Examining Neonicotinoid Resistance in the Honeybee

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A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science

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March 2021

Abstract

The genetic and molecular underpinnings of neonicotinoid (NNI) resistance are currently unknown in honeybees. Honeybees within the same colony exposed to clothianidin at the dose required to kill half of the population (LD50) exhibited different survival rates based on their genetics. The goal of my research was to explore transcriptomic differences in the brain, Malpighian tubules and ventriculus between non-resistant and resistant patrilines that had been exposed orally to a field realistic dose (4.6 ppb) of clothianidin for 2 hours. Transcriptomes were compared after 24 hours of exposure using RNA sequencing and the analysis revealed an upregulation in proteolysis and catabolic processes in the Malpighian tubules of non-resistant bees. This change in expression is likely a result of lack of neonicotinoid metabolism by the CYP9 enzymes. Finally, I compared the transcriptome of the Malpighian tubules, ventriculus and the brain and discovered that the majority of detoxification enzymes were highly upregulated in the Malpighian tubules. These findings shed light on the consequences of NNIs exposure and the molecular biology underlying NNI tolerance in honeybees.

Acknowledgements

I'd like to acknowledge my supervisor Dr. Amro Zayed for being patient with me, and helping guide me through this project (the first versions of this draft were very rough) and always giving me valuable advice on where I need to improve. I'd also like to acknowledge my other supervisory committee member Dr. Andrew Donini for overseeing my work and providing me with great constructive feedback. I'd also like to acknowledge Nadia Tsvetkov, seeing as without her, there would be no project. Her insights and advice helped tremendously when it came to analyzing and making sense of my data. I would also like to thank Tanushree Tiwari who helped me get on my feet when it came to using Linux servers and getting comfortable using computational methods in order to analyze my data. She was also someone who was incredibly patient with me, and her advice and support is something I never take for granted. I would also like to acknowledge the other members of the lab (in no particular order): Bandele Morrison, Arshad Imrit, Ida Conflitti, Dr. Clement Kent, KC Galang, Mateus Pepenelli, Rodney Richardson, Dova Suttner, Katie Dogantzis, and Taeyoon Yu (if I missed anyone, please know I thank you!). Though COVID-19 interrupted the time that I got to spend with them, they helped build up an extremely positive and inviting lab environment, and I will definitely miss our lunch breaks and chocolate tastings. Lastly, I'd like to acknowledge my dog Penny who became my unofficial lab partner when COVID hit. She helped me cope with the stress and loneliness that took place while doing my MSc during this time.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Supplementary Tables

Introduction

There are a number of factors implicated in the decline of honeybee health. One of the main contributors is the use of neonicotinoid insecticides (NNIs) on agricultural crops. While these pesticides have been banned in Europe, they are still widely used in North America (Gross, 2013). Neonicotinoids are a class of agricultural pesticides that are chemically similar to nicotine and can be subdivided into three classes: nitromethylenes, N-nitroguanidine and N-cyanoamidine neonicotinoids (Goulson et al., 2013). Their widespread usage as insecticides arises from their highly agonistic properties to insect nicotinic acetylcholine receptors (nAChRs), compared to the mammalian nAChRs (Tomizawa et al., 2005). In honeybees, acute levels of neonicotinoids can cause a variety of issues such as: impairing their immunity so that they are more susceptible to pathogens (Di Prisco et al., 2013); decreasing queen reproductive health (Williams et al., 2015); as well as causing learning and memory dysfunction in worker bees which can impact foraging (Henry et al., 2012). Chronic levels of neonicotinoids cause bee paralysis and eventually death (Blaquiere et al., 2012). Honeybees also tend to be more sensitive to N-nitro neonicotinoids (imidacloprid, clothianidin, thiamethoxam) compared to the N-cyano class (Iwasa et al., 2004).

Several researchers have shown that groups of bees within the same colony, or bees from different colonies or races, exhibit different health outcomes when exposed to NNIs. Laurino et al., found that there was a larger variation of honeybee acute oral toxicity of imidacloprid in the *Apis mellifera mellifera* colony compared to other honeybee genotypes *Apis mellifera ligustica*, and *Apis mellifera carnica* (2013). Suchail et al. (2000), also found similar results when comparing the LD50s of imidacloprid of A*pis mellifera mellifera* and *Apis mellifera caucasica*. *Apis mellifera mellifera* had a higher contact tolerance to imidacloprid (with an LD50 of 24 ng/bee compared to 14 ng/bee after contact application), however they both displayed similar oral toxicity results. A meta analysis was done in 2011 that also showed a wide variation in acute honeybee toxicity based on 13 studies (Cresswell, 2011). However, this large variation may be due to different methodologies, and environmental conditions rather than actual inherited resistance. Neonicotinoid resistance or tolerance has been previously documented in other insect species, and is mostly caused by an increase in expression in one or more of the cytochrome P450s that metabolize NNIs, or mutations in the nAChR which would reduce neonicotinoid binding (reviewed in Table 1.) (Bass et al., 2015).

Our group (Tsvektov and Zayed, in prep) carried out an experiment to understand the genetic and molecular biology underlying NNI sensitivity in honey bees. Briefly, Bees within the same colony were exposed to 29 ppb of clothianidin (the average LD_{50} obtained from the U.S. Environmental Protection Agency Pesticide Ecotoxicity database) and had shown a large variation of mortality based on their patriline (or dad's genetics), implying a certain level of inherited resistance to clothianidin. Specifically, they calculated a broad sense heritability value of 37.8% (Tsvetkov and Zayed, in prep). Honeybees are haplodiploid organisms, meaning in a typical honey bee colony, all worker bees are female and share the same mother, the queen bee. Because the queen is polyandrous (i.e. mate with many males), all worker bees sharing the same father are 75% related to one another, while workers with different fathers are 25% related. On a colony level any source of genetic variation could then only come from the males that the queen mates with. Paternal lineage can lead to phenotypic diversity within colonies, as demonstrated for sucrose responsiveness (Scheiner & Arnold, 2010), and hygienic behaviour (Perez-Sato et al., 2009). In Tsvetkov and Zayed's study, paternal lineage has been linked to the survivability of bees fed the LD_{50} dose of clothianidin.

The goal of my MSc is to explore the mechanisms responsible for the inherited differences in neonicotinoid resistance in honey bees. To achieve this, RNAseq was used to compare gene expression in the ventriculus, Malpighian tubules, and brains of honey bees from patrilines exhibiting high resistance and comparing them to patrilines with low resistance to NNIs. We used these datasets to test the hypothesis that inherited NNI resistance may be caused by an upregulation in detoxification enzymes in key tissues associated with detoxification. I also carried out an analysis to explore tissue-specific detoxification patterns of bees exposed to a sublethal dose of clothianidin.

Chapter 1

Transcriptomics of neonicotinoid tolerance in the honey bee

1.1 Introduction

Neonicotinoids (NNIs) are now the most widely used insecticides in the world and were developed in response to pest resistance to other chemical pesticides in agricultural crops (Jeschke et al., 2011). NNIs are systemic pesticides, where a coating is applied on the seed of a plant. The NNI is then absorbed by all parts of the plant as it continues to grow allowing it to be resistant to multiple pests at once, (Bromilow & Chamberlin, 1995). Honey bees are exposed to NNIs through multiple routes, such as pollen and nectar of agricultural crops, however presence of NNIs in wild plants near NNI treated crops has also been detected (Tsvetkov et al., 2017, Botlas et al., 2016). The literature on the effects of NNIs on honeybees has been very mixed. There are a multitude of studies linking NNI exposure of bees to altered learning and memory in foragers (Decourtye et al. 2004, Han et al. 2010, El Hassini et al. 2008). NNI exposure, even at sublethal doses have been linked to reduced immunity (Di Prisco et al., 2013) as well as increased mortality (Vidau et al., 2011). However there have also been studies that have contradicted

these findings (Williamson et al., 2013) and found no negative effects of NNI exposure on honeybees.

One possibility that may explain the variation in how NNIs influence bees is that some bee genotypes may be partly resistant to NNIs. Many insects have become resistant to neonicotinoids, either by an upregulation of their CYP enzymes, mutations in their CYP enzymes causing them to metabolize NNIs more effectively, or through mutations of their nAChRs (NNIs are no longer able to bind) (Table 1). There has been some indirect evidence of genetic predispositions that impact NNI survivability in honeybees. Woodcock et al. (2017) found that there were negative effects associated with honeybees near neonicotinoid treated crops in Hungary and the United Kingdom. In the same study, colonies in Germany had no effect associated with being near NNI treated crops, and experienced a higher number of egg cells. Laurino et al. (2013) also noted differences in LD50 doses between different *Apis mellifera* subspecies and colonies.

NNIs are first metabolized in the honeybee by cytochrome p450s. There are three P450 enzymes unique to the *Apis* family responsible for metabolizing neonicotinoids, CYP9Q1, CYP9Q2, and CYP9Q3. They're a family of cytochrome p450 mono oxidative genes which have a high affinity for the N-cyano NNIs, and have limited activity against nitro- substituted NNIs, with CYP9Q3 metabolizing the compounds the most efficiently (Manjon et al., 2018). The CYP9Q subfamily are highly expressed in the Malphghian tubules, ventriculus and the brain of the honeybee, which is consistent with the fact that these tissues are where xenobiotic detoxification would typically take place (Manjon et al., 2018, Vannette et al., 2015).

Tsvetkov & Zayed (in prep.) were able to link NNI resistance with patriline genetics in two separate honey bee colonies. Bees were given the LD50 dose, and after 24 hours, they were examined to see if they were dead or alive. Bees were then genetically fingerprinted to determine their patriline (dad), and patriline survival was then compared within each family (Figure 1A). There was a strong link between patriline and survival, thus leading them to the conclusion that NNI resistance is heritable in honeybees. They estimated broad sense heritability for LD50 survival at 37.8%. The CYP9Q1, CYP9Q2, and CYP9Q3 genes of the patrilines were also sequenced, and there was evidence of mutations present in the CYP9Q3 gene which was statistically associated with NNI resistance; bees with a specific group of haplotypes having 88% chance of surviving the LD50 dose of clothianidin. Tsvetkov & Zayed found that, for non-resistant bees (or bees which did not have the CYP9Q3 haplotype that corresponded to a higher survival), the CYP9Q1 haplotype still had an impact on survival. Non-resistant patrilines with a CYP9Q1 specific set of haplotypes have an average survival of 35%, while non-resistant patrilines with a different CYP9Q1 haplotype could have an average survival of 64%. Since we know which enzymes are responsible for NNI metabolism, and that a genetic predisposition exists which can alter NNI resistance, we can begin to explore tissue specific effects of NNIs, and the potential downstream effects caused by lower resistance to sublethal doses of NNIs. Sublethal doses of NNIs have been shown to cause morphological changes in the midgut and the Malpighian tubules (Catae et al., 2014, De Almeida Rossi et al., 2013, Oliveira et al., 2013). It has also been proven that field realistic doses can have an impact on the honeybee brain on a physiological level (reviewed by Cabirol and Haase, 2019) which can translate into altered honey bee behaviour. Our transcriptomic analysis will allow us to explore these effects between resistant and non-resistant bees by comparing differentially expressed genes between the two groups. It could give us insight into some of the molecular mechanisms involved in neonicotinoid toxicity, and will allow us to explore the effects that could potentially be causing the cellular changes associated with NNI resistance.

In this chapter, we aim to understand the molecular basis of NNI resistance by comparing global gene expression of resistant and non-resistant bees that have been exposed to a field realistic dose of clothianidin (Figure 1B). Additionally, we compared the transcriptomes of bees with different CYP9Q haplotypes. These analyses were conducted for 3 tissues previously known to be important for NNI detoxification: the Malpighian tubules, the brain and the ventriculus (Figure 2). By exploring different tissues, we can explore where the majority of the transcriptomics occur, and it allows us to explore other off-target impacts neonicotinoids may have, particularly on bees that are unable to metabolize them.

1.2 Materials and Methods

Experimental overview: Clothianidin exposure and bee collections

Bees were exposed to clothianidin in experiments carried out by Nadia Tsvetkov, a PhD student in the Zayed lab (Tsvetkov and Zayed, in prep). In summary, 9-day-old bees from two colonies were starved for 4 hours, and then fed 20 ul of sugar solution which either contained a field realistic dose (4.6 ppb), LD50 dose (29 ppb) or no (control) clothianidin for 2 hours. Only bees which had consumed 90% of their solutions were used in the analysis. The bees were then given a regular sugar solution (50% w/v) and after 24 hours, they were either scored as dead or alive before being frozen at -80ºC. They were then classified based on their patriline using microsatellite genotyping of 11 loci, following established methods (Tsvetkov & Zayed, in prep). Patrilines with the highest proportion of bees that survived the LD50 dose after 24 hours were classified as "Resistant". Similarly, patrilines with the highest proportion of bees that did not survive the LD50 dose after 24 hours were classified as "Non-Resistant". For the field realistic exposure, bees were exposed for 2 hours, and bees which had consumed over 90% of their sugar solutions were used in the analysis. After 24 hours these bees (they all had 100% survival due to the dose being sublethal) were then taken and frozen at -80ºC. A sublethal dose was chosen for transcriptomic analysis because it would be more representative of typical field exposure (Tsvetkov et al., 2017). As summarized in Figure 1B, we compared the transcriptomes from patrilines exhibiting the highest resistance from the LD50 studies to the patrilines exhibiting the lowest resistance with 3 patrilines within each group. Within each patriline we pooled the brain, Malpighian tubules, and ventriculus of 5 bees separately for each organ.

Dissections and RNA extractions

Frozen bees were placed in 1 ml of chilled RNAlater and thawed over ice. We performed dissections of the brain, Malphighian tubules, and ventriculus and kept them at -80ºC until RNA could be extracted. RNA extractions were completed using the Qiagen miRNeasy Mini kit. Briefly, we added 700ul of Qiazol lysis reagent to the tissues and homogenized the solution using a pipette. The protocol provided by the manufacturer was then followed in order to extract the RNA. We added 140 ul of chloroform to the mixture and shook the tubes before allowing them to sit at room temperature for 5 minutes. We then spun the mixture at 12 000 g for 15 minutes, and 1.5 volumes of 100% ethanol was added to the upper phase. Once a precipitate had formed, we ran the mixture through aRNeasy spin column. 700 ul of buffer RWT was then added, and once again run through the column. We added 500 ul of buffer RPE through the column twice before drying the membrane. 30ul of RNA-free water was then run through the column (this water now contains the RNA). RNA quality and quantity was assessed using a NanoDrop ND-1000 UV-Vis.

Pooling and Sequencing

Based on recommendations for illumina sequencing, we aimed for a minimum RNA concentration of 100ng/ul (or a total of 1500 ng of RNA). The ventriculus, brains and Malpighian tubules of 5 individual bees from each patriline were pooled separately based on their organ (i.e. all the brains of a patriline were pooled together). We had a total of 3 resistant patrilines, and 3 non-resistant patrilines and 18 pools in total. To ensure equal representation for each bee, we added 300 ng of RNA to each pool (1500ng/5bees). Samples were then kept at -80ºC until being shipped for sequencing at Genome Quebec. Libraries preparation and 100 bp pair-end sequencing using the Illumina NovaSeq 6000 S4, were carried out by Genome Quebec's sequencing facility (Montreal, QC). On average, we sequenced 233 million reads from each sample.

Assembly, annotation and transcriptomic analysis

Once the reads were received from Genome Quebec, they were trimmed using Trimmomatic, filtering out reads less than 50 base pairs (Bolger et al., 2014). Reads were aligned and indexed against the *Apis mellifera* genome (Amel_4.5) using STAR (Dober et al., 3013). MultiQC (Ewells et al., 2016) was run on all files to ensure quality alignments. Htcounts were used to count the aligned reads (Anders et al., 2014). Differential Expression analysis was carried out using EdgeR (Robinson et al., 2010). We used a Benjamini adjustment (Benjamini & Hochberg, 1995) of raw p-values, and genes with a False Discovery rate (FDR) of < 0.05 were considered to be differentially expressed. We first compared gene expression profiles of resistant vs non resistant patrilines (Figure 1). The bees were then compared based on their haplotypes. Because one of the haplotypes only had one biological replicate, we analyzed it using NOIseq (Tarazona et al., 2015). NOIseq simulates replicates under the assumption that the read counts follow a multinomial distribution. This allows the algorithm to make up for lack of replicates allowing us to view biological trends. Following the NOIseq recommendations (Tarazona et al., 2015), we normalized the data using the trimmed means methods and filtered out low counts (cpm <1). NOIseq does not output a p value, but rather an estimated probability value, they recommended a probability value of 0.9 for simulated data, however we chose a more stringent estimated probability of 0.95 to identify differentially expressed genes for this analysis. Since NOIseq simulates data, we view these results as biological trends rather than significantly expressed genes.

<u>GO analysis</u>

We used ShinyGO for enriched functional analysis (Ge et al., 2020) of Gene Ontology (GO) Processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) categories, using a false discovery rate cut off of 0.05. We also performed a brief literature search on genes that hadn't been grouped into any of the enriched GO functions.

1.3 Results

The survival rates from the LD50 trials of the patrilines are shown in Table 2, as well as their haplotypes (data provided by Nadia Tsvetkov). Non-resistant patrilines had an average survival of 40% while resistant patrilines had an average survival of 96% (Table 2, data provided by Nadia Tsvetkov). Transcriptomes were sequenced at an average read length of 100 bp and an average of 133 million reads per sample. Filtered reads had >99.9% alignment to the bee reference transcriptome (Figure 3).

Resistant (R) vs. Non-Resistant (NR)

We found no differentially regulated genes in the Brain between resistant and non-resistant honeybees. We did find 1 gene, XR_003306097.1, an uncharacterized protein (logFC =-3.35, $p<0.0001$, FDR < 0.0001), that was upregulated in the ventriculus in the NR honeybees.. Clustering by resistance was only seen in the Malpighian tubules (Figure 4). Resistant patrilines had 1 significantly upregulated gene in the Malpighian tubules: fatty acid amide hydrolase 2-B (logFC =1.12, $p<0.0001$, FDR < 0.0005). In the Malpighian tubules of NR patrilines, 95 genes were upregulated (Supplementary Table 1). Of the 95 upregulated genes there were 37 uncharacterized genes. Interestingly, caspase-3, one of the main regulatory genes involved in apoptosis, was upregulated in non-resistant bees (logFC =-1.19, $p<0.0005$, FDR < 0.05). Another gene, involved in osmotic regulation in the Malpighian tubules such as the sodium potassium calcium exchanger 3 was also upregulated (logFC =-1.50, $p<0.0005$, FDR < 0.0005). Three SOX transcription factors were also seen to be upregulated in the NR group such as Sox-2(logFC =-2.56, $p<0.0001$, FDR < 0.0001), and Sox- 13 (logFC =-3.56, $p<0.0001$, FDR < 0.001) and Sox 21-B (logFC =-1.71, $p<0.0005$, FDR < 0.05). CYP6A-4 was the only cytochrome P450 to be found to be upregulated in the NR group (logFC =-1.47, $p<0.0005$, FDR < 0.05). Dyenin-beta ciliary (involved in ciliary movement) (logFC =-4.30, $p<0.0001$, FDR < 0.005) , and uncharacterized LOC107964495 (logFC

=-4.24, $p<0.0001$, FDR < 0.001) were the most upregulated genes in our gene set (Supplementary Table 1).

Gene ontology analysis of the up-regulated genes in the Malpighian tubules of non-resistant bees indicated proteolysis (GO:0006508) as the only enriched biological process (BP) ($p < 0.016$). This group includes endothelin-converting enzyme homolog, trypsin, disintegrin and metalloproteinase domain-containing protein 10-like, putative serine protease K12H4.7, caspase-3, aminopeptidase N (Gene ID: 551180), aminopeptidase N (Gene ID: 551224), and serine protease 53. Up-regulated genes in the Malpighian tubules of non-resistant bees were also enriched for multiple molecular functions (Figure 5), including: peptidase activity (GO:0008233), peptidase activity-acting on L-amino acid peptides, beta-N-acetylhexosaminidase activity (GO:0004563), hydrolase activity (GO:0016787), hexosaminidase activity (GO:0015929), endopeptidase activity (GO:0004175), metallopeptidase activity (GO:0008237), catalytic activity, acting on a protein (GO:0140096), aminopeptidase activity(GO:0004177), catalytic activity (GO:0003824), serine-type peptidase activity (GO:0008236), and serine hydrolase activity (GO:0017171).

Expression differences between CYP9Q haplotypes

An analysis of differences in SNPs among CYP9Q1-3 genes revealed differences in survival between different CYP9Q haplotypes (Tsvetkov unpublished). The first indicator of survival was the SNPs present in the CYP9Q3 enzyme. Our resistant group (P7, P24, and P11) all had CYP9Q3 haplotypes that led to a survival rate in the LD50 study of 96% (Table 2). Our NR group (P21, P1, P8) all had CYP9Q3 haplotypes from the non-resistant group which lowered their survival. Within the NR group, P1 and P8 had SNPs in their CYP9Q1 gene leading to differences in survival as well. P1 and P8 had a survival average of 34.5% in the LD50 study (hereafter called the low survival haplotype), while P21 had a CYP9Q1 haplotype the led to a survival rate of 53% (hereafter called the intermediate survival haplotype). The purpose of this analysis was to compare the three haplotypes (noted R for high survival, P1P8 for low survival, and P21 for intermediate survival) individually in order to examine the molecular changes that occur as a result of their SNPs. Making transcriptomic comparisons between each NR haplotype (low vs intermediate) will allow us to study the biological response associated with having a lower tolerance compared to the haplotype that exhibits an intermediate tolerance. In this section, we compare low, intermediate and high survival groups to explore the range of responses associated with NNI exposure.

Low survival vs intermediate survival

When comparing the low survival group (34.5% survival) and the intermediate survival group (53% survival), we found the largest differences of gene expression in the Brain (Supplementary Table 3). 37 genes were upregulated in the intermediat group, including 3 P450s: CYP9Q2 (FC =1.10 , $p<0.05$), CYP6A17 (FC =1.75 , $p<0.05$) and cytochrome b5 (FC =1.84 , $p<0.05$). Other interesting differentially expressed genes include death associated protein 1, which is involved in apoptosis (logFC =1.41 , $p<0.05$) . The top 30 enriched biological processes included categories ($p<0.005$) such as transmembrane transport (GO:0090662) and establishment of localization (GO:0051234) (Supplementary Table 7). The intermediate survival group had upregulated genes enriched in 6 cellular component (CC) categories, most of them having to do with the membrane ($p<0.05$). Genes upregulated in this group were enriched for the following molecular functions: In catalytic activity (GO:0003824) ($p<0.005$), binding ($p<0.05$), 6 genes were also involved in oxidoreductase activity (GO:0016491) ($p<0.05$). Upregulated genes in the intermediate survival group were enriched for 6 KEGG pathways: MTOR signaling ($p<0.0005$), phagosome ($p<0.0005$), oxidative phosphorylation ($p<0.001$), metabolic pathways ($p<0.0005$), phagosome ($p<0.0005$) and carbon metabolism ($p < 0.005$).

In the Brain, 210 genes were found to be upregulated in the low survival group compared to the intermediate survival group including: the nACHRα-1 subunit (FC =-1.59 , $p<0.05$), AChE-2 (FC =-1.68, $p<0.05$) and choline-O- acetyl transferase (responsible for the synthesis of acetylcholine) (FC =-1.12 , $p<0.05$). Acetyl-coenzyme A synthetase (FC =-1.15 , $p<0.05$), muscarinic acetylcholine receptor DM1 (FC =1.72 , $p<0.05$), vesicular acetylcholine transporter (FC =-1.50 , $p<0.05$) were also upregulated in the low survival group. Other upregulated genes involved in neural signaling include: the octopamine receptor (FC =-1.23, $p<0.05$), the alpha-2A adrenergic receptor (FC =-1.45, $p<0.05$), vesicle associated membrane protein 2 (FC =-1.06, $p<0.05$), and neuroendocrine convertase 2 (FC =-1.45, $p<0.05$) (Supplementary Table 3). Our gene list had enrichment in 21 BP categories, the top ones being establishment of localization (GO:0051234) ($p<0.01$), transport ($p<0.01$), and localization ($p<0.01$) with 24 upregulated genes in each category. 21 cell component terms were enriched with 52 upregulated genes being involved with the membrane ($p<0.005$). Of the molecular function categories, binding was enriched with 77 genes being upregulated ($p<005$) as well as hydrolase activity (GO:0016787) ($p<0.05$). 2 KEGG pathways were enriched, the phagosome, and the longevity regulation pathway ($p<0.05$) (Supplementary Table 8).

In the Malpighian tubules, we saw less differences between the low survival and intermediate survival with only 11 genes upregulated in the intermediate survival group. The low survival group had 18 upregulated genes including upregulation of the CYP9Q3 protein (FC=1.35, $p<0.05$). We found no GO enrichment from the upregulated genes of the P21 haplotype. The only biological processes category that was enriched was proteolysis (GO:0006508) ($p<0.05$), with 4 genes in our list being involved. There was no significant enrichment in the cellular component GO category. 17 molecular function categories were enriched (Supplementary Table 9) with 9 genes being involved in catalytic activity(GO:0003824) ($p<0.05$) and 7 having hydrolase activity (GO:0016787) ($p<005$).

Only one gene, Mjrp1 (FC=7.54, $p<0.05$), was upregulated in the ventriculus of bees in the intermediate survival group. The low survival group had two upregulated genes in the ventriculus: vitellogenin (FC= 3.88, $p<0.05$) and LOC107963975 (FC =2.41 $p<0.05$).

Intermediate survival vs high survival

When comparing intermediate vs high survival, we found 2 upregulated genes in the high survival group: alpha-amylase (FC = 2.18, $p<0.05$) and NPC intracellular cholesterol transporter 2 homolog a (FC = 1.75, $p<0.05$). No upregulated genes were found in the intermediate group. In the ventriculus there were 2 genes upregulated in the high survival group: transcription factor SPT20 homolog, and take-out-like carrier protein. The intermediate survival group had 5 upregulated genes compared to the resistant group including CYP9Q3 and CYP336A1. Lastly, in the Malpighian tubules there were 19 genes upregulated in the intermediate group compared to the high survival group. These genes were not enriched for any gene ontology terms. The high survival group also had 5 upregulated genes in the Malpighian tubules: melitten (FC=4.58, $p<0.05$), hexamerin 70a (FC= 1.70, $p<0.05$), CYP9Q3 (FC = 2.11, $p<0.05$), facilitated trehalose transporter Tret1 (FC = 2.33, $p<0.05$), and transcription factor SPT20 homolog (FC = 2.17, $p<0.05$).

Low survival vs high survival

There were no differentially expressed genes in the brain between the low survival group and the high survival group. In the Ventriculus, we found one upregulated gene in the low survival group (uncharacterized LOC724439, FC = 2.29, $p<0.05$), and two genes upregulated in the high survival group (take-out-like carrier protein, FC =1.63, $p<0.05$, and trypsin-7 FC=1.32, $p<0.05$) (Supplementary Table 6). There were 22 upregulated genes in the Malpighian tubules of the low survival group and 3 genes upregulated in the high survival group: melittin (FC= 3.87, $p<0.05$), transcription factor SPT20 homolog (FC= 2.25 $p<0.05$), NPC intracellular cholesterol transporter 2 homolog a (FC= 1.87, $p<0.05$)

(Supplementary Table 6). In total there were 8 biological processes enriched in upregulated genes in the Malpighian tubules of the P1P8 group. Of these upregulated genes, 11 of the 22 were involved in metabolic processes($p<0.002$) and 7 were involved with proteolysis (GO:0006508) ($p<0.00001$) in the biological processes categories. In the 15 enriched molecular function categories, 11 genes were involved in hydrolase activity(GO:0016787) ($p<0.00001$), and 12 were involved in catalytic activity (GO:0003824) ($p<0.0001$) (Supplementary Table 10). 11 of the upregulated genes also overlapped with the genes that were upregulated in the intermediate survival group when compared to the low survival group.

1.4 Discussion

The goal of this experiment was to understand the molecular basis of neonicotinoid resistance, with our initial hypothesis being that we would see an upregulation in detoxification enzymes in our resistant patrilines. Our data shows that this is not the case, seeing as there were no upregulated detoxification genes found in our resistant patrilines when exposed to a sublethal dose of clothianidin. This suggests that it is allelic differences between patrilines that account for the majority of the resistance seen between our patrilines. The mutations in the CYP9Q enzymes can either allow more efficient metabolism of NNIs leading to resistance, or have the opposite effect and lower the metabolism of NNIs. That being said, analysis of the upregulated genes in the non-resistant group can still give us insight into the molecular mechanisms associated with NNI toxicity.

GO analysis revealed that there was an upregulation of proteolysis and catabolic processes in the Malpighian tubules of honeybees that are not resistant to neonicotinoids. Africanized honeybees that have been exposed to sublethal doses of thiamethoxam had altered Malpighian tubule cells, specifically in the basal labyrinth (Catae et al., 2014). The basal labyrinth is a specialized cellular structure in the Malpighian tubule cells that helps maximize contact with the hemolymph to increase the uptake of metabolized substances (Cruz-Landim,1998). The exposed bees had visible disorganization in this region, while the microvilli and mitochondria remained intact (Catae et al., 2014). After 3 days of exposure, there was a visible increase in smooth endoplasmic reticulum as a way to increase cellular detoxification. Finally, after 8 days of sublethal exposure, there were major alterations in the Malpighian tubules with the loss of basal labyrinth, the loss of mitochondria, and dilated microvilli (Catae et al., 2014). A similar effect was seen in De Almeida Rossi et al.'s study where they found a reduction in the basal labyrinth, after 3 days of sublethal exposure of the LD50/10 of imidacloprid in africanized honeybees (2013). The authors also found an increase of pyknotic nuclei, the irreversible condensation of the chromatin in the nucleus, and an increase in cytoplasmic vacuolization (De Almeida Rossi et al., 2013). Our RNAseq data seems to be consistent with these studies where the upregulation in genes involved in catalysis, and proteolysis in NR patrilines may be contributing to this reorganization of the Malpighian tubule cells in order to increase detoxification of clothianidin, which may have either non-functioning, or less effective, CYP9 enzymes because of mutations found in these patrilines.

Malpighian tubule cells have been known to self renew through multipotent stem cells (Singh et al., 2007). The upregulation of Sox transcription factors (known to regulate cell differentiation and development) in our NR group suggests a shift in cell differentiation in these cells as a result of

clothianidin exposure. This could have long term implications and may explain the major alterations in the Malpighian tubules seen in previous studies. There also was an upregulation in multiple genes involved in ciliary movement, LOC726309 protein artichoke, dynein beta chain, ciliary, hydrocephalus-inducing protein-like were all upregulated in NR bees. While there isn't cilia present in the honeybee Malpighian tubules, they do have the presence of microvilli (Noecelli et al., 2016). The upregulation of these genets suggests that there can be an increase in microvilli movement which may lead to an increased secretory activity of the Malpighian tubules. The sodium calcium potassium exchanger is involved with osmotic regulation and transepithelial secretion in the Malpighian tubules, and helps regulate the water secretion in the Malpighian tubule cells (Noecelli et al., 2016). We had found that this was upregulated in the Malpighian tubules of our bees. There could be a disruption in the ionic and fluid homeostasis in these cells and cells may be attempting to compensate for the lack of detoxification by increasing the propulsion of fluids from the hemolymph and body, into their waste. Due to their limited capacity to break down clothianidin, likely caused by mutations in CYP9Q enzymes, the non-resistant group appear to be experiencing altered Malpighian tubule detoxification. They may be trying to reorganize their cellular structures in order to increase detoxification capacity, or may be overwhelmed with the dose of clothianidin leading to cell death. We can also see this with the upregulation of caspase-3, a protein involved in apoptosis as well as the increase in proteolysis.

There have been many studies that have examined shifts in the brain as a response to NNIs (Table 3). In particular, Oliveira et al., 2013 looked at the side effects of the brain and midgut (ventriculus) of bees that were exposed to 1/10 and 1/100 of the LD50 of thiamethoxam for 3 days. Through histochemical analysis they found that the mushroom bodies and kenyon cells had significant morphological alterations indicative of cell death. Christen et al., looked at the transcriptomic profile of the brains of bees in response to 3 and 30 ppb of clothianidin (our bees had 4.6ppb). Bees were fed the solution ad libitum for 48 hours before being analyzed. They found 18 genes to be differentially expressed, compared to the control, 4 were upregulated while 14 were downregulated (Christen et al., 2014). There were no differences in gene expression in the brain however our bees had different exposure conditions which may have not been enough to cause a gene expression shift in the brain. Alternatively, because we had no unexposed group, gene expression shifts in the brain may be happening with all the bees, but is undetectable without an unexposed control.

With regard to the ventriculus, it has been reported that there has been a change in morphology to the midgut, such as cytoplasmic vacuolization, increased apocrine secretion and increased cell elimination., 1 day after being exposed to sublethal doses of thiamethoxam orally (Olivereira et al., 2013).

In Catae's et al.'s study they had also noticed a drastic increase in apoptosis in the cells in ventriculus of honeybees exposed to sublethal doses compared to the control group (2014). We did not see any difference between the R and NR groups. The ventriculus effect may be happening to all bees studied, regardless of resistance which is why we didn't see any changes and had we included a un-exposed control group, this phenomenon may have been detected in our data. We may also have not seen this effect due to the different exposure conditions between the studies.

Haplotype

Tsevetkov & Zayed (in prep.) found that CYP9Q sequence had an effect on honeybee survival to the LD50 dose of clothianidin. CYP9Q3 haplotype determined whether or not a bee could be considered resistant (88% survival to an LD50 dose of clothianidin) or non-resistant. Within the non-resistant group, CYP9Q1 played a role on whether a patriline had a low survival (34% survival to an LD50 dose of clothianidin), or an intermediate survival (53% survival to an LD50 dose of clothianidin). By comparing the differentially expressed genes between the NR haplotypes, it can give us information regarding the downstream effects of the CYP9Q1 mutations with regards to NNI toxicity.

We observed the largest changes in expression between the NR haplotypes (P1P8 vs P21, low vs intermediate survival) in the brain. The intermediate survival group was found to have an upregulation of mono-oxygenase activity with CYP9Q2 being upregulated. The data implies that in order to compensate for a less effective CYP9Q3 protein, this group may be upregulating CYP9Q2 and other P450s proteins to help breakdown clothianidin, even at sublethal doses. This difference between the CYP9Q1 haplotype may contribute to the higher survival rate between the intermediate and low groups. Our results aren't conclusive however, since we only had one patriline to explore this effect.

Another notable difference between the two NR haplotypes was that the intermediate survival group had an upregulation in genes involved with mTOR signaling. mTOR signaling has been proven to have involvement with learning and memory (Graber et al., 2013). The 3 upregulated genes involved with mTOR signalling were V-ATPases which are all involved with activating mTORC1. This suggests that bees in the low survival group might have altered learning and memory due to the fact that they have

lower expression of mTOR signalling genes which might help explain some of the learning dysfunctionalities seen in honeybees exposed to sublethal doses of neonicotinoids (Henry et al., 2012).

We found that the low survival compared to the intermediate survival had an upregulation of 210 genes in the brain. Based on our resistance analysis (R vs NR), there doesn't seem to be a major effect in the brain as a response to NNIs in a short period of time between resistant and non-resistant groups. Even when the low and intermediate survival groups were compared to the resistant group individually, there wasn't a large number of DEGs. Thus, the large differences may be a result of haplotypes within NR patrilines and could potentially play a role as to why the intermediate group has a higher survival rate when exposed to the LD50. We found that ACHe-2, the nAChRα subunit, and other neuroreceptors were upregulated in the low survival group. Christen et al., did notice a significant increase in the nAChRα subunit in the honeybee brain 72 hours after being orally exposed to 3 ppb of clothianidin, while there was a slight increase (n.s.) after 24 hours (2016). Seeing as clothianidin is an agonist to the nAChRα receptors a slight upregulation in the low survival as a response to small amounts of NNIs that could potentially play a larger role when it comes to more constant sublethal exposures, or at higher dosages. The upregulation of these receptors may indicate that the low survival group is having a harder time metabolizing the NNIs, and are thus upregulating these receptors in order to compensate. Additionally the upregulation of these receptors can also lead to behavioral changes associated with NNI exposure (Henry et al., 2012).

1.5 Conclusion

Our study shows that even sublethal doses can have an impact on honeybees that are not resistant to neonicotinoids. When comparing resistant and non-resistant patrilines, we found non-resistant patrilines had an upregulation in genes involved in proteolytic processes that may be involved in the structural damage of Malpighian tubules seen in previous studies. Our study gives insight into the toxicodynamics of honeybees that have different levels of resistance to neonicotinoids, and the mechanisms of how their susceptibility greatly increases depending on their genetics. We also found transcriptomic changes between non-resistant bees that had different CYP9Q1-3 haplotypes (which had previously resulted in different levels of survival (Tsvetkov & Zayed, in prep.). Once again, examining these molecular changes gives us insight into the mechanisms that result in long term NNI toxicity. This helps us understand the conflicting results in the literature regarding honeybee learning as a result of NNI exposure. Future studies should include what sort of impact these changes may have on a colony level, as well as long term expression studies based on resistance/haplotype.

Chapter 2

Differential expression analysis of detoxification enzymes in the brain, ventriculus and Malpighian tubules

2.1 Introduction

Most of the RNA-seq tissue specific data in response to NNIs has either been in the brain, or whole bee expression (Table 3). NNIs target the nACHr, however as shown by (Catae et al., 2014), there was a rise in apoptosis in the ventriculus after 24 hours as a response to sublethal doses of thiamethoxam, and the Malpighian tubules had suffered a major loss in their basal labyrinth. Comparing gene expression in different tissues of bees exposed to NNIs can help us understand which tissues are primarily involved in detoxifying NNIs as well as understand the downstream effects of NNI exposure on honey bees.

NNIs are detoxified in the honeybee through 3 major enzyme families: cytochrome P450s (P450s), glutathione transferases (GSTs) and carboxylesterases (CaEs) (Li et al., 2007). The P450s are involved with phase 1 metabolism, where they catalyze oxidation and demethylation reactions (Magesh et al., 2017). GSTs catalyze the metabolism of NNIs in phase 2 by conjugating the thiol group from glutathione, to the electrophilic center of toxic compounds or other reactive oxygen species. As a result, the compound is now more water soluble and targets the molecule to specific glutathione multidrug exporters (GSH) (Esther et al., 2015). Lastly, carboxylesterases are responsible for the hydrolysis of carboxyl esters into the corresponding alcohol and carboxyl acid, and have been shown to be involved with the formation of NNI metabolites. Suchail et al., has reported distribution of imidacloprid and its metabolites throughout the whole body of the honeybee suggesting metabolism occurs in multiple tissues, outside the midgut (the first line of defense when NNIs are orally ingested) (2004). Most of the studies looking at NNI detoxification pathways have used in-vitro metabolism assays, or toxicity bioassays with the presence/absence of certain enzyme inhibitors/inducers (reviewed by Magesh et al., 2017).

The goal of this chapter is to explore global gene expression in three tissues of honey bee workers exposed to a sublethal dose of clothianidin: the brain, Malpighian tubules and ventriculus. We chose these tissues based on previous research. For example, many studies have linked NNI exposure to altered honeybee behaviour and learning (El Hassani et al., 2008), suggesting that NNIs impact the honey bee brain transcriptome. We also examined the ventriculus (or midgut) as it constitutes the first lines of defense when NNIs are ingested orally (Kakamand et al., 2008). Finally, we also studied the Malpighian tubules because they play an important role in detoxification (Beyenbach et al., 2010). Moreover, one previous study (Manjon et al., 2018) clearly established that all three tissues play an important role in specifically detoxifying NNIs. We specifically characterized the expression patterns of three major detoxification enzyme families (GSTs, CaEs, and P450s) and compared them between the three above mentioned tissues.

2.2 Materials and methods

Dissections, RNA extractions and RNA sequences were performed as described in section 1.2, above. We took the RNAseq expression data of all bees (resistant and non-resistant) for each organ and compared the organs against one another.

Data analysis

We performed an ANOVA to compare tissue specific expressions of the midgut, brain and Malpighian tubules using the EdgeR program (Anders et al., 2014). We explored the functional role of differentially expressed genes by carrying out a gene ontology analysis using the ShinyGO software on uniquely upregulated genes (Ge et al., 2020). A gene was considered uniquely upregulated if it was upregulated relative to each other tissue. We then examined the top 30 enriched GO categories for each organ. Venn diagrams were created with the DEGs using an online software (http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl). We specifically focused on the following subsets of *Apis mellifera* genes involved in detoxification: cytochrome p450s, Glut-S-transferases (GST), and carboxylesterases (CAE).

2.3 Results

Tissue Specific expression:

We found 6,792 differentially expressed genes between the 3 Tissues. 855 transcripts were uniquely upregulated in the Ventriculus, 2406 were uniquely upregulated in the Malpighian tubules, and 3527 transcripts were uniquely upregulated in the Brain (Figure 5).

Gene ontology analysis:

We compared the GO categories enriched for uniquely upregulated genes of each organ (Supplementary Table 11). Enrichment analysis revealed that the uniquely upregulated genes in the ventriculus had overrepresentation in 20 biological processes with 204 genes being involved in metabolic processes, and 196 being involved in cellular processes Of the cellular components categories that were enriched, with 174 upregulated genes being involved in the membrane. In the top 30 enriched molecular function categories, 232 genes were involved with binding, 163 had catalytic activity and 93 were involved with protein binding.

The Malpighian tubules had significant enrichment in biological processes with 708 genes being expressed in the metabolic processes, 546 genes involving cellular metabolic processes and 118 being involved in peptide biosynthetic processes. In the cellular component category 492 genes were intracellular, 334 genes were categorized as being in the cytoplasm, while 289 were categorized in membrane bound organelles. Molecular function enrichment revealed 576 genes being involved in catalytic activity, and 467 genes were in the heterocyclic compound binding, and organic cyclic compound binding categories (Supplementary Table 12).

In the brain, there was enrichment in metabolic processes with 763 genes, 672 genes were a part of the organic substance metabolic process. In the cellular components, 582 genes were intracellular, and 385 genes were membrane bound. In the molecular function category, 1169 genes were involved in binding, with 592 being organic cyclic compound binding (Supplementary Table 13).

Detoxification enzymes

Of the detoxification enzymes analyzed, 4 out of the 6 GSTs were differentially expressed between tissues (Figure 7). Of the 51 cytochrome p450s analyzed, 22 were differentially expressed between tissues, with the majority being upregulated in the Malpighian tubules. Lastly 5 out of 8 carboxylesterases analyzed were differentially expressed between tissues. CYP6AS4 was the only P450 that was the most highly expressed in the brain, compared to the other tissues. Catalase was also the only

enzyme that was the most highly expressed in the brain relative to the Malpighian tubules and the ventriculus. Lastly, Carboxylesterase clade I, member 1 was the only carboxylesterase that was the most highly expressed in the brain. CYP18A1, CYP9Q1, and cytochrome p450 9-e2 like were the only P450s that were up-regulated in the ventriculus relative to the brain and Malpighian tubules. All the other detoxification enzymes were up-regulated in the Malpighian tubules.

2.4 Discussion

The brain had the highest number of uniquely upregulated genes compared to the Malpighian tubules and the ventriculus. This makes sense seeing as the function of the brain is different compared to the Malpighian tubules and ventriculus (both organs belonging to the digestive tract). In terms of gene enrichment, it was surprising to see that the Malpighian tubules and the brain had more molecular functions in common compared to the ventriculus (Figure 7). Many of these functions involved binding of substrates such as cyclic molecules and a variety of nucleotides. The Malpighian tubules are responsible for the detoxification in the body, and much of that involves the breakdown of organic molecules. In the brain, these molecules (such as octopamine, dopamine etc.) are used to induce signals between neurons. So, though the MF functions may be similar, as shown in the BP categories, the outcome of these functions is very different. KEGG analysis revealed that the Malpighian tubules are enriched for metabolic processes, which is inline with its known role in filtering and detoxifying substances from the hemolymph (Nocelli et al., 2016).

In terms of phase I detoxification, our data had the highest levels of CYP450 expression in the Malpighian tubules followed by the ventriculus and brain having varying amounts of expression, depending on the P450 (Figure 8). This is interesting seeing as our data gives more insight into understanding which P450 is more highly expressed in each tissue. Decourtye et al., (2004) found that CYP expression increased in the honeybee brain 30 minutes after a sublethal dose (0.12 ng) of imidacloprid was fed. However there was no follow-up study done to compare the levels 24 hours later. While we weren't able to get an unexposed/control tissue to determine the baseline levels of detoxification enzymes, a honeybee protein atlas has been published which allows us to compare DEG data (Chan et al., 2013). When looking at the P450 expression, they found the highest expression in the Malpighian tubules, followed by the brain, and then the ventriculus. However it's important to note that they had calculated the P450s as a fraction of all proteins across castes (workers tend to have higher P450 expression compared to queens and drones). When comparing organs in just the digestive tract (of bees of mixed ages), Vannette et al. (2015), found a higher number of P450s to be expressed in the Malpighian tubules, followed by high expression in the midgut, which is consistent with our findings.

With regard to phase II detoxification, we found that GST expression was highest in the Malpighian tubules, however the brain had higher Gst-1, Gst-U1, and Gst-T1 expression compared to the ventriculus (figure 7). Higher phase II metabolism in the brain is also seen when comparing GST levels of the brain to the Malpighian tubules as there was only a slight upregulation in Gst-U1 and GST-1 in the Malpighian tubules (the detoxification center of the bee) compared to the brain. Chan et al. (2013), found high GST levels in the Malpghian tubules, ventriculus and the brain compared to the rest of the honeybee tissues in the protein atlas. Because of the nature of their study, it wasn't really clear which of the three tissues had the most amount of GST expression, but they were higher compared to other honeybee tissues. Vannette et al. (2015) used qPCR to determine DEGs involved in detoxification in the honeybee digestive tracts and found 4 GSTs were upregulated in the Malpighian tubules, while 5 were upregulated in the midgut.

Our data implies that there may be a downregulation in GST activity in the ventriculus, seeing as we had lower levels compared to what has been published (Chan et al., 2013, Vanette et al., 2015). This could be a result of NNI damage to the ventriculus, similar to the effects seen by Oliveira et al., 2014. This data also implies that the honeybee brain is more equipped to deal with NNI metabolites rather than the original compounds. This supports the idea that longer exposure to NNIs, even at sublethal doses, becomes more toxic to bees. As bees continue to ingest NNIs, first damage to their ventriculus is done limiting their phase I detoxification (Oliveira et al., 2014, Catae et al., 2014). Though their ventriculus recovers in the long term (Catae et al., 2014), long term damage is done to the Malpighian tubules (Oliveira et al., 2014) which have the highest phase I and phase II activity (based on their enzymes). Due to the limited CYP expression in the brain, honeybees aren't able to metabolize NNIs, or other exogenous substances as efficiently leading to altered behaviour seen as a result of NNI exposure (Tvetkov et al., 2017, Decourtye et al., 2004).

In terms of tissue specific expression of the CYP9 enzymes, the main NNI metabolizers, we found that CYP9Q2 and CYP9Q3 was the most expressed in the Malpighian tubules compared to the other tissues. Once again this makes sense considering a major role of the Malpighian tubules is to detoxify xenobiotic substances from the body. Manjon et al. (2018) quantified CYP9Q baseline expression in the brain, Malpighian tubules and ventriculus of honeybees using qPCR and found similar levels of CYP9Q1 in the midgut and the Malpighian tubules, while the brain had significantly low expression of CYP9Q1. The midgut had the lowest expression of CYP9Q2, followed by a significant increase in the brain, while the Malpighian tubules had the highest expression. CYP9Q3 expression levels were significantly higher in the brain and Malpighian tubules compared to the midgut (Manjon et al., 2018). CYP9Q1 expression patterns in our study were similar to Manjon et al. where the brain had the lowest expression followed by

the MT and ventriculus. CYP9Q2 was also significantly expressed in the Malpighian tubules relative to the brain and ventriculus, however we did not see a difference between the brain and ventriculus. Manjon et al. had found that the brain had similar CYP9Q3 expression levels as the Malpighian tubules, however in our data, CYP9Q3 expression was significantly higher in the Malpighian tubules compared to the brain. We also did not see a difference in CYP9Q3 expression between the brain and ventriculus, with the brain only having a slight upregulation.As a result of clothianidin exposure in our study, the CYP9Q3 gene could potentially be upregulated in the ventriculus and Malpighian tubules in all patrilines, however because of the nature of our experiment, we have no negative control that could confirm this. Looking at the specific expression patterns of these enzymes allow us to see where the effects of NNI exposure could have an impact. Long term damage to the Malpighian tubules (Catae et al., 2014) would result in a drastic reduction of clothianidin metabolism, seeing as that is where the CYP91-3 genes are the most highly expressed. The potential upregulation of CYP9Q3 in the ventriculus and Malpighian tubules indicates that phase I metabolism of clothianidin does increase, and could help defend against the damage that could be caused by short-term sublethal doses.

2.5 Conclusion

Our data provides insight into the detoxification mechanisms present in honeybee tissues, and helps explain the long-term toxicity seen as a result of NNI field-realistic exposure on a molecular basis. Damage to the ventriculus and Malpighian tubules can lead to altered phase I metabolism, making it difficult for honeybees to break down NNIs, thus leading to long-term damage. Additionally, looking at the specific patterns of CYP9Q1-3 enzymes allow us to determine which tissues are the most susceptible to NNI toxicity damage. Follow up studies looking at longer-term detoxification enzyme expression in different honeybee tissues as a result of sublethal NNI exposure should be done in order to validate these predictions.

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TABLES

Table 1: Summary of neonicotinoid resistant insects and their mechanisms based on a review done by Bass et al., 2015

Species	Upregulation (↑)/ Downregulation (↓)/ mutation	Enzyme/gene
B. tabaci	↑	CYPCM1 Glut –S transferases CYP4C64
M. Persicae	↑ mutation	CYPCY3-caused by modification in promoter region β1 nAChR subunit –point mutation in loop D region (R81T)
A Gasyppi	mutation	nAChR – R81T
N. Lugens	mutation ↑	nAChR- N1α1 + N1α3 CYP6EA1, CYP6AY1
M. Domestica	↑	CYP6A1, CYP6D1, CYP6D3, CYP6G4
T. vaporariorum	↑	CYP6CM1-4

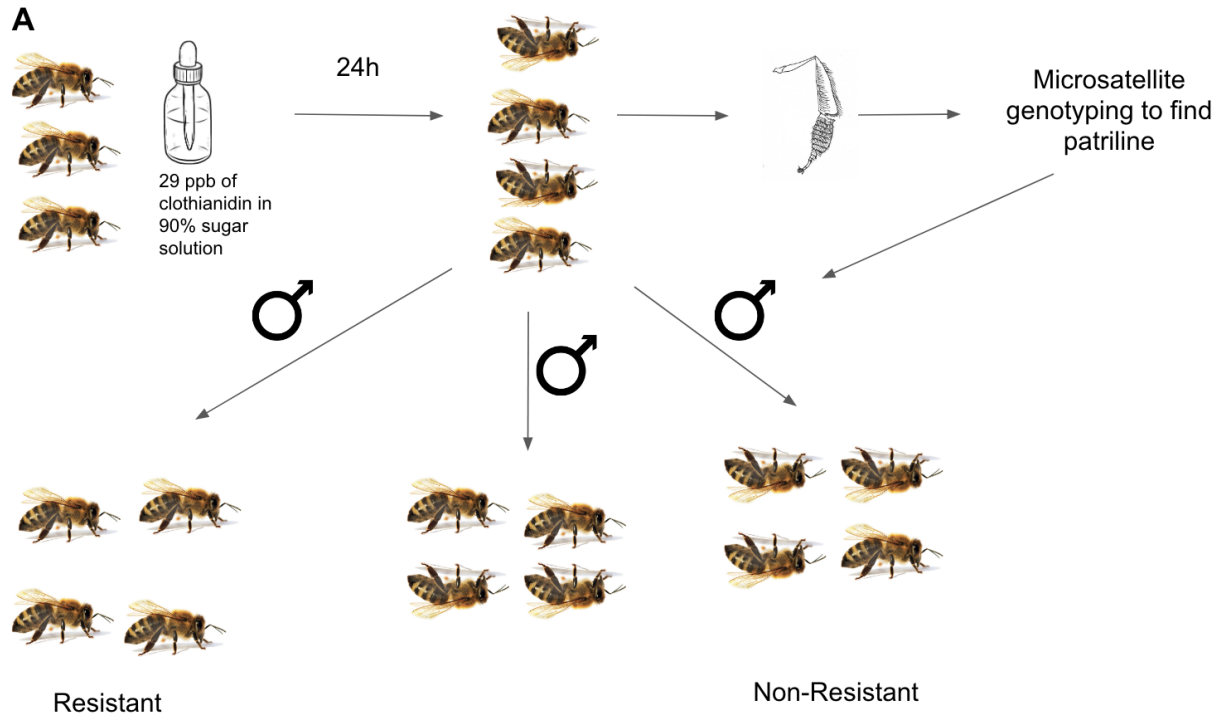
Table 2: Summary of LD50 trials of Patrilines being used in RNAseq analysis. Bees were fed a 29 ppb clothianidin-sugar solution for 1 hour. After 24 hours, bees were checked to see if they were dead or alive, and survival rates based on Patrilines were determined. CYP9Q1 and CYP9Q3 genes were sequenced and haplotype was determined, Data provided by Nadia Tsvetkov.

Patriline	Tox	freq	N	Survival(%)	Classification	CYP9Q3 Haplotype	CYP9Q1 Haplotype
P24	Alive	21	22	95	Resistant	O	C
P7	Alive	10	10	100	Resistant	K	C
P11	Alive	16	17	94	Resistant	M	C
P1	Alive	4	13	31	Non-Resistant	N	B
P8	Alive	5	13	38	Non-Resistant	P	E
P21	Alive	8	15	53	Non-Resistant	L	D

Table 3: Summary of RNAseq studies done in honeybees as a result of neonicotinoid exposure.

Author	Year	Bee Age	Organ	Neonic + Dose	DEG	GO Enrichment
				3, 30* ppb Imidacloprid		BP:male mating behavior and imaginal disc-derived wing vein specification, N-acetylneuraminate catabolic process, protein refolding MF:alpha-amylase and amylase activity
				clothianidin 30* ppb		BP:N-acetylneuraminate catabolic process and protein refolding in biological processes MF: N-acetylneuraminate lyase activity and unfolded protein binding CC: chorion and nucleosome in cellular components
Christen et al	2018	Foragers of mixed age came from a site with no agricultural/pesticide activity	brain, and hypopharyngeal gland	thiamethoxam 1 ppb, 10* ppb		BP: 3′-phosphoadenosine 5′-phosphosulfate metabolic process and carbohydrate metabolic process MF: 3′(2′), 5′- bisphosphate nucleotidase activity and alpha-amylase activity
Wu et al	2017	1 week old bees + 1d, 2d, 4d, 8d	whole bee	imidacloprid- 0.1, 1 10 ppb	160 UR 349 DR	Down regulation of muscle related genes
Shi et al	2017	4 day old bees, exposed for 10 days	whole bee	thiamethoxam-10 ppb	225 UR 384 DR	BP:Protein metabolism, biosythetic processes CC: ribosome, ribonucleprotein complex MF: ribosome, structural molecule activity, oxidoreductase activity
Li et al	2019	11 day old bees from hive -> exposed for 11 days in cage	Brain	imidacloprid - 20 ppb (0.02 ng/µl)	130 DR 1 UR	Downregulated in imidacloprid treated bees GO:0055114 Oxidation-reduction process GO:0005506 Iron ion binding GO:0016491 Oxidoreductase activity KEGG : phototransduction pathway - involved in memory formation
Derecka et al	2013		larvae	imidacloprid (2 ppb)	300 DEG	lipid-carbohydrate-mitochondrial metabolic network, CYP450, lipid homeostasis, sugar metabolism
Wu, Chang et al.,	2017		Heads of newly emerged bees	500 ppb	578 DEG	MF: monooxygenase activity, oxidoreductase activity and steroid hydroxylase activity BP: fatty acid metabolic process, lipid biosynthetic process, steroid metabolic process, small molecule biosynthetic process and monocarboxylic acid metabolic process CC: extracellular matrix

FIGURES


A
24h
29 ppb of clothianidin in 90% sugar solution
Microsatellite genotyping to find patriline
♂
♂
♂
Resistant
Non-Resistant

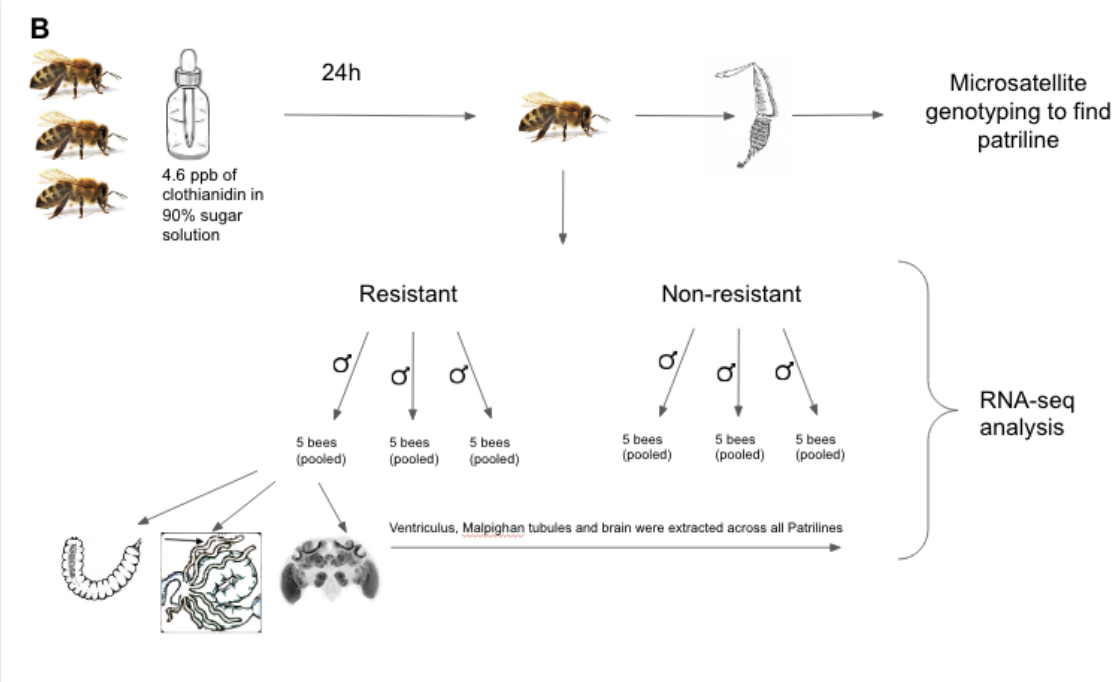


Figure 1: Overview of LD50 study (A) and RNA seq study design (B). Bees from the 3 patrilines who exhibited the most resistance to the LD50 dose of clothianidin are being compared to bees from 3 patrilines who exhibited the least amount of resistance. The brains, Malpighian tubules, or ventriculus of 5 bees from each patriline will be pooled together. Each patriline represents a biological replicate for the resistant/ non-resistant phenotype.

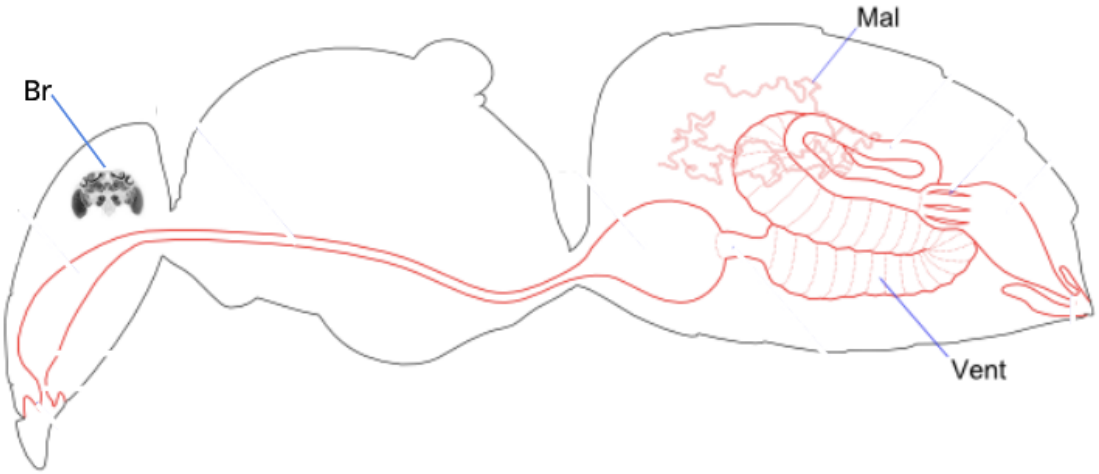


Figure 2: Honeybee tissues used in the transcriptomic analysis. RNA of the Brains (Br), Malpighian tubules (Mal) and Ventriculus (V) of 5 bees exposed to 4.6 ppb of clothianidin were pooled for each patriline.

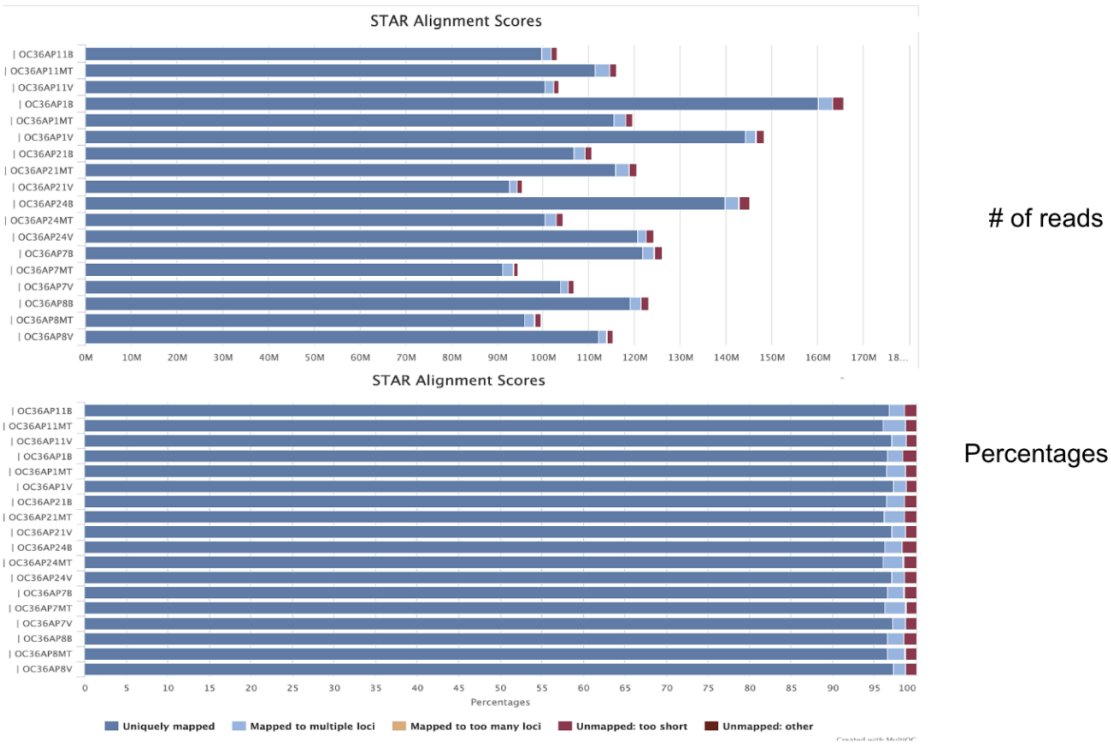


Figure 3: RNA seq number of reads an alignment as determined by MultiQC. Reads were trimmed using the Trimmomatic software before being aligned using STAR. Average read length was 100 bp, and average alignment was 99%.

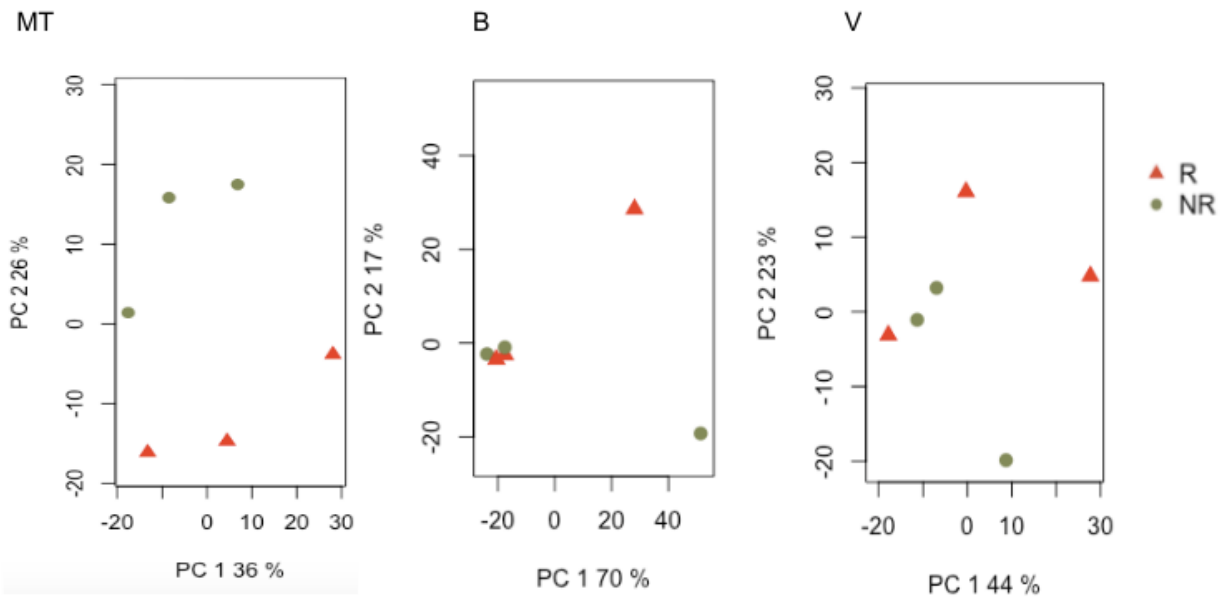


Figure 4: PCA plots of the Malpighian tubules, Brain and Ventriculus of transcriptomic data of honeybees exposed to 4.6 ppb of clothianidin. Clustering based on resistance could only be seen in the Malpighian tubules.

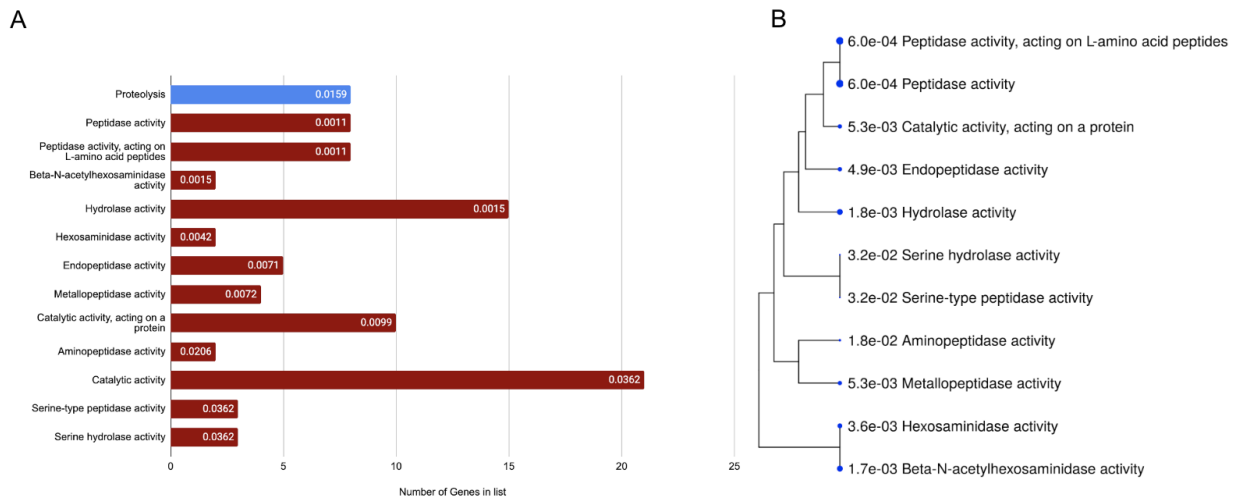


Figure 5: Significantly Enriched Biological Processes (blue) Molecular Function (red) Gene Ontology (GO) terms represented in the significantly upregulated DEGs in the Malpighian tubules of non-resistant honeybees exposed to 4.6 ppb of clothianidin. A) Number of differentially expressed genes associated with the GO process. A total of 95 genes were found to be upregulated ($p<0.05$). B) Hierarchical clustering of Enriched Molecular Function processes.

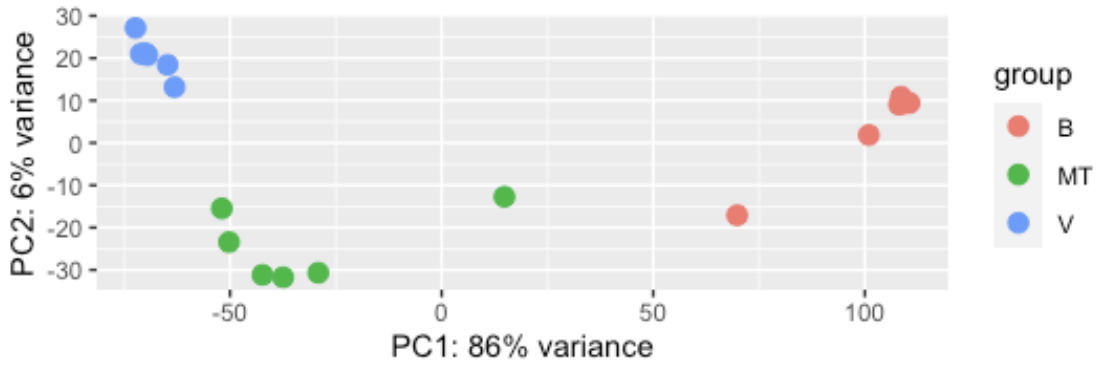


Figure 6: RNAseq data PCA of the Brain, Malpighian tubules and Ventriculus. Data clustered strongly by organ

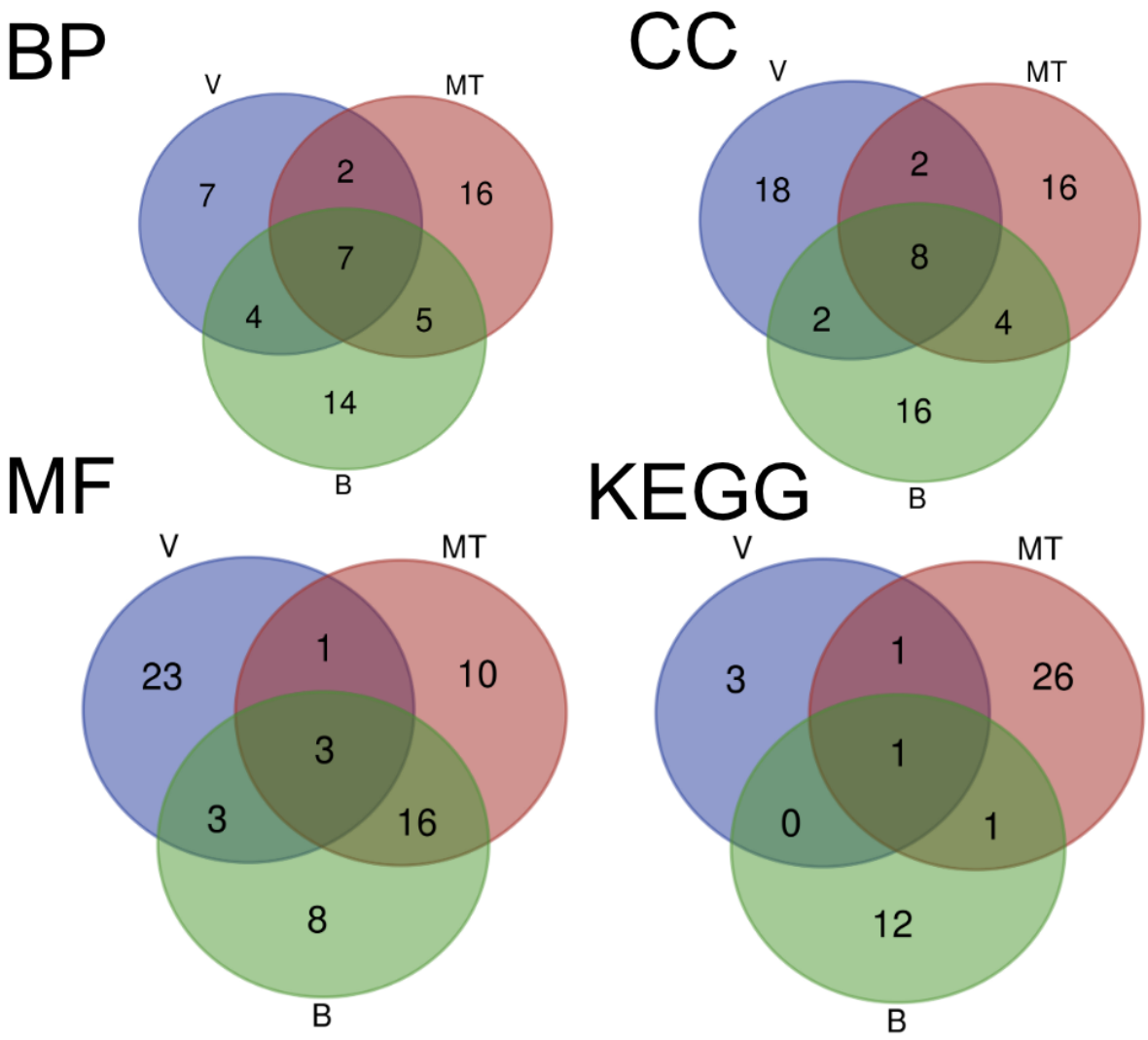


Figure 7: Venn diagram of Enriched Biological Processes (BP), Cellular Compartments (CC), Molecular Function (MF) and KEGG of uniquely upregulated genes in the Ventriculus (V) Malipighian Tubules (MT) and the Brain (B) The Associated GO lists can be found in Supplementary Tables !4-17

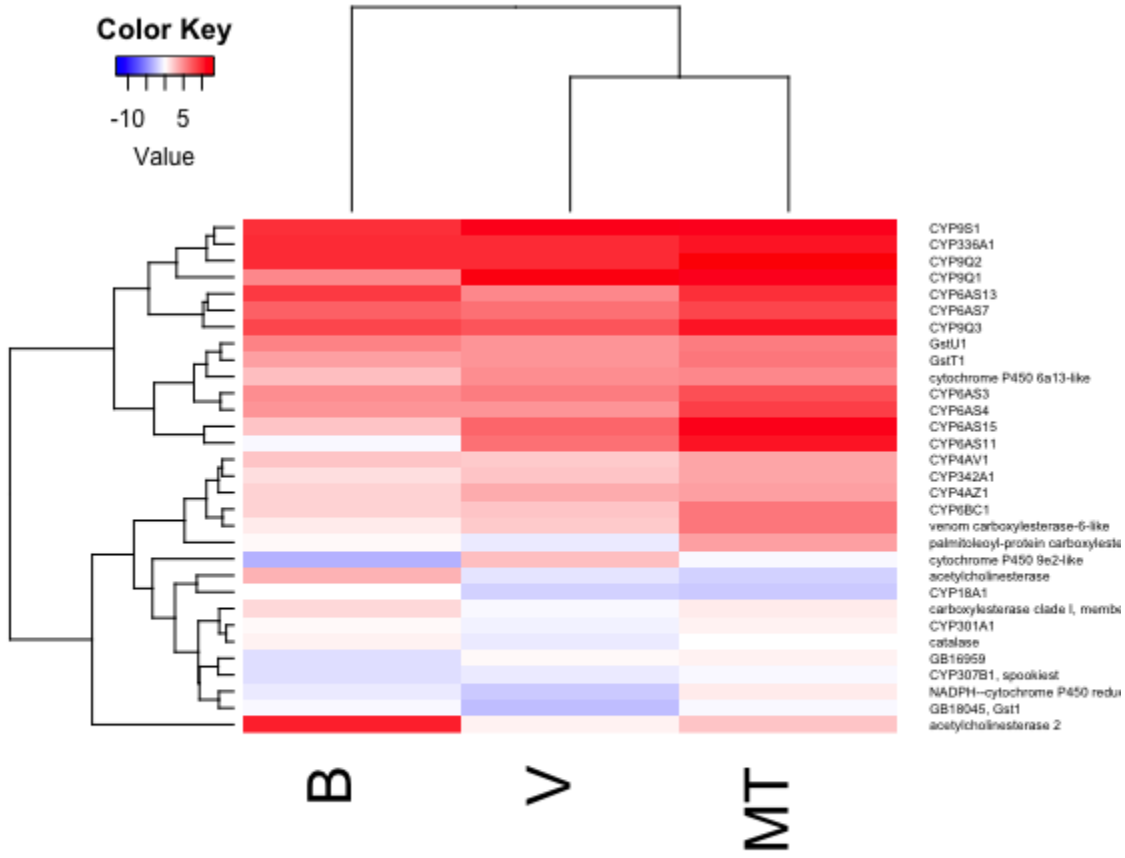


Figure 8: Differential gene expression of detoxification enzymes in the Malpighian tubules, ventriculus, and brain.

Supplementary Tables

Supplementary Table 1: List of DEGs in the Malpighian tubules between Resistant and non-resistant patrilines exposed to a field realistic dose of clothianidin

Gene	logFC	logCPM	PValue	FDR	GeneID	Protein
XM_026445507.1	-4.30	4.43	4.6E-06	0.001	551562	dynein beta chain, ciliary
XR_001703097.2	-4.24	2.67	2.2E-06	0.001	107964495	uncharacterized LOC107964495
XM_392226.7	-3.84	-0.82	4.0E-04	0.040	408690	calmodulin
XM_006563260.2	-3.81	3.32	3.8E-05	0.006	550785	Apis mellifera fructose-bisphosphate aldolase (LOC550785), transcript variant X2, mRNA
XM_006564738.3	-3.76	4.43	6.0E-09	0.000	412969	uncharacterized LOC412969
XM_006559239.3	-3.49	-2.17	4.2E-06	0.001	724242	protein neuralized
XM_026444499.1	-3.29	-2.05	2.1E-04	0.025	410363	uncharacterized LOC410363
XM_016912656.2	-3.16	-2.86	3.5E-06	0.001	408884	transcription factor Sox-13
XM_026445881.1	-3.12	3.66	7.9E-05	0.012	100577769	Apis mellifera uncharacterized LOC100577769
XR_001702612.2	-3.02	-0.54	4.3E-06	0.001	107964233	uncharacterized LOC107964233
XM_016911272.2	-3.02	-0.81	2.5E-05	0.004	412883	Allatostatin C receptor
XM_394827.6	-2.99	7.97	4.1E-09	0.000	411353	lipase 3
XM_392043.7	-2.90	0.49	6.0E-12	0.000	408500	endothelin-converting enzyme homolog [Apis mellifera (honey bee)
XM_003249830.4	-2.84	-2.96	6.6E-05	0.010	100577110	Apis mellifera uncharacterized LOC100577110

XM_026440879.1	-2.81	8.20	2.8E-07	0.000	726309	LOC726309 protein artichoke
XR_001703293.2	-2.81	-0.69	2.0E-05	0.004	107964569	uncharacterized LOC107964569
XR_408967.3	-2.77	-2.44	1.1E-06	0.000	102656563	uncharacterized LOC102656563
XM_006565484.3	-2.77	2.80	8.7E-07	0.000	551224	aminopeptidase N
XM_016913279.2	-2.75	4.58	3.9E-14	0.000	100578995	vanin-like protein 1
XM_016913467.2	-2.71	-1.00	2.6E-04	0.030	413052	uncharacterized LOC413052
XM_016914011.2	-2.68	-3.44	3.0E-04	0.033	411490	probable beta-hexosaminidase fdl
XM_006572315.3	-2.66	0.79	2.8E-04	0.032	100578112	adenylate kinase isoenzyme 5
XM_006561437.3	-2.61	0.22	5.7E-09	0.000	408960	protein slit [Apis mellifera (honey bee)]
XM_394067.6	-2.59	-1.38	2.1E-06	0.001	410589	semaphorin-5A
XM_026444698.1	-2.56	3.48	8.6E-08	0.000	408411	transcription factor Sox-2
XR_003306397.1	-2.48	-0.98	1.4E-04	0.018	113219352	uncharacterized LOC113219352
NM_001011636.1	-2.48	5.71	1.2E-07	0.000	409798	Fabp FABP-like protein
XM_003251822.4	-2.45	3.10	6.1E-09	0.000	100577161	uncharacterized LOC100577161
XM_016916443.2	-2.44	1.81	7.1E-05	0.011	100576136	Apis mellifera zinc finger BED domain-containing protein 1
XR_003306009.1	-2.44	-1.50	6.7E-08	0.000	107965414	uncharacterized LOC107965414
XM_006557650.3	-2.43	-1.03	8.1E-07	0.000	102654685	uncharacterized LOC102654685
XM_006565481.3	-2.40	10.48	2.6E-07	0.000	551180	aminopeptidase N
XM_026442484.1	-2.40	-0.44	1.3E-04	0.017	410791	mitochondrial uncoupling protein 2
NR_039429.1	-2.38	-1.52	1.1E-04	0.015		Apis mellifera microRNA mir-1175

XM_006564914.3	-2.37	1.55	2.9E-04	0.032	552592	facilitated trehalose transporter Tret1
XR_003306097.1	-2.33	3.00	1.2E-08	0.000	107965483	uncharacterized LOC107965483
XM_393060.7	-2.29	2.85	2.8E-07	0.000	409553	pancreatic triacylglycerol lipase
XM_026439414.1	-2.27	6.56	1.8E-04	0.022	551714	cGMP-dependent protein kinase, isozyme 1
XM_003249136.4	-2.25	6.48	5.0E-04	0.049	100578100	uncharacterized LOC100578100
NM_001013361.1	-2.24	3.81	1.8E-09	0.000	410902	18-w 18-wheeler
XM_026446208.1	-2.21	0.68	2.0E-04	0.023	412949	ionotropic receptor 25a
XM_026444170.1	-2.18	0.06	4.8E-04	0.046	410398	insulin-like growth factor 2 mRNA-binding protein 1
XM_006565067.3	-2.17	0.80	3.9E-07	0.000	410470	putative polypeptide N-acetylgalactosaminyl transferase 9 [
XM_003250107.4	-2.15	1.98	1.3E-05	0.003	100576163	short neuropeptide F
XM_003250921.4	-2.14	1.58	6.0E-07	0.000	100578548	uncharacterized LOC100578548
XM_001120112.5	-2.13	9.86	1.9E-05	0.004	724308	serine protease 53
XM_026443683.1	-2.11	-0.56	1.2E-05	0.003	727052	mucin-17-like
XM_026439740.1	-2.10	-1.14	2.4E-07	0.000	100578706	uncharacterized LOC100578706
XM_006559465.3	-2.09	0.57	3.0E-04	0.033	100577488	uncharacterized LOC100577488
XM_623673.6	-2.09	5.10	1.7E-06	0.000	410729	putative serine protease K12H4.7
XM_006561640.3	-2.07	1.15	5.1E-04	0.049	725671	homeobox protein Nkx-2.8
XM_026442221.1	-2.03	1.49	3.3E-04	0.033	725670	mast/stem cell growth factor receptor Kit
NM_001327967.1	-2.01	0.11	1.3E-05	0.003	724216	juvenile hormone acid O-methyltransferase
XM_016910864.2	-2.01	-1.64	1.9E-06	0.001	408694	plastin-2

XR_407492.3	-2.00	3.01	3.2E-04	0.033	100578394	uncharacterized LOC100578394
XM_001121077.5	-1.98	7.88	1.6E-04	0.019	725202	chymotrypsin inhibitor
XM_003251239.4	-1.97	5.83	6.3E-05	0.010	100578412	Apis mellifera uncharacterized LOC100578412
NM_001134949.1	-1.97	4.71	3.5E-05	0.006	406107	Apis mellifera glucosamine-fructose-6-phosphate aminotransferase 2 (Gfat2), mRNA
XM_003250751.4	-1.96	5.85	3.7E-07	0.000	100578731	uncharacterized LOC100578731
XM_026444761.1	-1.95	3.08	3.4E-08	0.000	551796	cationic amino acid transporter 4
XM_392630.6	-1.95	4.38	1.9E-11	0.000	408500	uncharacterized LOC409105
XR_120331.4	-1.94	-0.78	2.8E-05	0.005	100576585	uncharacterized LOC100576585
XM_394855.6	-1.91	7.48	1.3E-04	0.017	411381	Apis mellifera caspase-3
XM_016913943.2	-1.88	6.52	7.6E-10	0.000	408992	NPC intracellular cholesterol transporter 1 homolog 1b
XR_119746.4	-1.88	1.72	1.3E-06	0.000	100576397	uncharacterized LOC100576397
XR_003305949.1	-1.83	0.07	1.4E-05	0.003	113219194	uncharacterized LOC113219194
XM_001121102.5	-1.83	1.93	4.0E-04	0.040	725225	ninjurin-2
XR_001705202.1	-1.83	0.13	2.7E-04	0.031	107965347	uncharacterized LOC107965347
XM_006561800.3	-1.80	7.25	1.6E-05	0.003	726818	beta-hexosaminidase subunit beta
XM_016914797.2	-1.79	8.19	2.2E-05	0.004	408320	uncharacterized LOC408320
XM_003251355.4	-1.78	2.95	1.3E-04	0.017	100577128	Apis mellifera uncharacterized LOC100577128
XM_026445002.1	-1.78	2.41	2.0E-05	0.004	102653748	growth/differentiation factor 8

XM_026444640.1	-1.77	4.59	8.3E-07	0.000	107965746	uncharacterized LOC107965746
XM_001121910.4	-1.71	-0.48	1.3E-04	0.017	726150	transcription factor Sox-21-B
XM_016916502.2	-1.71	6.98	8.4E-05	0.012	408534	Apis mellifera trypsin
XM_026445397.1	-1.69	-1.14	4.2E-04	0.042	410609	disintegrin and metalloproteinase domain-containing protein 10-like
XM_006566014.3	-1.69	2.67	1.7E-05	0.003	410211	four and a half LIM domains protein 2
XM_026442934.1	-1.68	-1.52	3.2E-05	0.005	113218571	hydrocephalus-inducing protein-like
XM_006560978.3	-1.66	-0.52	2.4E-04	0.028	412774	uncharacterized LOC412774
XM_026442948.1	-1.65	5.79	3.2E-04	0.033	409465	uncharacterized LOC409465
XM_016911303.2	-1.63	4.75	4.5E-05	0.007	107964189	Apis mellifera uncharacterized LOC107964189
XM_001120425.5	-1.63	7.99	8.3E-05	0.012	724536	Apis mellifera uncharacterized LOC724536
XM_006562350.3	-1.60	0.00	1.4E-04	0.018	409057	uncharacterized LOC409057
XM_006570213.3	-1.58	2.18	3.3E-04	0.033	411126	broad-complex core protein isoforms 1/2/3/4/5
XM_003249633.4	-1.58	5.41	2.4E-07	0.000	100578090	uncharacterized LOC100578090
XM_395905.6	-1.54	3.82	3.2E-06	0.001	412448	zinc transporter ZIP1
XM_016917454.2	-1.50	4.91	1.3E-06	0.000	412316	sodium/potassium/calcium exchanger 3
XM_026442378.1	-1.48	5.19	1.3E-04	0.017	726315	sphingomyelin phosphodiesterase 1
XM_026444746.1	-1.47	3.98	1.4E-04	0.018	107965400	probable cytochrome P450 6a14
XM_006564895.3	-1.44	3.13	5.8E-05	0.009	100578813	Apis mellifera uncharacterized LOC100578813

XR_001702082.2	-1.28	3.27	1.7E-05	0.003	107964028	uncharacterized LOC107964028
XM_026445495.1	-1.26	2.16	3.3E-04	0.033	411463	PH and SEC7 domain-containing protein 2
XM_026439095.1	-1.26	5.44	6.0E-06	0.001	408661	uncharacterized LOC408661
XM_003249567.4	-1.24	5.21	9.0E-08	0.000	100578068	lateral signaling target protein 2 homolog
XM_026439235.1	-1.24	5.19	5.5E-05	0.009	725588	Apis mellifera uncharacterized LOC725588
XM_026444401.1	1.32	4.92	1.5E-06	0.000	551094	fatty-acid amide hydrolase 2-B

Supplementary Table 2: List of DEGs in the Brain between the Intermediate Survival group and the Low Survival group.

Gene	Low Survival mean	Intermediate Survival mean	FC	Probability	GeneID	Protein
XM_026446194.1	7378.5	30580	-2.05	0.991	725343	alkyldihydroxyacetonephosphate synthase
XM_001120613.5	4600	18827	-2.03	0.985	724721	farnesol dehydrogenase
XM_393996.7	7121.5	28785	-2.02	0.991	410517	extracellular serine/threonine protein CG31145
XM_394018.7	3948	15211	-1.95	0.978	410539	protein 5NUC
XM_016913496.2	2358.5	8677	-1.88	0.956	107964838	probable uridine nucleosidase 2
NM_001011583.2	30909.5	113604	-1.88	0.997	406094	chemosensory protein 3
NM_001011598.1	31466	114680	-1.87	0.997	406114	alpha-amylase
XM_006559407.3	4479	16160	-1.85	0.979	409638	elongation of very long chain fatty acids protein AAEL008004
XM_392401.7	22253	79930	-1.84	0.996	408871	sorbitol dehydrogenase
XM_001120801.4	10941	39273	-1.84	0.992	726860	cytochrome b5
XM_395153.7	3478.5	11943	-1.78	0.969	411685	ATP-binding cassette sub-family D member 1
XM_001120140.5	7475	25430	-1.77	0.987	725038	NPC intracellular cholesterol transporter 2 homolog a
XM_395671.6	2690.5	9097	-1.76	0.956	412209	probable cytochrome P450 6a17

XM_001121061.5	2885	9712	-1.75	0.960	726978	homeodomain-only protein
XM_003249082.4	3073.5	10073	-1.71	0.961	100578929	uncharacterized LOC100578929
XM_026439627.1	2822	8725	-1.63	0.950	726529	ankyrin repeat domain-containing protein 29
XM_026442250.1	7982	24659	-1.63	0.986	412694	uncharacterized LOC412694
XM_026438947.1	9002	27577	-1.62	0.988	724919	mitochondrial amidoxime reducing component 2
XM_026441700.1	9872	28329	-1.52	0.986	725936	titin homolog
XM_016914951.2	11746	31306	-1.41	0.986	727593	UDP-glucuronosyltransferase 2C1
XM_392446.7	5313	14137	-1.41	0.969	408918	death-associated protein 1
XM_006564534.3	3791	9865	-1.38	0.950	411272	putative acyl-CoA-binding protein
XM_006565307.3	8224.5	21216	-1.37	0.978	551241	peroxisomal membrane protein PMP34
XM_006564945.3	16123	41186	-1.35	0.988	724293	yellow-x1
XM_006566923.2	8312.5	20626	-1.31	0.977	552712	6-phosphogluconate dehydrogenase, decarboxylating
XM_392591.7	5860	14457	-1.30	0.966	409066	pyridoxine-5'-phosphate oxidase
XM_003250766.4	13743.5	30990	-1.17	0.981	726268	uncharacterized LOC726268
XM_393342.7	5970.5	13449	-1.17	0.959	409852	transcription factor SPT20 homolog
XM_003249259.4	17072.5	38267	-1.16	0.983	100578226	zinc finger protein GLI1
XM_392000.7	98499	212329	-1.11	0.988	408452	cytochrome P450 9e2

XM_006557449.3	8820	17677	-1.00	0.958	102654369	la-related protein 6
XM_393750.7	8094	16106	-0.99	0.952	410269	probable multidrug resistance-associated protein lethal(2)03659
XM_016911661.2	30796	58434	-0.92	0.970	408818	hexokinase-1
XM_026443298.1	26066	48708	-0.90	0.967	412458	farnesol dehydrogenase
XM_624343.6	28387	51600	-0.86	0.962	551961	V-type proton ATPase subunit G
XM_625095.6	26867	48248	-0.84	0.958	552720	V-type proton ATPase subunit E
XM_624109.5	53663.5	95311	-0.83	0.959	551721	V-type proton ATPase subunit B
XM_003249180.4	49640	28363	0.81	0.951	726804	protein BTG2
XM_006572061.3	46117.5	26120	0.82	0.952	725680	vesicle-fusing ATPase 1
XM_003249641.4	79325	44846	0.82	0.956	724405	protein lethal(2)essential for life
XM_006572019.3	33461.5	18883	0.83	0.950	412245	acidic mammalian chitinase
XM_006559974.3	172585.5	96676	0.84	0.964	408547	toxin 3FTx-Lei1
XM_392359.7	36510.5	20399	0.84	0.954	408827	carbonic anhydrase 1
NM_001011635.1	156665	87509	0.84	0.964	409376	yellow-f
XM_026442033.1	29943	16563	0.85	0.952	412701	vacuolar protein sorting-associated protein 13D
XM_392611.7	67106.5	37108	0.85	0.961	409086	caprin homolog
XR_001704393.2	40387	21724	0.89	0.963	107965036	uncharacterized LOC107965036
XM_623217.6	65468	35027	0.90	0.968	550827	tubulin alpha-1 chain
XM_393558.7	48098.5	25643	0.91	0.968	410071	glycerol-3-phosphate phosphatase

XM_006569857.3	75268	40015	0.91	0.970	410905	sodium/potassium-transporting ATPase subunit beta-2
XM_395236.7	60474.5	31969	0.92	0.970	411769	kinesin heavy chain
XM_026442980.1	44797	23619	0.92	0.968	411347	peripheral plasma membrane protein CASK
XM_026443772.1	23805.5	12498	0.93	0.957	551941	uncharacterized LOC551941
NM_001256037.1	30930	16180	0.93	0.964	727274	PHD and ring finger domains 1
XM_392313.7	171625	89649	0.94	0.977	408782	tubulin beta-1
XM_393699.7	23446.5	12198	0.94	0.958	410216	reticulocalbin-2
XM_623210.6	41466	21541	0.94	0.969	408971	ras-related protein Rab-3
XM_026442948.1	19827	10285	0.95	0.955	409465	uncharacterized LOC409465
XM_625109.6	296021	153408	0.95	0.979	552734	poly(U)-specific endoribonuclease homolog
NM_001278330.1	42051.5	21757	0.95	0.971	726003	Rab escort protein
XM_392799.7	100719.5	52079	0.95	0.977	409278	apolipoprotein D
XM_026439176.1	51703.5	26609	0.96	0.974	551687	uncharacterized LOC551687
XM_006567146.2	35964	18466	0.96	0.970	409559	probable protein S-acyltransferase 23
XM_016914717.2	24685.5	12617	0.97	0.963	107965147	uncharacterized LOC107965147
XM_393267.7	57454.5	29317	0.97	0.975	409774	proteoglycan Cow
XM_392759.7	52130.5	26562	0.97	0.974	409235	neurotrimin
XM_006560676.3	19131.5	9740	0.97	0.956	726728	anaphase-promoting complex subunit 1
XM_006565857.3	106120.5	53892	0.98	0.979	726924	synapse-associated protein 1
XM_006562570.3	25887	13135	0.98	0.965	409212	cadherin-87A

XM_026442738.1	104800.5	52915	0.99	0.980	409020	inaD-like protein
XM_001119962.5	63931	32273	0.99	0.977	724192	titin
XM_394217.5	17363.5	8765	0.99	0.955	410741	proton-coupled amino acid transporter-like protein pathetic
XM_625067.6	20636	10392	0.99	0.959	552693	uncharacterized LOC552693
XM_026441761.1	22347.5	11220	0.99	0.963	107963990	mucin-5AC
XM_026446479.1	37484.5	18788	1.00	0.974	409034	uncharacterized LOC409034
XM_001120297.5	32569.5	16289	1.00	0.972	724440	calcium-transporting ATPase type 2C member 1
XM_006569936.3	23200.5	11590	1.00	0.965	100576346	protein PFC0760c
XM_026439652.1	55715	27788	1.00	0.978	408725	protein sickie
XM_001121323.5	18550.5	9245	1.00	0.959	725480	innexin inx1
XM_396806.6	18282	9111	1.00	0.958	413361	TBC1 domain family member 16
XR_003305294.1	19101	9514	1.01	0.961	113218980	uncharacterized LOC113218980
XM_003250216.4	55016.5	27199	1.02	0.980	100576569	uncharacterized LOC100576569
XM_395173.7	14570.5	7202	1.02	0.953	411705	synaptojanin-1
XM_006559222.3	33076.5	16339	1.02	0.975	413510	SNF-related serine/threonine-protein kinase
XM_003249827.4	14506	7124	1.03	0.954	100576471	uncharacterized LOC100576471
XM_624108.6	13679	6706	1.03	0.951	551720	activator of 90 kDa heat shock protein ATPase homolog 1
XM_001121525.5	29905.5	14614	1.03	0.974	725708	uncharacterized LOC725708
XM_001122027.5	13905	6766	1.04	0.952	726276	uncharacterized LOC726276
XM_026441396.1	14877.5	7228	1.04	0.955	102654344	glutamine-rich protein 2

XR_003305570.1	24096	11701	1.04	0.970	494508	serine/threonine-protein kinase pakE
XM_392602.7	964759.5	468453	1.04	0.987	409077	aminopeptidase N
XM_393174.7	23797	11531	1.05	0.970	409676	kinesin 2A
XM_001121946.5	21935	10617	1.05	0.967	726190	arginine/serine-rich protein PNISR
XM_001123131.5	20772	10017	1.05	0.965	727423	uncharacterized LOC727423
XM_392231.7	268748.5	129225	1.06	0.987	408695	uncharacterized LOC408695
XM_001120194.5	21802.5	10483	1.06	0.967	724488	protein lethal(2)essential for life
XM_006558616.3	16242.5	7787	1.06	0.959	411808	proline dehydrogenase, mitochondrial
XM_026445110.1	36928.5	17696	1.06	0.978	408465	vesicle-associated membrane protein 2
XM_001120658.5	34230	16385	1.06	0.977	724761	uncharacterized LOC724761
XM_394578.6	17371	8289	1.07	0.962	411104	homeobox protein Nkx-2.4
XR_410156.3	17101.5	8126	1.07	0.961	726065	uncharacterized LOC726065, transcript variant X8
XM_016916593.2	352153	166884	1.08	0.988	102656354	uncharacterized LOC102656354
XM_026444509.1	13622.5	6449	1.08	0.954	409539	microtubule-associated protein 2
XM_026445497.1	25952	12268	1.08	0.973	413784	rabphilin
XM_006568615.3	17580	8299	1.08	0.962	725150	uncharacterized LOC725150
XM_006567389.3	95621	45115	1.08	0.985	408987	sortilin-related receptor
XM_397465.7	58177.5	27311	1.09	0.982	414029	ABC transporter A family member 1

XM_006564472.3	15173	7111	1.09	0.958	100578890	uncharacterized LOC100578890
XM_393616.7	16571.5	7753	1.10	0.962	410133	PDZ domain-containing RING finger protein 4
XM_026440223.1	13999.5	6546	1.10	0.956	411209	uncharacterized LOC411209
XM_026442495.1	39065	18212	1.10	0.980	100578826	protein strawberry notch-like
XM_016916072.2	29472	13672	1.11	0.977	724594	ADP-ribosylation factor-like protein 4C
XM_006561263.3	13059.5	6043	1.11	0.953	551658	anillin
XM_026444930.1	45132	20771	1.12	0.983	726997	uncharacterized LOC726997
XM_393631.7	15415	7090	1.12	0.961	410148	carboxypeptidase Q
XM_026441587.1	19361.5	8887	1.12	0.967	408934	choline O-acetyltransferase
XM_392257.7	390487.5	178098	1.13	0.990	408722	sodium/potassium -transporting ATPase subunit beta-2
XM_392256.7	27412	12413	1.14	0.977	408721	neurocalcin homolog
XM_394484.6	17425	7856	1.15	0.966	411011	guanine nucleotide-binding protein G(s) subunit alpha
NM_001012962.1	21079	9502	1.15	0.972	503861	nitric oxide synthase
XM_006561241.3	23317	10508	1.15	0.975	550870	uncharacterized LOC550870
XM_006562299.3	38701.5	17413	1.15	0.983	552017	alpha-catulin
XM_026441982.1	13052	5867	1.15	0.957	409624	acetyl-coenzyme A synthetase
XM_026445301.1	29153	13094	1.15	0.979	409502	uncharacterized LOC409502

XM_026440932.1	23715.5	10643	1.16	0.975	552545	division abnormally delayed protein
XM_003249567.4	17095.5	7663	1.16	0.966	100578068	lateral signaling target protein 2 homolog
XM_016917438.2	13707.5	6138	1.16	0.959	413578	U2 snRNP-associated SURP motif-containing protein
XM_026441624.1	17560	7863	1.16	0.966	100577986	probable sodium/potassium/ calcium exchanger CG1090
XM_026440829.1	63637	28459	1.16	0.986	726101	uncharacterized LOC726101
XM_026440160.1	37501	16753	1.16	0.983	113218723	fibrous sheath CABYR-binding protein-like
XM_006558996.3	43322	19353	1.16	0.984	409323	uncharacterized LOC409323
XM_016911949.2	15967.5	7032	1.18	0.965	107964349	leucine-rich repeat protein soc-2 homolog
XM_006559821.3	111481	48793	1.19	0.990	408951	14-3-3 protein epsilon
XM_026440032.1	13292.5	5806	1.19	0.960	406139	ankyrin-3
XM_392630.6	18648	8141	1.20	0.970	409105	uncharacterized LOC409105
XM_006570090.3	12945	5593	1.21	0.959	551031	uncharacterized LOC551031
XM_006566860.3	15669	6752	1.21	0.966	410379	uncharacterized LOC410379
XM_393966.5	26443.5	11389	1.22	0.980	410487	uncharacterized LOC410487
XM_026440227.1	48156	20584	1.23	0.987	408797	nidogen-2
XM_003251147.4	81604.5	34837	1.23	0.990	410867	uncharacterized LOC410867

XM_001120611.5	16686	7122	1.23	0.968	726411	immunoglobulin domain-containing protein oig-4
NM_001011565.1	11821.5	5042	1.23	0.955	406068	octopamine receptor
XM_026439735.1	42598.5	18069	1.24	0.987	411536	E3 ubiquitin-protein ligase HERC2
XM_026439113.1	165979.5	70069	1.24	0.992	102655100	protein Cep78 homolog
XM_006561089.3	134002.5	56462	1.25	0.992	552020	neuromodulin
XM_392431.5	53847.5	22534	1.26	0.988	408902	F-box/LRR-repeat protein 16
XM_394464.7	12972.5	5426	1.26	0.961	410989	menin
XM_006566073.3	112065	46767	1.26	0.992	100576882	uncharacterized LOC100576882
XM_026446329.1	12176	5078	1.26	0.958	100577959	uncharacterized LOC100577959
XM_026439116.1	11856.5	4941	1.26	0.957	725294	tyrosine-protein phosphatase 99A
XM_006567583.3	21294.5	8856	1.27	0.976	409073	equilibrative nucleoside transporter 4
XM_001122612.5	12747	5280	1.27	0.961	726896	zinc finger and BTB domain-containing protein 47
NM_001172718.1	39222.5	16239	1.27	0.986	100379261	uncharacterized LOC100379261
XM_396815.6	19449.5	8026	1.28	0.974	413370	hemicentin-2
XM_393156.7	13752	5671	1.28	0.964	409658	uncharacterized LOC409658
XM_006561834.3	35306.5	14554	1.28	0.985	409004	ras-related protein Rab6
XR_003304637.1	17440	7163	1.28	0.971	409957	synaptotagmin 4, transcript variant X2
XM_026445082.1	13503.5	5526	1.29	0.963	550801	ATP-dependent RNA helicase dbp2

XM_624701.6	191434	77942	1.30	0.993	552326	uncharacterized LOC552326
XR_003304346.1	45280.5	18363	1.30	0.987	113218632	uncharacterized LOC113218632
XM_026444693.1	10678	4320	1.31	0.952	408408	uncharacterized LOC408408
XM_001120296.5	27317.5	11035	1.31	0.982	724439	uncharacterized LOC724439
XM_026439426.1	11318.5	4559	1.31	0.956	410918	protein tramtrack, beta isoform
XM_397519.7	163461.5	65791	1.31	0.993	408696	uncharacterized LOC408696
XM_006564471.3	41678.5	16752	1.31	0.987	409390	cAMP-dependent protein kinase type II regulatory subunit
XM_016916296.2	10651.5	4248	1.33	0.952	411617	inactive rhomboid protein 1
XM_392464.7	200130.5	79654	1.33	0.993	408935	high-affinity choline transporter 1
XM_006559511.3	13503	5333	1.34	0.964	552576	activated Cdc42 kinase-like
XM_006569316.3	76190	30060	1.34	0.991	409949	agrin
NM_001160064.1	355547.5	139813	1.35	0.994	408928	heat shock protein 90
XM_001120452.5	22136.5	8690	1.35	0.979	724563	uncharacterized LOC724563
XM_006564167.3	115546.5	45083	1.36	0.993	724432	ribosomal RNA processing protein 36 homolog
XM_006566310.3	19113.5	7451	1.36	0.975	410317	small conductance calcium-activated potassium channel protein
XM_394673.7	23274	9052	1.36	0.981	411199	sodium-dependent neutral amino acid transporter B(0)AT3

XM_026439677.1	26090.5	10107	1.37	0.983	113218647	gamma-aminobutyric acid type B receptor subunit 1
XM_001119931.5	29554	11382	1.38	0.984	724178	uncharacterized LOC724178
XM_006562668.3	123933.5	47681	1.38	0.993	408809	diacylglycerol kinase theta
XM_026445260.1	33891	12944	1.39	0.987	406096	type I inositol 1,4,5-trisphosphate 5-phosphatase
XR_001706525.2	165934	63298	1.39	0.994	107966103	uncharacterized LOC107966103
NM_001327957.1	16387.5	6234	1.39	0.971	551454	voltage-dependent calcium channel subunit alpha-2/delta-3
XM_006560749.3	13499.5	5135	1.39	0.966	409022	fruitless
XM_006564049.3	106170	40252	1.40	0.993	410467	uncharacterized LOC410467
XM_026445233.1	30607.5	11584	1.40	0.986	100576461	uncharacterized LOC100576461
XM_003251160.4	37496	14132	1.41	0.988	724220	synaptotagmin-10
XM_006567266.3	44588	16795	1.41	0.989	408920	long-chain fatty acid transport protein 4
XM_006566196.3	128085.5	48213	1.41	0.994	726321	uncharacterized LOC726321
XM_006564912.3	12123	4548	1.41	0.963	410527	probable cation-transporting ATPase 13A3
XR_003306559.1	16852	6320	1.41	0.973	102654200	uncharacterized LOC102654200
XM_001121838.5	28121.5	10509	1.42	0.985	726068	uncharacterized LOC726068
XM_393712.6	12135.5	4534	1.42	0.963	410229	toll-like receptor 6
XM_026443706.1	154285	57449	1.43	0.995	413428	uncharacterized LOC413428
XM_016912597.2	11583	4313	1.43	0.962	413473	GTPase-activating Rap/Ran-GAP

						domain-like protein 3
XM_026441696.1	602555	223949	1.43	0.996	725131	microtubule-associated protein futsch
XM_006567785.3	10391	3822	1.44	0.955	411113	solute carrier family 12 member 4
XM_001122075.5	23414	8555	1.45	0.983	726331	alpha-2A adrenergic receptor
XM_026440031.1	61533.5	22195	1.47	0.992	409981	titin
XM_001122204.5	53487	19289	1.47	0.990	726472	prohormone-3
XM_001121746.5	840891	303139	1.47	0.996	725960	neurofilament heavy polypeptide
XM_026444761.1	16909.5	6077	1.48	0.974	551796	cationic amino acid transporter 4
NM_001011650.1	15620.5	5551	1.49	0.972	408879	soluble guanylyl cyclase alpha 1 subunit
XM_016913943.2	147105	52030	1.50	0.995	408992	NPC intracellular cholesterol transporter 1 homolog 1b
XM_016915547.2	19110	6757	1.50	0.979	551869	vitamin K-dependent gamma-carboxylase
XM_006562557.3	57054.5	20162	1.50	0.991	725064	vesicular acetylcholine transporter
XM_026446268.1	37535	13188	1.51	0.989	410756	regulating synaptic membrane exocytosis protein 1
XM_026444198.1	49077.5	17237	1.51	0.990	724546	sodium-coupled monocarboxylate transporter 1
XM_026443704.1	27499	9658	1.51	0.986	552619	homeobox protein onecut

XR_003305813.1	13341.5	4680	1.51	0.968	413211	SIFamide receptor, transcript variant X1
XM_392545.7	34594.5	12065	1.52	0.989	409016	uncharacterized LOC409016
XM_026442500.1	301095	104778	1.52	0.996	408961	apolipophorins
XM_016918056.2	23979	8296	1.53	0.984	408699	high affinity cAMP-specific and IBMX-insensitive 3',5'-cyclic phosphodiesterase 8
XM_006568046.3	17548.5	6057	1.53	0.977	100577322	cell wall integrity and stress response component 2
XM_392742.7	10561	3625	1.54	0.960	409218	neural Wiskott-Aldrich syndrome protein
XM_392366.7	78340.5	26805	1.55	0.994	408835	neuroendocrine convertase 2
NM_001256040.1	123774.5	42156	1.55	0.995	100859932	uncharacterized LOC100859932
XM_006560851.3	13501.5	4586	1.56	0.970	411494	cyclin-dependent kinase 5 activator 1
XM_026445737.1	17269.5	5834	1.57	0.977	552490	uncharacterized LOC552490
XM_016911769.2	9515.5	3195	1.57	0.954	102654965	uncharacterized LOC102654965
XM_006568175.3	168637	56599	1.58	0.995	410857	protein lethal(2)essential for life
XM_026440891.1	14609.5	4882	1.58	0.972	102654994	uncharacterized LOC102654994
XM_006561070.3	20797.5	6939	1.58	0.982	409781	uncharacterized LOC409781
XM_026444841.1	31197	10399	1.58	0.988	724592	calmodulin-binding transcription activator 2

XM_016911303.2	12483	4156	1.59	0.968	107964189	uncharacterized LOC107964189
NM_001327949.1	13788.5	4582	1.59	0.971	100577273	uncharacterized LOC100577273
XM_394701.7	28633	9498	1.59	0.988	411227	tyrosine-protein phosphatase non-receptor type 9
XM_006569213.3	15346	5087	1.59	0.974	413503	TWiK family of potassium channels protein 18-like
XM_026442626.1	30087	9969	1.59	0.988	726214	nicotinic acetylcholine receptor alpha1 subunit
XM_016914729.2	19306	6381	1.60	0.981	100577364	FERM, ARHGEF and pleckstrin domain-containing protein 1
NM_001145740.1	10451.5	3427	1.61	0.961	724217	neurexin 1
XM_026439692.1	11475.5	3759	1.61	0.965	726283	neuronal calcium sensor 2
XM_393772.6	13552.5	4376	1.63	0.971	410291	dipeptidase 1
XM_003249754.4	18037	5798	1.64	0.980	100578822	protein IWS1 homolog
NM_001011636.1	81200.5	25912	1.65	0.995	409798	FABP-like protein
XM_026440916.1	49613	15770	1.65	0.992	408865	uncharacterized LOC408865
XM_016915816.2	10715.5	3401	1.66	0.964	410689	ELAV-like protein 2
XM_006563127.3	48166.5	15240	1.66	0.992	408554	neural-cadherin
XM_026439304.1	10435	3280	1.67	0.963	411871	dual specificity tyrosine-phosphorylation-regulated kinase 2-like
NM_001167836.1	17316.5	5401	1.68	0.979	409314	prohormone-2
XM_394342.7	25024	7792	1.68	0.987	410866	LIM domain only protein 3

NM_001040230.1	193270.5	60172	1.68	0.997	406104	acetylcholinestera se 2
XM_026445881.1	78834.5	24261	1.70	0.995	100577769	uncharacterized LOC100577769
XM_397501.6	15420.5	4690	1.72	0.975	408473	uncharacterized LOC408473
XR_003304193.1	168209	51148	1.72	0.997	113218601	uncharacterized LOC113218601
XM_006571435.3	75995	23099	1.72	0.995	725415	BTB/POZ domain-containing protein KCTD16
XM_026440863.1	30123.5	9145	1.72	0.990	551385	frequenin-1
XM_394686.7	31176	9442	1.72	0.990	411212	discoidin domain-containing receptor 2
XM_395760.7	12902.5	3904	1.72	0.971	412299	muscarinic acetylcholine receptor DM1
XM_394423.7	9522	2881	1.72	0.957	410947	tyrosine decarboxylase
XM_026443497.1	10258	3064	1.74	0.963	107965041	solute carrier organic anion transporter family member 4A1-like
XM_393285.7	36857	10978	1.75	0.991	409791	cAMP-dependent protein kinase catalytic subunit
XR_003305178.1	17667	5243	1.75	0.980	113218944	uncharacterized LOC113218944
XM_026441660.1	11956	3525	1.76	0.969	100576948	GTP-binding protein RAD-like
XM_392530.7	10151	2745	1.89	0.965	409000	cell adhesion molecule 2
XM_006558502.3	9423	2405	1.97	0.962	552578	uncharacterized LOC552578
XM_001120327.5	9391	2363	1.99	0.962	724465	enhancer of split mbeta protein
XM_396310.6	10506	2596	2.02	0.967	412858	hemicentin-2
XM_006566861.3	17778.5	4264	2.06	0.983	102654959	myb-like protein Q

XM_026440983.1	29784	7116	2.07	0.991	100576683	uncharacterized LOC100576683
XM_006558575.3	25775	5678	2.18	0.990	113218523	uncharacterized LOC113218523
XR_003305923.1	14912.5	3233	2.21	0.979	113219182	uncharacterized LOC113219182
XM_006562260.3	24663	3820	2.69	0.990	410368	cadherin-23

Supplementary Table 3: List of DEGs in the Malpighian tubules between the Low Survival Group and Intermediate Survival Group.

Gene	Low Survival mean	Intermediate Survival mean	FC	Probability	GeneID	Protein
NM_001011572.1	14284.5	58093	-2.02	0.983	406078	transferrin 1
XM_001120220.5	12415.5	3686	1.75	0.951	724386	NPC intracellular cholesterol transporter 2
XM_001120296.5	6778.5	20288	-1.58	0.960	724439	uncharacterized LOC724439
XM_001121746.5	22374	90525	-2.02	0.986	725960	neurofilament heavy polypeptide
XM_001122299.4	92085	44341	1.05	0.952	726571	venom acid phosphatase Acph-1
XM_003250091.4	41061.5	11028	1.90	0.979	100577527	uncharacterized LOC100577527
XM_006559813.3	49006.5	18512	1.40	0.968	102655332	protein orai-2
XM_006561089.3	5252.5	17230	-1.71	0.960	552020	neuromodulin, transcript variant X1
XM_006562300.3	180160	70644	1.35	0.974	408453	cytochrome P450 9e2
XM_006562785.3	176859.5	88071	1.01	0.951	726592	proteoglycan 4
XM_006563203.3	5116.5	15194	-1.57	0.951	503505	venom protein 2, transcript variant X1
XM_006568066.3	3860.5	12521	-1.70	0.950	413047	venom carboxylesterase-6 -like
XM_006569857.3	2413.5	10823	-2.16	0.955	410905	sodium/potassium -transporting ATPase subunit beta-2

XM_006570570.3	101904.5	18914	2.43	0.990	102654405	uncharacterized LOC102654405
XM_006571296.3	46496.5	5436	3.10	0.988	413575	facilitated trehalose transporter Tret1
XM_016915694.2	178163	85856	1.05	0.956	726352	transmembrane protease serine 11B-like protein
XM_016916549.2	71083	19755	1.85	0.982	410626	sodium-coupled monocarboxylate transporter 1
XM_016917117.2	60622.5	24711	1.29	0.964	102654520	putative uncharacterized protein DDB_G0282133
XM_026438943.1	56810.5	25516	1.15	0.955	100577377	uncharacterized LOC100577377
XM_026439113.1	5934.5	22522	-1.92	0.972	102655100	protein Cep78 homolog
XM_392319.7	69016.5	25937	1.41	0.971	408788	UDP-glucuronosyltransferase 1-3
XM_392799.7	3767	15222	-2.01	0.962	409278	apolipoprotein D
XM_394092.7	257960.5	128238	1.01	0.953	410614	tubulin alpha chain
XM_394370.5	110695.5	33979	1.70	0.982	410894	chymotrypsin-1
XM_394827.6	59086.5	28120	1.07	0.951	411353	lipase 3
XM_397017.6	11335.5	1981	2.52	0.960	413576	facilitated trehalose transporter Tret1-2 homolog
XM_623724.5	48967	17129	1.52	0.972	551327	carboxypeptidase B
XM_623919.5	58711	25773	1.19	0.959	551524	zinc carboxypeptidase
XR_001706525.2	5593	21471	-1.94	0.971	107966103	uncharacterized LOC107966103
XR_003304193.1	5807.5	18518	-1.67	0.961	113218601	uncharacterized LOC113218601

Supplementary Table 4: List of DEGs in the Ventriculus between the Intermediate survival group and the Low survival group

Gene	Low Survival mean	Intermediate Survival mean	FC	Probability	GeneID	Protein
NM_001011579.1	47	8786	-7.55	0.984	406090	major royal jelly protein 1
NM_001011578.1	5308.5	361	3.88	0.969	406088	vitellogenin
XM_006570574.3	101493.5	19061	2.41	0.960	107963975	uncharacterized LOC107963975

Supplementary Table 5: List of DEGs in all organs between the Intermediate Survival group and the High Survival group

Organ	Gene	Intermediate Survival Mean	High Survival Mean	FC	Probability	GeneID	Protein
Brain	NM_001011598.1	12517	56680.33333	-2.18	0.987	406114	alpha-amylase
	XM_001120140.5	6349	21400	-1.75	0.961	725038	NPC intracellular cholesterol transporter 2 homolog a
Ventriculus	NM_001011640.1	2640	12092	-2.20	0.972	449496	take-out-like carrier protein
	XM_026445951.1	41202	14163	1.54	0.970	107965822	retrovirus-related Pol polyprotein from type-1 retrotransposable element R2
	XM_393342.7	5716	18001	-1.65	0.967	409852	transcription factor SPT20 homolog
	XM_001119981.5	178229	71441	1.32	0.964	724211	cytochrome P450 9e2
	XM_006562300.3	41401	15620	1.41	0.964	408453	cytochrome P450 9e2
	NM_001160064.1	50816	20152	1.33	0.960	408928	heat shock protein 90
	XM_026442500.1	20236	8023	1.33	0.953	408961	apolipophorins
Malpighian Tubules	NM_001011607.2	719	16816	-4.55	0.982	406130	melittin
	NM_001011636.1	13869	1593.333333	3.12	0.971	409798	FABP-like protein
	NM_001110764.1	8715	28616	-1.72	0.950	726848	hexamerin 70a
	NM_001160064.1	43680	13427.66667	1.70	0.952	408928	heat shock protein 90
	XM_001120116.5	31387	4214	2.90	0.978	724312	vanin-like protein 1

	XM_001121077.5	31581	9410.666667	1.75	0.952	725202	chymotrypsin inhibitor
	XM_001121746.5	90525	22486	2.01	0.966	725960	neurofilament heavy polypeptide
	XM_001122571.5	304097	102093.6667	1.57	0.951	726855	vegetative cell wall protein gp1
	XM_003251842.4	36057	9827	1.88	0.957	100577143	uncharacterized LOC100577143
	XM_006562300.3	70644	305785.3333	-2.11	0.971	408453	cytochrome P450 9e2
	XM_006565481.3	216107	45502.66667	2.25	0.974	551180	aminopeptidase N
	XM_006566073.3	17346	4007.666667	2.11	0.957	100576882	uncharacterized LOC100576882
	XM_006571296.3	5436	27421.66667	-2.33	0.967	413575	facilitated trehalose transporter Tret1
	XM_016913943.2	23437	4036.333333	2.54	0.971	408992	NPC intracellular cholesterol transporter 1 homolog 1b
	XM_026439414.1	13136	3180.666667	2.05	0.950	551714	cGMP-dependent protein kinase, isozyme 1
	XM_026440879.1	50443	7240	2.80	0.980	726309	protein artichoke
	XM_026442948.1	12992	2719	2.26	0.956	409465	uncharacterized LOC409465
	XM_026443036.1	36047	9463.333333	1.93	0.959	107964964	uncharacterized LOC107964964
	XM_026443101.1	89860	16973	2.40	0.976	113219028	pancreatic

							triacylglycerol lipase-like
	XM_393127.6	237939	30475.66667	2.96	0.987	409626	chymotrypsin-2
	XM_393342.7	17319	78234	-2.18	0.970	409852	transcription factor SPT20 homolog
	XM_394827.6	28120	5688	2.31	0.967	411353	lipase 3
	XR_001706525.2	21471	5541	1.95	0.956	107966103	uncharacterized LOC107966103
	XR_003304193.1	18518	4444.333333	2.06	0.957	113218601	uncharacterized LOC113218601

Supplementary Table 6: List of DEGs in all organs between the Low Survival group and High Survival group

Organ	**Gene**	**Low Survival**	**High Survival**	**FC**	**prob**	**GeneID**	**Protein**

		Mean	Mean				
Malpighian tubules	NM_001011607.2	1149.5	16816	-3.87	0.979	406130	melittin
	XM_393342.7	16426	78234	-2.25	0.976	409852	transcription factor SPT20 homolog
	XM_001120140.5	4689	17125.66667	-1.87	0.953	725038	NPC intracellular cholesterol transporter 2 homolog a
	XM_623919.5	58711	17245.66667	1.77	0.962	551524	zinc carboxypeptidase
	XM_001120425.5	43715	12500	1.81	0.962	724536	uncharacterized LOC724536
	XM_003251842.4	37434.5	9827	1.93	0.964	100577143	uncharacterized LOC100577143
	XM_016916502.2	24216	6084.333333	1.99	0.962	408534	trypsin
	XM_016914797.2	53913	13325	2.02	0.969	408320	uncharacterized LOC408320
	XM_623724.5	48967	12074	2.02	0.968	551327	carboxypeptidase B
	XM_006561800.3	28320.5	6929	2.03	0.965	726818	beta-hexosaminidase subunit beta
	XM_026443036.1	38735	9463.333333	2.03	0.967	107964964	uncharacterized LOC107964964
	XM_001122571.5	431425.5	102093.6667	2.08	0.976	726855	vegetative cell wall protein gp1
	XM_394855.6	35250	7510.666667	2.23	0.970	411381	caspase-3
	XM_003250751.4	11475.5	2420.666667	2.25	0.954	100578731	uncharacterized LOC100578731
	XM_001121077.5	45165.5	9410.666667	2.26	0.973	725202	chymotrypsin inhibitor
	XM_003251239.4	11329	2347.333333	2.27	0.954	100578412	uncharacterized LOC100578412
	XM_001120112.5	194495	35491.33333	2.45	0.982	724308	serine protease 53
	XM_026439414.1	18790	3180.666667	2.56	0.970	551714	cGMP-dependent protein kinase, isozyme 1
	XM_003249136.4	18053	3033.6666	2.57	0.970	100578100	uncharacterized

			67				LOC100578100
	XM_006565481.3	282473	45502.66667	2.63	0.985	551180	aminopeptidase N
	XM_026443101.1	110100.5	16973	2.70	0.984	113219028	pancreatic triacylglycerol lipase-like
	XM_026440879.1	60616	7240	3.07	0.986	726309	protein artichoke
	XM_393127.6	284252	30475.66667	3.22	0.991	409626	chymotrypsin-2
	XM_001120116.5	39394.5	4214	3.22	0.984	724312	vanin-like protein 1
	XM_394827.6	59086.5	5688	3.38	0.987	411353	lipase 3
	XM_026445507.1	6060	204.3333333	4.89	0.955	551562	dynein beta chain, ciliary
Ventriculus	XM_001120296.5	9511.5	1948	2.29	0.970	724439	uncharacterized LOC724439
	NM_001011640.1	3895	12092	-1.63	0.962	449496	take-out-like carrier protein
	XM_016912939.2	6907.5	17262.33333	-1.32	0.954	724565	trypsin-7

Supplementary Table 7: Enriched GO processes of Upregulated genes in the brain of the Intermediate Survival group compared when compared to the Low Survival group

GO Category	Functional Category	Enrichment FDR	Genes in list
Biological Processes	Small molecule metabolic process	0.002	7
	Pyridine-containing compound metabolic process	0.002	3
	Small molecule biosynthetic process	0.002	4
	Cellular aldehyde metabolic process	0.002	2
	Transmembrane transport	0.002	7
	Cation transmembrane transport	0.002	4
	Inorganic ion transmembrane transport	0.002	4
	Inorganic cation transmembrane transport	0.002	4
	Proton transmembrane transport	0.002	3
	Nucleoside phosphate metabolic process	0.002	4
	Nucleotide metabolic process	0.002	4
	Monovalent inorganic cation transport	0.002	4
	Organophosphate metabolic process	0.002	5
	Monocarboxylic acid metabolic process	0.002	3
	Establishment of localization	0.002	8
	Nucleobase-containing small molecule metabolic process	0.002	4
	Oxidation-reduction process	0.002	6
	ATP hydrolysis coupled transmembrane transport	0.002	2
	ATP hydrolysis coupled ion transmembrane transport	0.002	2
	Coenzyme metabolic process	0.002	3
	Transport	0.002	8
	Cation transport	0.002	4
	Energy coupled proton transmembrane transport, against electrochemical gradient	0.002	2
	ATP hydrolysis coupled proton transport	0.002	2
	Localization	0.003	8
	Nucleotide catabolic process	0.003	2
	Nucleoside phosphate catabolic process	0.004	2

	Ribose phosphate metabolic process	0.004	3
	Pyridine-containing compound biosynthetic process	0.004	2
	Ion transmembrane transport	0.005	4
Cellular Components	Proton-transporting two-sector ATPase complex	0.001	3
	Proton-transporting two-sector ATPase complex, catalytic domain	0.003	2
	Proton-transporting V-type ATPase complex	0.003	2
	Membrane	0.020	13
	Membrane part	0.020	13
	Membrane protein complex	0.020	3
Molecular Function	Cofactor binding	0.000	8
	Primary active transmembrane transporter activity	0.000	4
	P-P-bond-hydrolysis-driven transmembrane transporter activity	0.000	4
	ATPase activity, coupled to transmembrane movement of substances	0.000	4
	ATPase activity, coupled to movement of substances	0.000	4
	Ion binding	0.000	15
	Coenzyme binding	0.000	5
	ATPase activity, coupled	0.000	4
	Active transmembrane transporter activity	0.000	4
	Organic cyclic compound binding	0.000	15
	Heterocyclic compound binding	0.000	15
	Catalytic activity	0.000	16
	Small molecule binding	0.000	10
	Nucleotide binding	0.001	9
	ATPase activity	0.001	4
	Nucleoside phosphate binding	0.001	9
	Oxidoreductase activity	0.001	6
	Anion binding	0.001	9
	Heme binding	0.002	3
	Tetrapyrrole binding	0.002	3
	Transmembrane transporter activity	0.004	5
	Transporter activity	0.007	5

	Carbohydrate derivative binding	0.009	7
	Binding	0.011	18
	Cation binding	0.011	7
	Monooxygenase activity	0.013	2
	Nucleoside-triphosphatase activity	0.015	4
	Pyrophosphatase activity	0.015	4
	Oxidoreductase activity, acting on CH-OH group of donors	0.015	2
	Hydrolase activity, acting on acid anhydrides	0.015	4
KEGG	Metabolic pathways	0.000	8
	Phagosome	0.000	3
	MTOR signaling pathway	0.000	3
	Oxidative phosphorylation	0.001	3
	Peroxisome	0.004	2
	Carbon metabolism	0.008	2

Supplementary Table 8: Enriched GO processes of Upregulated genes in the brain of the Low Survival group when compared to the Intermediate Survival group

GO Category	Functional Category	Enrichment FDR	Genes in list
Biological Processes	Regulation of vesicle-mediated transport	0.006	3
	Establishment of localization	0.008	24
	Transport	0.008	24
	Localization	0.009	24
	Cell-cell adhesion via plasma-membrane adhesion molecules	0.027	3
	Phosphorus metabolic process	0.027	17
	Phosphate-containing compound metabolic process	0.027	17
	Homophilic cell adhesion via plasma membrane adhesion molecules	0.027	3
	Regulation of exocytosis	0.027	2
	Regulation of secretion	0.030	2
	Cell-cell adhesion	0.030	3
	Regulation of secretion by cell	0.030	2
	Potassium ion transport	0.030	4
	Dephosphorylation	0.030	6
	Phospholipid dephosphorylation	0.034	2
	Phosphatidylinositol dephosphorylation	0.034	2

	Cell adhesion	0.034	4
	Biological adhesion	0.034	4
	Response to drug	0.041	2
	Regulation of transport	0.044	3
	Regulation of molecular function	0.050	7
Cellular Processes	Microtubule	0.000	6
	Intrinsic component of plasma membrane	0.000	6
	Supramolecular complex	0.000	6
	Supramolecular polymer	0.000	6
	Supramolecular fiber	0.000	6
	Polymeric cytoskeletal fiber	0.000	6
	Integral component of plasma membrane	0.001	5
	Sodium:potassium-exchanging ATPase complex	0.002	2
	Plasma membrane part	0.002	6
	Cation-transporting ATPase complex	0.002	2
	Plasma membrane	0.002	10
	Membrane	0.002	52
	Intrinsic component of membrane	0.002	49
	Cell periphery	0.002	10
	Membrane part	0.003	50

	Microtubule cytoskeleton	0.003	6
	ATPase dependent transmembrane transport complex	0.005	2
	Integral component of membrane	0.006	47
	Cytoskeleton	0.009	7
	Cytoskeletal part	0.011	6
	Anchored component of membrane	0.021	2
Molecular Function	Binding	0.019	77
	Calcium ion binding	0.019	8
	Phosphoric ester hydrolase activity	0.019	7
	Ion binding	0.019	41
	Nucleotide binding	0.022	23
	Transporter activity	0.022	14
	Hydrolase activity	0.022	25
	Hydrolase activity, acting on ester bonds	0.022	10
	Enzyme regulator activity	0.022	6
	Ribonucleotide binding	0.022	21
	Small molecule binding	0.022	24
	Carbohydrate derivative binding	0.022	22
	Nucleoside phosphate binding	0.022	23

	Lipid transporter activity	0.022	3
	Cytoskeletal protein binding	0.023	7
	Purine nucleotide binding	0.027	20
	Active transmembrane transporter activity	0.027	5
	Purine ribonucleotide binding	0.027	20
	Cation binding	0.027	22
	Phosphatase activity	0.029	5
	Calmodulin binding	0.030	2
	Dipeptidase activity	0.030	2
	Lyase activity	0.030	5
	Protein binding	0.031	33
	Guanylate cyclase activity	0.033	2
	Anion binding	0.033	22
	Rab GTPase binding	0.034	2
	Metal ion binding	0.034	21
	Molecular function regulator	0.034	7
	Purine nucleoside binding	0.040	6
KEGG	Longevity regulating pathway	0.013	3
	Phagosome	0.047	3

Supplementary Table 9: Enriched GO processes of upregulated genes in the Malpighian tubules of Low Survival group when compared to the Intermediate Survival group

GO Category	Functional Category	Enrichment FDR	Genes in list
Biological Process	Proteolysis	0.013	4
Molecular Function	Proteolysis	0.013	4
	Metallocarboxypeptidase activity	0.002	2
	Carboxypeptidase activity	0.002	2
	Peptidase activity	0.002	4
	Metalloexopeptidase activity	0.002	2
	Hydrolase activity	0.002	7
	Peptidase activity, acting on L-amino acid peptides	0.002	4
	Exopeptidase activity	0.007	2
	Catalytic activity	0.012	9
	Serine-type endopeptidase activity	0.012	2
	Serine-type peptidase activity	0.014	2
	Serine hydrolase activity	0.014	2
	Metallopeptidase activity	0.014	2
	Transition metal ion binding	0.023	3
	Catalytic activity, acting on a protein	0.023	4
	Transmembrane transporter activity	0.025	3
	Endopeptidase activity	0.028	2
	Transporter activity	0.030	3

Supplementary Table 10: Enriched GO processes of upregulated genes in the Malpighian tubules of Low Survival group when compared to the High Survival group

GO Category	Functional Category	Enrichment FDR	Genes in list
Biological Processes	Proteolysis	0.000	7
	Protein metabolic process	0.000	8
	Organonitrogen compound metabolic process	0.001	8
	Primary metabolic process	0.002	10
	Organic substance metabolic process	0.002	10
	Metabolic process	0.002	11
	Nitrogen compound metabolic process	0.003	9
	Macromolecule metabolic process	0.006	8
Molecular Function	Peptidase activity	0.000	7
	Hydrolase activity	0.000	11
	Peptidase activity, acting on L-amino acid peptides	0.000	7
	Catalytic activity, acting on a protein	0.000	8
	Catalytic activity	0.000	12
	Exopeptidase activity	0.000	3
	Endopeptidase activity	0.000	4
	Metallocarboxypeptidase activity	0.000	2
	Serine-type endopeptidase activity	0.001	3
	Carboxypeptidase activity	0.001	2
	Serine-type peptidase activity	0.001	3
	Serine hydrolase activity	0.001	3
	Metallopeptidase activity	0.001	3
	Metalloexopeptidase activity	0.001	2
	Zinc ion binding	0.015	3
	Transition metal ion binding	0.033	3

Supplementary Table 11: Enriched GO processes of uniquely upregulated genes in the Ventriculus

GO Category	Functional Category	Enrichment FDR	Genes in list
Biological Processes	Metabolic process	1E-08	204
	Establishment of localization	3E-06	71
	Organic substance metabolic process	3E-06	168
	Transport	3E-06	71
	Localization	5E-06	71
	Primary metabolic process	1E-05	158
	Organonitrogen compound metabolic process	1E-05	101
	Nitrogen compound metabolic process	1E-05	147
	Protein metabolic process	2E-05	85
	Macromolecule metabolic process	3E-05	133
	Proteolysis	2E-04	34
	Cellular process	7E-04	196
	Ion transport	7E-03	26
	Transmembrane transport	9E-03	37
	Dephosphorylation	1E-02	13
	Biological regulation	1E-02	87
	Cellular metabolic process	2E-02	133
	Macromolecule modification	3E-02	47

	Regulation of cellular process	4E-02	77
	Regulation of biological process	4E-02	79
Cellular Components	Integral component of membrane	5E-08	167
	Intrinsic component of membrane	5E-08	167
	Membrane part	9E-08	170
	Membrane	2E-07	174
	Nucleosome	2E-04	8
	Protein-DNA complex	2E-04	8
	DNA packaging complex	4E-04	8
	Endomembrane system	7E-03	20
	Chromatin	9E-03	8
	Cell	1E-02	127
	Chromosomal part	2E-02	10
	Intracellular part	2E-02	102
	Intracellular	3E-02	110
	Golgi cisterna	3E-02	3
	Golgi cisterna membrane	3E-02	3
	Organelle	3E-02	87
	Cytoplasm	3E-02	52
	Cell part	3E-02	121
	Golgi stack	3E-02	3
	Intracellular organelle	3E-02	85
	Chromosome	3E-02	10
	Endoplasmic reticulum	4E-02	9
	Actin cytoskeleton	4E-02	6

	Non-membrane-bounded organelle	4E-02	28
	Intracellular non-membrane-bounded organelle	4E-02	28
	Bounding membrane of organelle	4E-02	10
	Peptidase complex	4E-02	5
	Organelle subcompartment	4E-02	11
	Membrane-bounded organelle	5E-02	69
	COPII vesicle coat	5E-02	2
Molecular Function	Catalytic activity	2E-05	163
	Hydrolase activity	2E-05	81
	Peptidase activity	5E-05	31
	Peptidase activity, acting on L-amino acid peptides	5E-05	30
	Catalytic activity, acting on a protein	7E-05	57
	Hydrolase activity, hydrolyzing O-glycosyl compounds	1E-03	10
	Transporter activity	2E-03	37
	Transmembrane transporter activity	2E-03	34
	Cysteine-type peptidase activity	3E-03	10
	Binding	4E-03	232
	Hydrolase activity, acting on glycosyl bonds	4E-03	10
	Protein heterodimerization activity	4E-03	8

	Endopeptidase activity	4E-03	17
	Serine-type peptidase activity	4E-03	12
	Serine hydrolase activity	4E-03	12
	Phosphatase activity	5E-03	12
	Actin binding	1E-02	11
	Protein dimerization activity	1E-02	13
	DNA binding	1E-02	37
	Cation binding	1E-02	61
	Cysteine-type endopeptidase activity	1E-02	4
	Phosphoric ester hydrolase activity	1E-02	13
	DNA-binding transcription factor activity	1E-02	16
	Metal ion binding	1E-02	60
	N-acetyl-beta-D-galactosaminidase activity	1E-02	2
	Transcription regulator activity	3E-02	18
	Serine-type endopeptidase activity	3E-02	9
	Cytoskeletal protein binding	4E-02	14
	Chloride transmembrane transporter activity	4E-02	3
	Protein binding	4E-02	93
KEGG	Lysosome	2E-04	10
	Protein processing in endoplasmic reticulum	9E-04	12
	Amino sugar and nucleotide sugar metabolism	8E-03	6
	Metabolic pathways	9E-03	36
	Other glycan degradation	3E-02	3

Supplementary Table 12: Enriched GO processes of uniquely upregulated genes in the Malpighian Tubules

GO Category	Functional Category	Enrichment FDR	Genes in list
Biological Processes	Metabolic process	6E-60	708
	Cellular metabolic process	7E-44	546
	Peptide biosynthetic process	9E-43	118
	Translation	4E-42	116
	Organonitrogen compound biosynthetic process	4E-42	176
	Peptide metabolic process	8E-42	120
	Amide biosynthetic process	1E-41	119
	Cellular amide metabolic process	2E-39	122
	Cellular process	2E-38	704
	Organic substance metabolic process	6E-38	567
	Organonitrogen compound metabolic process	4E-37	359
	Primary metabolic process	5E-35	538
	Nitrogen compound metabolic process	5E-35	505
	Cellular nitrogen compound metabolic process	1E-32	334
	Cellular nitrogen compound biosynthetic process	4E-32	244
	Biosynthetic process	5E-31	295
	Organic substance biosynthetic process	4E-30	288
	Cellular biosynthetic process	6E-30	284
	Cellular protein metabolic process	5E-26	239
	Cellular macromolecule metabolic process	2E-24	349
	Gene expression	7E-24	241
	Protein metabolic process	1E-23	274
	Macromolecule metabolic process	2E-23	428
	Macromolecule biosynthetic process	2E-21	214
	Cellular macromolecule biosynthetic process	1E-20	210

	Oxidation-reduction process	3E-18	130
	Small molecule metabolic process	4E-17	119
	Generation of precursor metabolites and energy	4E-14	46
	Purine nucleoside triphosphate metabolic process	7E-13	28
	Purine ribonucleoside triphosphate metabolic process	7E-13	28
Cellular Components	Cell	3E-50	546
	Cell part	6E-48	532
	Intracellular	5E-59	518
	Intracellular part	2E-63	492
	Intracellular organelle	3E-42	388
	Organelle	4E-41	388
	Cytoplasm	1E-85	334
	Membrane-bounded organelle	8E-24	289
	Intracellular membrane-bounded organelle	4E-24	286
	Protein-containing complex	2E-43	260
	Cytoplasmic part	7E-67	249
	Intracellular organelle part	5E-24	197
	Organelle part	2E-23	197
	Non-membrane-bounded organelle	8E-21	127
	Intracellular non-membrane-bounded organelle	8E-21	127
	Ribonucleoprotein complex	2E-44	108
	Mitochondrion	1E-30	90
	Ribosome	4E-38	78
	Organelle membrane	8E-15	65
	Mitochondrial part	4E-23	61
	Mitochondrial envelope	1E-16	47
	Organelle envelope	4E-13	47
	Envelope	6E-13	47
	Mitochondrial membrane	1E-16	44
	Mitochondrial inner membrane	9E-15	36
	Organelle inner membrane	3E-14	36

	Mitochondrial protein complex	1E-10	25
	Ribosomal subunit	2E-13	21
	Proton-transporting two-sector ATPase complex	3E-12	20
	Small ribosomal subunit	9E-11	14
Molecular Function	Catalytic activity	2E-43	576
	Structural constituent of ribosome	1E-39	80
	Binding	6E-34	825
	Heterocyclic compound binding	6E-29	467
	Organic cyclic compound binding	7E-29	467
	Ion binding	6E-27	405
	Structural molecule activity	3E-24	90
	Small molecule binding	2E-21	247
	Anion binding	2E-21	243
	Nucleotide binding	5E-20	231
	Nucleoside phosphate binding	5E-20	231
	Ribonucleotide binding	4E-18	205
	Purine nucleotide binding	1E-17	203
	Purine ribonucleotide binding	1E-17	202
	Purine ribonucleoside triphosphate binding	2E-17	200
	Carbohydrate derivative binding	3E-17	214
	Oxidoreductase activity	2E-16	119
	RNA binding	1E-15	103
	Drug binding	1E-12	176
	Adenyl nucleotide binding	1E-11	158
	ATP binding	1E-11	156
	Adenyl ribonucleotide binding	1E-11	157
	Proton transmembrane transporter activity	2E-10	23
	Hydrolase activity	8E-10	211
	Transferase activity	2E-08	171
	Translation factor activity, RNA binding	3E-08	22
	Metal ion binding	7E-08	186

	Hydrolase activity, acting on acid anhydrides	7E-08	88
	Pyrophosphatase activity	8E-08	87
	Nucleoside-triphosphatase activity	8E-08	85
KEGG	Metabolic pathways	2E-43	186
	Ribosome	5E-36	68
	Oxidative phosphorylation	6E-26	50
	Proteasome	8E-13	22
	Carbon metabolism	1E-10	29
	Ribosome biogenesis in eukaryotes	2E-08	23
	Phagosome	3E-08	22
	Valine, leucine and isoleucine degradation	3E-08	15
	Cysteine and methionine metabolism	8E-07	14
	Protein processing in endoplasmic reticulum	2E-06	27
	Glutathione metabolism	6E-06	13
	Citrate cycle (TCA cycle)	3E-05	12
	RNA transport	1E-04	25
	Biosynthesis of amino acids	3E-04	14
	Protein export	3E-04	9
	Drug metabolism	4E-04	7
	2-Oxocarboxylic acid metabolism	4E-04	7
	Metabolism of xenobiotics by cytochrome P450	9E-04	7
	Peroxisome	3E-03	13
	Pyruvate metabolism	4E-03	7
	Drug metabolism	4E-03	8
	Glyoxylate and dicarboxylate metabolism	6E-03	7
	Propanoate metabolism	6E-03	7
	Glycolysis / Gluconeogenesis	7E-03	9
	Fatty acid metabolism	1E-02	9

	MTOR signaling pathway	1E-02	14
	Porphyrin and chlorophyll metabolism	1E-02	7
	ABC transporters	1E-02	5
	Pentose phosphate pathway	2E-02	6
	Fructose and mannose metabolism	2E-02	5

Supplementary Table 13: Enriched GO processes of uniquely upregulated genes in the Brain

GO Category	Functional Category	Enrichment FDR	Genes in list
Biological Processes	Cellular process	2E-41	914
	Metabolic process	1E-23	763
	Organic substance metabolic process	4E-25	672
	Primary metabolic process	4E-25	647
	Cellular metabolic process	1E-28	636
	Nitrogen compound metabolic process	6E-24	598
	Macromolecule metabolic process	4E-25	553
	Cellular macromolecule metabolic process	3E-24	440
	Cellular nitrogen compound metabolic process	1E-19	374
	Biological regulation	3E-13	366
	Organic cyclic compound metabolic process	3E-24	350
	Heterocycle metabolic process	3E-24	347
	Cellular aromatic compound metabolic process	7E-24	345
	Regulation of biological process	1E-12	343
	Nucleobase-containing compound metabolic process	4E-24	337
	Regulation of cellular process	7E-13	334
	Nucleic acid metabolic process	6E-27	309
	Gene expression	1E-14	270
	RNA metabolic process	1E-20	251
	Response to stimulus	1E-11	241

	Cellular response to stimulus	3E-11	229
	Macromolecule biosynthetic process	1E-09	225
	Cellular macromolecule biosynthetic process	9E-10	223
	Macromolecule modification	1E-13	207
	Protein modification process	1E-12	187
	Cellular protein modification process	1E-12	187
	Cellular component organization or biogenesis	4E-12	158
	Cellular component organization	8E-12	147
	Response to stress	3E-10	69
	Cellular response to DNA damage stimulus	9E-10	49
Cellular Components	Cell	2E-35	640
	Cell part	2E-35	629
	Intracellular	1E-36	582
	Intracellular part	4E-32	521
	Organelle	2E-30	451
	Intracellular organelle	1E-29	445
	Membrane-bounded organelle	6E-31	385
	Intracellular membrane-bounded organelle	3E-29	374
	Nucleus	4E-27	282
	Protein-containing complex	2E-14	239
	Organelle part	1E-20	236
	Intracellular organelle part	5E-20	232
	Cytoplasm	2E-05	203
	Nuclear part	7E-16	104
	Catalytic complex	8E-11	85
	Membrane-enclosed lumen	1E-08	70
	Organelle lumen	1E-08	70
	Intracellular organelle lumen	1E-08	70

	Nuclear lumen	5E-10	64
	Endomembrane system	5E-05	63
	Transferase complex	7E-10	51
	Nucleoplasm	5E-08	45
	Nucleoplasm part	4E-07	42
	Chromosome	2E-05	36
	Microtubule cytoskeleton	6E-04	32
	Transferase complex, transferring phosphorus-containing groups	2E-05	20
	DNA-directed RNA polymerase complex	2E-06	18
	RNA polymerase complex	2E-06	18
	Nuclear DNA-directed RNA polymerase complex	2E-06	18
	RNA polymerase II, holoenzyme	2E-05	15
Molecular Function	Binding	8E-64	1169
	Organic cyclic compound binding	1E-28	592
	Heterocyclic compound binding	1E-28	592
	Protein binding	7E-27	476
	Ion binding	3E-22	495
	Nucleic acid binding	1E-18	349
	Catalytic activity	9E-17	622
	Purine nucleotide binding	3E-15	246
	Ribonucleotide binding	3E-15	247
	Purine ribonucleotide binding	3E-15	245
	Nucleotide binding	4E-15	272
	Nucleoside phosphate binding	4E-15	272
	Purine ribonucleoside triphosphate binding	4E-15	242
	Small molecule binding	7E-15	285
	Anion binding	7E-15	280

	Carbohydrate derivative binding	6E-13	253
	Transferase activity	6E-11	229
	Adenyl nucleotide binding	2E-09	190
	Adenyl ribonucleotide binding	3E-09	189
	ATP binding	5E-09	186
	DNA binding	7E-09	147
	Metal ion binding	4E-08	240
	Ribonucleoside binding	4E-08	59
	Nucleoside binding	5E-08	59
	Cation binding	5E-08	241
	Purine nucleoside binding	9E-08	57
	GTP binding	9E-08	57
	Purine ribonucleoside binding	9E-08	57
	Guanyl ribonucleotide binding	1E-07	57
	Guanyl nucleotide binding	2E-07	57
KEGG	Spliceosome	7E-09	37
	Longevity regulating pathway	5E-04	13
	RNA polymerase	4E-03	11
	Basal transcription factors	4E-03	12
	Endocytosis	4E-03	24
	MAPK signaling pathway	4E-03	17
	Glycerophospholipid metabolism	5E-03	13
	RNA degradation	6E-03	16
	Metabolic pathways	9E-03	113
	Fanconi anemia pathway	9E-03	7
	MTOR signaling pathway	9E-03	18
	Notch signaling pathway	9E-03	8
	Wnt signaling pathway	2E-02	15
	Purine metabolism	4E-02	13

Supplementary Table 14: List of Enriched Biological Processes in common between the Brain (B), Malpighian Tubules (MT) , and Ventriculus (V) of uniquely upregulated genes.

Organs	Total	Biological Process
B MT V		Metabolic process
		Organic substance metabolic process
		Cellular metabolic process
		Primary metabolic process
		Cellular process
		Macromolecule metabolic process
	7	Nitrogen compound metabolic process
MT V		Organonitrogen compound metabolic process
	2	Protein metabolic process
B V		Regulation of biological process
		Macromolecule modification
		Biological regulation
	4	Regulation of cellular process
B MT		Cellular nitrogen compound metabolic process
		Gene expression
		Cellular macromolecule metabolic process
		Macromolecule biosynthetic process
	5	Cellular macromolecule biosynthetic process
V		Transmembrane transport
		Proteolysis
		Localization
		Dephosphorylation
		Transport
		Establishment of localization
	7	Ion transport
MT		Generation of precursor metabolites and energy
		Organonitrogen compound biosynthetic process
		Organic substance biosynthetic process
		Purine ribonucleoside triphosphate metabolic process
		Purine nucleoside triphosphate metabolic process
	16	

		Cellular protein metabolic process
		Peptide metabolic process
		Peptide biosynthetic process
		Translation
		Cellular amide metabolic process
		Cellular nitrogen compound biosynthetic process
		Biosynthetic process
		Amide biosynthetic process
		Cellular biosynthetic process
		Small molecule metabolic process
		Oxidation-reduction process
B		Response to stimulus
		Nucleic acid metabolic process
		Heterocycle metabolic process
		Cellular protein modification process
		Organic cyclic compound metabolic process
		Cellular aromatic compound metabolic process
		Cellular component organization
		Protein modification process
		Response to stress
		RNA metabolic process
		Cellular component organization or biogenesis
		Nucleobase-containing compound metabolic process
		Cellular response to DNA damage stimulus
	14	Cellular response to stimulus

Supplementary Table 15: List of Enriched Cellular Compartments in common between the Brain (B), Malpighian Tubules (MT), and Ventriculus (V) of Uniquely upregulated genes.

Organs	Total	Cellular Components
B MT V		Cytoplasm
		Intracellular organelle
		Intracellular part
		Organelle
		Intracellular
		Membrane-bounded organelle
		Cell part
	8	Cell
MT V		Non-membrane-bounded organelle
	2	Intracellular non-membrane-bounded organelle
B V		Chromosome
	2	Endomembrane system
B MT		Intracellular membrane-bounded organelle
		Protein-containing complex
		Organelle part
	4	Intracellular organelle part
V		Organelle subcompartment
		Intrinsic component of membrane
		Integral component of membrane
		Golgi cisterna
		Actin cytoskeleton
		Peptidase complex
		DNA packaging complex
		Bounding membrane of organelle
		Chromosomal part
		Golgi cisterna membrane
		Protein-DNA complex
		COPII vesicle coat
		Endoplasmic reticulum
		Membrane
		Golgi stack
		Nucleosome
		Membrane part
	18	

		Chromatin
MT		Organelle membrane
		Mitochondrial part
		Mitochondrial inner membrane
		Mitochondrial envelope
		Ribosome
		Envelope
		Mitochondrial protein complex
		Small ribosomal subunit
		Cytoplasmic part
		Organelle envelope
		Organelle inner membrane
		Mitochondrial membrane
		Proton-transporting two-sector ATPase complex
		Ribonucleoprotein complex
	16	Mitochondrion
		Ribosomal subunit
B		Nucleoplasm
		Catalytic complex
		Transferase complex, transferring phosphorus-containing groups
		Nucleoplasm part
		Nuclear part
		Membrane-enclosed lumen
		RNA polymerase complex
		Microtubule cytoskeleton
		Intracellular organelle lumen
		Nuclear DNA-directed RNA polymerase complex
		Organelle lumen
		Nuclear lumen
		DNA-directed RNA polymerase complex
		RNA polymerase II, holoenzyme
		Nucleus
	16	Transferase complex

Supplementary Table 16: List of Enriched Molecular Functions in common between the Brain (B), Malpighian Tubules (MT), and Ventriculus (V) of Uniquely upregulated genes.

Organs	Total	Molecular Functions
B MT V		Catalytic activity
		Binding
	3	Metal ion binding
MT V	1	Hydrolase activity
B V		Protein binding
		Cation binding
	3	DNA binding
B MT		Purine nucleotide binding
		Ribonucleotide binding
		Nucleoside phosphate binding
		Adenyl ribonucleotide binding
		Transferase activity
		Ion binding
		ATP binding
		Nucleotide binding
		Anion binding
		Heterocyclic compound binding
		Organic cyclic compound binding
		Small molecule binding
		Purine ribonucleoside triphosphate binding
		Carbohydrate derivative binding
		Adenyl nucleotide binding
	16	Purine ribonucleotide binding
V		DNA-binding transcription factor activity
		Actin binding
		Serine-type endopeptidase activity
		Transporter activity
		Peptidase activity, acting on L-amino acid peptides
		Catalytic activity, acting on a protein
		Endopeptidase activity
	23	

		Cytoskeletal protein binding
		Chloride transmembrane transporter activity
		Hydrolase activity, acting on glycosyl bonds
		Serine-type peptidase activity
		Transmembrane transporter activity
		N-acetyl-beta-D-galactosaminidase activity
		Transcription regulator activity
		Phosphoric ester hydrolase activity
		Phosphatase activity
		Protein heterodimerization activity
		Cysteine-type endopeptidase activity
		Peptidase activity
		Cysteine-type peptidase activity
		Protein dimerization activity
		Hydrolase activity, hydrolyzing O-glycosyl compounds
		Serine hydrolase activity
MT		Pyrophosphatase activity
		Oxidoreductase activity
		RNA binding
		Structural molecule activity
		Nucleoside-triphosphatase activity
		Structural constituent of ribosome
		Hydrolase activity, acting on acid anhydrides
		Proton transmembrane transporter activity
		Translation factor activity, RNA binding
	10	Drug binding
B		Nucleic acid binding
		GTP binding
		Purine nucleoside binding
		Ribonucleoside binding
		Guanyl nucleotide binding
		Purine ribonucleoside binding
		Guanyl ribonucleotide binding

8

		Nucleoside binding

Supplementary Table 17: List of Enriched KEGG in common between the Brain (B), Malpighian Tubules (MT), and Ventriculus (V) of Uniquely upregulated genes.

Organs	Total	KEGG
B MT V	1	Metabolic pathways
MT V	1	Protein processing in endoplasmic reticulum
B MT	1	MTOR signaling pathway
V	3	Amino sugar and nucleotide sugar metabolism
		Lysosome
		Other glycan degradation
MT	26	Porphyrin and chlorophyll metabolism
		Drug metabolism
		Glyoxylate and dicarboxylate metabolism
		2-Oxocarboxylic acid metabolism
		Fructose and mannose metabolism
		Pyruvate metabolism
		Citrate cycle (TCA cycle)
		Oxidative phosphorylation
		Ribosome biogenesis in eukaryotes
		Peroxisome
		Proteasome
		Glycolysis / Gluconeogenesis
		Biosynthesis of amino acids
		Metabolism of xenobiotics by cytochrome P450
		RNA transport
		ABC transporters
		Glutathione metabolism
		Valine, leucine and isoleucine degradation
		Ribosome
		Phagosome
		Cysteine and methionine metabolism
		Pentose phosphate pathway

		Carbon metabolism
		Protein export
		Propanoate metabolism
		Fatty acid metabolism
B		Wnt signaling pathway
		RNA polymerase
		Glycerophospholipid metabolism
		RNA degradation
		Purine metabolism
		Spliceosome
		Endocytosis
		Basal transcription factors
		Longevity regulating pathway
		MAPK signaling pathway
		Notch signaling pathway
	12	Fanconi anemia pathway