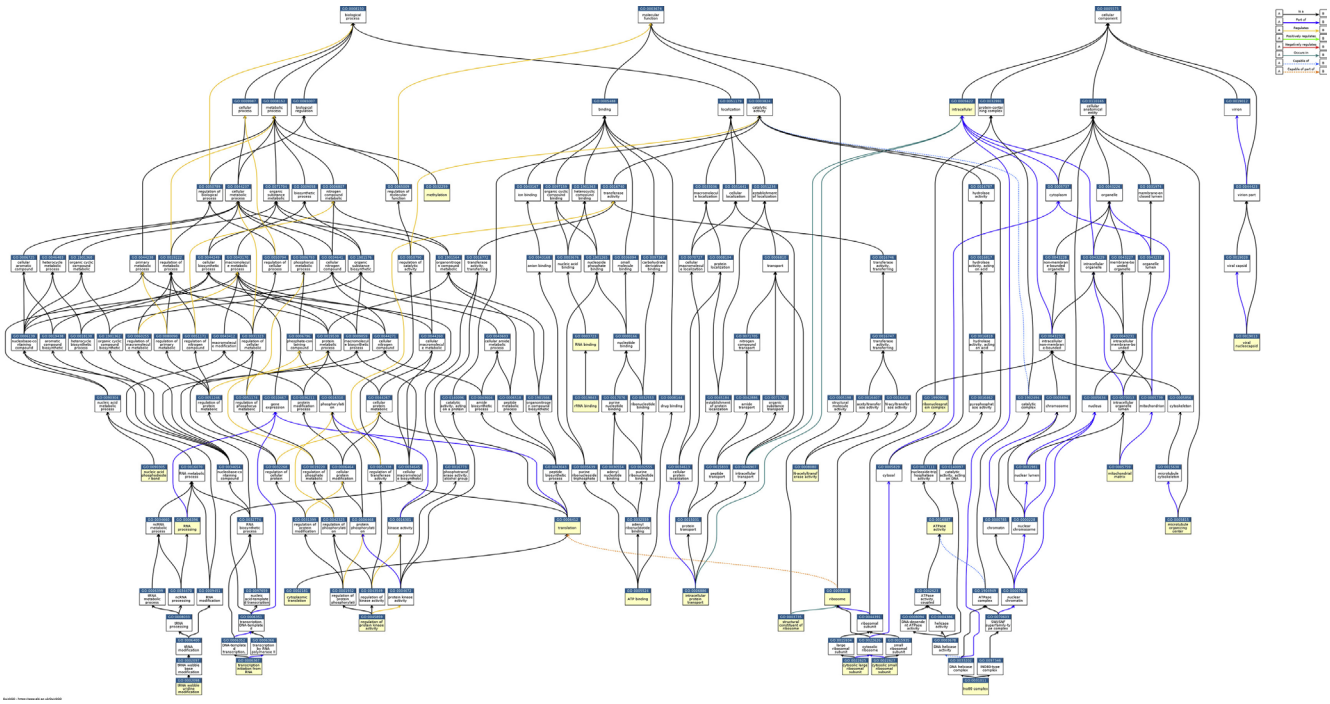

Fig S4. Ancestor chart of overrepresented GO terms associated with genes down-regulated 12 hours after exposure to esfenvalerate.

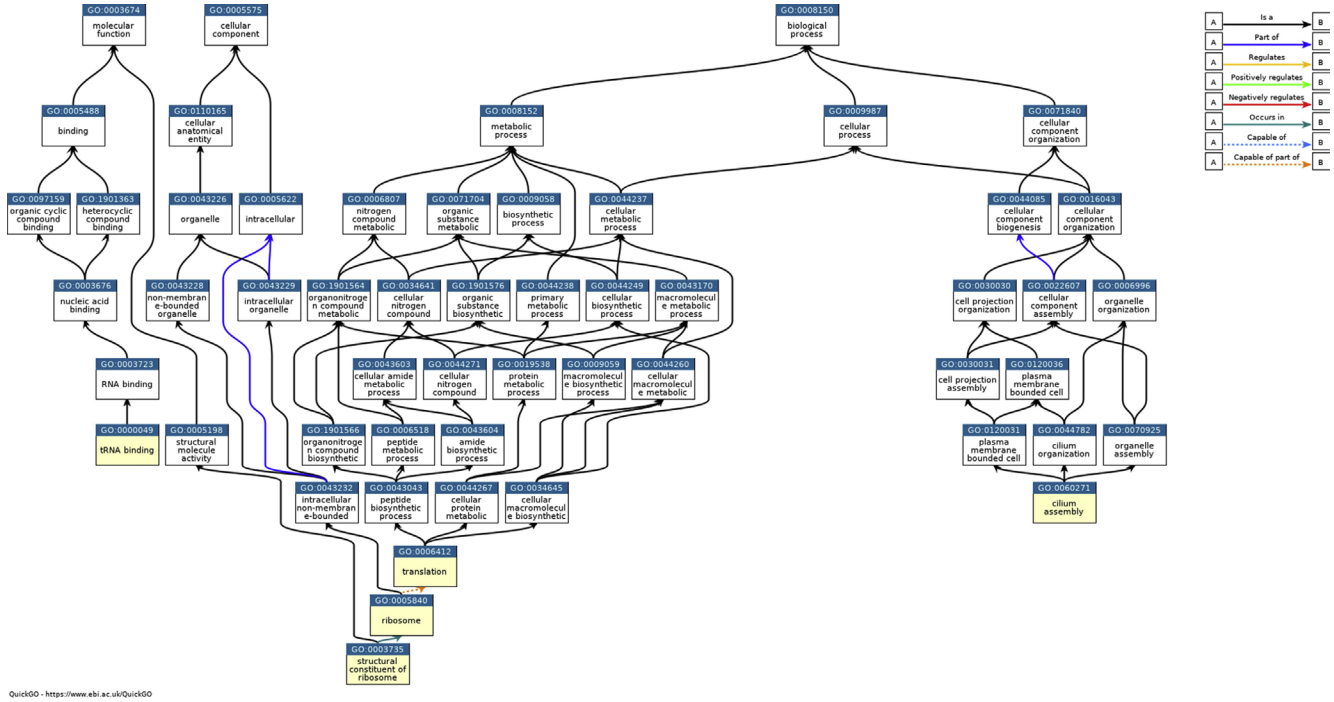

Fig S5. Ancestor chart of overrepresented GO terms associated with genes down-regulated 12 hours after exposure to thiamethoxam.

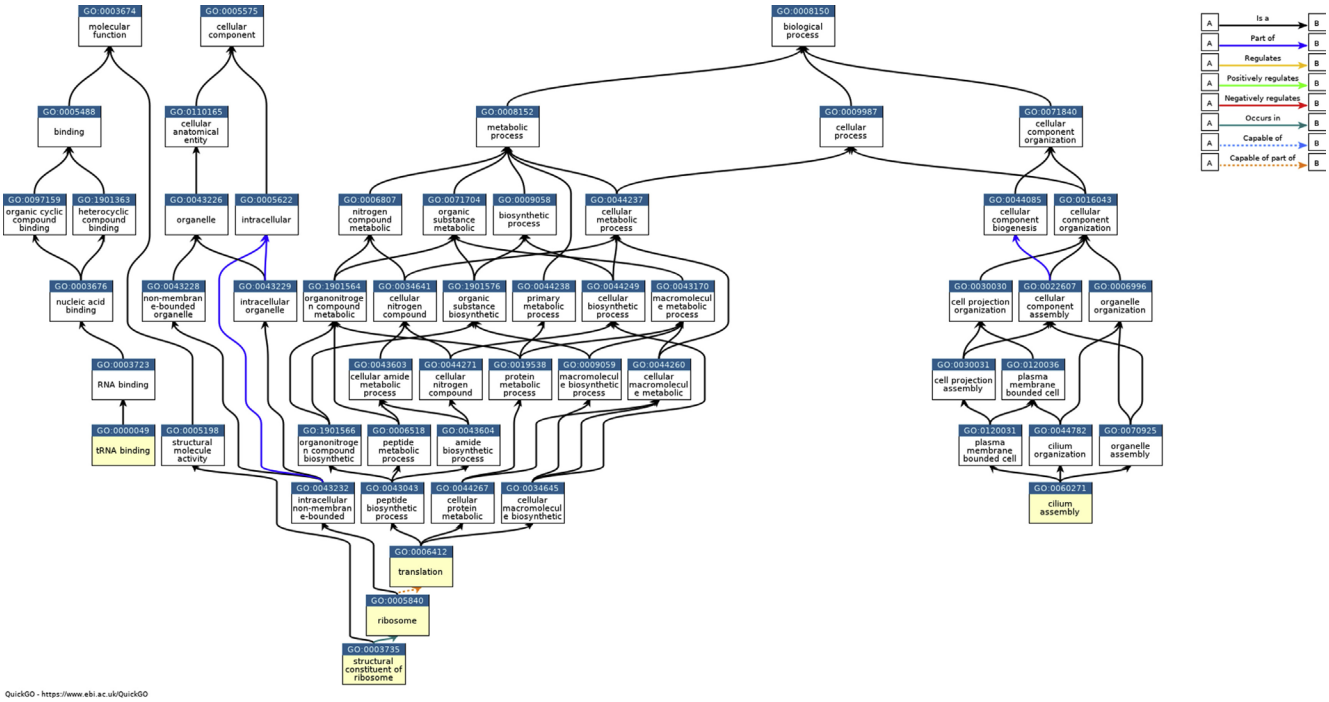

Fig S6. Ancestor chart of overrepresented GO terms associated with genes down-regulated 12 hours after exposure to esfenvalerate and 12 hours after exposure to thiamethoxam.

Supplemental Table S1. Inferred functions of the 20 most strongly up-regulated genes (relative to control) 12h after exposure to esfenvalerate.

AG6014660	Putative hexokinase. GO terms suggest possible role in glycolysis or glucose homeostasis. Maybe involved in energy metabolism, often seen as a common part of general stress response. Also strongly upregulated in response to thiamethoxam at 12h.
AG6034535	GO terms suggest membrane protein, possibly interacts with steroids?. Function unclear.
AG6026920	Putatively involved in alpha-tocopherol (vitamin E) transport. Alpha-tocopherol helps protect against oxidative stress in Drosophila, maybe general stress response.
AG6025731	Unknown.
AG6025720	IPS indicates a transmembrane protein, otherwise unknown.
AG6004183	Unknown
AG6029987	Probably a membrane protein, otherwise unknown.
AG6020723	Unknown
AG6031115	Unknown, B2G GO term suggests a DNA binding protein.
AG6022723	Unknown
AG6026921	Possibly involved in alpha tocopherol transport. *NOTE* Gene number is sequential with AG6026920, The two are adjacent in the genome. Blastx suggests 2 parts of the same gene.
AG6005892	Unknown.
AG6025759	Unknown.
AG6021535	Membrane-bound alkaline phosphatase.
AG6027850	The blast2Go "name" and interpro scan indicate tropomyosin, involved in muscle contraction. Blast2GO GO terms also include "nucleic acid binding" and "alanine-tRNA ligase activity". Also strongly upregulated in response to thiamethoxam at 12h.
AG6013434	Unknown.
AG6023616	Unclear, B2G GO terms hint at both nucleic acid binding and myosin complex / motor function.
AG6032677	Membrane protein. Possibly involved in G-protein based signaling.
AG6038717	Unknown.
AG6024921	Possible membrane-bound glycosyl hydrolase.

Supplemental Table S2. Inferred functions of the 20 most strongly up-regulated genes (relative to control) 12h after exposure to thiamethoxam.

AG6034535	Unidentified membrane protein
AG6014660	Putative hexokinase. GO terms suggest possible role in glycolysis or glucose homeostasis. Maybe involved in energy metabolism, often seen as a common part of general stress response. Also strongly upregulated in response to esfenvalerate at 12h.
AG6013188	Unidentified transmembrane protein.
AG6029987	Unknown.
AG6013778	Unknown.
AG6010228	GO terms indicate 1) hydrolysis of glycosidic bonds and/or glucose dehydrogenase activity, 2) FAD binding (a quick search on web of science indicates that FAD-dependent glucose dehydrogenases exist, although they mostly seem to have been isolated from fungi), 3) membrane protein, 4) structural constituent of cuticle. No obvious function, but could point to something in the integument, possibly involved in chitin metabolism / remodeling.
AG6022723	Unknown.
AG6031274	Unknown.
AG6031274	Unknown.
AG6027850	The blast2Go "name" and interpro scan indicate tropomyosin, involved in muscle contraction. Blast2GO GO terms also include "nucleic acid binding" and "alanine-tRNA ligase activity". Also strongly upregulated in response to esfenvalerate at 12h.
AG6008644	Unknown.
AG6020826	Unknown.
AG6034507	Probable membrane-bound Ras protein involved in GTP-mediated signal transduction.
AG6023670	Unknown.
AG6025517	Unknown.
AG6004602	Kiran Donthu's B2G GO terms are a mixed bag, membrane component, zinc ion binding, and DNA integration (ie.integration of one DNA molecule into another). Not obvious what this might be.
AG6032677	Probable membrane-bound Ras protein involved in GTP-mediated signal transduction.
AG6032370	Kiran Donthu's B2G GO terms point to both nucleic acid binding and endopeptidase activities. Not at all sure what to make of that.
AG6018719	Probable transcription factor.
AG6036254	Unknown.

Supplemental Table S3. Inferred functions of the 20 most strongly down-regulated genes (relative to control) 12h after exposure to esfenvalerate.

AG6026208	Metallopeptidase, possibly membrane-bound.
AG6028839	Unknown.
AG6000779	Unidentified transmembrane protein.
AG6029001	Unknown.
AG6039807	Unknown.
AG6039777	Unknown.
AG6036823	Unknown.
AG6007025	Unidentified membrane protein.
AG6023047	Unknown.
AG6023137	Unknown.
AG6041832	Unknown.
AG6004421	Unknown.
AG6042054	Unknown.
AG6021652	Unknown.
AG6002463	Unknown.
AG6042240	Unknown.
AG6021895	Unknown.
AG6003346	Unknown.
AG6012178	Unknown.

Supplemental Table S4. Inferred functions of the 20 most strongly down-regulated genes (relative to control) 12h after exposure to thiamethoxam.

AG6007487	Unknown.
AG6039732	Unknown.
AG6015398	Unidentified transmembrane protein.
AG6019493	Unknown.
AG6023047	Unknown.
AG6041137	Unknown.
AG6039777	Unknown.
AG6040592	Unknown.
AG6003610	Unknown.
AG6041833	Unknown.
AG6037173	Unknown.
AG6028214	Unknown.
AG6029325	Unknown.
AG6013956	Unknown.
AG6025557	Unknown.
AG6019953	Unidentified transmembrane protein.
AG6005035	Unknown.
AG6041257	Unknown.
AG6025708	Unknown.
AG6003610	Unknown.

Supplemental Table S5. Inferred functions of the 20 most strongly up-regulated genes (relative to control) 24h after exposure to esfenvalerate.

AG6035448	Unknown.
AG6015336	B2G GO terms suggest lipid binding/transport.
AG6001561	B2G terms seem a bit inconsistent, pointing to either FAD-dependent oxidoreductase *or* neuropeptide signalling. The latter is supported by IPS annotating as a pyrokinin, a family of insect neuropeptides that mediate visercal muscle contractions.
AG6006453	IPS finds fibronectin type III / immunoglobulin-like fold domain and an epidermal growth factor-like domain. These are found in a wide range of proteins so the function is unclear, possibly cell adhesion.
AG6025377	Probable transcription factor.
AG6025439	Looks like a member of the SMP-30/regulacin family. These proteins regulate cellular Ca^{2+}, could be involved in signaling but this is a big family with a wide variety of functions.
AG6025438	Probably a membrane-bound endopeptidase.
AG6024969	Unknown.
AG6015929	B2G GO terms point to heterocyclic compound binding. A lot of proteins associated with this GO term bind nucleotides or nucleic acids. Could be a transcription factor or other transcriptional regulator (supported by the b2g "name" being af4 fmr2 family member 4-like).
AG6035449	Possible membrane-bound protease.
AG6028764	Unknown.
AG6020067	Putative glucuronidation involved in phase II xenobiotic metabolism.
AG6006634	Unknown.
AG6013434	Unknown.
AG6008259	Probable actin-binding protein.
AG6008259	Membrane protein, possibly an acyl transferase.
AG6013625	Unknown.
AG6013554	Transcription factor.
AG6018859	Unidentified transmembrane protein.
AG6024968	Unknown.

Supplemental Table S6. Inferred functions of the 20 most strongly down-regulated genes (relative to control) 24h after exposure to esfenvalerate.

AG6010560	Unknown.
AG6010580	Unknown.
AG6010557	Unknown.
AG6008446	Possible protein kinase, maybe membrane-bound. This could just be a coincidence, but the B2G name "repetitive proline-rich cell wall protein 2" matches a Uniprot entry for a soybean protein!
AG6010559	Unknown.
AG6010564	Unidentified membrane protein.
AG6010562	Unknown.
AG6019022	Unknown.
AG6039736	Mitochondrial NADH dehydrogenase, part of ATP synthesis.
AG6014330	Unidentified membrane protein.
AG6010558	Unknown.
AG6015086	Unknown.
AG6029798	Unidentified membrane protein.
AG6029798	Transmembrane sugar transporter. B2G also gives a couple of GO terms related to processing small nuclear RNAs, which seems contradictory.
AG6003000	Possible cytochrome P450 monoxygenase.
AG6009434	Cuticle protein.
AG6025422	Unidentified membrane protein.
AG6006591	Unknown.
AG6002747	Peroxidase.

Supplemental Table S7. Expression levels of putative cytochrome P450 monooxygenase genes, relative to controls (log_2 fold-change). Asterisks indicate significant differential expression at a false-discovery rate of 0.1. “NA” indicates gene expression too low to be measured in either control or treatment conditions.

Gene	Esfenvalerate, 12h	Esfenvalerate, 24h	Thiamethoxam, 12h	Thiamethoxam, 24h
AG6008728	0.534	0.173	0.561	-0.057
AG6018178	1.301*	0.065	0.981	-0.018
AG6036432	-0.309	0.012	-0.122	-0.034
AG6027734	1.309*	0.034	1.141*	-0.1
AG6030583	0.673*	0.266	0.512	-0.053
AG6027735	0.816*	-0.004	0.348	-0.053
AG6026478	0.137	0.086	0.204	-0.118
AG6002505	0.384	0.117	0.409	-0.043
AG6019116	0.535*	0.535*	0.313	-0.028
AG6014576	-0.793*	0.292	-0.898*	-0.137
AG6013195	-0.884*	-0.012	-0.43	0.125
AG6030586	0.624*	0.409	0.375	-0.081
AG6018153	0.711	0.13	0.615	-0.044
AG6004782	-0.21	0.13	-0.202	0.056
AG6018487	0.697*	0.333	-0.203	-0.048
AG6002506	-0.25	-0.371	-0.503	0.039
AG6026424	0.409*	0.057	0.317*	-0.121
AG6018923	1.357*	0.636*	0.662*	0.055
AG6000572	-0.754*	0.083	-0.519	0.05
AG6011084	NA	0.052	0.006	-0.04
AG6030591	-0.401	-0.031	-0.494	0.118
AG6014305	-0.111	0.332	-0.053	0.105
AG6023746	1.413*	0.624*	0.723*	-0.011
AG6011346	-0.058	0.231	-0.112	-0.137
AG6035846	1.32*	0.057	0.948*	-0.096
AG6012485	1.105*	0.214	0.65*	-0.094
AG6022965	-0.368	0.208	0.155	0.028
AG6035848	1.231*	0.578*	0.303	-0.076

Supplemental Table S7, continued.

AG6011050	-0.607*	-0.17	-0.303	-0.013
AG6034428	0.672	0.389	0.752*	0.07
AG6003000	0.394	-0.638*	0.547	-0.225
AG6003479	0.303	0.135	-0.263	0.069
AG6036333	1.835*	0.201	0.812*	-0.011
AG6006129	-0.662*	-0.269	-0.468*	0.043
AG6016439	1.496*	0.428	0.613	0.058
AG6036334	1.005*	0.116	0.671*	-0.115
AG6002794	0.554	0.221	0.574	0.048
AG6023846	-0.031	0.078	-0.241	0.045
AG6002795	0.468	0.117	0.245	-0.087
AG6001356	-0.263	0.1	0.072	-0.002
AG6018477	1.087*	0.403	0.706*	0.002
AG6005201	0.813*	0.223	0.686*	-0.035
AG6018927	0.391	0.191	0.055	-0.086
AG6000576	0.799*	0.473	0.811*	-0.09
AG6005202	-0.668*	-0.156	-0.456	-0.067
AG6030787	0.988*	0.079	0.717	0.026
AG6018478	-0.22	-0.01	-0.058	-0.038
AG6001358	-0.48*	-0.183	-0.365	-0.067
AG6019229	0.549	0.256	0.233	-0.074
AG6036430	0.95*	-0.247	0.677*	-0.068
AG6018481	0.878*	0.31	0.449	-0.031
AG6019231	1.488*	0.509	0.839*	0.034
AG6018931	-0.191	0.082	-0.105	-0.032
AG6039218	0.363	-0.041	0.141	-0.044

Supplemental Table S8. Expression levels of putative glutathione-S-transferase genes, relative to controls (log_2 fold-change). Asterisks indicate significant differential expression at a false-discovery rate of 0.1.

Gene	Esfenvalerate, 12h	Esfenvalerate, 24h	Thiamethoxam, 12h	Thiamethoxam, 24h
AG6027400	-0.361	-0.133	-0.115	0.045
AG6022120	0.374	-0.079	0.323	-0.05
AG6022314	-0.195*	0	-0.118	0.074
AG6025610	-0.164	-0.174	-0.126	-0.125
AG6018942	-0.221	-0.243	-0.057	-0.076
AG6013975	0.07	-0.059	0.078	0.027
AG6018943	0.941*	0.155	0.582	-0.074
AG6003443	0.443*	0.02	0.451*	-0.043
AG6024616	-0.023	0.031	-0.123	-0.085
AG6011298	-0.392*	-0.231	-0.248	-0.193
AG6024617	1.09*	-0.198	0.761*	-0.124
AG6011300	-0.05	0.027	0.219	-0.147
AG6021936	0.102	-0.022	0.02	-0.091
AG6003576	0.078	0.03	-0.066	-0.111
AG6013069	-0.323*	-0.181	-0.231	0.032

Supplemental Table S9. Expression levels of putative carboxylesterase genes, relative to controls (log_2 fold-change). Asterisks indicate significant differential expression at a false-discovery rate of 0.1.

Gene	Esfenvalerate, 12h	Esfenvalerate, 24h	Thiamethoxam, 12h	Thiamethoxam, 24h
AG6004867	0.64*	-0.227	0.867*	-0.091
AG6032899	1.399*	0.392	1.02*	0.022
AG6010355	-0.541*	-0.222	-0.139	-0.041
AG6032900	0.458	0.133	0.128	-0.068
AG6010358	-0.168	0.212	-0.2	0.149
AG6007844	-0.441*	-0.338	-0.092	0.005
AG6007846	-0.447*	-0.036	-0.239	0.069
AG6033369	0.084	-0.127	0.158	-0.07
AG6002269	-0.137	0.017	-0.087	-0.082
AG6015193	0.809*	0.252	0.493*	-0.052
AG6015032	0.432	0.257	0.132	-0.005
AG6007848	0.737*	0.194	0.52	-0.007
AG6002272	0.42	0.155	-0.126	-0.032
AG6023925	0.522	0.288	0.338	0.034
AG6007850	-0.473*	-0.444	0.008	-0.054
AG6007582	1.176*	0.205	1.001*	-0.038
AG6023928	0.523	0.297	0.188	0
AG6023929	0.484	0.147	0.142	-0.003
AG6023930	1.102*	0.147	0.661	0.021
AG6023931	0.684*	0.435	0.326	0.126
AG6015464	0.008	0.05	-0.021	-0.087
AG6025282	0.47*	0.021	0.311	-0.201
AG6015465	-0.392*	-0.221	-0.277	-0.049
AG6011356	0.133	0.262	0.028	0.034
AG6016049	-0.066	-0.334	0.096	-0.11
AG6015196	0.018	0.037	0.165	-0.024
AG6025283	0.898*	0.093	0.194	-0.083
AG6016051	0.552	-0.031	0.445	-0.042
AG6008253	-1.04*	-0.254	-0.57*	0.049
AG6015019	1.176*	0.568*	0.856*	0.023
AG6001208	1.379*	0.461	0.998*	0.038
AG6013217	0.349	0.225	0.14	-0.081
AG6007836	0.578*	0.087	0.388	-0.071
AG6010351	-0.824*	-0.071	-0.391	0.018
AG6015021	1.605*	0.409	1.221*	0.051

Supplemental Table S10. Expression levels of putative ABC-transporter genes, relative to controls (log_2 fold-change). Asterisks indicate significant differential expression at a false-discovery rate of 0.1.

Gene	Esfenvalerate, 12h	Esfenvalerate, 24h	Thiamethoxam, 12h	Thiamethoxam, 24h
AG6015836	-0.543*	-0.072	-0.019	0.093
AG6005556	-0.889*	-0.11	-0.438*	0.003
AG6024387	0.367	0.119	-0.055	-0.102
AG6015816	-0.863*	-0.062	-0.662*	-0.071
AG6015846	0.16	0.24	0.044	0.149
AG6022731	0.965*	0.293	0.4	-0.036
AG6006018	-0.351	-0.032	-0.161	-0.027
AG6018120	-0.289	0.307	-0.085	0.128
AG6008081	-0.126	-0.122	-0.084	-0.079
AG6015849	0.093	-0.065	0.216	-0.192
AG6028993	-0.15	-0.135	0.006	-0.044
AG6018122	0.628*	0.288	0.577*	-0.056
AG6005630	-0.637*	0.015	-0.465	-0.021
AG6022734	-0.002	0.052	-0.036	0.051
AG6021094	0.099	-0.001	0.202	-0.029
AG6024391	0.749*	0.188	0.457	-0.107
AG6003072	-0.666*	-0.261	-0.197	-0.094
AG6018123	0.658*	0.425	0.722*	-0.012
AG6012176	-0.356	-0.105	-0.367	-0.134
AG6004243	0.464*	0.14	0.431	-0.039
AG6002809	-0.863*	-0.03	-0.385	0.019
AG6005634	0.034	0.031	-0.128	-0.049
AG6013168	0.332*	0.117	0.044	0.032
AG6016394	-0.536*	-0.073	-0.314	0.039
AG6015854	-0.224	-0.287	-0.115	0.015
AG6010062	0.185	0.137	0.231	-0.048
AG6021099	0.54*	0.089	0.221	-0.074
AG6026114	-0.097	-0.119	0.265	0.004
AG6007859	0.513*	0.022	0.308	-0.075

Supplemental Table S10, continued.

AG6019810	-0.161	0.001	0.015	-0.051
AG6002811	0.196	0.087	0.186	-0.039
AG6038833	-0.693*	-0.292	-0.459*	-0.001
AG6005637	-0.849*	-0.159	-0.359	0.018
AG6005007	0.009	0.088	0.02	-0.07
AG6028243	0.609*	0.198	0.288	-0.056
AG6020653	-0.857*	-0.172	-0.538*	0.036
AG6005639	-0.86*	-0.343	-0.383	-0.069
AG6005640	-1.206*	-0.331	-0.426	0.051
AG6000325	-0.398*	-0.135	-0.146	-0.046
AG6013619	-0.774*	-0.194	-0.266	-0.065
AG6007314	-0.862*	-0.229	-0.39	0.018
AG6010039	-0.371	0.259	-0.452	-0.058
AG6002818	0.177	0.066	0.306	0.007
AG6024759	0.21	0.122	-0.008	0.098
AG6001018	0.2	0.264	0.31	-0.022
AG6011620	0.294*	0	0.174	-0.121
AG6025931	0.081	0.062	0.056	-0.012
AG6011141	-0.231	0.078	-0.115	0.05
AG6015834	0.123	-0.197	0.408	-0.015
AG6014740	-0.569*	-0.136	-0.301	0.024
AG6023872	-0.44*	-0.087	-0.229	-0.001
AG6015838	0.044	0.036	-0.048	0.015
AG6019811	0.158	0.144	0.414	-0.043
AG6019812	-0.083	-0.386	0.159	-0.179
AG6018416	0.228	0.237	0.003	-0.064
AG6015840	-0.484	-0.245	0.109	0.131
AG6005560	-0.674*	-0.214	0.046	-0.079
AG6012164	1.339*	0.294	0.965*	-0.038
AG6008223	0.103	-0.025	0.114	-0.004
AG6013156	-1.13*	-0.293	-0.859*	0.054
AG6015842	-0.459	-0.109	-0.277	0.057
AG6005562	-0.335*	-0.011	0.312	-0.041
AG6019109	-1.104*	-0.159	-0.575*	-0.019
AG6030986	-0.325	-0.006	-0.111	0.181
AG6003905	0.188	0.083	0.189	-0.067
AG6019757	-0.438*	-0.132	-0.292*	-0.033
AG6026683	-0.075	0.048	-0.002	-0.09
AG6018420	0.823*	0.165	0.527*	-0.113
AG6013159	-0.88*	-0.246	-0.692*	0.069