Development of exposure biomarkers for the honey bee (*Apis mellifera*): neonicotinoids versus traditional pesticides

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Abstract

Pollination is a vital ecosystem service crucial for reproduction of flowering plants, including agricultural crops. The western honey bee, *Apis mellifera,* is the most used managed pollinator worldwide. The use and overuse of agrochemicals is hypothesized to have played a role in increasing rates of colony mortality in Canada and globally. The identity of stressors affecting a colony is difficult to discern; information critical for diagnosing and managing honey bee colony health. Here, I explored the potential of using gene expression profiles as diagnostic biomarkers for exposure to various agrochemicals in honey bees. I found genes differentially expressed unique to each stressor, which could be putative biomarkers for specific agrochemical exposure. I found genes common between pesticides, which could be a putative general agrochemical stress signal. My research indicates that gene expression profiles can be an excellent tool for discovering stressor-specific biomarkers and diagnosing stressors found in honey bee colonies.

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Introduction

Pollination is a key process in agriculture because it is vital for the reproduction of flowering plants (Calderone, 2012). The eusocial western honey bee, *Apis mellifera,* is the only honey bee in North America and is the most common managed pollinator in the world (Delaplane et al, 2000). Globally, managed pollination is a massive and growing industry, expanding from $200 billion USD in 1993 to $300 billion USD in 2009 (Lautenbach et al, 2012). Through their pollination services, Canadian honey bees contribute $3.97 to $5.5 billion to the national economy each year (Agriculture and Agri-Food Canada, 2016). In addition to pollination, honey bees also produce honey. In 2020, Canada's almost 12,000 beekeepers produced 82 million pounds of honey which is valued at $208 million (Statistics Canada, 2020). However, recent declines in honey bee populations have created a global concern for both the well-being of the species as well as our food security (Ollerton, 2017).

Unfortunately, honey bees are susceptible to a wide range of stressors, which include parasites, pathogens, toxicants such as pesticides used in agriculture, and lastly malnutrition (Ratnieks & Carreck, 2010; Goulson et al, 2015). Furthermore, these stressors interact with each other, which in some cases can increase their detrimental effects (O'Neal et al, 2018). For instance, pesticides can impair the honey bee's immune system, reducing their ability to defend against parasites (Goulson et al, 2015). These stressors have resulted in increasing rates of colony mortality worldwide (Gregorc, 2020). Canadian beekeepers have been losing more than a quarter of their colonies annually since 2006 (CAPA, 2019).

Pesticide exposure plays an important role in honey bee health (Johnson, 2015). Traditional pesticides are typically applied to crops via a spray to crop foliage or in some cases, by drenching the soil (Bateman, 2003). Emerging systemic pesticides are often applied as a coating to seeds prior to planting (Blacquière et al, 2012). If applied as a seed coating, systemic pesticides are spread through the plant as it develops, including its pollen and nectar (Fairbrother et al, 2014). Systemic pesticides are typically water soluble (Sanchez-Bayo et al, 2013) and consequently, they can easily spread throughout the environment via surface water runoff from agricultural fields, leading to widespread uptake and expression of these pesticides by off-target plants (Simon-Delso et al, 2015). A honey bee worker can directly encounter agrochemicals while foraging, and then expose the colony upon her return (Blacquière et al, 2012).

One recent class of insecticides, called neonicotinoids, have been implicated in reducing honey bee health in the field (Tsvetkov et al, 2017; Schneider et al, 2012; Sandrock et al, 2014; Straub et al 2021). Developed in the 1990's, neonicotinoids are related to the natural insecticide nicotine (Mencke & Jeschke, 2002). Like nicotine, neonicotinoids target the nicotinic acetylcholine receptor (nAChR), and act as agonists by binding permanently to the receptor (Tomizawa & Casida, 2003). This results in neural dysfunction leading to changes in behaviour (Ludicke & Nieh, 2020), altered development (Siefert et al, 2020), or death (Baines et al, 2017). The systemic nature of neonicotinoids also presents the issue of off-target plants becoming exposed (Botías et al, 2015). This environmental contamination provides an enduring exposure resulting in a significant reduction in several honey bee traits (Tsvetkov et al, 2017). Clothianidin and thiamethoxam represent two of the most common neonicotinoids used in agriculture

(Bass et al, 2015). Interestingly, clothianidin and thiamethoxam were among several agrochemicals banned in the European Union in 2013 (Stokstad, 2018). Unfortunately, both chemicals, among hundreds of others, are still registered for use in Canadian agriculture (Health Canada, 2019a, b).

Current pesticide monitoring methods include predicting exposure from a crop application/landscape perspective (Simon-Delso et al, 2017), as well as using a hazard quotient to estimate risk from contaminated pollen and nectar (Wen et al, 2021). Although these methods are well developed, there is recent warning against the use of the hazard quotient to measure exposure (Thompson, 2021). Transcriptomic methods may provide a more immediate picture of what stressors are affecting the colony at the time of sampling. Utilizing genomics is the leading edge of bee stewardship (Grozinger & Zayed, 2020); Wang et al (2019) published a proof-of-concept metabolomics approach to biomarker development for nutritional stress in the bumblebee, *Bombus terrestris*.

A central goal of my research is the development of honey bee biomarkers based on gene expression. The use of biomarkers offers an avenue for rapid and accurate determination of stressors affecting organisms (Griffiths et al, 2002). Biomarkers are a common tool used in human diagnostics and facilitate accurate prediction of specific conditions or diseases (Califf, 2018). A common example of the use of biomarker in human healthcare is the measurement of cholesterols to assess cardiovascular health (Gordon et al 1977; Assmann et al 1996). Furthermore, gene expression-based biomarkers are widely used

in humans to evaluate risks in prescribing medication (Liu et al, 2019) as well as detecting novel cancer subtypes from biopsies (Cieślik & Chinnaiyan, 2018).

There have been a few instances of honey bee gene expression being used as potential biomarkers in the past. *Defensive* and *relish* are two immune genes proposed to be differentially regulated under pesticide stress (Badiou-Beneteau et al., 2012; Skaldina and Sorvari, 2017). Detox enzymes, *esterase* and *glutathione S-transferase* have been used as biomarkers in other insects but aren't great for biomarkers in honey bees (Lopez-Uribe et al, 2020), as validation of these returned conflicting results (Carvalho et al, 2013; Pokhrel et al 2018). Perhaps, our focus on using highly studied genes involved in detox as biomarkers prohibits the discovery of more robust biomarkers. Even more recently, Decio et al (2021) tested the effect of thiamethoxam exposure on honey bees using the expression of only 7 genes, including *esterases* and *glutathione S-transferase*. There exists an untapped potential in the understudied genes for use as potential biomarkers. Studies that use whole transcriptome to screen for potential biomarkers are much more likely to detect robust biomarkers.

The objective of my thesis is to discover molecular biomarkers for pesticide exposure. I will accomplish this by experimentally exposing honey bees to agrochemicals, followed by transcriptomic analyses to discover genes that are differentially regulated in the exposed honey bees. I have chosen four insecticides to study. Thiamethoxam and clothianidin are two common and systemic neonicotinoid insecticides, while spinetoram and spirotetramat are non-neonicotinoid insecticides. Spinetoram is a spinosyn whose

mode of action is similar to neonicotinoids because it also targets the nAChR, except at an allosteric site (different from the active binding site) (Bacci et al, 2016). Spirotetramat is a tetramic acid derivative that inhibits lipid biosynthesis (Brück et al, 2009). I predict that exposure to the above-mentioned insecticides will elicit a gene expression response in honey bees. Differentially regulated genes (DEG) may be related to either the symptoms that agrochemicals are exerting on the honey bees, or they could reflect the activation of the honey bee's detoxification system to respond to exposure. There may be common DEGs across different chemicals as well as DEGs that are specific to individual compounds. These unique DEGs could arise if the different insecticides cause different symptoms or are detoxified by different enzymes. I plan to investigate the functional role of DEGs based on knowledge gleaned from *Drosophila melanogaster* (Gramates et al, 2022).

The enzymes responsible for detoxification in animals are categorised into the following gene families: *cytochrome P450's* (*P450*), *carboxylesterases* (*COE*), *glutathione transferases* (*GST*), *uridine 5'-diphospho-glucuronosyltransferases* (*UGT*), and *ATP-binding cassette* (*ABC*) transporters (Li et al., 2007; Berenbaum & Johnson, 2015). These enzymes participate in the 3 phases of insect detoxification: functionalization, conjugation, and excretion. During functionalization, enzymes, mostly the P450's (Claudianos et al, 2006), modify toxicants to prevent them from reacting with their lipophilic sites (Berenbaum & Johnson, 2015). Afterwards, in the conjugation phase, the products from the first phase are further modified for solubilization and transport (Xu et al, 2013). *GSTs* are primarily responsible for conjugation but can also engage in

functionalization (Berenbaum & Johnson, 2015). The *GST* enzymes work to detoxify agrochemicals and metabolites via conjugation of the thiol functional group from glutathione into reactive oxygen species; this acts to increase the molecules' water solubility and targets them for removal via multidrug exporters (Li et al, 2007). Lastly, in the third phase, products from the previous phase are targeted for excretion from the cell (Dermaauw & Van Leeuwen, 2014).

The honey bee has far fewer detox genes than other sequenced insects such as the silk moth *Bombyx mori*, the parasitoid wasp *Nasonia vitripennis*, and the fruit fly *Drosophila melanogaster* (Ahn et al, 2012). The honey bee's lack of detoxification genes may render honey bees more susceptible to pesticide toxicity (Claudianos et al, 2006). However, this corollary has caused some debate in the literature. A review by Hardstone & Scott (2010) found that honey bees have pesticide resistance comparable with other insects, however, others have hypothesized that a smaller reservoir of detox genes may result in a reduced capacity to metabolize multiple agrochemicals simultaneously (Johnson et al, 2009; Johnson et al, 2012). While honey bees have a reduced complement of detoxification genes, they still contain members of the three major enzyme families (*P450s, GSTs, COEs, UDPs*) (Johnson et al, 2006; Johnson et al, 2009; Johnson et al, 2012; Johnson et al, 2018; Mao et al, 2009; Mao et al 2011). Notably, three *P450* genes *CYP9Q1*, *CYP9Q2*, and *CYP9Q3* were the first honey bee *P450's* to be functionally characterized (Mao et al. 2009); and polymorphisms in these genes have been implicated in differential detoxification properties (Tsvetkov, 2021).

Currently there is a knowledge gap in biomarkers for specific pesticide exposures. Developing such a tool could promote more beekeepers to discover if their bees have been poisoned by pesticides. Filling this gap would provide help for beekeepers to better manage their hives, which would benefit the Canadian agricultural system. The experiments carried out herein will also build on a much larger effort – the BeeCSI project (BeeCSI.ca) – to develop stressor-specific biomarkers to diagnose honey bee health.

Methods

Cage experiments for acute and sublethal exposure

We carried out experiments on nurse bees (i.e., young workers who feed the developing brood) for several reasons. Firstly, they are easy to identify and represent a distinct subcaste of worker bees that have similar behaviour and age (Winston, 1991). Previous research has shown that within a subcaste like nurses, gene expression is relatively insensitive to age (Whitfield et al, 2006) – that is old nurses and young nurses share very similar gene expression profiles. Lastly, nurse bees act as a central hub for pesticide transmission given their roles of food digestion, jelly synthesis, and feeding other castes of the hive (Winston, 1991). Nurse bees were collected into cages with soft forceps from healthy hives that have laying queens. For each agrochemical, we sampled from five distinct hives representing 5 biological replicates, which surpasses the minimum set for gene expression studies (N = 3) (Conesa et al, 2016). Following standard methods (Williams et al, 2013), we set up 3 experimental cages filled with 60 bees each for each replicate for each agrochemical. These cages were either exposed to the control dose, the low dose (sublethal, equal to ¼ high dose), and the high (acute, equal to LD50 or

greatest amount that can be dissolved in acetone) dose. The doses were delivered by oral exposure via spiked 50% sugar syrup. The cages were placed in an incubator at 33 °C for 2 hours in order to starve the bees. The bees were assessed for mortality and deceased bees were removed. Next, two pre-weighed feeder tubes were added to each cage to allow the bees to consume either the control dose, the high dose, or the low dose (Table 2). After 6 hours of exposure, we replaced all feeders with 50% sugar syrup for an additional 2 hours, after which the bees were again assessed for mortality and live bees were euthanized on dry ice and then stored at -80 °C for gene expression analysis. Only bees that survived the trials were used to avoid RNA degradation in dead tissues, and to avoid transcription signals associated with the near-death condition. The latter accomplished by feeding the bees on only 50% sugar syrup for the final 2 hours to not accidentally sample bees that are near death. The feeders were weighed before and after to calculate consumption. The above experimental procedure was conducted for 4 agrochemicals (Table 1). The doses for the treatments were selected to represent the LD50 (high dose) or ¼ of the LD50 (low dose); the exception to this is spirotetramat whose high dose is the greatest concentration that can be dissolved, and the low dose is ¼ of that.

Dissection, RNA extraction, and sequencing

From each biological replicate, 10 honey bees were dissected into three tissue categories: the head (antennae removed), the abdomen (gut and stinger removed), and the midgut. The head was chosen as it contains the brain, a major sensory organ and main target of neonicotinoids (Moffat et al, 2016). The abdomen tissue is important for

hemolymph circulation (Schwab et al, 1991) and contains fat bodies which function in protein synthesis and pesticide detoxification (Arrese & Soulages, 2010). Lastly, the midgut was chosen due to its role in pesticide metabolism (Mao et al, 2011). Within each replicate and tissue type, we pooled dissections from the 10 individuals to generate a single sample for each tissue and biological replicate. Pooling is important to reduce within-group variation; thereby generating a balanced representation of the population of bees within a hive (Takele Assefa et al, 2020). To that end, Tekele Assefa et al. (2020) found that variance decreases non-linearly when pool size increases and thus increasing pool sizes beyond 10 offers little variance reduction. These pools were then homogenized with ceramic beads in a Fisherbrand Bead Mill 24 (Thermo Fisher) according to the RNAeasy (Qiagen) manual and then RNA was extracted with the KingFisher Flex system according to the NucleoMag RNA kit (Thermo Fisher). Purified RNA samples were then sent to Genome Quebec (Montreal, Quebec) for library preparation and paired-end sequencing on a NovaSeq 6000 with an average read depth of 50 million.

Statistical analysis and visualization

To process the raw sequencing data, we aligned the reads to the current honey bee genome (Amel HAv3.1; Wallberg et al, 2019) using STAR v2.9.7a (Dobin et al, 2013) with default parameters. Afterwards, the generated binary alignment and map (BAM) files were used to generate count matrices via HTSeq-Count v0.13.5 (Anders et al, 2014) with parameters: non-strandedness and the feature counting mode was intersection-nonempty. Count files are human readable, easily interpreted and serve as input files for differential expression statistical analysis. The count files were downloaded and imported into RStudio (RStudio Team, 2020) to perform differential expression analysis with

DESeq-2 v1.32.0 (Love et al, 2014). The model used to explain expression included the hive genetics as a blocking factor (Equation 1).

$$Expression \sim Treatment + Hive\ genetics \quad (1)$$

Here the expression term represents the normalized gene counts vector for each sample, the treatment term categorizes samples as exposed or control, and the hive genetics term controls for the batch effect of the different hives. This was important because not only did we found an association of treatment and control samples based on the hive they were sampled from, but also there has been documented effects of genetics on pesticide sensitivity (Rinkevich et al, 2015). DEG's were determined from the model if after a Benjamini-Hochberg correction they had an adjusted p-value (FDR) less than 0.1. We used an FDR of <0.1 instead of 0.05, as it is an increasingly common practice in RNA-Seq studies (Wiśniewska et al, 2022; Xiao et al 2022; Simmons et al, 2022), and is the default and recommended setting in DESeq-2 (Love et al, 2014). DEG lists from each experiment were used in several overlap and distinction analyses, including both union sets across tissues and tissue specific subsets. Additionally, during the final analysis for putative biomarkers all subsets were considered: tissue and dose and pesticide.

DEGs (FDR 0.1) and background gene lists (significant and non-significant combined) were extracted from the analysis of each agrochemical experiment. The honey bee gene lists were converted to *Drosophila melanogaster* homologues using HymenopteraMine (Walsh et al, 2022). The homologues were then converted to Flybase gene IDs using the Flybase ID Validator webtool (Grammates et al, 2022). These gene lists were entered into the Database for Annotated, Visualization and Integrated Discovery (DAVID) for

functional enrichment analysis (Huang et al, 2009; Sherman et al, 2021). The DAVID platform produced enrichment associations between DEGs and gene ontology functional terms.

The Venn diagrams were computed and drawn with the VENN Diagram webtool (Van de Peer Lab, n.d.). The bar charts and the principal components analysis were performed and plotted in RStudio (RStudio Team, 2022) with the ggplot2 package (Wickham 2016). The volcano plots were generated in RStudio (RStudio Team, 2022) using base R (R Core Team, 2022) functions. The gene ontology summary visualizations were generated and plotted using the python library GO-Figure! (Reijnders & Waterhouse, 2021), where the gene ontology results were plotted onto semantic space where terms with similar functions are clustered, and terms are redundantly reduced.

Results

We exposed western honey bee nurses (*Apis mellifera*) to four different pesticides dissolved in 50% sugar water (clothianidin, thiamethoxam, spirotetramat, and spinetoram) at two doses each under controlled cage conditions and measured their RNA expression in different tissues (Table 2). Bees in control cages were fed only 50% sugar water. The gene expression of the exposed bees for each experiment was compared to that of the control bees. Overall, the high dose experiments elicited a stronger gene response (respective to the controls) than their low dose counterparts for every pesticide (Table 3). In total there were 4, 47, 124, and 306 DEG's that had adjusted p values below the 0.1 threshold during the low dose clothianidin, thiamethoxam, spirotetramat, and spinetoram experiments, respectively. For the high dose experiments, the total number

of DEG's that had adjusted p values below the 0.1 threshold were 319, 116, 328, and 1503 during the clothianidin, thiamethoxam, spirotetramat, and spinetoram experiments, respectively. The $\log_2$ fold changes of the DEGs ranged from -0.772 to 3.872 for clothianidin exposure, from -2.759 to 1,316 for thiamethoxam exposure, from -3.072 to 5.365 for spirotetramat exposure, and from -2.245 to 4.494 in spinetoram exposure. During the low dose clothianidin and thiamethoxam experiments, the greatest number of DEGs were found in the gut (Table 3). For the low dose exposure to spirotetramat, the greatest number of DEGs were found in the abdomen (Table 3). At both doses, exposure to spinetoram resulted in the greatest number of DEGs in head (Table 3). For the high dose clothianidin and thiamethoxam experiments, the greatest number of DEGs were found in abdomen (Table 3). Lastly, for the high dose spirotetramat experiment, the greatest number of DEGs were found in the head (Table 3).

To visualize the effect of exposure on gene expression more broadly, we plotted the expression data using volcano plots (Figures 1-2), where the y-axis represents the $-\log_{10}$ p values of the differential expression analysis for each gene after Benjamini-Hochberg correction for multiple testing, and the x-axis depicts the range of differential expression in units of $\log_2$ fold change. The low dose experiments exhibited relatively fewer differentially expressed genes for clothianidin and thiamethoxam, more from spirotetramat and most for spinetoram, with no overall trend for enrichment of DEGs in any specific tissue. For the high dose experiments, all pesticides generated volcano plots with greater y-axis peaks than their low dose counterparts. The majority of DEG's associated with neonicotinoid exposure were observed in abdomen tissues; whereas the

majority of DEG's associated with non-neonicotinoid insecticide exposure were observed in the head tissues.

We conducted a principal component analysis (PCA) on the full gene count data from each experimental replicate (Figures 3-6). The replicates (hives) from the control groups tended to be more tightly clustered than replicates from the treatment groups. There were no complete separations between control and treatment; as was expected since the PCA's were generated on the full list of genes, not just significant or pesticide related genes. This trend was true for all experiments, however the PCA for the head tissues exposed to high dose of spinetoram was fully separated for all but one sample point (CNd2H); this separation is intuitive because of the substantive number of DEG.

We combined DEG lists across tissues to generate a single list of DEG's per agrochemical and dose level to explore the overall gene expression response while still maintaining the tissue level resolution. Theoretically, this approach is superior to conducting RNA-Seq on whole bees and then generating DEG lists because of the assumption that there are tissue specific signals of gene expression that would get minimized if they were averaged across all tissues. We conducted a two-way overlap analysis with these lists for each pesticide at the low dose (Table 4) and high dose (Table 5). This analysis revealed the pair-wise similarities between the combined DEG lists for each pesticide. At the low dose every possible pesticide pair exhibited at least one shared differentially expressed gene except for the clothianidin-thiamethoxam and clothianidin-spirotetramat pairs (Table 4). At the low dose, the spinetoram DEG list had the greatest overlap with other chemicals

since 25% of clothianidin DEGs, 15% of thiamethoxam DEGs, and 8% of spirotetramat DEGS were also significant in the spinetoram DEG list (Table 4). At the high dose every pair shared at least one differentially expressed gene (Table 5). At the high dose, the trend of spinetoram having the greatest overlap continued, except for thiamethoxam; spinetoram DEGs were similar in 27% of clothianidin DEGs, 26% of thiamethoxam DEGs, and 63% of spirotetramat DEGs, but clothianidin DEGs were similar in 32% of thiamethoxam DEGs (Table 5). This means that the thiamethoxam DEG list had greatest overlap with clothianidin but clothianidin had greatest overlap with the spinetoram DEG list.

To further investigate the multi-pesticide overlaps of the tissue combined gene sets we created 4-way Venn diagrams for the low dose experiments (Figure 7) and high dose experiments (Figure 8). At the low dose there were no overlaps to a greater degree than the two-way overlaps described in Table 4; genes are present in the same three out of a possible 6 two-way overlaps. Unlike the low dose 4-way overlap analysis, the high dose 4-way overlap provided more information than a simple pairwise comparison: genes occupied 3-way and 4-way subsets. At the high dose, all six possible two-way overlap sets contained genes, three out of a possible four three-way overlap sets contained genes, and there were 3 genes that were common to all pesticides (Figure 8). The only subset that was empty in the high dose 4-way analysis was the 3-way intersection of clothianidin, thiamethoxam and spirotetramat. Finally, the subset with the greatest number of DEGs was the intersection of clothianidin and spinetoram which contained 54 DEGs. Since that intersection was a subset of only two pesticides, we can compare its

size to the corresponding subset that was found in the previous pairwise comparison; when only considering pairs the clothianidin-spinetoram intersection contains 83 DEGs.

At both doses, there is a subset of genes that are unique to each chemical. In total, during the low dose experiments bees expressed 3, 35, 110, and 252 unique DEG's during exposure to clothianidin, thiamethoxam, spirotetramat, and spinetoram, respectively (Figure 7). In total, during the high dose experiments bees expressed 200, 55, 106, and 1185 unique DEG's during exposure to clothianidin, thiamethoxam, spirotetramat, and spinetoram, respectively (Figure 8). We found a trend where for most pesticide exposures, its DEGs were more likely to be unique than similar. For the low dose experiments, 75% of clothianidin DEGs, 76% of thiamethoxam DEGs, 89% of spirotetramat DEGs, and 93% of spinetoram DEGs were found only within their respective unique subsets. Likewise, for the high dose experiments, 65% of clothiandin DEGs, 49% of thiamethoxam DEGs, 56% of spirotetramat DEGs, and 81% of spinetoram DEGs were unique. Overall, exposure to spinetoram was associated with the greatest number of unique DEGs and exposure to thiamethoxam was associated with the least (Figures 7 & 8).

We used gene ontology (GO) enrichment analysis to explore the biological processes, molecular functions, and cellular components associated with the DEG's. Unsurprisingly, the larger DEG lists were able to generate more significant GO enrichments, with several DEG lists not returning any significant enrichment. Fittingly, transcriptional control was a common biological process enriched among DEGs (Figure 9). DEGs found in head

tissues of bees exposed to spinetoram and spirotetramat were enriched for terms associated with behaviour and nucleosome assembly (Figure 9). Whereas in abdomen and gut tissues exposure to spirotetramat were associated with DEGs related to fatty acid biosynthetic processes (Figure 9). DEGs associated with muscle and wing development and developmental growth were only found in tissues exposed to spinetoram (Figure 9). Consistent with the result of the ubiquitous enrichment for transcriptional control as a biological function; across all chemical exposures, the DEGs found were associated with the cellular components of nucleosome/chromatin or the extracellular (Figure 10). Likewise, across all chemical exposures, the DEGs found were associated with the molecular functions of chromatin structure and protein binding (Figure 11). Taken altogether, the biological processes, cellular components, and molecular function GO terms associated with the DEGs shared a commonality of transcriptional regulation.

To investigate specific genes as potential biomarkers, we sorted the DEG lists by adjusted p value for each sample, tissue, and dose to generate lists of the top ten most significantly differentially expressed genes (Tables 6-9). This revealed a wide range of genes involved in the expression response of pesticides, the majority of which are either uncharacterized or don't have common names in the current version of the honey bee genome. Of the named genes *Pgrp-s2* appeared the most frequently (4) across the various samples, followed by *nAChR subunit* genes (3) and *Inr-2* (3).

To uncover specific DEG's that were significant in multiple experiments we combined all 24 gene lists (4 chemicals x 2 doses x 3 tissues) and sorted it by gene occurrence. The

3 DEGs that were most commonly found across all experiments are all uncharacterized (Table 11). Finally, to further investigate the magnitude of the differential expression of the most commonly associated DEGs (Table 11) we sorted the $\log_2$ fold change of their mean normalized expression. Out of a possible 24 experiments (4 chemicals x 2 doses x 3 tissues), two genes were found to be most commonly differentially expressed: *LOC102656472* and *LOC100577522*, both significant in 7 experiments (Table 11). For both of those genes, exposure to neonicotinoids was associated with upregulation relative to controls, while exposure to non-neonicotinoids was associated with down regulation relative to controls (Figures 12-13).

The most common cytochrome p450 gene differentially expressed was *CYP6AQ1*; it was upregulated in head tissues exposed to thiamethoxam and down regulated in head tissues exposed to spinetoram and spirotetramat (Figure 14). *Carboxylesterase*, which is involved in detoxification, was down regulated in at least one tissue exposed to each chemical (Figure 15). The most common immune related gene differentially expressed by exposure to pesticides was *Pgrp-s2*; it was upregulated in head tissues exposed to spirotetramat and down regulated in head and abdomen tissues exposed to thiamethoxam, and down regulated in abdomen and gut tissues exposed to spinetoram (Figure 16).

Finally, to discover robust biomarkers for pesticide stress at the tissue level we compared the unique DEGs from each pesticide against the total gene expression count data of the other pesticides. To prune genes that may have been almost significant in other

chemicals, and therefore not unique to the chemical in question, DEGs were only considered if they had an adjusted p value of greater than 0.5 in the other pesticide gene sets. This analysis revealed putative biomarkers for all pesticides during both high and low dose exposures (Table 12). These putative biomarkers were also present in all tissue types for all pesticides, except for clothianidin exposure which only had putative biomarker genes in abdomen and gut tissues. The descriptions of these genes fit into the ongoing trend of DEGs have a wide range of functions as well as several being uncharacterized (Table 13).

Discussion

We set out with the goal of discovering unique signatures of exposure to the four agrochemicals tested. We were successful in finding a trend where genes are more likely to be uniquely differentially expressed than to be shared between stressors (Figures 7 & 8). This bodes well for future research that would include more agrochemicals in the analysis – signifying that gene expression is an excellent choice for biomarker discovery.

For my thesis we exposed honey bees to four different pesticides (clothianidin, thiamethoxam, spinetoram, and spirotetramat) and quantified global gene expression in 3 tissues associated with detoxification. Generally, the higher the dose the bees were subjected to, the greater the change in gene expression. All four chemicals each had their own subset of genes that were uniquely differentially expressed. Genes that were significantly differentially regulated in this study had a wide range of functions including behaviour, immune function, development, and expectedly, detoxification. Interestingly, the top ten most significant genes of each experiment revealed were related to not only

detoxification processes, but also other molecular functions such as metabolism and sensory perception (Table 10).

Clothianidin and thiamethoxam are both systemic neonicotinoids that have been used in agriculture since 2003 and 1991, respectively (Table 1). Both compounds have similar modes of action in terms of how they influence honey bees (Bass et al, 2015). Despite this, our results show that while the DEGs associated with exposure to each NNI do overlap to some extent, the majority of DEGs are unique to each NNI (Figures 7-8). In the low dose experiment, the DEG lists of clothianidin and thiamethoxam shared 0 DEGs, although this is likely a result of the low number of significant genes due to clothianidin exposure (4) (Table 4). They had a greater overlap in the high dose experiments, where 32% of thiamethoxam genes were also found in clothianidin and 12% of clothianidin genes were found in the thiamethoxam set.

The most significant gene expressed in thiamethoxam treated gut tissues at both doses was *transferrin* (*Tsf-1*), a gene involved in iron sequestration, that has been recently implicated honey immunology where higher expression of *Tsf-1* allowed the microsporidian parasite *Nosema ceranae* to access more nutritional iron and results in greater proliferation (Rogdriguez-Garcia et al, 2021) (Table 7). The high dose thiamethoxam treatment resulted in immune gene *peptidoglycan recognition protein S2* (*Pgrp-s2*) being significantly differentially expressed in head tissues (Table 7). *Pgrp-s2* has been suggested to be necessary for the initiation of the Toll pathway in defence against the Israeli acute paralysis virus (IAPV) (Deng et al, 2022), its expression has been

positively associated Deformed wing virus-A (DWV-A) (Traniello et al, 2020) and Sindbis virus (SINV) infections (Brutscher et al, 2017). Clothianidin exposure has been shown to down regulate the expression of *apidaecin* (Di Prisco et al, 2013), an antimicrobial gene whose expression is used as a measure of Toll pathway activation (Evans et al, 2006). In the former study, Di Prisco et al (2013) also showed that honey bees exposed to clothianidin experienced a greater infection of DWV. In this study, we weren't able to detect an effect on *apidaecin* expression due to clothianidin exposure, however we did find that thiamethoxam down regulates *apidaecin* in both head and gut tissues, These results add to the growing body of knowledge regarding the synergistic interactions between pesticides and the honey bee's immune system (O'Neal et al, 2018).

Spirotetramat and spinetoram are the two non-neonicotinoid pesticides we tested. Spirotetramat, is a newer pesticide than the neonicotinoids and is classified as a tetramic acid derivative; its mode of action involves inhibition of lipid synthesis (Brück et al, 2009). Unsurprisingly, the gene ontology results for spirotetramat included terms for fatty acid biosynthesis. Additionally, *apolipophorin-III* (*a4*) was significantly differentially expressed in spirotetramat gut tissues (Table 8). *A4* has mainly been characterized as a lipid transport protein although has been also implicated in innate immunity (Weers & Ryan, 2006). Unlike the three other chemicals, spirotetramat had the smallest proportion of unique genes (33%), which is surprising given its distinct mode of action. Spinetoram, a spinosyn with the same target molecule as the neonicotinoids (nAChR) although at a different site, elicited the strongest gene expression response. With a large number

(1185) of total unique genes significantly differentially expressed during the high dose exposure (Figure 8).

We found that pesticide exposure has effects on a wide range of biological functions. In particular, I was interested if I could identify any signals of developmental and/or nutritional stress caused by pesticide exposure. Hypopharyngeal glands are the centres of royal jelly production (Painter & Biesele, 1966; Knecht & Kaatz, 1990), a nutrient dense secretion fed to worker larvae and queens at both larval and adult life stages (Haydak, 1943). Gupta et al (1995) showed that honey bees treated with insecticide diflubenzuron displayed reduced development of the hypopharyngeal glands. This has been hypothesized to result in abnormal larval development and issues with queen rearing (Desneux et al, 2007). In this study we found, honey bees treated with spinetoram exhibited a reduction expression of several major royal jelly proteins: *mrjp1, mrjp4, mrjp5, mrjp6, mrjp7,* and *mrjp9* in head tissues. Interestingly, *mrjp6* was upregulated in gut tissues during that same spinetoram exposure. Additionally, *mrjp6* was down regulated in thiamethoxam treated head tissues, but up regulated in spirotetramat treated head tissues. This is in line with Shi et al, (2017) who found that thiamethoxam exposure reduced expression of *mrjp1, mrjp3,* and *mrjp4*. While we didn't find a significant effect of clothianidin on the expression of a major royal jelly protein gene in this study, Christen et al (2018) found that clothianidin exposure reduced the expression of *mrjp1, mrjp2,* and *mrjp3*. This data taken together suggests that perhaps pesticides that target the nAChR reduce the expression of major royal proteins in head tissue; and that it may not matter if the pesticide targets the nAChR at its active site, as with clothianidin and thiamethoxam,

or its allosteric site as with spinetoram. In addition to their nutritional importance, major royal jelly proteins also have anti-microbial functions (Fontana et al, 2004) and thus their down regulation may also have effects on the honey bee's immune capacity.

Cytochrome P450 is a family of genes heavily involved in pesticide detoxification in honey bees (Berenbaum & Johnson, 2015; Claudinianos et al, 2006). While the upregulation of *CYP6AQ1* in thiamethoxam treated heads may be a function of the detoxification system, its down regulation in head tissues exposed to spinetoram or spirotetramat is concerning (Figure 14). If the down regulation would have causal effects on mortality this would add to the growing body of literature of synergistic interactions between pesticide stressors. A recent meta-analysis on bee mortality found that agrochemicals are more likely to synergize with other agrochemicals rather than with parasites or nutritional stress (Siviter et al, 2021) The trend continues overall with the significant expression of genes related to detoxification were more often reduced in bees exposed to pesticides (Figures 14, 15, S1, S2). Future research should investigate whether this is a result of a toxic effect on the bee rather a shifting in transcriptional priorities to detoxify by a currently unknown mechanism.

The two most common DEG's: *LOC102656472* and *LOC100577522* (Table 11) are both uncharacterized genes; although the latter has been putatively described with chemical sensory and perception (Tsuruda et al, 2012). They are both great candidates for markers for general neonicotinoid exposure due to not only being significant in upregulation due

to neonicotinoid stress but also down regulation from exposure to the non-neonicotinoid pesticides; and should merit further research (Figures 12-13).

Heat shock proteins are a family of proteins responsible for the maintenance of other proteins under various distinct stressors (Zhao & Jones, 2012). The expression of heat shock proteins has been used as a biomarker environmental stress from pesticides (Lewis et al, 1999). More recently, Koo et al (2015) suggested to use heat shock protein expression as a biomarker to distinguish between stress caused by exposure to neonicotinoid imidacloprid, flower-thinning agents, or heat shock. They found that exposure to neonicotinoid imidacloprid for 6 hours reduced expression of heat shock protein encoding genes *hsp70*, and *hsp90* (Koo et al, 2015). On the contrary, a later study found no effect of 7-day exposure to imidacloprid on expression of these genes in neither head nor abdomen tissues (Kim et al 2019). In our study, we found down regulation of 3 heat shock genes: *Hsc70-4*, *Hsp beta-1*, and *Hsp83*, all found in head tissues, the first gene in bees exposed to spinetoram and the latter two genes with spirotetramat exposed bees. As well as an up regulation of *Hsp70Ab-like* in abdomen tissues of bees exposed to clothianidin. While heat shock proteins are appropriate markers of general stress, we did not find evidence to support their role as insecticide specific biomarkers. The discrepancy between Koo et al (2015) and Kim et al (2019) may be attributed to the variation in length of exposure. Sustained and dynamic transcriptional responses to pesticide stress remains to be an interesting topic for future research.

Finally, our list of pesticide specific putative biomarkers provided several insights. First, we were able to detect robust biomarkers at a high dose of exposure, and perhaps more importantly also at a low dose (Table 12). Second, we found that the DEGs were present in all tissue types, (except clothianidin head tissues), providing for flexibility in developing a biomarker assay. Additionally, not only were there genes with a variety of functions, but also there were several uncharacterized genes that may be suited as competent biomarkers (Table 13). Even though the honey bee is well studied, there still exists a gap in the complete functional response to pesticide exposure. Hopefully this thesis illuminates the need for further research into the toxification biology of the honey bee.

Conclusion

The impact of pesticides on pollinators including the economically important western honey bee continues to be an active focus of research and development. It can be a difficult subject to investigate with many opposing stakeholders, novel pesticide creation, and various regulations and governing bodies. However, it remains imperative to conduct robust and unbiased experiments to determine the effects of pesticides. Herein, we have detailed numerous consequences that pesticides have on honey bee's gene expression - some of which may reduce their resistance against pathogens or other toxicants. We have observed that there are many DEGs that are affected commonly across chemicals, as well as unique DEGs for each chemical. Lastly, we found that DEGs belong to several different functional groups throughout the honey bee genome.

Understanding how the honey bee responds to pesticides can reveal the impacts on their lifespan as well as their interaction with their environment. Additionally, this research can also be used as a model for other off-target pollinators. In conclusion, research into the genetics of honey bee detoxification systems and the development of robust and specific biomarkers is an important step towards the stewardship of our modern agricultural environment.

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Appendix A: Tables and Figures

Table 1. Details of pesticides used in the study.

	Introduced	Classification	Mode of Action
Thiamethoxam	1991	neonicotinoid	nAChR competitive agonist (Bass et al, 2015)
Clothianidin	2003	neonicotinoid	nAChR competitive agonist (Bass et al, 2015)
Spirotetramat	2007	tetramic acid derivative	lipid synthesis inhibition (Brück et al, 2009)
Spinetoram	2007	spinosyn	nAChR allosteric modulator (Bacci et al, 2016)

Table 2. Doses of pesticides administered in a 50% sugar solution.

	Control (ng/μL)	Low Dose (ng/μL)	High Dose (ng/μL)
Clothianidin	0	0.1425	0.57
Thiamethoxam	0	0.115	0.46
Spirotetramat	0	25	100
Spinetoram	0	0.215	0.859

Table 3. Number of genes statistically (FDR 0.1) differentially upregulated (Up) or downregulated (Down) for each pesticide, tissue, and dose.

	Low Dose						High Dose					
	Heads		Guts		Abdomen		Heads		Guts		Abdomen	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Clothianidin	1	0	2	1	0	0	6	9	22	31	108	143
Thiamethoxam	3	8	2	20	10	4	7	18	8	5	34	44
Spirotetramat	34	72	3	1	6	8	81	182	29	5	18	13
Spinetoram	11	20	61	47	112	55	565	821	20	51	12	34

Table 4. Number of genes significantly differentially expressed (FDR = 0.1) after bees were exposed to the low dose. Tissue gene sets were combined within chemicals. Matrix details where genes overlap between chemicals. The diagonal represents the total number of genes for each chemical.

	Clothianidin	Thiamethoxam	Spirotetramat	Spinetoram
Clothianidin	4	0	0	1
Thiamethoxam	0	46	4	7
Spirotetramat	0	4	124	10
Spinetoram	1	7	10	270

Table 5. Number of genes significantly differentially expressed (FDR = 0.1) after bees were exposed to the high dose. Tissue gene sets were combined within chemicals. Matrix details where genes overlap between chemicals. The diagonal represents the total number of genes for each chemical.

	Clothianidin	Thiamethoxam	Spirotetramat	Spinetoram
Clothianidin	310	36	23	83
Thiamethoxam	36	112	16	29
Spirotetramat	23	16	320	203
Spinetoram	83	29	203	1461

Table 6. Top 10 most significant genes expressed during exposure to clothianidin in each tissue and dose.

Low dose			High dose		
Head	Gut	Abdomen	Head	Gut	Abdomen
LOC102656359	*LOC100577819*	-	*LOC102654285*	*LOC410626*	*LOC550965*
-	*LOC100576498*	-	*LOC412829*	*LOC412092*	*LOC410614*
-	*LOC550964*	-	*LOC726914*	*LOC413377*	*LOC100578437*
-	-	-	*LOC726289*	*LOC551566*	*LOC724367*
-	-	-	*LOC550828*	*LOC100576418*	*LOC725621*
-	-	-	*LOC551758*	*LOC411186*	*LOC724187*
-	-	-	*LOC100578068*	*LOC725217*	*LOC408844*
-	-	-	*LOC552421*	*LOC726215*	*LOC551250*
-	-	-	*LOC410006*	*nAChRa9*	*LOC410509*
-	-	-	*LOC100578608*	*LOC100577135*	*LOC113219243*

Table 7. Top 10 most significant genes expressed during exposure to thiamethoxam in each tissue and dose.

Low dose			High dose		
Head	Gut	Abdomen	Head	Gut	Abdomen
LOC100578816	*Tsf1*	*LOC100577614*	*LOC100578156*	*Tsf1*	*LOC100576758*
LOC551401	*Apid1*	*LOC102653599*	*Pgrp-s2*	*LOC413101*	*Pgrp-s2*
LOC100578777	*LOC410780*	*LOC552190*	*LOC725164*	*LOC725053*	*ILP-2*
LOC102653584	*LOC727110*	*LOC100577717*	*Mrjp6*	*LOC552149*	*LOC550655*
LOC410398	*Obp14*	*Cpap3-c*	*LOC102656472*	*LOC726421*	*LOC724861*
LOC725400	*LOC100576555*	*LOC100577522*	*LOC409633*	*LOC408867*	*LOC410626*
LOC551465	*LOC408817*	*LOC410734*	*LOC100577777*	*LOC100578156*	*LOC552024*
Dat	*Uvop*	*LOC726672*	*LOC409648*	*LOC411288*	*LOC552313*
LOC725164	*LOC100577163*	*LOC102653678*	*LOC552811*	*Hex110*	*LOC725407*
LOC100576994	*LOC410566*	*LOC100578347*	*Apid1*	*LOC100578865*	*LOC408643*

Table 8. Top 10 most significant genes expressed during exposure to spirotetramat in each tissue and dose.

Low dose			High dose		
Head	Gut	Abdomen	Head	Gut	Abdomen
LOC113219378	*LOC412815*	*LOC726292*	*LOC408462*	*LOC412815*	*LOC107963967*
InR-2	*LOC100577054*	*LOC100577268*	*LOC726101*	*Obp3*	*LOC551837*
LOC409801	*LOC100578608*	*LOC551837*	*LOC107964589*	*LOC412401*	*LOC551098*
LOC408462	*LOC412401*	*LOC107964258*	*LOC551360*	*A4*	*LOC409624*
LOC724946	-	*LOC725670*	*LOC724946*	*LOC550686*	*LOC725260*
LOC413995	-	*LOC726424*	*LOC100577198*	*LOC725305*	*LOC725671*
LOC113219382	-	*LOC100577629*	*InR-2*	*LOC725387*	*LOC551465*
LOC411564	-	*LOC107964189*	*LOC100577883*	*LOC100577163*	*LOC100576738*
Ancr-1	-	*LOC100578347*	*LOC107965787*	*LOC552712*	*LOC552073*
Dscam	-	*LOC725209*	*LOC411564*	*LOC412503*	*LOC100578329*

Table 9. Top 10 most significant genes expressed during exposure to spinetoram in each tissue and dose.

Low dose			High dose		
Head	Gut	Abdomen	Head	Gut	Abdomen
LOC551863	*Pban*	*LOC100576540*	*LOC724867*	*LOC412209*	*LOC411290*
LOC725400	*LOC725158*	*LOC409791*	*LOC100577883*	*LOC409628*	*LOC411058*
LOC100576277	*LOC100576555*	*Crzr*	*LOC724717*	*Cyp6as5*	*LOC100576169*
LOC100576935	*LOC409053*	*nAChRa9*	*LOC411983*	*LOC107965822*	*LOC726879*
LOC552512	*Fabp_1*	*LOC100577995*	*LOC408462*	*Pgrp-s2*	*Y-e3*
LOC410462	*LOC107965005*	*LOC412161*	*LOC411564*	*LOC410626*	*LOC724654*
LOC107965822	*LOC413569*	*nAChRb2*	*LOC550885*	*LOC100578929*	*LOC412263*
LOC100576540	*LOC413101*	*LOC100577614*	*LOC724946*	*LOC409515*	*LOC100577827*
LOC100577522	*LOC552206*	*LOC409039*	*InR-2*	*LOC100578356*	*LOC494509*
LOC102654257	*Pgrp-s2*	*LOC100579026*	*LOC413995*	*LOC552797*	*LOC102656472*

Table 10. The count and descriptions of selected genes found in the top ten most significant sets of each experiment.

Count	Gene	Description
4	*pgrp-s2*	peptidoglycan recognition protein S2
3	*inr-2*	insulin-like receptor-like
2	*apid1*	apidaecin 1
2	*tsf-1*	transferrin 1
2	*nAChRa9*	nicotinic acetylcholine receptor alpha9 subunit
1	*ilp-2*	insulin-like peptide 2
1	*mrjp6*	major royal jelly protein 6
1	*hex110*	hexamerin 110
1	*cpap3-c*	cuticular protein analogous to peritrophins 3-C
1	*dat*	dopamine transporter
1	*uvop*	ultraviolet-sensitive opsin
1	*obp14*	odorant binding protein 14
1	*ancr-1*	AncR-1 non-coding nuclear RNA
1	*dscam*	Down syndrome cell adhesion molecule
1	*obp3*	odorant binding protein 3
1	*a4*	apolipophorin-III-like protein
1	*pban*	pheromone biosynthesis-activating neuropeptide
1	*fabp_1*	fatty acid binding protein
1	*Crzr*	corazonin receptor
1	*nAChRb2*	nicotinic acetylcholine receptor beta2 subunit
1	*Cyp6as5*	cytochrome P450 6AS5
1	*Y-e3*	yellow-e3

Table 11. The count and descriptions of the 15 most common DEGs across all experimental conditions (N=24).

Count	Gene	Description
7	*LOC102656472*	Uncharacterized
7	*LOC100577522*	Uncharacterized
6	*LOC113219243*	Uncharacterized
5	*Pgrp-s2*	peptidoglycan recognition protein S2
5	*Mir3759*	microRNA 3759
5	*LOC726134*	carboxylesterase
5	*LOC413596*	receptor-type guanylate cyclase gcy-4
5	*LOC410732*	3-phosphoinositide-dependent protein kinase 1
5	*LOC410626*	sodium-coupled monocarboxylate transporter 1
5	*LOC113219378*	large subunit ribosomal RNA
5	*LOC100576555*	cytochrome b561 domain-containing protein 2
5	*CYP6AQ1*	cytochrome P450 6AQ1
4	*Obp19*	odorant binding protein 19
4	*Mrjp6*	major royal jelly protein 6

Table 12. Putative biomarkers for each pesticide sorted by most significantly differentially expressed genes during each pesticide exposure. Details include the exposure dose and the exposed tissue. Genes were excluded if the same tissue had an adjusted p value less than 0.5 after exposure from a different pesticide.

	Gene	Dose	Tissue	Adj. P value	Log2 Fold Change
Thiamethoxam	*Tsf1*	Low	Gut	1.28E-18	-0.869
	Apid1	Low	Gut	4.84E-14	-0.812
	LOC100578816	Low	Head	3.89E-07	-0.683
	LOC100578156	High	Head	4.31E-07	-0.883
	LOC102653599	Low	Abdomen	1.01E-04	0.302
	LOC552190	Low	Abdomen	1.06E-03	-0.777
	LOC100577717	Low	Abdomen	1.14E-03	0.516
	Cpap3-c	Low	Abdomen	2.37E-03	-0.745
	LOC552149	High	Gut	2.68E-03	0.387
	LOC727110	Low	Gut	2.89E-03	-1.234
Clothianidin	*LOC550965*	High	Abdomen	2.13E-09	0.782
	LOC724367	High	Abdomen	1.04E-08	0.979
	LOC410509	High	Abdomen	1.55E-07	-0.772
	LOC552217	High	Abdomen	8.74E-07	-1.003
	LOC410087	High	Abdomen	1.70E-06	0.512
	LOC552758	High	Abdomen	2.73E-06	-0.566
	LOC100577028	High	Abdomen	2.89E-06	-0.462
	LOC551566	High	Gut	7.73E-05	-0.586
	LOC725217	High	Gut	2.45E-04	-0.511
	LOC100576498	Low	Gut	3.78E-02	1.129
Spirotetramat	*LOC107963967*	High	Abdomen	1.46E-12	0.952
	LOC726292	Low	Abdomen	1.14E-06	-1.424
	LOC412401	High	Gut	1.78E-06	0.756
	LOC550686	High	Gut	1.02E-05	0.488
	LOC551623	High	Head	1.25E-05	-0.474
	LOC725305	High	Gut	1.32E-05	1.477
	LOC100577268	Low	Abdomen	1.50E-05	0.430
	LOC100577717	High	Head	2.18E-05	0.687
	LOC409456	High	Head	8.42E-05	-0.324
	LOC113218601	High	Head	9.84E-05	-0.287
Spinetoram	*LOC412209*	High	Gut	3.72E-14	-0.562
	LOC724480	High	Head	1.04E-11	-0.517
	LOC724642	High	Head	3.69E-11	-0.668
	LOC726737	High	Head	9.97E-10	-0.752
	LOC552836	High	Head	3.21E-09	-0.612
	LOC406081	High	Head	5.15E-09	-0.504
	LOC409791	Low	Abdomen	3.32E-08	0.431
	LOC725732	High	Head	6.62E-08	-0.376
	LOC725967	High	Head	6.62E-08	-0.559
	LOC100576169	High	Abdomen	7.02E-08	-0.922

Table 13. Descriptions of putative biomarkers for each pesticide.

	Gene	Description
Thiamethoxam	*Tsf1*	transferrin 1
	Apid1	apidaecin 1
	LOC100578816	uncharacterized LOC100578816
	LOC100578156	uncharacterized LOC100578156
	LOC102653599	uncharacterized LOC102653599
	LOC552190	prisilkin-39
	LOC100577717	uncharacterized LOC100577717
	Cpap3-c	cuticular protein analogous to peritrophins 3-C
	LOC552149	aquaporin AQPAn.G
	LOC727110	yellow-x2
Clothianidin	*LOC550965*	probable cytochrome P450 6a14
	LOC724367	protein lethal(2)essential for life
	LOC410509	mucin-2
	LOC552217	uncharacterized LOC552217
	LOC410087	uncharacterized LOC410087
	LOC552758	retinol dehydrogenase 12
	LOC100577028	insulin-like growth factor I
	LOC551566	uncharacterized LOC551566
	LOC725217	uncharacterized protein PFB0145c
	LOC100576498	uncharacterized LOC100576498
Spirotetramat	*LOC107963967*	serine protease inhibitor 3
	LOC726292	natterin-3
	LOC412401	lipophorin receptor
	LOC550686	ATP-citrate synthase
	LOC551623	nucleolysin TIAR
	LOC725305	uncharacterized LOC725305
	LOC100577268	uncharacterized LOC100577268
	LOC100577717	uncharacterized LOC100577717
	LOC409456	AT-rich interactive domain-containing protein 4B
	LOC113218601	uncharacterized LOC113218601
Spinetoram	*LOC412209*	probable cytochrome P450 6a17
	LOC724480	asparagine synthetase [glutamine-hydrolyzing]
	LOC724642	F-box/LRR-repeat protein 3
	LOC726737	venom acid phosphatase Acph-1
	LOC552836	uncharacterized LOC552836
	LOC406081	glucose oxidase
	LOC409791	cAMP-dependent protein kinase catalytic subunit
	LOC725732	uncharacterized LOC725732
	LOC725967	D-3-phosphoglycerate dehydrogenase
	LOC100576169	uncharacterized LOC100576169

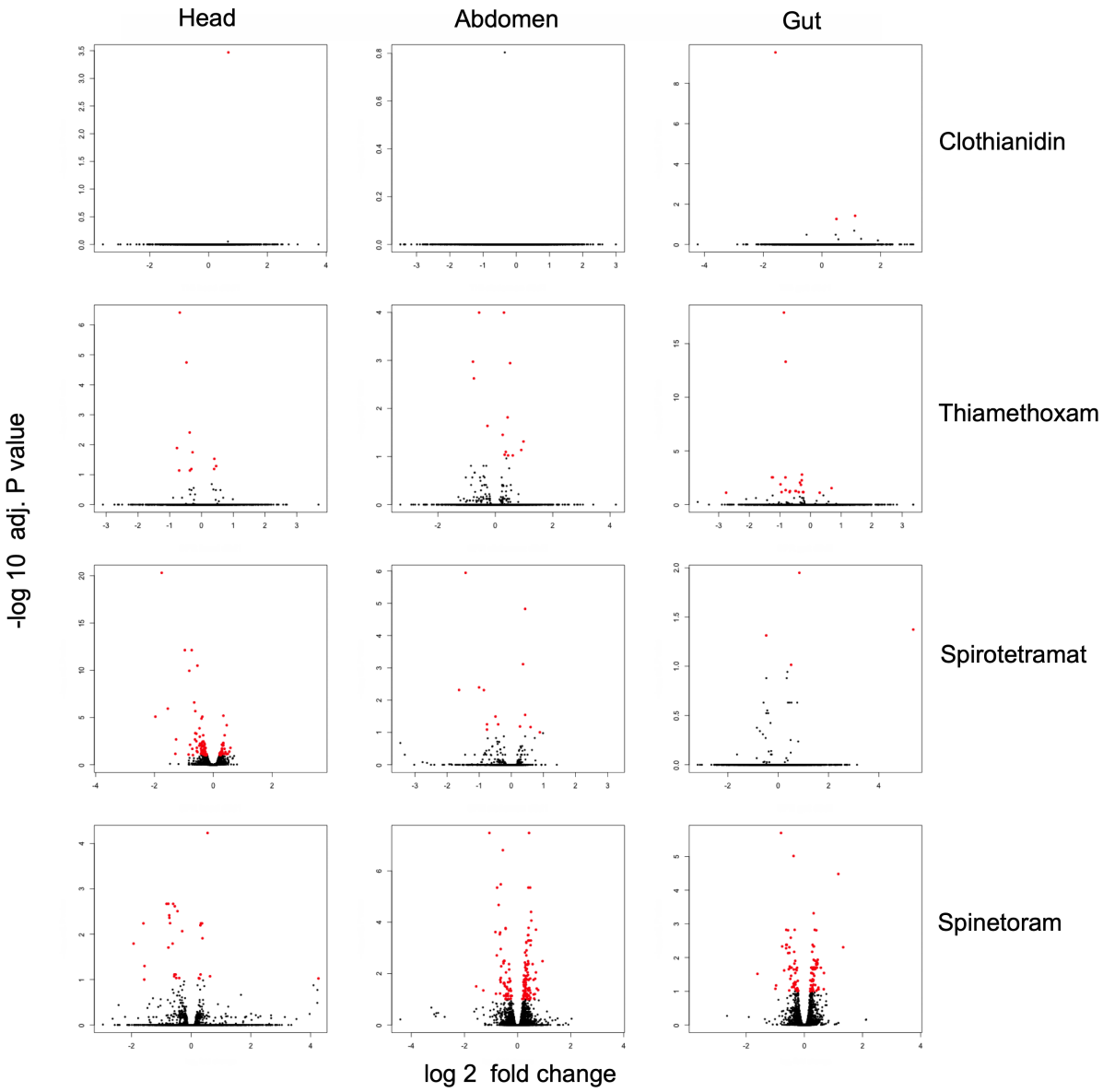

Figure 1. Volcano plots of differentially expressed genes (FDR 0.1) during the low dose experiment.

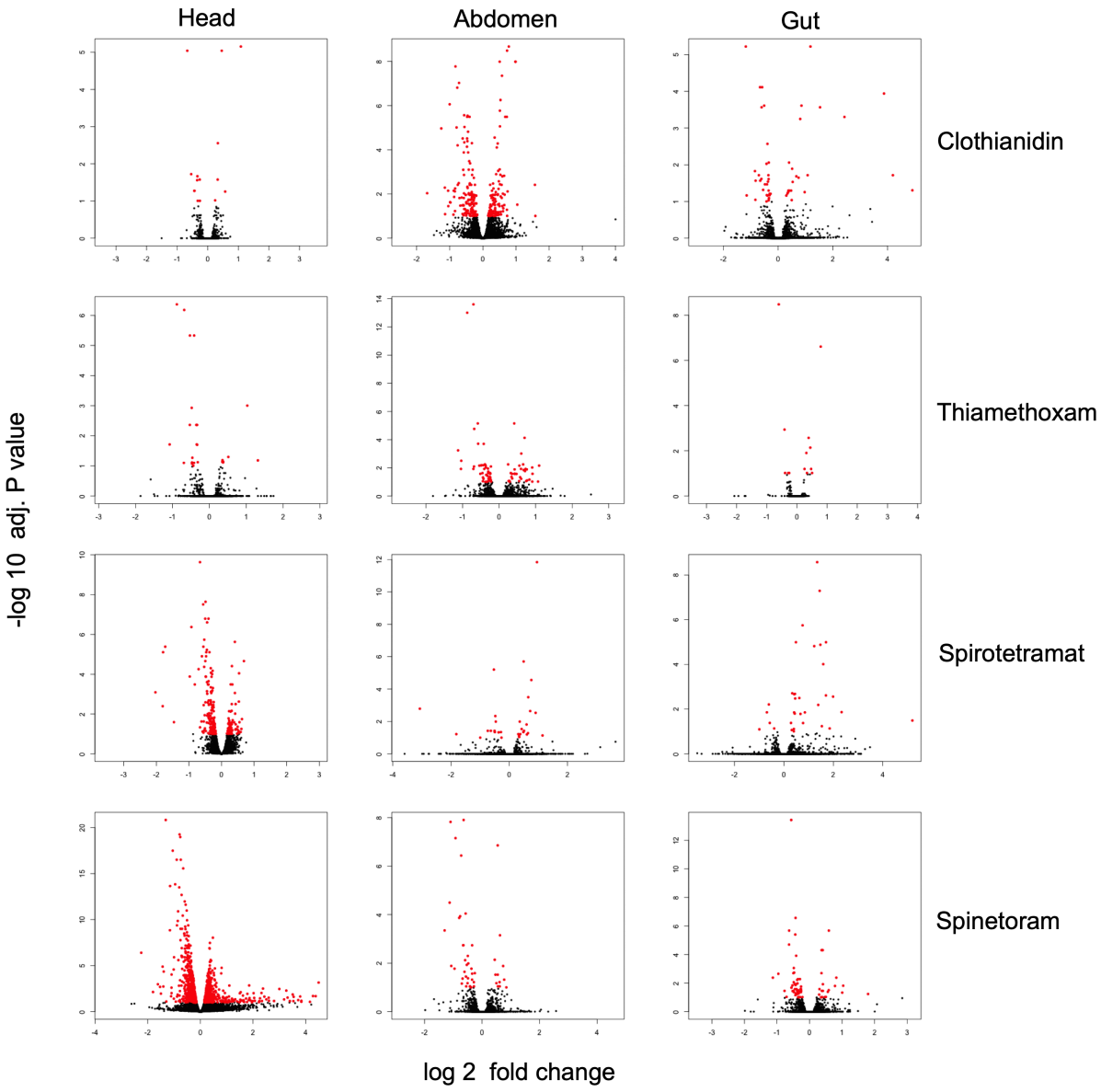

Figure 2. Volcano plots of differentially expressed genes (FDR 0.1) during the high dose experiment.

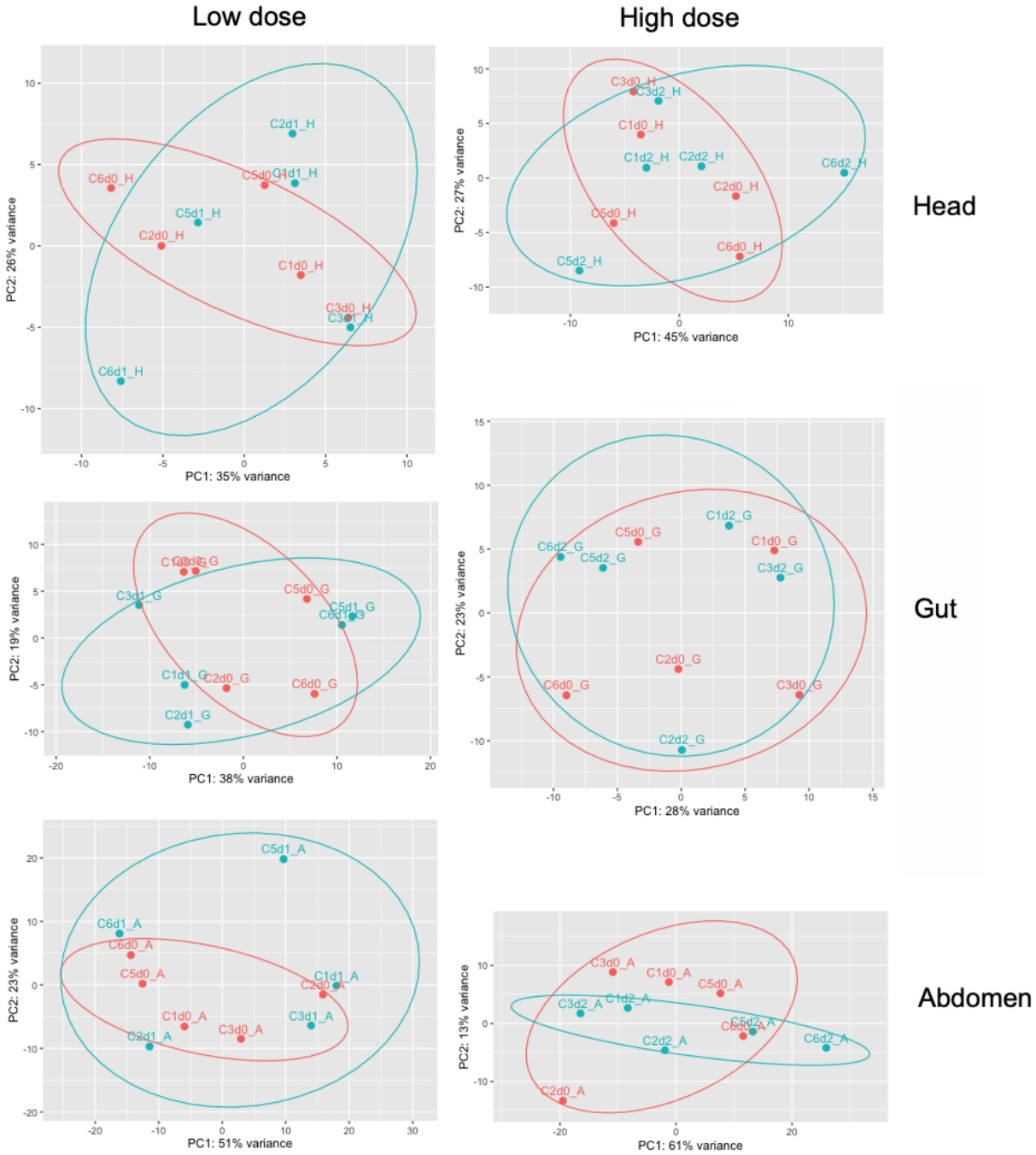

Figure 3. Principal component analysis of total gene count data from the clothianidin experiments. The red points represent the control data, and the blue are treated samples.

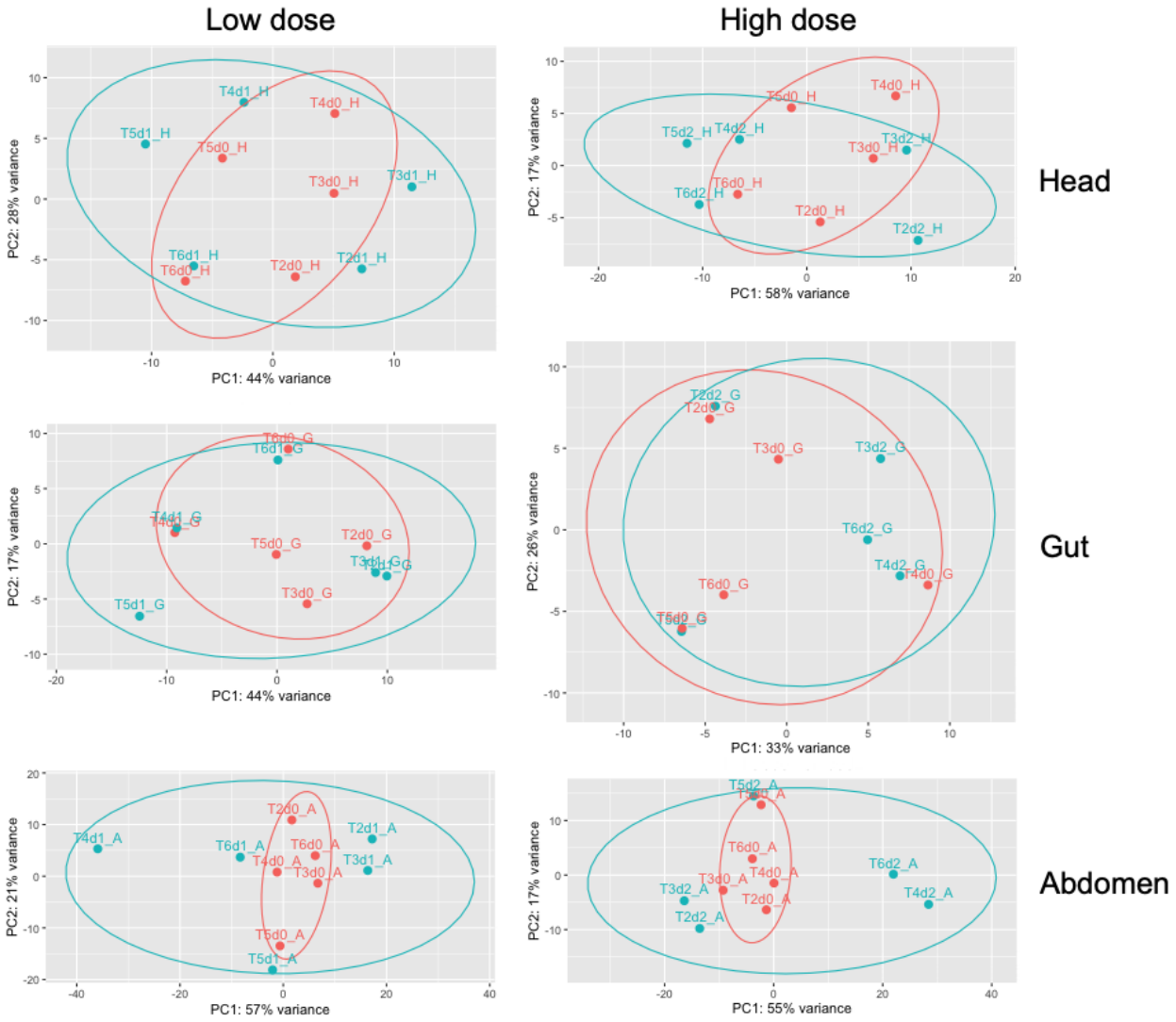

Figure 4. Principal component analysis of total gene count data from the thiamethoxam experiments. The red points represent the control data, and the blue are treated samples.

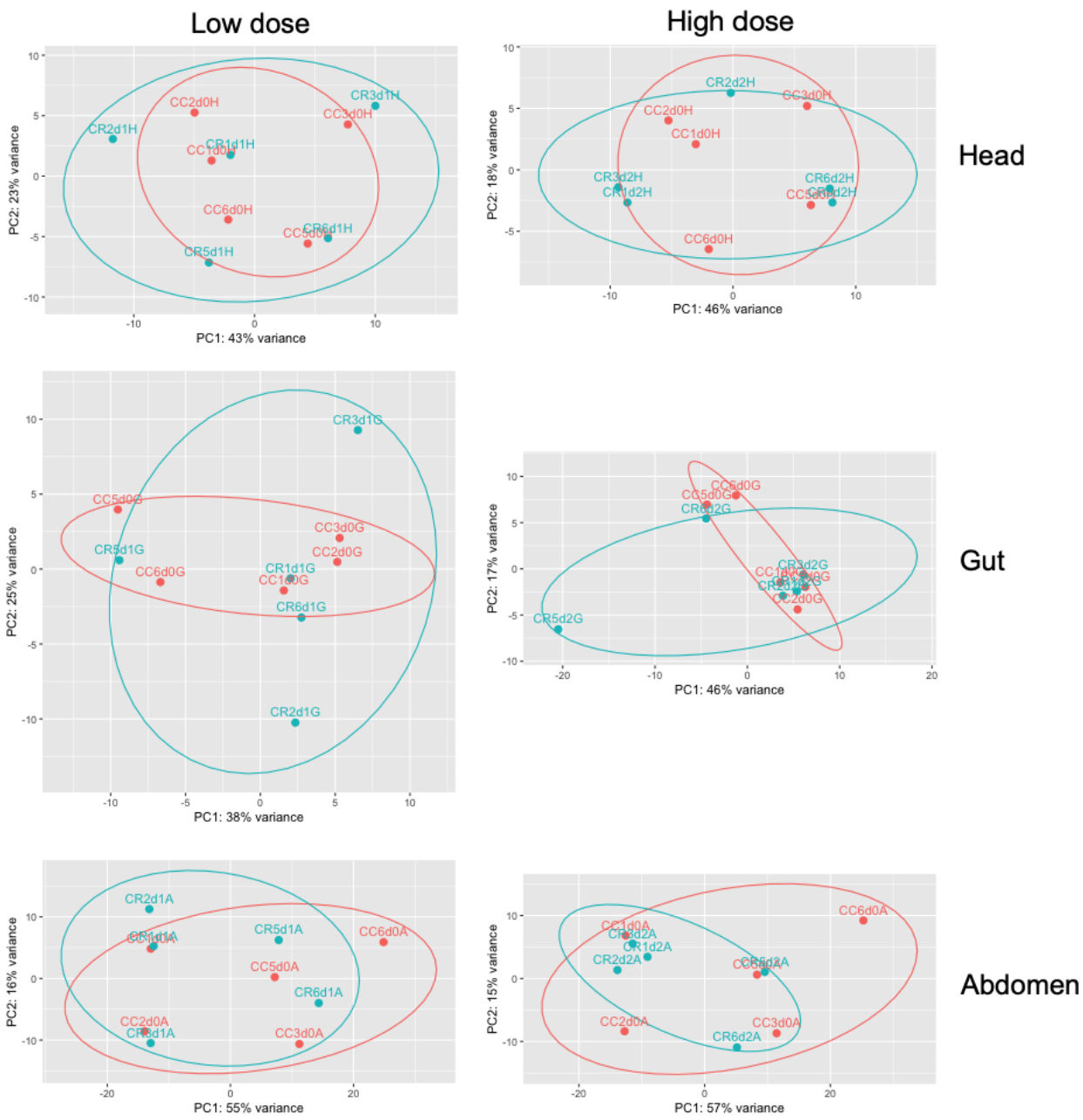

Figure 5. Principal component analysis of total gene count data from the spirotetramat experiments. The red points represent the control data, and the blue are treated samples.

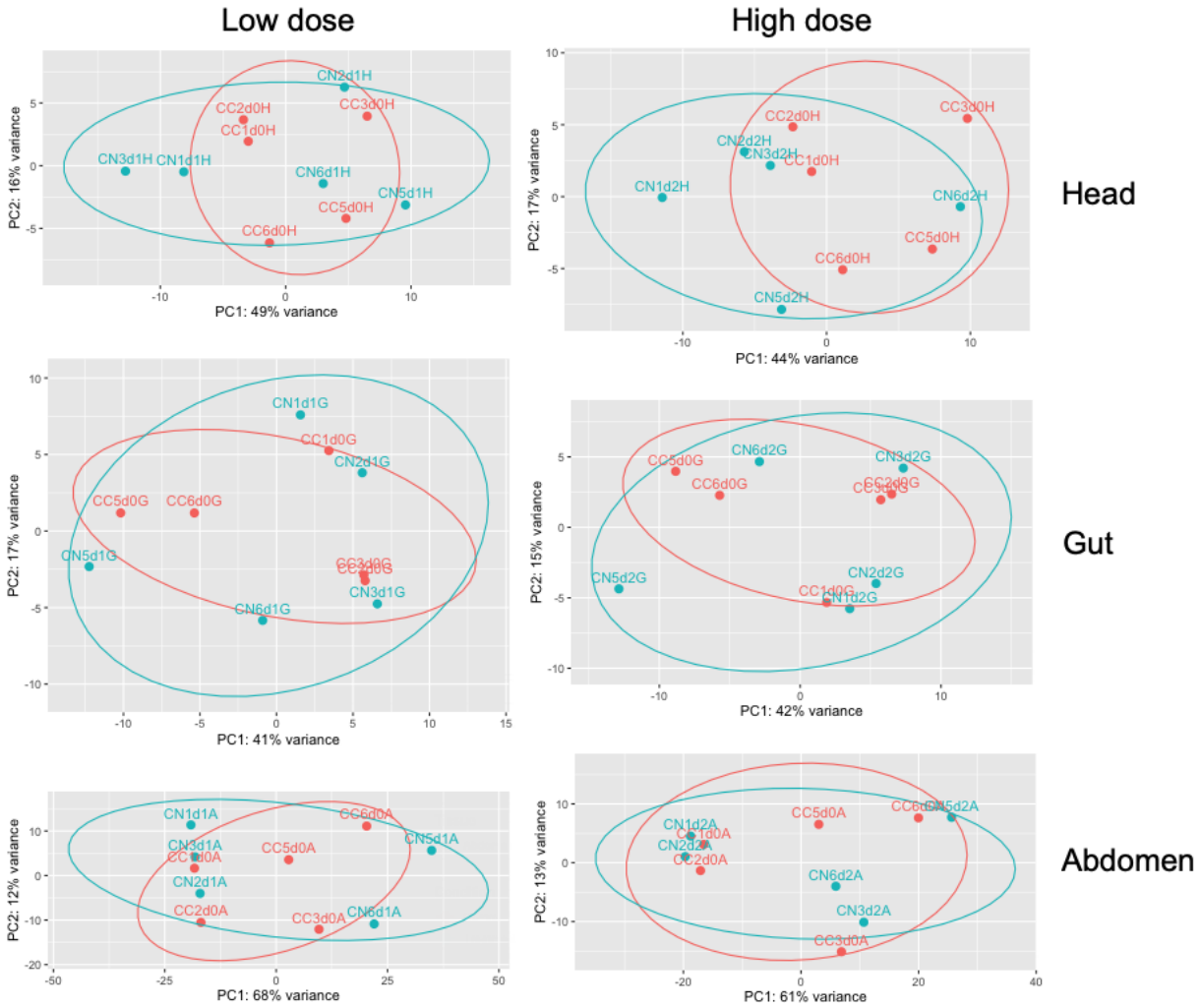

Figure 6. Principal component analysis of total gene count data from the spinetoram experiments. The red points represent the control data, and the blue are treated samples.

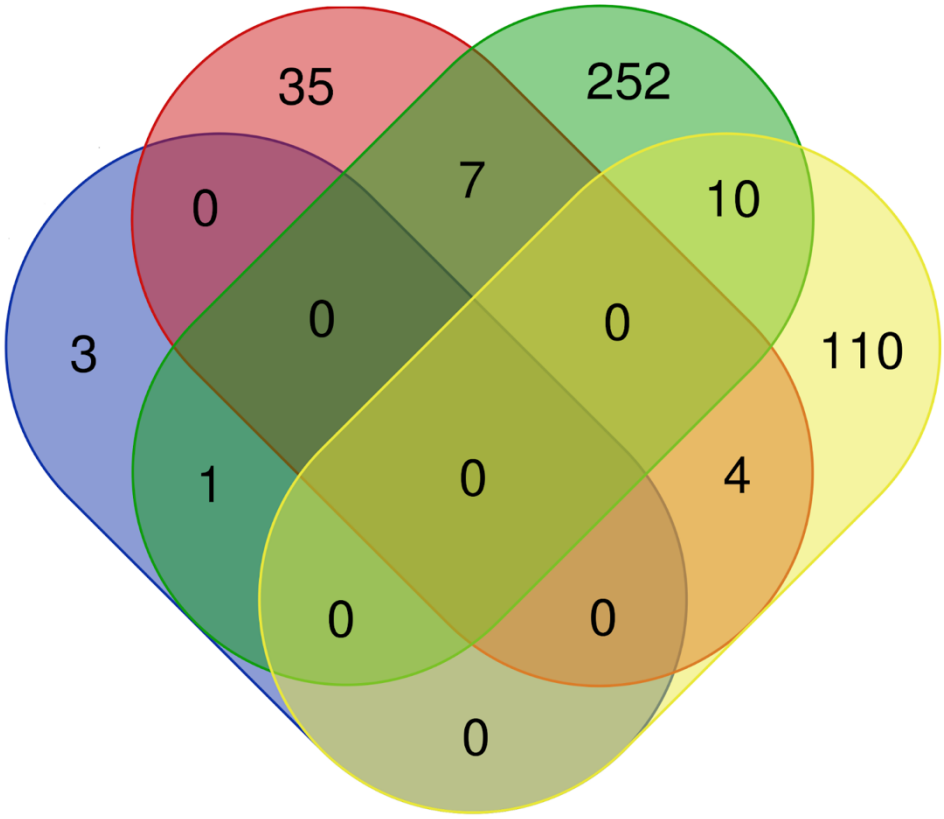

Figure 7. A four-way Venn diagram depicting the number of genes differentially expressed (FDR 0.1) in response to the low dose treatment and their overlapping patterns between chemicals. Blue represents clothianidin, red is thiamethoxam, green is spinetoram, and yellow is spirotetramat. Genes unique to each chemical are positioned on the periphery.

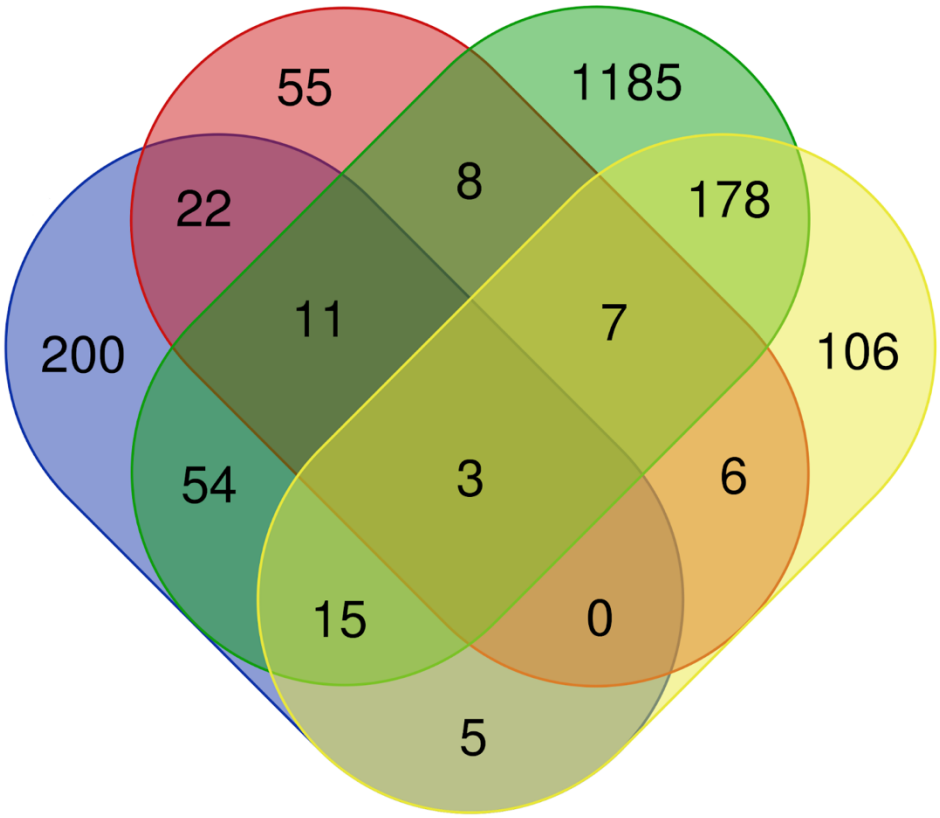

Figure 8. A four-way Venn diagram depicting the number of genes differentially expressed (FDR 0.1) in response to the high dose treatment and their overlapping patterns between chemicals. Blue represents clothianidin, red is thiamethoxam, green is spinetoram, and yellow is spirotetramat. Genes unique to each chemical are positioned on the periphery.

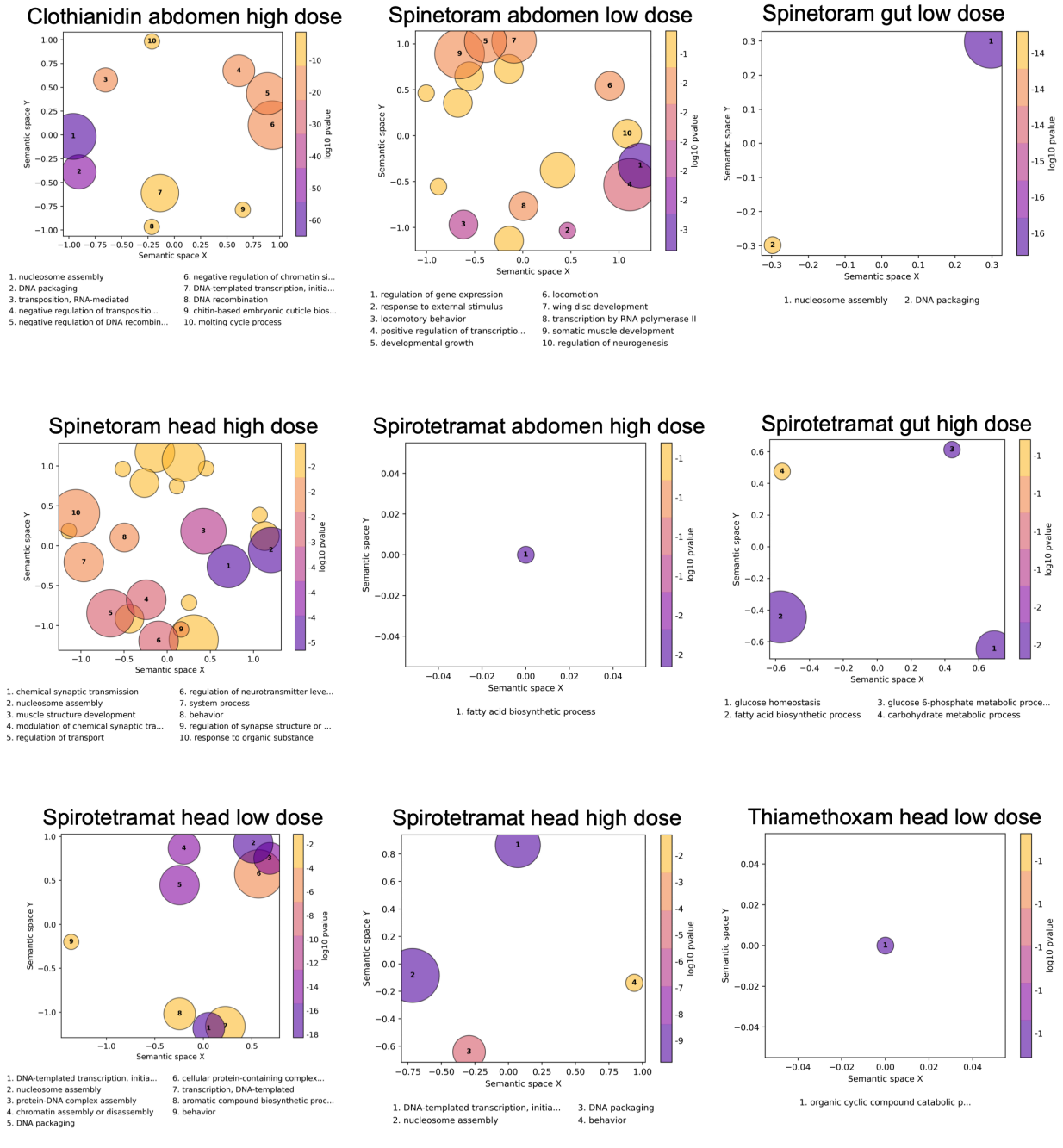

Figure 9. Scatterplots of biological process gene ontology terms onto semantic space. The size of the circles represent enrichment, and the colour represents significance.

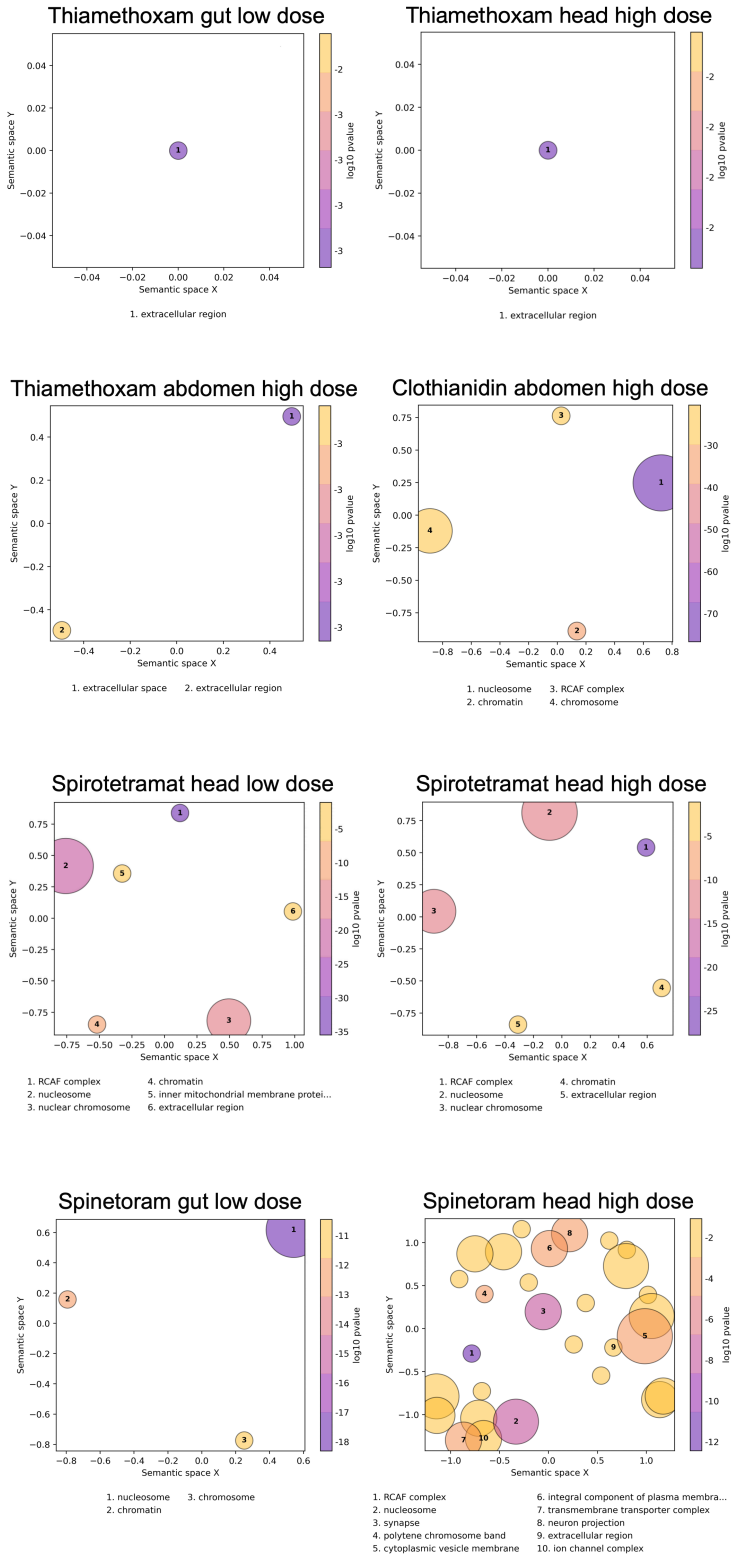

Figure 10. Scatterplots of cellular component gene ontology terms onto semantic space. The size of the circles represent enrichment, and the colour represents significance

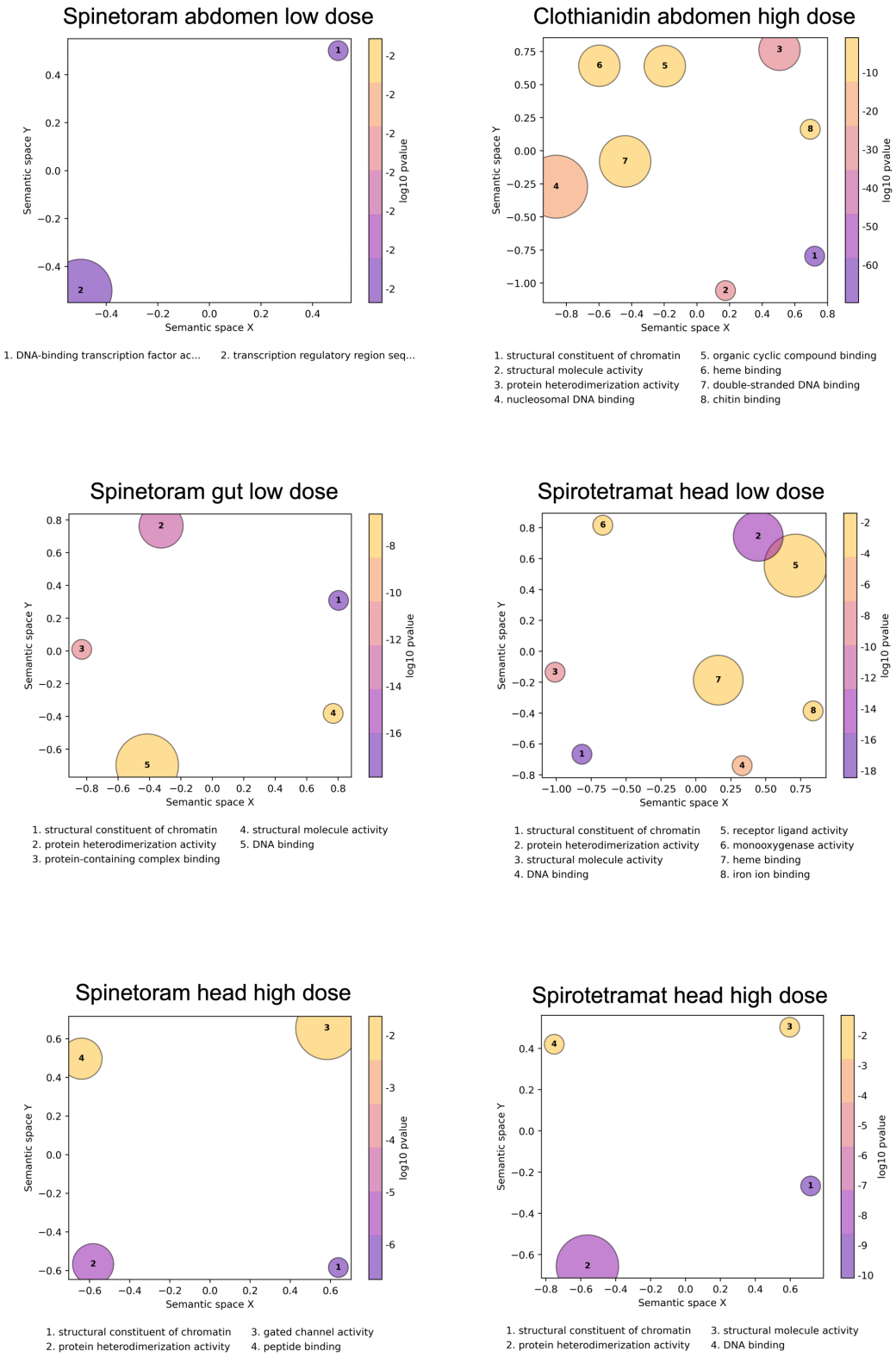

Figure 11. Scatterplots of molecular function gene ontology terms onto semantic space. The size of the circles represent enrichment, and the colour represents significance.

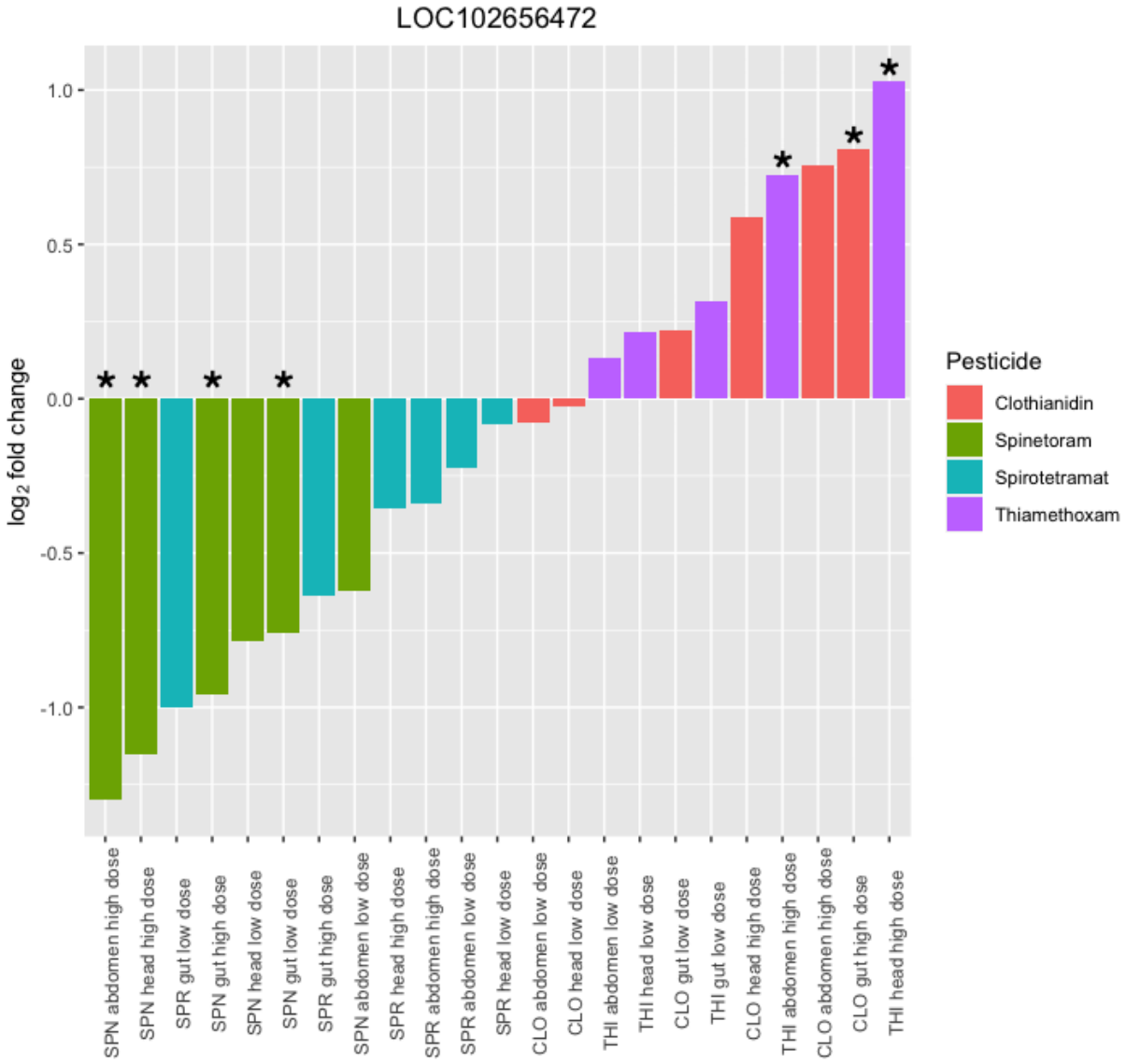

Figure 12. A bar plot of the $\log_2$ fold change of the gene expression of *LOC102656472*. Bars with an asterisk indicate samples that had significant (FDR 0.1) differential expression.

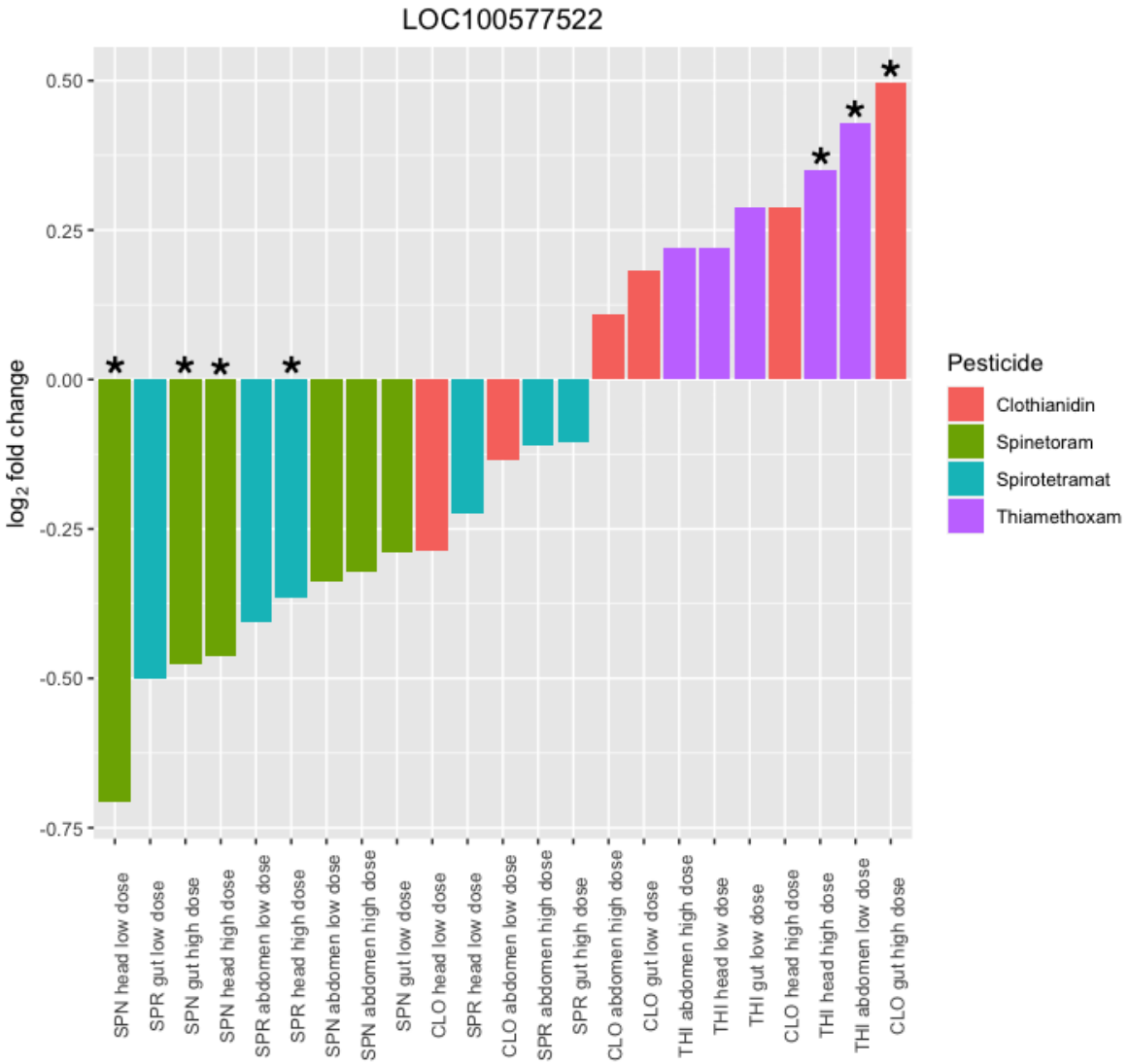

Figure 13. A bar plot of the $\log_2$ fold change of the gene expression of *LOC100577522*. Bars with an asterisk indicate samples that had significant (FDR 0.1) differential expression.

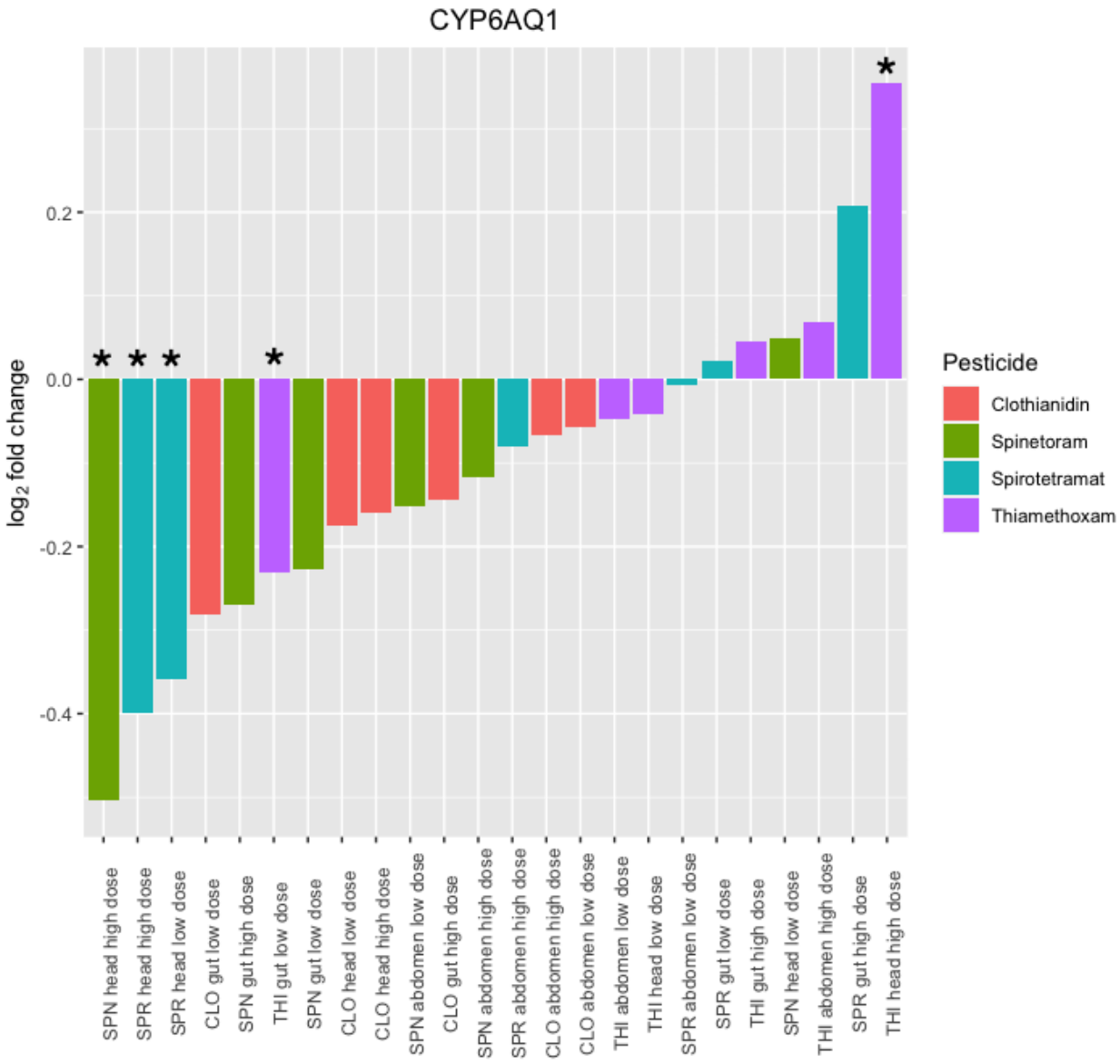

Figure 14. A bar plot of the log_2 fold change of the gene expression of *CYP6AQ1*. Bars with an asterisk indicate samples that had significant (FDR 0.1) differential expression.

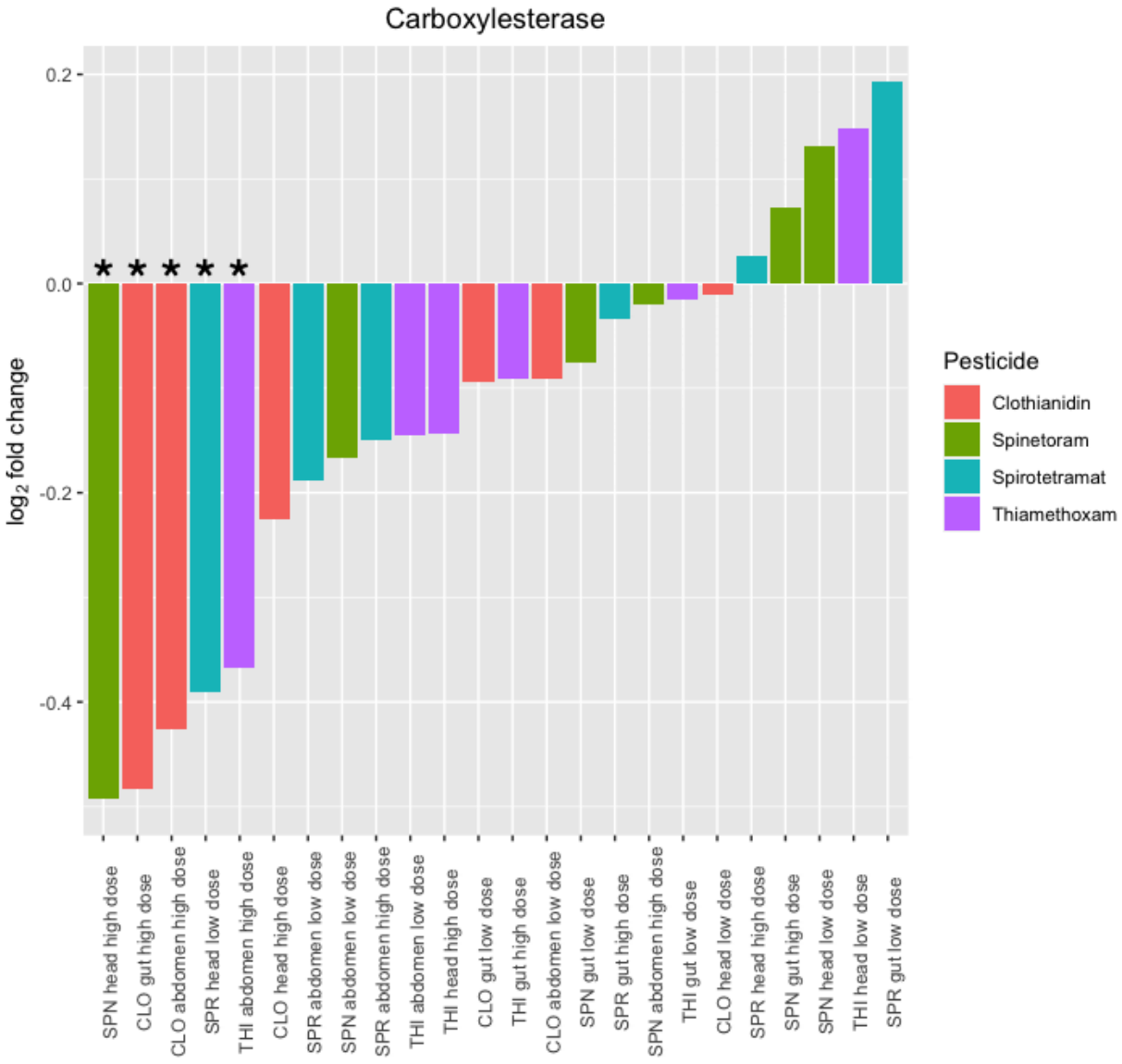

Figure 15. A bar plot of the $\log_2$ fold change of the gene expression of *Carboxylesterase*. Bars with an asterisk indicate samples that had significant (FDR 0.1) differential expression.

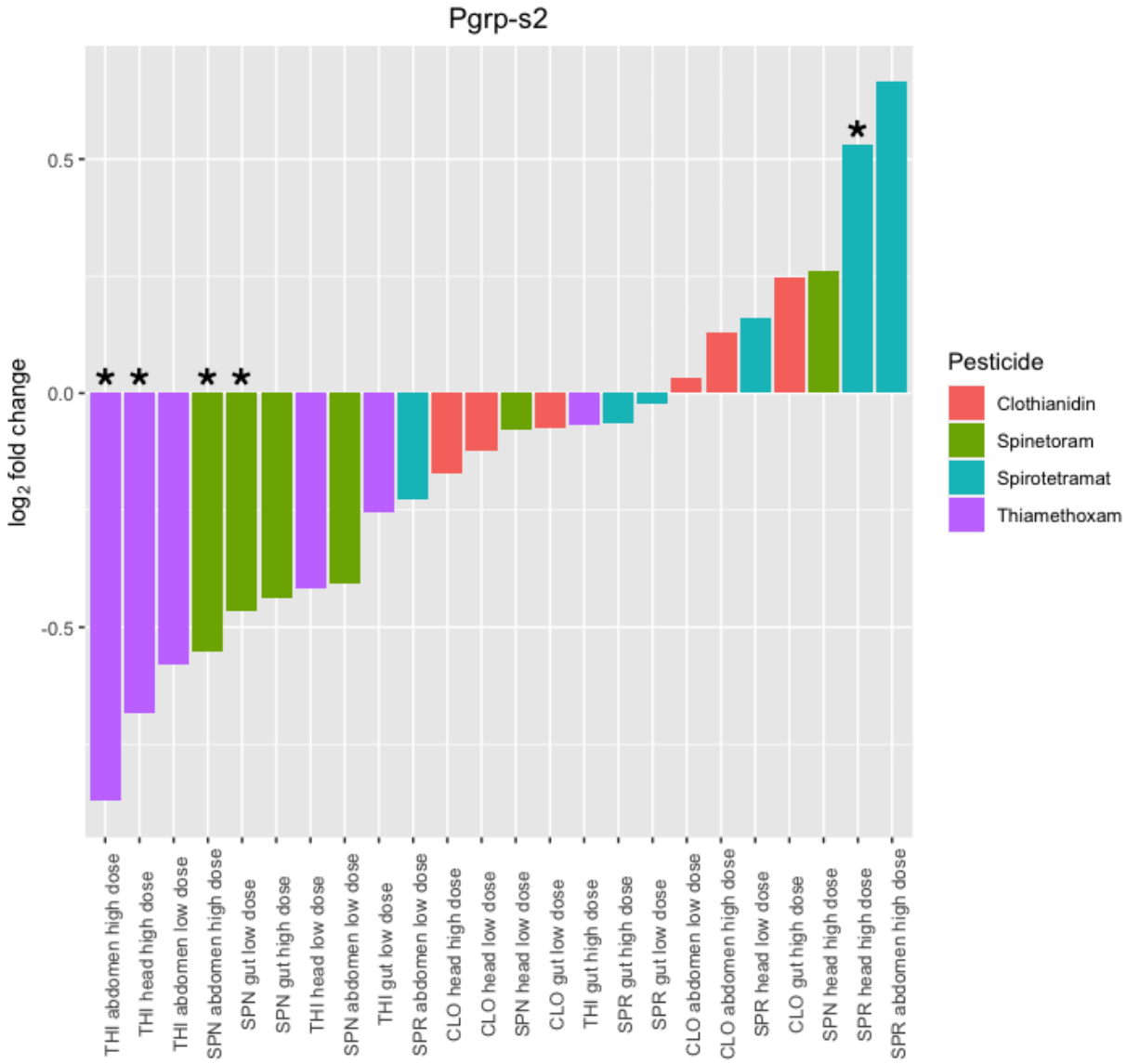

Figure 16. A bar plot of the $\log_2$ fold change of the gene expression of *Pgrp-s2*. Bars with an asterisk indicate samples that had significant (FDR 0.1) differential expression.

Appendix B: Supplementary Figures

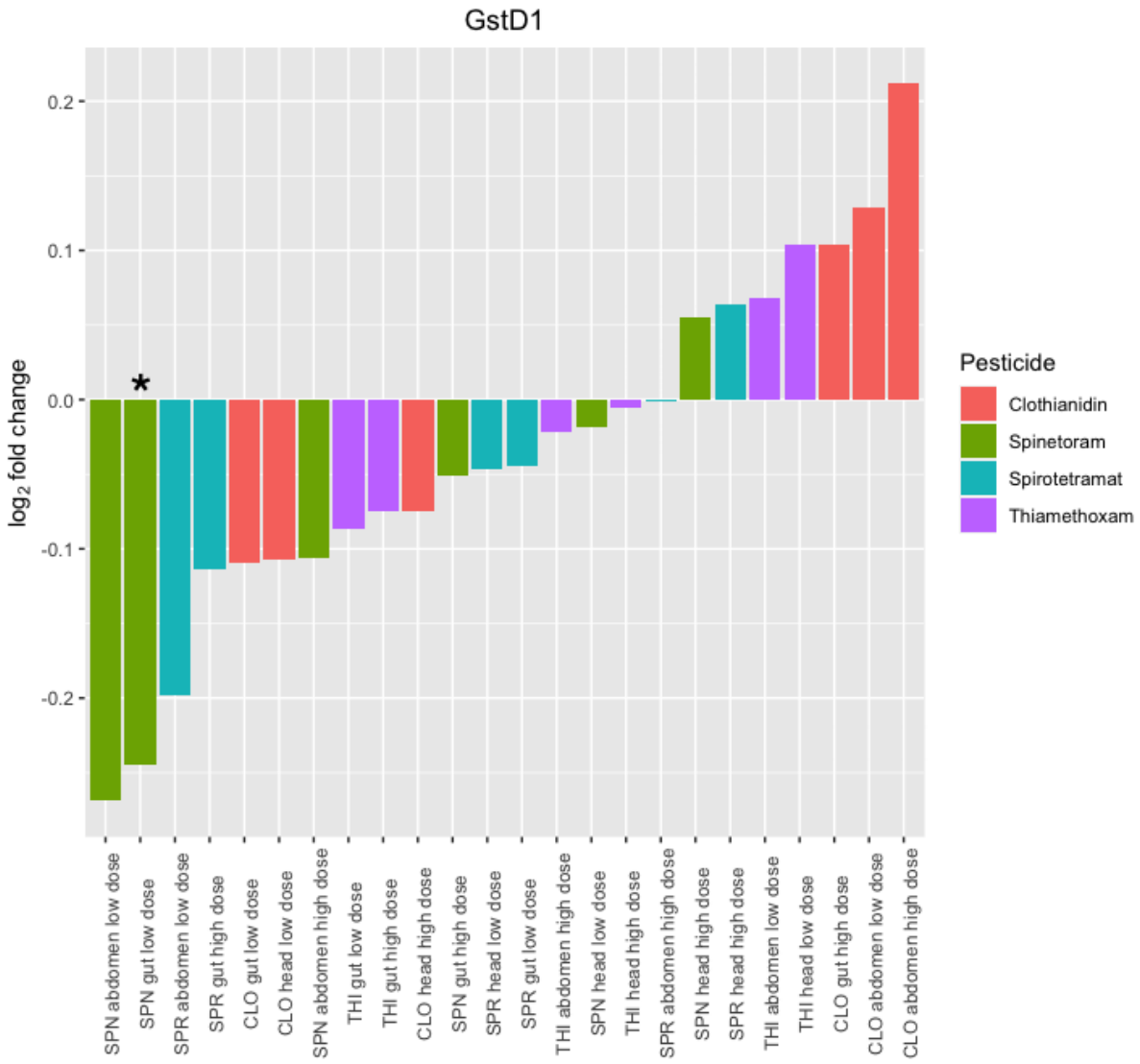

Figure S1. A bar plot of the $\log_2$ fold change of the gene expression of *GstD1*. Bars with an asterisk indicate samples that had significant (FDR 0.1) differential expression.

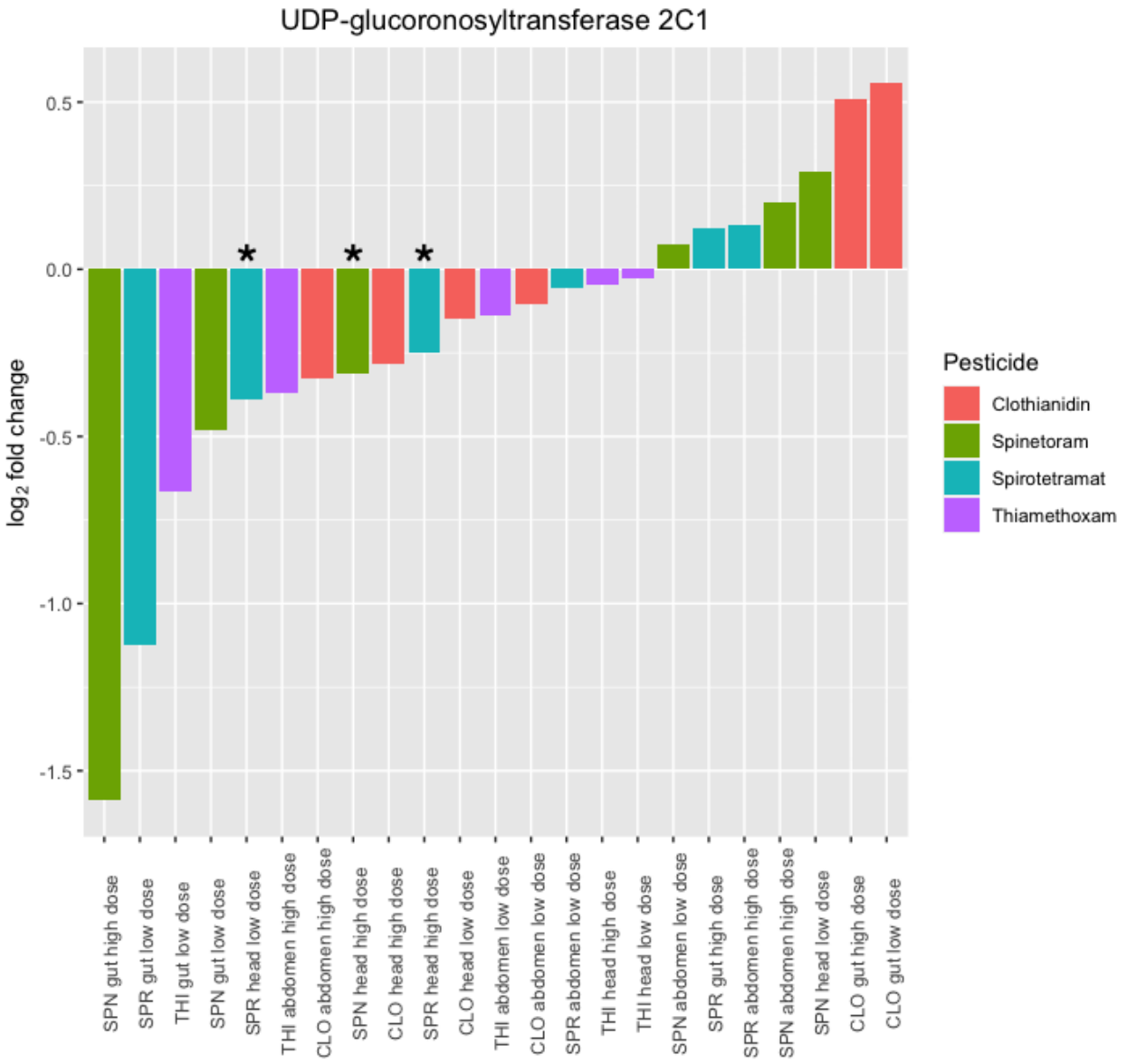

Figure S2. A bar plot of the log_2 fold change of the gene expression of *UDP-glucoronosyltransferase 2C1*. Bars with an asterisk indicate samples that had significant (FDR 0.1) differential expression.