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Antioxidant Genes in *Apis mellifera*, Their Implication in Pesticide Detoxification

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by

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ABSTRACT

The effect of pesticides on *Apis mellifera* mortality as well as their effect on the transcriptional regulation of antioxidant genes was the aim of this study. Unlike previous studies, the work here shows the combined effects of pesticides imidacloprid and coumaphos on worker bees at levels likely encountered by workers using both toxological and molecular analysis. Bee brood were collected from hives and incubated until hatching. The 1 day old bees were then segregated and subjected to varying concentrations of coumaphos and imidacloprid both independently and in concert. Workers from each treatment group were removed and stored in RNA-later until they were used for molecular analysis. The 10 antioxidant genes monitored here comprised of both primary and secondary antioxidants. Four of the secondary antioxidants used were seleno-like genes found in the genome. These showed differential expression throughout the bee's development as well in the presence of pesticides.

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CHAPTER I - INTRODUCTION

Honey bees are some of the most important pollinators in the United States and around the world. In the United States most honey bees are *Apis mellifera*, the European honey bee. They were introduced to North America 400 years ago by the European settlers (Urbana, 2006). Honey bees are estimated to pollinate 35% of the human diet (Klein *et al.*, 2007), while insect pollination attributes $20 billion to the GDP (Gallai, 2009). For the last 60 years the honey bee population in the United States has decreased more than 50%, see Fig 1, (vanEngelsdorp and Meixner, 2010). Initially the population decline was attributed to pesticides (Atkins, 1975). However, in the last decade the idea that no one factor alone is responsible for the consistent honey bee decline or colony collapse disorder, CCD (vanEngelsdorp *et al*., 2009). Though some countries have seen an incline managed honey bees the United States has reported consistent losses in managed honey bee colonies, see Figure 2 (Bruckner *et al.,* 2018).

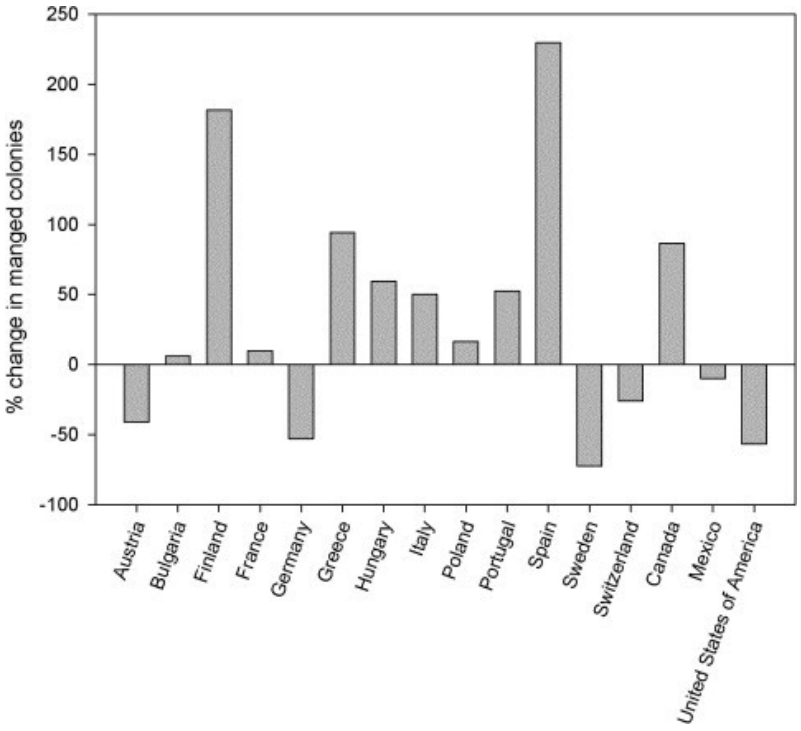

Figure 1: Percent change in number of managed bee colonies between 1961 and 2006 in selected countries in Europe and North America (vanEngelsdorp and Meixner, 2010).

HONEY BEE BIOLOGY

Apis mellifera are a eusocial species; like other social insects, all members of the hive serve special roles to better the hive. Honey bees live in a hive comprised mainly of a wax comb. The hive contains a honeycomb made up of thousands of hexagon cells. These cells are used for the development of young bees, as well as honey and pollen stores. The constituents of the hive are comprised of the 3 types of adult bees and the developing brood. The adult bees present in a hive are a queen, about 60,000 workers and a few hundred drones depending on the season. The queen's main purpose is to lay eggs

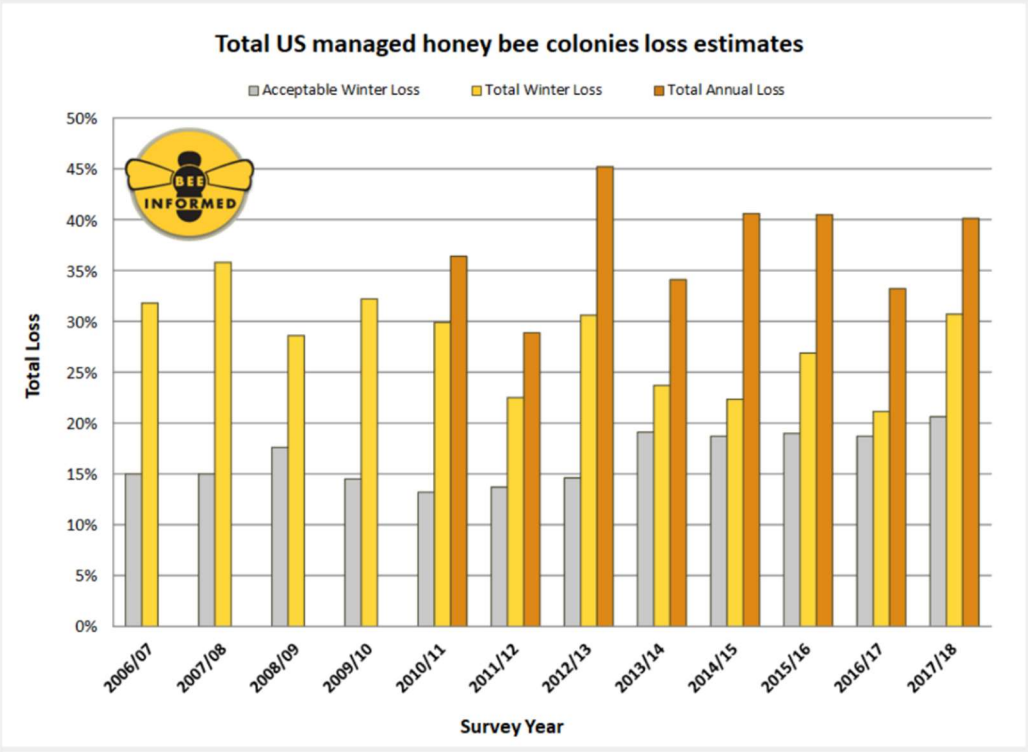

Figure 2: Managed honey bee colony loss reported by the Bee Informed network. Yellow bars show reported winter losses from October 1st to April 1st. The orange bars represent the total colony loss for a given year and include the losses reported from April 1st to October 1st. Grey bars represent the accepted anticipated colony loss by those surveyed (Bruckner *et al.,* 2018).

and is the only sexually developed female in the hive (Hoover *et al.* 2003). The workers are non-sexually developed females who have specialized features that allow them to collect pollen, feed the developing brood and queen, build the honeycomb, thermoregulate the hive, and perform all activities to maintain the hive. Worker bees are the engine of the hive and play numerous roles depending on their age and the need of the hive (Page and Peng, 2001). The drones are sexually developed males whose purpose is to mate with the queen.

HONEY BEE DEVELOPMENT

All 3 types of adult bees undergo the same stages of development however, the duration of these stages vary based on the type of bee that is developing. Honey bee development has 3 phases; egg, larva, and pupae. The term brood is used to collectively refer to developing bees in any of these stages.

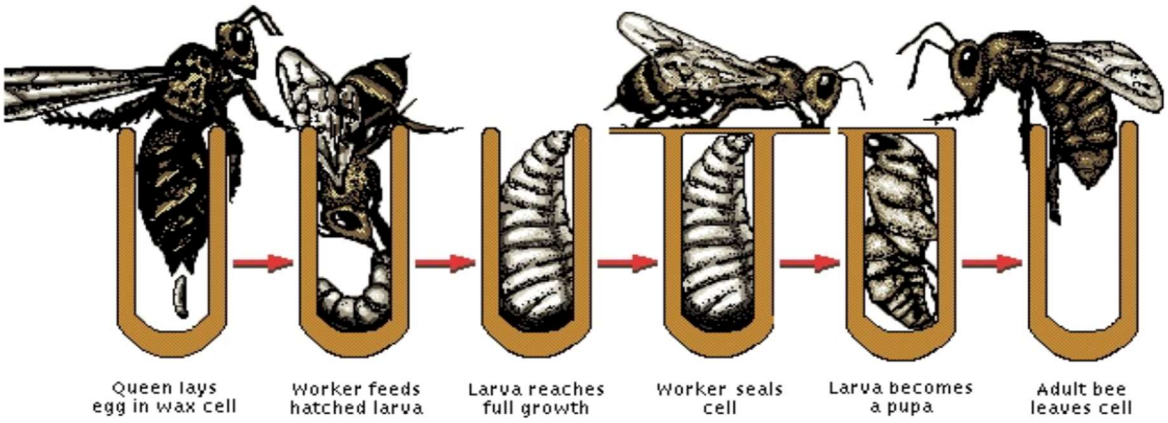

Figure 3: Honey bee development from egg to adult (left to right). The queen lays the egg in the wax cell made by the workers. As the larva develops the workers feed the larvae until it is fully grown. Once fully grown the worker caps the cell. The pupa undergoes metamorphosis and emerges as an adult.

The queen will lay 1 egg per cell in the honeycomb. After a few days the egg hatches and at that time the larval stage begins. For the next 5 or 6 days the nurse worker bees will feed the larvae using specialized brood food glands then the worker will cap the cell, sealing the larvae in. Once the cell is capped the developing young is in the pre-pupal stage. Within the cell the pupae begins to take on its adult form and develop pigment.

SEASONAL CHANGES

Throughout the year the population of the hive is dynamic. In the spring the hive is awakened from its more dormant winter period and the main goal of the hive is reproduction. The queen lays eggs and the workers begin collecting pollen and water to liquefy thick honey for developing brood. Once the brood develop; the hive population begins to bloom and the scarce drones become more prevalent. As the population grows so does the work done by the hive. By mid-spring the worker bees are at full force and surpluses of honey and pollen begin to accumulate. As the population continues to grow the hive starts getting crowded and can sometimes lead to swarming and the creation of a new hive.

The formation of a new hive is brought about just before the virgin queen emerges. The old queen and the majority of the hive constituents rush out of the hive and will cluster together while scouts look for a site to construct a new hive (Seeley 2010). Once found, the queen and her entourage migrate there and quickly begin building combs, laying eggs and gathering nectar and pollen. In the old hive, the remaining bees continue to care for developing brood and collect resources. Once the new queen emerges she must secure her position as the queen. She searchers the hive for any other queens and will fight to the death until only one remains. Once the surviving queen is a week or so old she will emerge from the hive to mate with one or more drones in the air. Upon returning and only after mating the nurse bees care for the queen who begins laying eggs shortly thereafter.

By summer the population is at its highest numbers and the focus of the hive shifts toward winter survival. The day length is the longest so workers are able to forage

for longer lengths of time. In these long summer days the worker bees have their shortest expected life span of five to six weeks (Amdam and Omholt, 2002). In late summer and early fall there is less pollen available in the environment so to conserve resources for the winter the remaining drones are removed from the hive and the workers prevent them from re-entering. At this point, the queen does not lay as many eggs and the workers collect propolis from trees to seal any cracks. In the winter months, like in the summer, the worker thermoregulate the hive and care for the queen and brood. In these months the older adult bees die and fewer numbers of adults are emerging however, young adult workers in the colder months can live up to 6 months (Page and Peng, 2001).

COLONY COLLAPSE DISORDER

Unlike normal winter losses in the spring of 2007 and again in 2008 the United States experienced a severe loss of managed honey bee colonies (vanEngelsdorp *et al.,* 2007; vanEngelsdorp *et al.,* 2008). A large portion of these colonies had a few common characteristics: (1) excessive brood populations with a sudden loss of adult worker bees, (2) lack of dead worker bees in or around the affected hive, (3) delayed invasion of pests into the hive as well as delayed cleptoparasitism from other colonies (Cox *et al.*, 2007). Since then, these symptoms have been used to characterize the phenomenon referred to as Colony Collapse Disorder, or CCD. Though annual colony losses of managed hives have remained high >23%, the number of CCD cases responsible has been decreased from 60% in 2008 to just over 30% in 2013 (EPA).

ANTIOXIDANTS

Apis mellifera like most social insects they rely on grooming and hygienic interactions rather than a robust immune system. Honey bees encode fewer genes involved in immune and antioxidant pathways than most other insects (Wilson, 2006; Corona and Robinson, 2006). Upon sequencing the honey bee genome, it was also found that *Apis mellifera* has evolved more slowly than most other insects including *Drosophila* (Wilson,

2006). The honey bee innate immune system strongly relies on reactive oxygen species, or ROS, which serve as the first line of defense to protect the bee from a pathogenic state.

The honey bee immune system can be triggered by tissue injury when the exoskeleton is breached or the presence of pathogen associated molecular patterns, PAMPs. The innate immune system's cellular response is triggered and uses cytokines and cytokine like signals to recruit phagocytic cells to the site; many of these signals are thought to be reactive species. Specialized phagocytic cells in the insect, haemocytes, are recruited to the site and will engulf and enzymatically degrade any cells they do not recognize as self or determine are terminally damaged. As a result of these signals and by the action of the haemocytes different immune pathways can be activated based on the PAMPs encountered (Hoffman and Reichhart, 2002; Chain and Anderson, 1983; Brutscher *et al.*, 2015).

ROS AND THE HONEY BEE

Antioxidants can be categorized as primary and secondary antioxidants. Primary antioxidants are those that act directly on reactive oxygen species, or ROS. Secondary antioxidants function to recycle those primary antioxidants and to repair damage brought about by oxidative stress. Currently, there are 39 antioxidants that have been uncovered in the genome and represent 10 protein groups (Corona and Robinson, 2006).

Reactive oxygen species is generated in the honey bee as a byproduct of aerobic respiration as well as in response to biotic and abiotic stressors. The main source of ROS in the honey bee is mitochondrial respiration. Manganese containing SOD, or SOD2, is responsible for regulating ROS in the mitochondria; which through the formation of H_2O_2, is one of the most important contributors to regulating and maintaining the ROS gradient between the cytosol and the mitochondrial matrix (Hauptman *et al*., 1996). Reactive oxygen species can be very harmful to cells by damaging proteins, disrupting gradients and interfering or altering the cells basic biochemistry and pathways.

Cytochrome P450, or CYP, is the main component responsible for the microsomal detoxification of xenobiotics. CYPs are cytoplasmic and the specific isoforms present are required for the breakdown of different toxic compounds (McDonnell and Dang, 2013). Upon degradation by CYP, ROS and other charged substrates are produced. These reactive products are inactivated by constituent antioxidants such as the GST to prevent cellular damage (McDonnell and Dang, 2013).

The immune system of the honey bee also makes use of the aggressive nature of ROS molecules. Phagocytic hemocytes serve as the first line of defense, when activated it initiates a signaling cascade via the phenoloxidase system. Phenoloxidase is the main oxidase responsible for arming the immune response through a large oxygen influx. To prevent damage to host tissue antioxidant expression is also activated initially in the form of catalase but uses peroxidase as pathogenesis persists.

Primary Antioxidants

Superoxide dismutase catalyzes the disassembly of superoxide's into oxygen and hydrogen peroxide. Honey bees contain two types of superoxide dismutase proteins, SOD1 and SOD2. SOD1, AKA copper/zinc SOD or cu/znSOD, is the cytoplasmic SOD and is highly prevalent in the thorax and muscles of worker bees due to foraging (Schippers *et al.*, 2006; Williams *et al*., 2008). SOD2 is the mitochondrial version, which will be discussed with more detail below.

Catalase acts on hydrogen peroxide to produce oxygen and water. In the honey bee, its highest levels of expression have been found in the intestine of a newly fertilized queen. The presumption is that it is due to the high oxygen demand of the developing embryo (Li J *et al*., 2009). The queen has a higher basal expression of antioxidants than the other hive constituents and is thought to be the reason why the life expectancy of a queen is up to 10 times longer than the rest of the bees (Page and Ping, 2001; Weirich *et al.*, 2002).

Peroxidase acts on hydrogen peroxide like catalase but uses a secondary antioxidant as an electron donor, such as thioredoxin or glutathione. Insects code for 3 genera of peroxidases; thioredoxin peroxidase peroxidoredoxins or TPX's (Radyuk *et al.,* 2001), phospholipid-hydroperoxide or GTPX (Missirlis *et al.,* 2003), and glutathione S-

transferase or GST (Taba and Aigki, 2000). Honey bees lack antioxidant genes in the glutathione S-transferase (GST) family epsilon while the delta class only has one representative (Collins *et al*., 2004; Corona *et al*., 2005; Yee-Tung *et al.,* 2017). These two GSTs, delta and epsilon, are highly important for pesticide detoxification. Unlike delta and epsilon classes the honey bee has more sigma GSTs; these are primary thought to play a role in lipid peroxidation and are localized to tissue that has a large metabolic load (Singh, 2001).

Secondary Antioxidants

Methionine sulfoxide reductase, Msr, is involved in protein repair by catalyzing the TRX-dependent reduction of methionine sulphoxide to methoionine (Moskovitz, 1996; Kumar, 2002). MsrA can act on both free and protein bound methionine but MsrB exclusively acts on protein bound methionion.

TrxR1 or thioredoxin reductase functions to recycle the primary antioxidants, thioreduction and glutathione S-transferase (Kanok, 2001). The genome of the honey bee shows a reduction in the number of TrxR1 transcripts compared to other insects (Corona and Robinson, 2006). In humans; TrxR1 is a selenoprotein containing selenocystine

SELENIUM AND SELENOPROTEIN

Selenium is commonly found in environment in low concentrations and its distribution varies greatly ranging from 0.01-2 mg/kg (Winkel *et al.,* 2015). In alkaline soils; agriculture runoff can lead to a buildup in its bioavailable forms, selenate SeO_4^{2-} and selenite SeO_3^{2-} (Wu, 2004). Some plants will hyperaccumulate selenium in selenium rich soils and can have concentrations of 15,000 mg/kg by their dry weight (Winkel *et al.,* 2015). Selenium is an interesting micronutrient in that the even for larger organisms like humans the tolerance window is narrow with a recommended dietary allowance of only 55 µg to 400 µg per day (institute of medicine, 2000). Moving outside the dietary window can have drastic consequences weather it be selenium deficiency or selenium toxicity.

Selenate has been shown to cause increased mortality in honey bees most strongly affecting the larval stages. Treatment with as little as 0.6 mg/L resulted in a reduction in capped brood, increased pupal development time, and as a result lower total worker weight for the colony (Hladun *et al*., 2013). In some cases, after chronic exposure to Selenium no capped brood were present in the hive. However, adult bees were able to withstand a 5x increase in selenium accumulation (Hladun *et al*., 2016). Acute feeding of both predominate forms of selenium show that selenate is more toxic to larvae, LD50 0.72 mg/L, than selenite LD50 of 1.0 mg/L. While in adult foragers, both forms of selenium have an LD50 of 58 mg/L (Hladun *et al*., 2013).

Further characterization of uncharacterized Seleno-like proteins is needed in *Apis mellifera.* According to the literature *Apis mellifera* does not possess Selenoproteins (Chapple and Guigo, 2008). However, its close relative *Apis cerana* has been shown to possess them. These Seleno-like proteins possess a high degree of similarity to known selenoproteins found in other species in both coding sequence as well as conserved domains.

In a recent study comparing the expression of antioxidant genes, TrxR1 and SOD1, in *Apis mellifera* and *Apis cerana* it was shown that in response to both chemical and environmental stressors both species exhibited very similar expression profiles. However, the magnitude of the response was greater in *A. mellifera* when exposed to increased, 37°C, or decreased temperature, 4°C, compared to the control, 27°C. When injected with the oxidants, hydrogen peroxide and paraquat, the profiles as well as their relative expression were proportional over the course of 9 hours in both species (Koo *et al.,* 2016).

Selenoproteins have been functionally classified into 6 categories, which include their role as peroxidases and reductaces, protein folding, redox signaling, hormone metabolism, and selenium synthesis and transport (Gonzalez-Flores *et al.,* 2013). It should be noted that non-selenum containing homologues have been found across almost all domains in life (Gonzalez-Flores *et al.,* 2013). When comparing the functionality of selenocysteine, Sec, containing proteins to identical homologues containing cysteine, Cys, in place of sec it was found that functionality was retained but the loss of sec

resulted in a lower enzymatic activity (Johansson *et al.,* 2006; Gonzalez-Flores *et al.,* 2013). The advantage for incorporating Sec into the active site of a protein is a result of the innate properties of selenium. When Cys containing proteins are overoxidized they inactivated but the Sec versions of these proteins prevent overoxidation and can be recycled even after reaching a more oxidized state and is more reactive than its non Sec containing relative (Hondal and Ruggles*,* 2011; Gonzalez-Flores *et al.,* 2013).

If the classical selenosystem is found to be intact in *Apis mellifera* it would go against the popular assumption that the necessary selenomachinery is absent. If the classical selenosystem is absent but selenoproteins are present; it could prove to broaden our understanding into the underlying machinery or alternative regulatory network of selenoproteins as well as provide new insight into the detoxification genes found in the honey bee. If both the selenosystem and selenoproteins are absent then selenoproteins could serve as a target in the varroa mite. Further investigation of the seleno-system in *Apis melliferia* may prove to be crucial in not only understanding how the bee's cope with pollinating selenium rich areas but also may provide an alternative for the treatment of *Varroa destructor* by targeting the selenoprotein dependent machinery.

VARROA MITE (*Varroa destructor)*

Varroa destructor is the most harmful parasite to the honey bee. *Varroa destructor* is a mite that not only requires the honey bee for feeding but also for reproduction. The female mite can feed on adult or pupae honey bees by ingesting the honey bees hemolymph causing physiological and developmental issues. In addition, the exchange of fluids can provide a vector for the transmission of viruses such as DWV and the wound created from feeding can cause secondary infections.

PESTICIDES (COUMAPHOS AND IMIDACLOPRID)

Pesticides used in agriculture are designed to target specific pests while remaining relatively benign to non-target organisms. Unlike laboratory conditions, bees may come in contact with numerous pesticides that when combined could cause responses more drastic than exposure to any of the individual chemicals alone. Unintended exposure to pesticides is one of the many suspected causes for CCD, along with other environmental stressors may attribute separately or synergistically have potential effects on bees (Cox *et al*., 200; vanEngelsdrop and Meixner, 2010). Coumaphos is an organophosphate with acaricidic properties and is commonly used by beekeepers to control the parasitic mite (*Varroa destructor*). Coumaphos acts systemically by irreversibly binding the enzyme acetylcholinesterase, AChE, at its active site and results in over stimulation of the receptor, AChR. (TOXNET, 1975-1978). A fraction of the coumaphos is consumed by a bee, the majority is spread throughout the colony by eusocial interactions such as trophallaxis (Bevk *et al.,* 2012; van Buren *et al*., 1992a; van Buren *et al.,* 1993) and has been reisolated from honey, wax, and royal jelly (Wallner, 1999; Tremolada *et al*., 2004; Martell *et al*., 2007; Smodis Sker *et al*., 2010).

The acute toxicity of coumaphos becomes more potent to bees as they age with the lethal dose (LD_{50}) varying from 3 to 6 μg (van Buren *et al*. 1992b). Chronic exposure to coumaphos has been demonstrated to negatively impact foraging behavior, cause a reduced size in the hypopharyngeal glands, increase the rate of apoptosis, and increase mortality in brood (Smodis Sker *et al.*, 2010). Though coumaphos is reported as being weakly toxic to honey bees even at low concentrations it has been shown to impair the honey bee immune system (Desneux *et al.*, 2009), hinder mobility and increase involuntary gut movements (Williams *et al.,* 2013) more work is needed to determine its affects when combined with other commercially available pesticides that would normally or possibly be encountered during foraging (Extension Toxicology Network, 2001). In a study conducted on honey bees demonstrating symptoms of CCD; residues of 121 pesticides, and coumaphos concentrations ranging from 1.0 μg/Kg to 919 mg/Kg were isolated from 98 % of wax samples (Mullin *et al*., 2010).

Imidacloprid is one of the most widely used pesticides and is the active ingredient in nearly all commercially available pesticides. Like coumaphos, it acts on the central nervous system by interfering with the transmission of the neurotransmitter acetylcholine. Though coumaphos mode of action is accomplished by inhibiting AChE, imidacloprid works by mimicking acetylcholine, ACh. Imidacloprid has been shown to act by binding to the post-synaptic nicotinic acetylcholine receptors, nAChRs, in the central nervous system of insects (Matsuda and Sattelle, 2005) and has been found to be highly toxic to honey bees, with acute oral LD50 from 0.004 to 0.005 μg per bee (EFSA, 2012). Imidacloprid has been shown to alter development of the honey bees hypopharyngeal glands resulting in a reduced diameter of the acinal ducts and increased cell death (Smodis *et al.*, 2010). Both contact and oral administration of imidacloprid have been conducted to evaluate its toxicity on honey bees (Suchail *et al.*, 2000; Decourtye *et al*., 2004). Residues of imidacloprid in treated crops can exceed this LD50, with levels of 2–3.9 μg/kg in pollen and less than 2 μg/kg in nectar (Bonmatin *et al.,* 2003: Schmuck *et al.*, 2001). The working hypothesis here is that; upon exposure to pesticides detoxification genes/enzymes will be upregulated in the bee to alleviate its effects.

Pesticides can be found in the environment can have ranging concentration. To assess the lethality of coumaphos from environmentally reported samples concentrations ranging from 11,500 PPB to 185,200 PPB will be used. To assess the effect of both coumaphos and imidacloprid independently and in concert imidacloprid will be used in concentrations of 5 PPB and 20 PPB.

CHAPTER II – HYPOTHESIS AND SPECIFIC AIMS

Hypothesis:

Upon exposure to pesticides detoxification genes/enzymes will be upregulated in the bee to alleviate its effects.

Specific Aims:

1) Evaluate bee mortality rate when chronically exposed to varying concentrations of pesticides through their diet.
2) Study how chronic exposure to pesticides regulates select antioxidant genes and if uncharacterized possible antioxidant genes show differential expression in response to exposure.

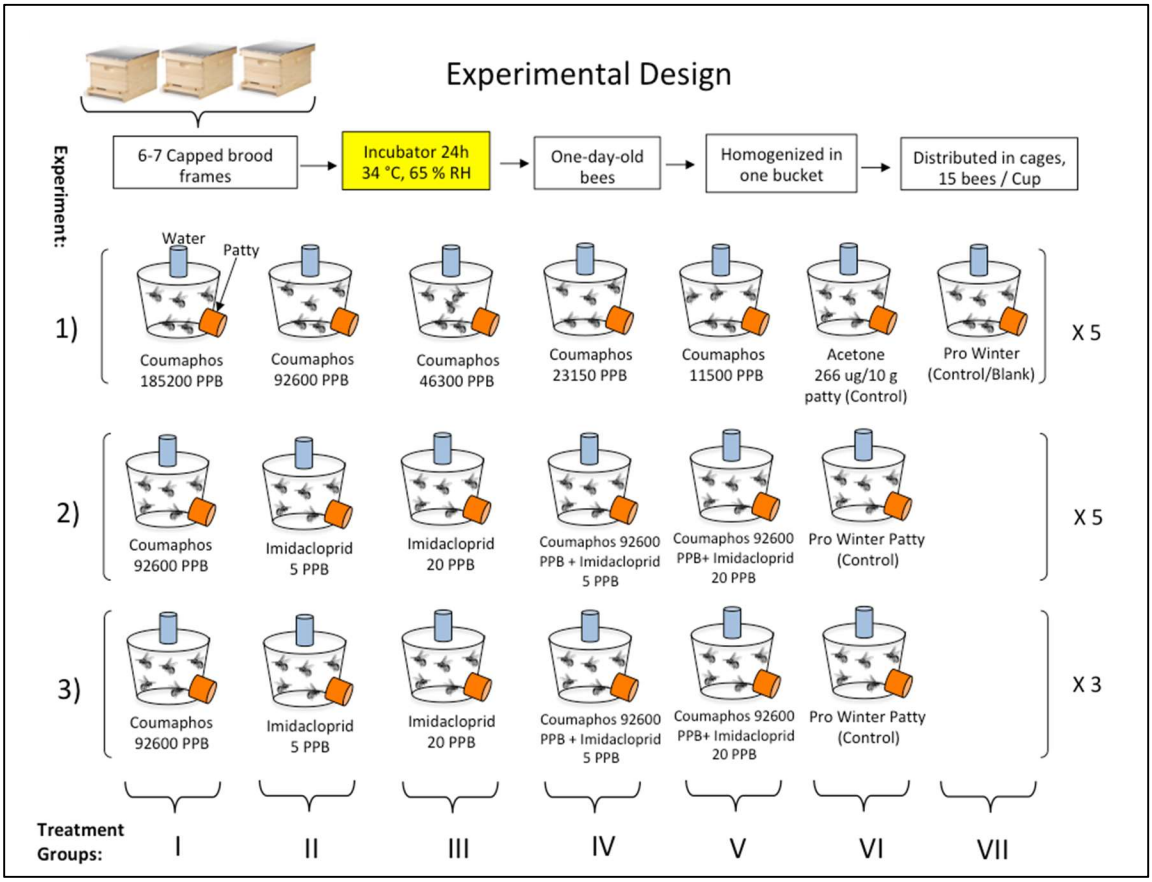

Figure 4: Experiential design (1-3) and the number of treatment groups in each experiment. Experiments 1 and 2 were used to evaluate the mortality rates of worker bees when chronically exposed to pesticides. Experiment 1 was used to evaluate the mortality rates of worker bees when chronically exposed to coumaphos. Experiment 2 was conducted to determine the mortality rates when both coumaphos and imidacloprid were consumed at concentrations likely encountered while foraging. Samples for the antioxidant gene study were polled from experiment 3. Additives to the diet were mixed to the Pro Winter patty (patty) as base sugar candy.

Specific Aim 1:

Evaluate bee mortality rate when chronically exposed to varying concentrations of pesticides through their diet.

Experiment 1: Caged bees were exposed to coumaphos concentrations ranging 185,200; 92,600; 46,300; 23,150 and 11,500 PPB with 2 control cages. Coumaphos was administered by incorporation into Pro Winter Patties by dissolving into 266 μL of acetone for each 10 g paddy. Each group will be given 1 g of patty initially with more added as needed. 15; 3-day-old worker bees were used for each treatment group. The control groups consisted of two treatment groups. The first contained only acetone without the addition of coumaphos, the other did not contain acetone or coumaphos (see Figure 4 Experiment 1). Each treatment group was replicated 5 times, dead bees were collected daily, and food consumption was recorded.

Experiment 2 and 3: ProWinter Patties were again used here to assess the mortality and transcriptional effect of the pesticides coumaphos and imidacloprid. Six treatment groups were used here coumaphos at 92,600 PPB, imidacloprid at 5 PPB, imidacloprid at 20 PPB as well as both coumaphos at 92,600 PPB and imidacloprid at 5 PPB and coumaphos at 92,600 PPB and imidacloprid at 20 PPB as well as one control group with no pesticides (See Figure 4 Experiment 2 and 3). For experiment 2 each treatment group will be replicated 5 times, For experiment 3 each treatment group will be replicated 3 times. For both experiments dead bees were collected daily, and food consumption was recorded.

Specific Aims 2:

Study how chronic exposure to pesticides regulates select antioxidant genes and if uncharacterized possible antioxidant genes show differential expression in response to exposure.

The transcriptional expression of primary and secondary antioxidants in honey bees was assessed in multiple life stages, when exposed to pesticides, infected with the parasitic mite *Varroa destructor* as well as when exposed to pesticides, coumaphos and imidacloprid. Worker bees were collected from hives managed by the Mississippi State University. The baseline expression of antioxidants for each life stage (adult, pupae, and larvae) as well as select tissue types (midgut, and brain) in those stages were used to determine if the antioxidant genes are being differentially regulated throughout their development or within the specific tissue. Three biological replicates of each sample were preserved in RNAlater. Upon collection samples were kept on ice while transported to the University of Southern Mississippi in Hattiesburg, MS. Upon receipt samples were kept at -80°C until analysis was preformed. To determine the effect of pesticides on the transcriptional activity of antioxidants 1-day-old worker bees were treated with Coumaphos at 92,600 PPB, Imidacloprid at 5 PPB, Imidacloprid at 20 PPB as well as both Coumaphos at 92,600 PPB and Imidacloprid at 5 PPB and Coumaphos at 92,600 PPB and Imidacloprid at 20 PPB (See Figure 4 Experiment 3). Living adult workers were collected at both 10 and 20 days after exposure to determine the impact of the pesticides on the select antioxidant genes transcriptional activity. **RNA Extraction and qRT-PCR:**

Each sample collected was manually homogenized via pestle in lysis solution. The homogenized product was then quickly spun down and supernatant was extracted using a 20g needle to avoid removing cellular debris. All proceeding steps were done in accordance with the manufacturers provided protocol (inventogen RNA extraction). RNA concentration and purity was assessed using nanodrop. Samples containing contaminants as determined by their 260/280 and 260/230 ratios were discarded or re-purified.

Isolated mRNA was set to a concentration of 500 ng before being used to generate cDNA (iscript kit, BioRad). Technical triplicates containing 7.5 ng of cDNA was used to asses transcriptional expression by SYBR green using qRT-PCR. The results were then normalized against four reference genes, see table 1. The data was then normalized to housekeeping genes (Rp49, Rps5a, Tbp-f and RpL32) to determine the relative gene expression by the CFX maestro software (Biorad) using the calculated ΔΔCq. The

graphic used to depict the transcriptional expression of genes were also generated using the CFX maestro software.

Table 1. Shows the identification, description and NCBI accession numbers for both housekeeping and target genes to be used in this study. Sequences of the primers used for each gene are also provided.

Gene ID	Gene description	Accession ID	Primers F and R	
Housekeeping Genes				
Rp49	Ribosomal Protein 49	AF441189	GTCACCAGAGTGATCGTTACA GGGCATCAAATATTGTCCCTTAAA	
Rps5a	Ribosomal protein S5a	GB11132	GTACCTACCACGACGACATTA CACAATTCCAGCGACCAAATAA	
Tbp-f	TATA box binding factor	XM_393492	GGAGGAGATACTCCAGCTATGTA CATCTGGTACCGTTGGTGTATAA	
RpL32	Ribosomal protein L32	AF441189.1	GAGAAACTGGCGTAAACCTAAAG GTTGGCAACATATGACGAGTTT	
Target Genes				
Primary Antioxidants				
Cat	Catalase	NP_001171540.1	TCCACTCATTCCTGTTGGTAAG GCCGGATCGAAGGCTATTT	
Sod 1	Superoxide dismutase 1	NP_001171498.1	CGTTCCGTGTAGTCGAGAAAT GGTACTCTCCGGTTGTTCAAA	
Sod 2	Superoxide dismutase 2	NP_001171519.1	TGCAGCAAGACGTATCCTATTT CATGGTGCTTTGAATGGTGAAG	
Secondary Antioxidants				
MsrA	Methionine sulphoxide reductase A	NM_001178047.1	GGGCCGGTGATTGTTTATTTG CAACGACTTCTGTATGATCACCT	
MsrB	Methionine sulphoxide reductase B	XM_006569172.1	GTATTAGATCAGGGACGAGTCAAG CATCCATCGTAGTTCTCTCCAA	
Trxr1	Thioredoxin reductase	AY329357.1	CGTCCACCAACTCGTAGATTAG CTAGTACAACTTCTACATCCTCCAAA	
SelK	Selenoprotein K-like	NM_001278332.1	CGTCCACCAACTCGTAGATTA CTAGTACAACTTCTACATCCTCCAAA	
SelT	Selenoprotein T-like	XM_623426.5	ACAGCCACCAGCATCATT GGACCACACAGGAACATCATT	
SelS	Small VCP/p97-interacting protein-like	XM_006559143.1	TGGGTGATGGTTCTAGAGGATA CACATTTCCTCAGCCTCGAATA	
SelM	15kDa selenoprotein M like protein	XM_006557387.2	CGATATCCACGTGCTGTTCT TCGGATCTAAACCTCTAACGTATTT	

Data Analyses.

Mortality difference and variance (ANOVA) within and between the treatment groups were analyzed using Statgraphic software. By Dr. Alec Gregorc at the USDA-

ARS Thad Cochran Southern Horticultural Research Laboratory in Poplarville, MS. Variances in bee mortality across the different levels of treatment were compared using Fischer's F-tests or Tukey tests.

Bee Rearing Conditions:

Experiments were conducted at the USDA-ARS Thad Cochran Southern Horticultural Research Laboratory in Poplarville, MS. Combs with capped brood were collected from 3 colonies and incubated at 35°C in darkness. 1-day-old workers were collected and placed into cups labeled for each treatment group. Cups and cages were modified to allow air movement and provide access to remove dead bees. Each treatment group was given supplementary water and Pro Winter sugar Paddy (Mann Lake LTD). Analytical standard grade coumaphos (Pestanal, CAS #56-72-4) and imidacloprid (Pestanal, CAS #138261-41-3) were used. ProWinter Patties are made at the beginning of the experiment and stored in the at 4 °C until needed.

CHAPTER IV – RESULTS

Begin a new chapter here. When comparing the larval to the pupal stages of development; the primary antioxidants Catalase SOD1 and SOD2 were strongly expressed with greater than five fold regulation than was seen in the pupae (5.87, P<0.0001; 6.56, P<0.0001; and

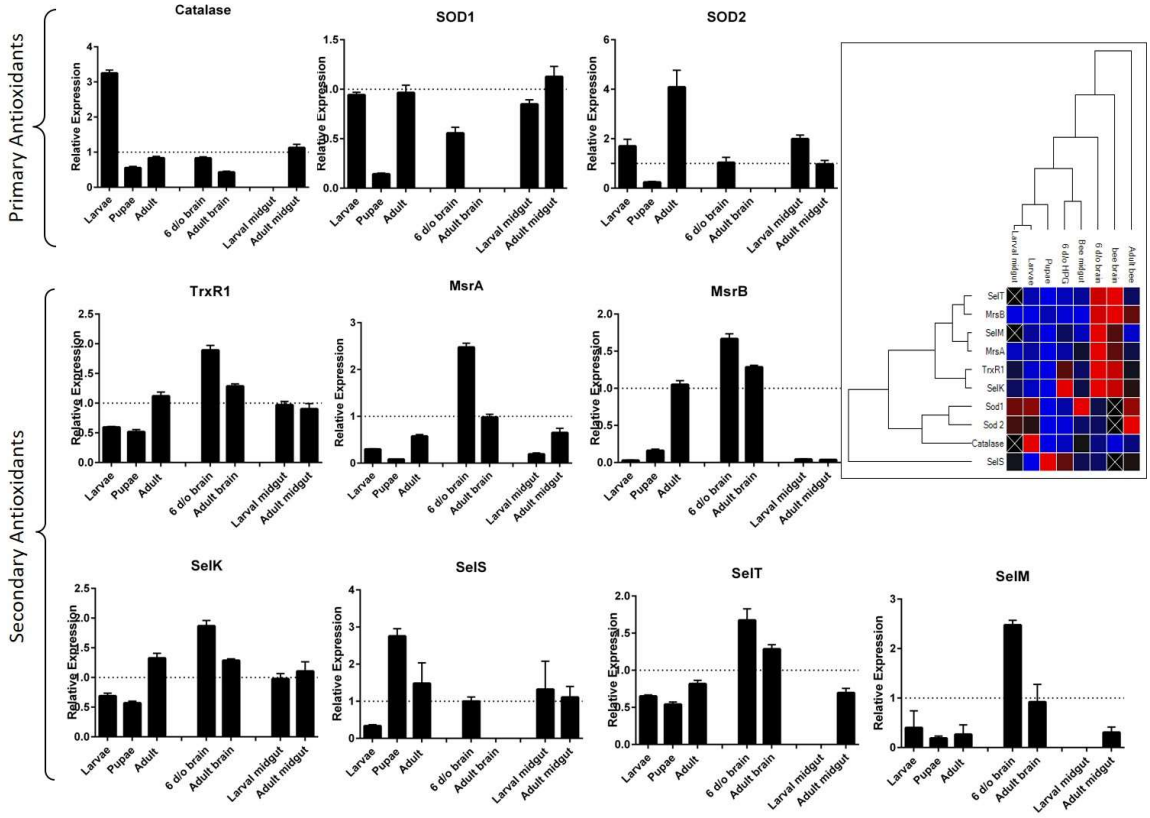

Figure 5: The transcriptional expression and heat map of Antioxidant genes per life stage and within select tissues. The primary and secondary antioxidant genes relative gene expression are grouped horizontally for the larval, pupal and adult stages of development. The relative expression is also shown for the midgut of a young adult and larvae, and the brain of a young adult and six day old adult. The relative expression of antioxidants found in these life phases and specific tissues are represented in the heat map to right. The qPCR regulation threshold value was set at P = 0.05, gene expression below this value were not considered up or down-regulated. Error bars represent the Standard Errors SE.

6.99, P<0.005 respectively). The pupae showed a higher expression of the secondary antioxidants MsrB 5.57, P<0.005 and SelS 8.27, P<0.0001 while SelK and SelT were slightly down regulated by 1.2, P<0.05; P<0.005.

When comparing the pupae to the young worker both primary antioxidants SOD1 and SOD2 showed a marked increase of regulation (6.72, P<0.0001 and 16.78 P<0.005). The secondary antioxidants MsrA and MsrB were also highly increased (6.88, P<0.0001 and 6.53, P<0.0001) with SelK and TrxR1 both showing just over a two fold increase (P<0.0001) compared to the pupal phase.

Within in the isolated tissues the midgut and brain remained rather consistent. The most notable, and only found to be statically significant, difference being MrsA which showed a expression of 3.49 higher in the midgut of the adult compared to that of the larvae P<0.005, see Fig 5.

Specific Aims 1:

Evaluate bee mortality rate when chronically exposed to varying concentrations of pesticides through their diet.

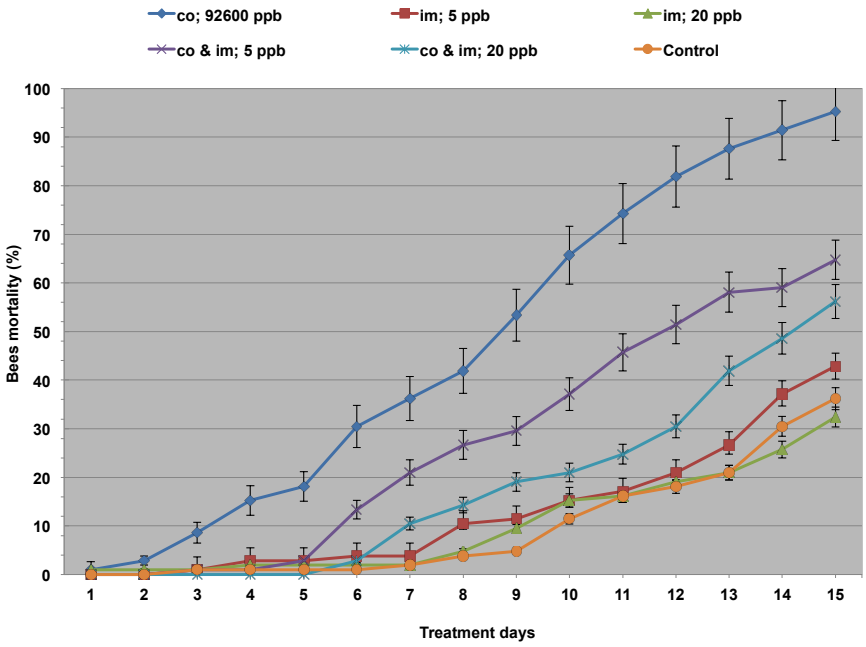

Figure 6. Bee mortality (%) after 9 days of feeding on five coumaphos concentrations**.** Incrementally halved concentrations of coumaphos are: 185,200 ppb; 92,600 ppb; 46,300 ppb; 23,150 ppb and 11,500 PPB, incorporated in Pro Winter sugar patties. Group VI control bees received a diet of patty with the acetone solvent. Group VII control bees were fed only patty. Letters that are the same indicate means that are not significantly different according to the Tukey HSD test ($P < 0.05$). Bars indicate mean ±1 standard deviation.

Coumaphos effects on bee survival (Experiment 1). Caged bees exposed to coumaphos concentrations of 185,200; 92,600; 46,300; 23,150 and 11,500 ppb (185,200 mg/kg; 92,600 mg/kg; 46,000 mg/kg; 23,150 mg/kg and 11,500 mg/kg) remained active throughout the experiment. Even in the bees exposed to the highest levels of coumaphos behavior such as grooming appeared normal for worker bees in all treatment groups. Coumaphos concentration had a high correlation on bee mortality in the first 9 days ($F = 12.38$; $df = 6$; $P < 0.001$) with the highest mortality being represented by the two highest coumaphos concentration of 92,600 and 185,200 ppb, Fig. 6. Mortality rates for the three lower concentrations

11,500 to 46,300 ppb were relative non toxic to the bees compared to the control treatment whose ProWinter patty was only supplemented with the coumaphos solvent acetone, Fig. 6.

Coumaphos and imidacloprid interaction (Experiment 2).

In the first 15 days the addition of both coumaphos and imidacloprid treatments induced significant levels of bee mortality ($F = 6.18$, $dF = 53$, $P < 0.01$), Fig. 7. Coumaphos had the highest rate of mortality in all treatment groups. When coumaphos at the same concentration was amended with imidacloprid at both 5ppb and 20 ppb a lower mortality rate was observed compared to the bees treated with coumaphos alone. However, when administered alone imidacloprid at 5 ppb imidacloprid and imidacloprid at 20 ppb were relatively non-toxic compared to any coumaphos treatment groups, Fig. 7 and 8. Again, the addition of

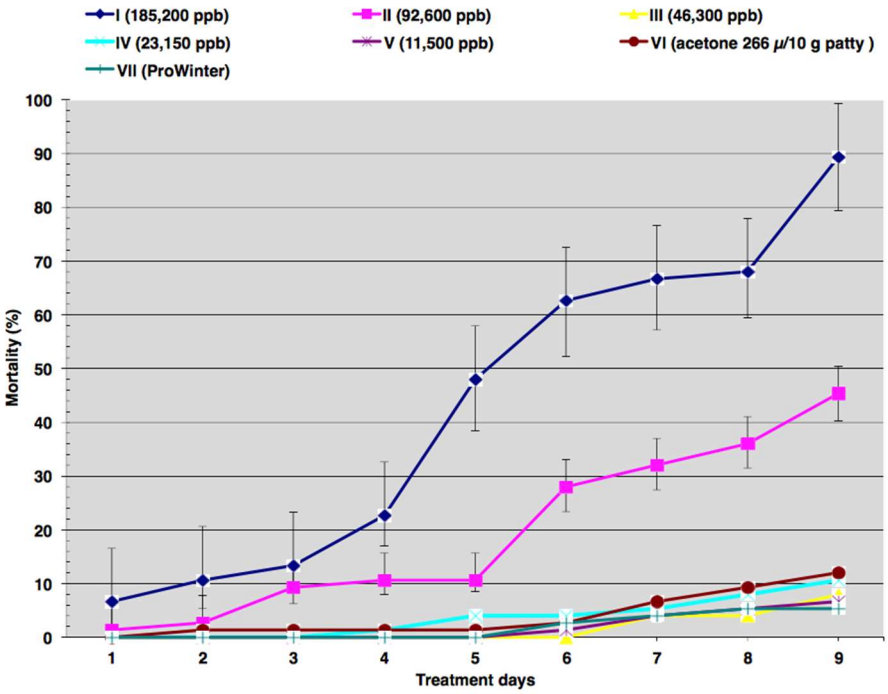

Figure 7. Cumulative bee mortality across six treatment groups. Treatments included coumaphos (92,600 ppb), imidacloprid (5 and 20 ppb), and their combinations. All treatments incorporated Pro Winter sugar patty (patty). Control bees received only patty, no other additives were used.

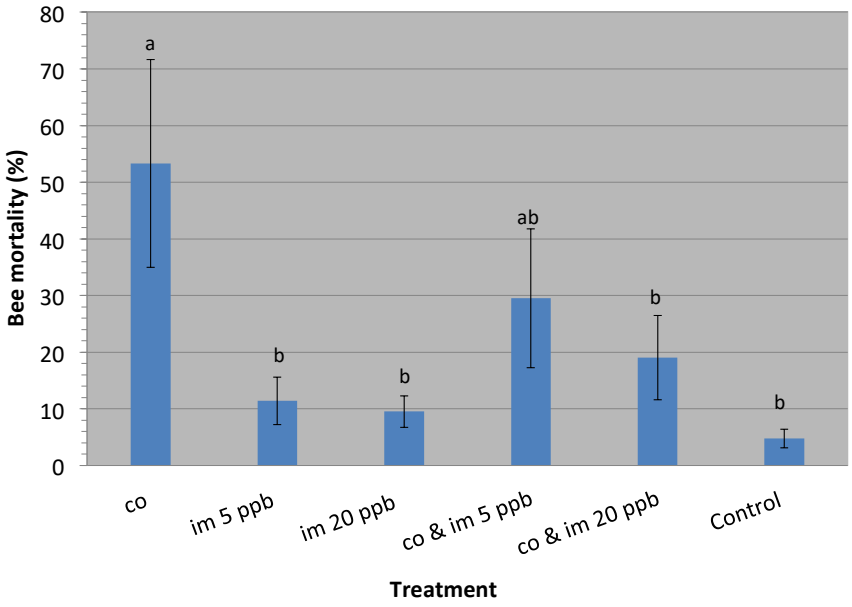

Figure 8. Cumulative mortality after 9 days of feed on patty tented with pesticides. Caged-bees were fed Pro Winter sugar patty (patty) containing 92,600 ppb coumaphos (co); 5ppb imidacloprid (im), 20 ppb imidacloprid, 92,600 ppb coumaphos & 5 ppb imidacloprid, 92,600 ppb coumaphos + 20 ppb imidacloprid, and a control where caged bees received patty without additives. Letters that are the same indicate means that are not significantly different according to the Tukey HSD test ($P<0.05$). Bars indicate mean ±1 standard deviation.

coumaphos at 92,600 ppb was very toxic killing more than half of the workers by 9 days and almost all by day 15, Fig. 6 and 7. The addition of imidacloprid to bees fed coumaphos at 92,600 ppb seemed to alleviate the toxic effects of coumaphos alone while proving more harmful than imidacloprid alone at both imidacloprid at 5 ppb imidacloprid and imidacloprid at 20 ppb, Figs. 7 and 8.

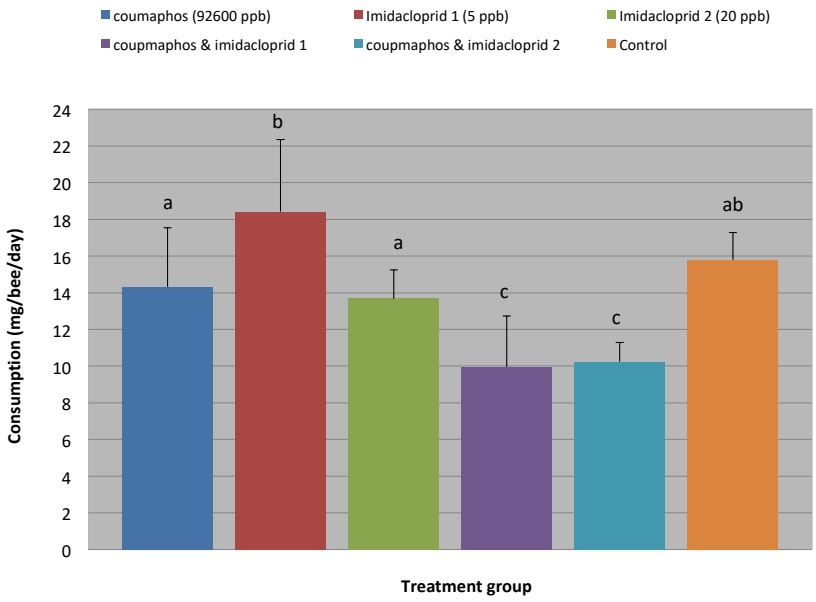

Figure 9: Consumption rate of Pro Winter sugar patty amended with or without coumaphos and imidacloprid insecticides, or their combinations. Letters that are the same indicate means that are not significantly different according to the Tukey HSD test ($P < 0.05$). Bars indicate mean +1 standard deviation.

Specific Aims 2:

Study how chronic exposure to pesticides regulates select antioxidant genes and if uncharacterized possible antioxidant genes show differential expression in response to exposure.

Antioxidant gene expression.

The expression of 10 antioxidant genes varied significantly among treatments during the ten-day feeding period. Bees fed with 5 PPB imidacloprid for ten days showed significant down regulations in three different target genes: Cat ($P < 0.001$), MsrA and TrxR1 ($P < 0.01$), Fig.

10A. Both SelT and MsrB genes expressed up-regulations but were not considered significant based on the normalization of our dataset while conducting the gene study,

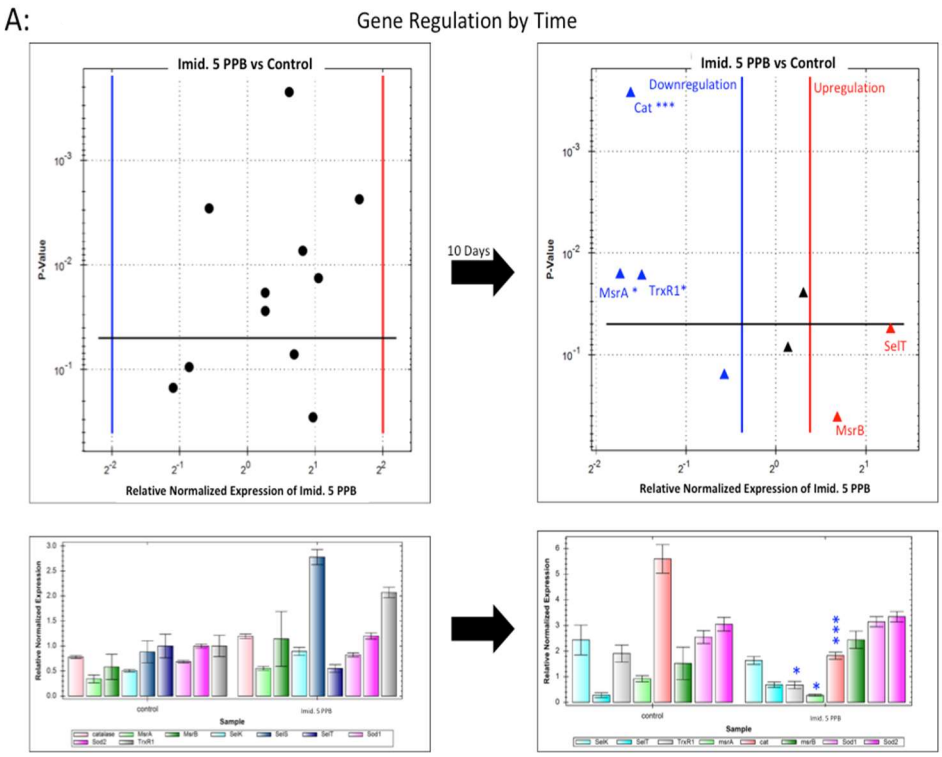

Figure 10A . Volcano plot and bar graph for the expression of antioxidant genes at 10 and 20 days after exposure to imidacloprid 5PPB.. All gene studies were normalized using the housekeeping genes mentioned in Table 1. The qPCR regulation threshold value was set at P = 0.05, gene expression below this value was not considered up or down-regulated. Level of significances are P < 0.05 *, P< 0.01 **, P < 0.001 *** and error bars are the Standard Errors SE.

because they exceeded the critical P-value of 0.05. The higher concentration of imidacloprid (20 PPB) led to a significant up-regulation in catalase activity (Cat: P < 0.001) along with three other genes (TrxR1, SelK, MsrB; P < 0.05), while Sod2 was down-regulated (P < 0.001), Fig. 10B.

The latter figure shows down and up regulations of the SelT and MsrA genes, respectively. Coumaphos showed no changes in the first sampling date (day 10),

B:

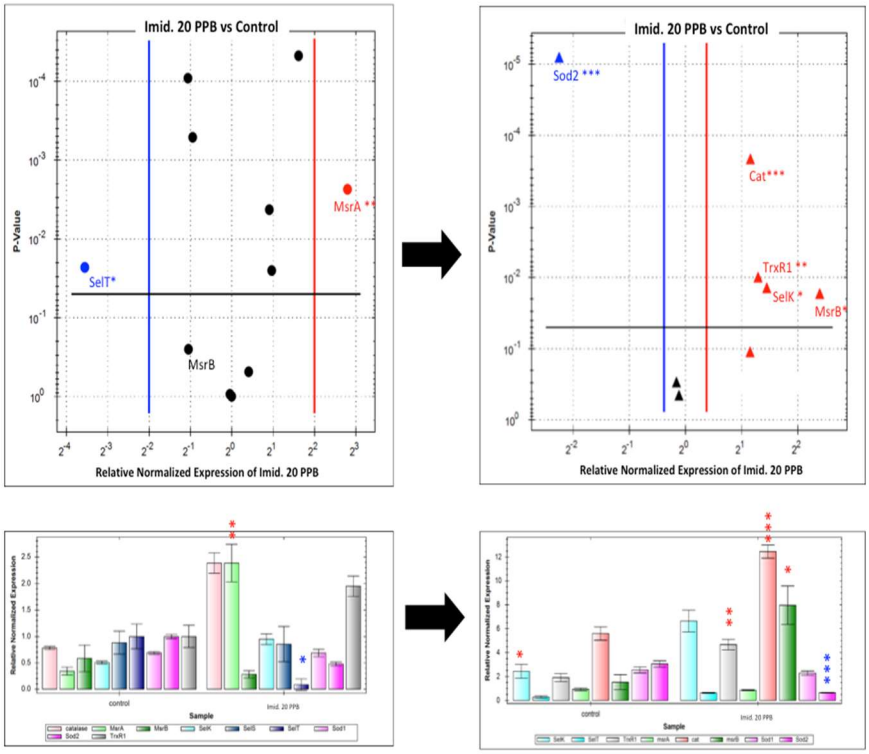

Figure 10B. Volcano plot and bar graph for the expression of antioxidant genes at 10 and 20 days after exposure to coumaphos 92,600 PPB.. All gene studies were normalized using the housekeeping genes mentioned in Table 1. The qPCR regulation threshold value was set at P = 0.05, gene expression below this value was not considered up or down-regulated. Level of significances are P < 0.05 *, P< 0.01 **, P < 0.001 *** and error bars are the Standard Errors SE.

however, at day 20, two genes were down regulated (Cat; P < 0.001) and (TrxR1; P < 0.05), Fig. 10C. Cat and MsrA were both down regulated in bees fed for 10 days on coumaphos and 5 ppb imidacloprid mixture, Fig. 8D. The final treatment (coumaphos and 20 ppb imidacloprid) showed significant down regulation in a single target gene (Sod2; P < 0.001), Fig. 10E. Figure 10E's Clustergram classifies the data and generates a hierarchal tree based on the degree of similarity of expression for both target genes and

treatments. This dendrogram revealed close similarities between (control vs imidacloprid 5PPB), (coumaphos vs imidacloprid 20 PPB), while the mixture of coumaphos and imidacloprid, regardless the imidacloprid concentrations, seemed to have similar effects on the regulation of the target genes, Fig. 10E.

C:

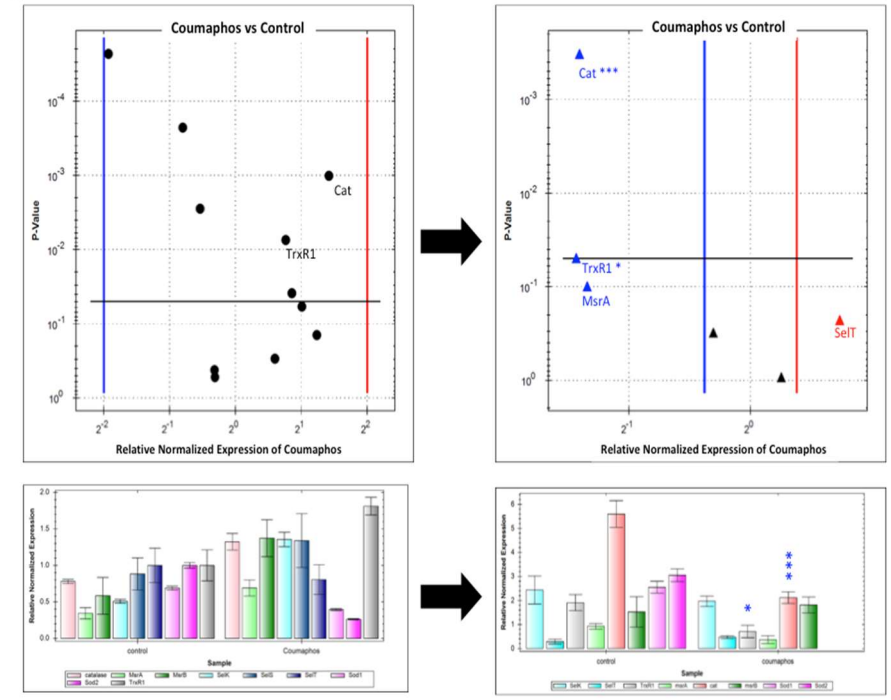

Figure 10C . Volcano plot and bar graph for the expression of antioxidant genes at 10 and 20 days after exposure to coumaphos 92,600 PPB.. All gene studies were normalized using the housekeeping genes mentioned in Table 1. The qPCR regulation threshold value was set at P = 0.05, gene expression below this value was not considered up or down-regulated. Level of significances are P < 0.05 *, P< 0.01 **, P < 0.001 *** and error bars are the Standard Errors SE.

D:

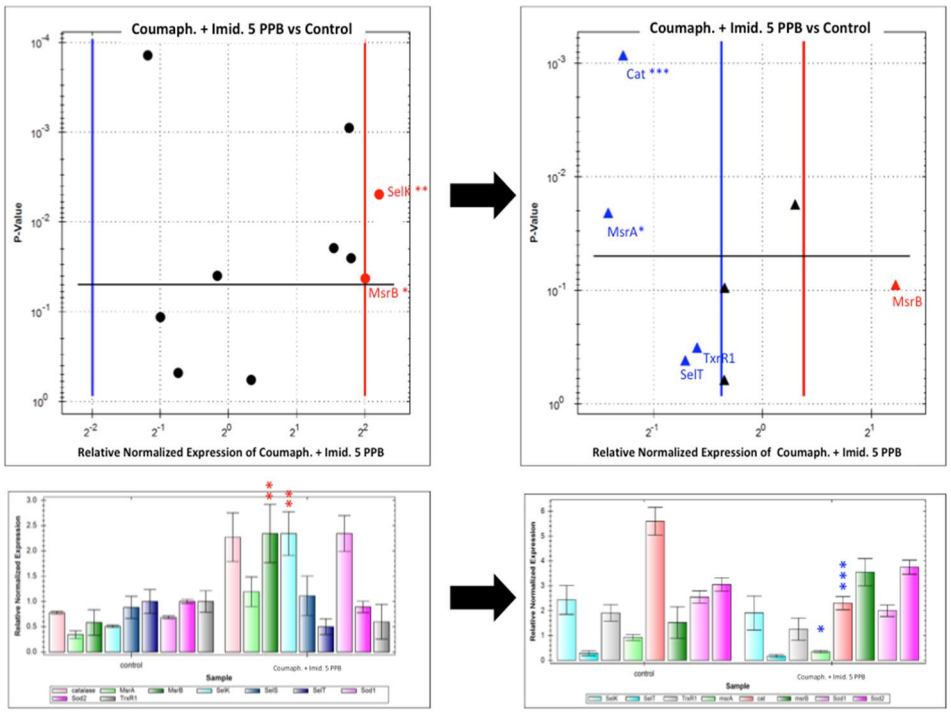

Figure 10D. Volcano plot and bar graph for the expression of antioxidant genes at 10 and 20 days after exposure to coumaphos 92,500 PPB and imidacloprid at 5 PPB.. All gene studies were normalized using the housekeeping genes mentioned in Table 1. The qPCR regulation threshold value was set at P = 0.05, gene expression below this value was not considered up or down-regulated. Level of significances are P < 0.05 *, P< 0.01 **, P < 0.001 *** and error bars are the Standard Errors SE.

E:

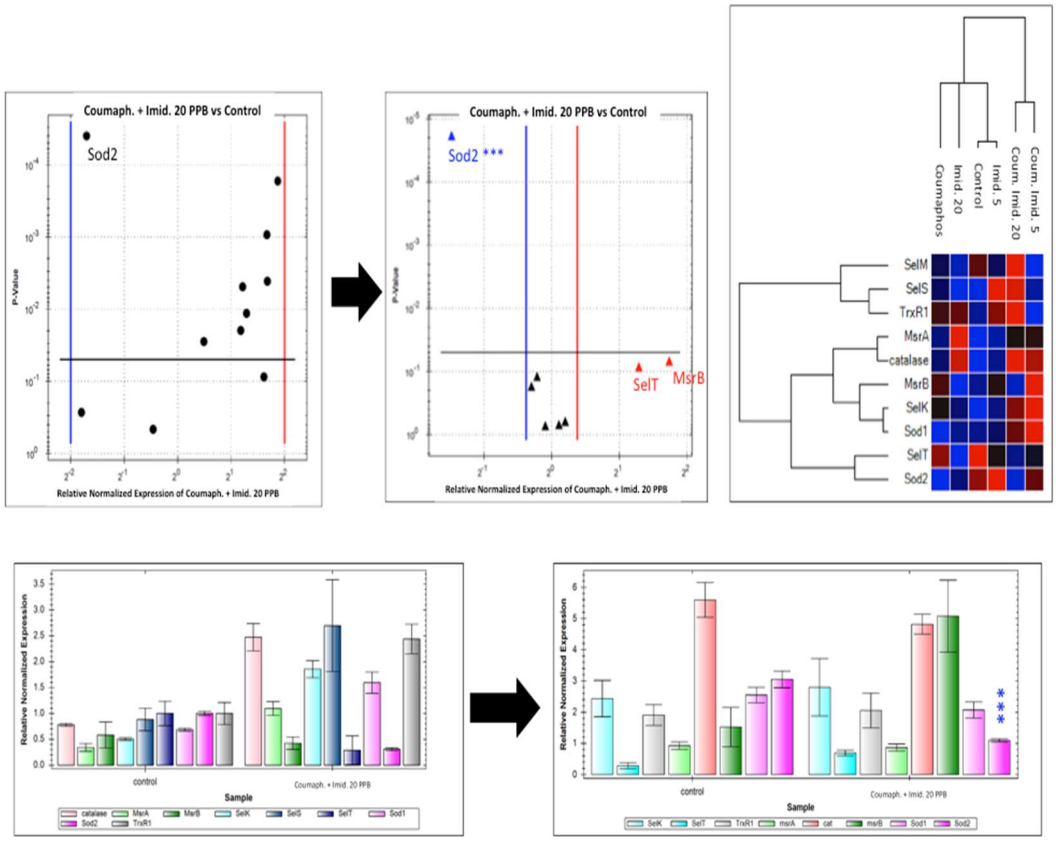

Figure 10E. Volcano plot, bar graph and clustergram for the expression of antioxidant genes at 10 and 20 days after exposure. The volcano plot on the top left shows the differential expression of genes when exposed to coumaphos and imidacloprid 20 PPB plot after 10 and 20 days. The clustergram on the top right shows the heat map for the expression of each treatment group and depicts the similarity of the expression profiles using a dendrogram. All gene studies were normalized using the housekeeping genes mentioned in Table 1. The qPCR regulation threshold value was set at P = 0.05, gene expression below this value was not considered up or down-regulated. Level of significances are P < 0.05 *, P< 0.01 **, P < 0.001 *** and error bars are the Standard Errors SE.

CHAPTER V – DISCUSSION

In Figure 5, we determined that our uncharacterized seleno-genes are being expressed in the honey bee and are being done at varying rates depending on the developmental stage of the bee. The heat map depicting the expression profiles of the genes of antioxidants tested show the expression of the uncharacterized seleno-transcripts are grouped with the antioxidants tested, with the exception of SelS whose expression pattern is the least like any others seen here. This suggests that they may be co-regulated along with other antioxidants. When treated with pesticides, as seen in Fig 10, the trend of the uncharacterized seleno-transcripts grouping with characterized antioxidants is again seen however the relatedness of the individual genes changes.

Taking both distributions into account it can be inferred that the when the worker is exposed to pesticides these uncharacterized seleno like genes are being differentially regulated by upstream mechanism resulting in similar profiling in an attempt to compensate for the toxic effects imposed by the pesticides. Based on the expression and function of known selenoproteins in other organisms the interaction between antioxidants and these possible selenoproteins is proposed, Fig 11. It is also possible that the well characterized antioxidants are being independently regulated of each other or in a reciprocal fashion with a fixed minimum thus creating large gaps in their expression profiles and the seleno-transcripts are showing profiles similar to the antioxidants because of the innate programed regulation between the characterized antioxidant profiles.

Here we have shown that these Seleno-transcripts are expressed and are differentially expressed depending on the life stage as well as the tissue type. It seems

that these seleno-transcripts most likely code for a functional protein that under oxidative stress fits the characteristics of secondary antioxidants being up regulated to mitigate oxidative stress induced by pesticides. The information generated here cannot determine nor refute that these are co-regulated with other antioxidants. However, it does seem they are involved in oxidative stress based on the related expression to other antioxidants. Further research would be needed incorporating a broader population of target transcripts to determine the degree of relatedness and possible co-regulation of the uncharacterized seleno-transcripts and the well characterized antioxidant transcript. It can safely assumed they also possess a more diverse roll outside of their antioxidant activity in development as well as modulating cellular processes. Further characterization of their activity as well as the regulation is needed to understand what role they are playing in the honey bee.

The mortality of bees administered coumaphos at range of concentrations from 11,500 PPB to 185,200 PPB, Fig 6. This range is relevant when considering concentrations as high as 30,000 PPB have been found in bees with bee products being found to contain 43,400 PPB (Tremolada *et al.,* 2004). In addition, coumaphos is commonly added to bee hives by beekeepers in the form of CheckMite strips to treat and prevent *Varroa destructor* infestation. If the presence of coumaphos induced a metabolic burden or resulted in an immune response by the phenyl oxidase system the antioxidant expression should also be increased as a function of increased activity in the endoplasmic reticulum. The molecular data obtained here at both 10 and 20 days after exposure shows that catalase activity is down regulated in all treatment groups receiving the coumaphos imidacloprid cocktail which could be a result of an innate antioxidant response seen in insects upon pathogen exposure and results in a shift to favor the use of peroxidases, to

prevent lipid peroxidation, while downregulating Catalase (Dubovskiy *et al.,* 2008). This phenomenon is thought to promote the opportunity of mutations that can give rise to increased resistance. And may be the what is observed here. This seems likely, especially in the treatment groups receiving imidacloprid at 5 PPM, since the down regulation of catalase is accompanied by an up regulation of secondary antioxidants that are needed for peroxidase recycling. This mechanism may also be the reason that upon infestation by *Varroa destructor* a downregulation of primary antioxidants was observed accompanied by an up regulation in SelS, SelM, and SelT, see Fig S1.

Though, seemingly unapparent in the molecular assays we found that the toxic effects of Coumaphos when amended with imidacloprid conferred increased resistance to coumaphos, Fig. 7. When comparing this finding to the consumption rates it is apparent that at least one mechanism this is accomplished through is decreased consumption, Fig 9. Though the mechanism of detection is unclear it seems that the bees may be more prone to detecting the presence of the pesticides when encountered together as opposed to alone.

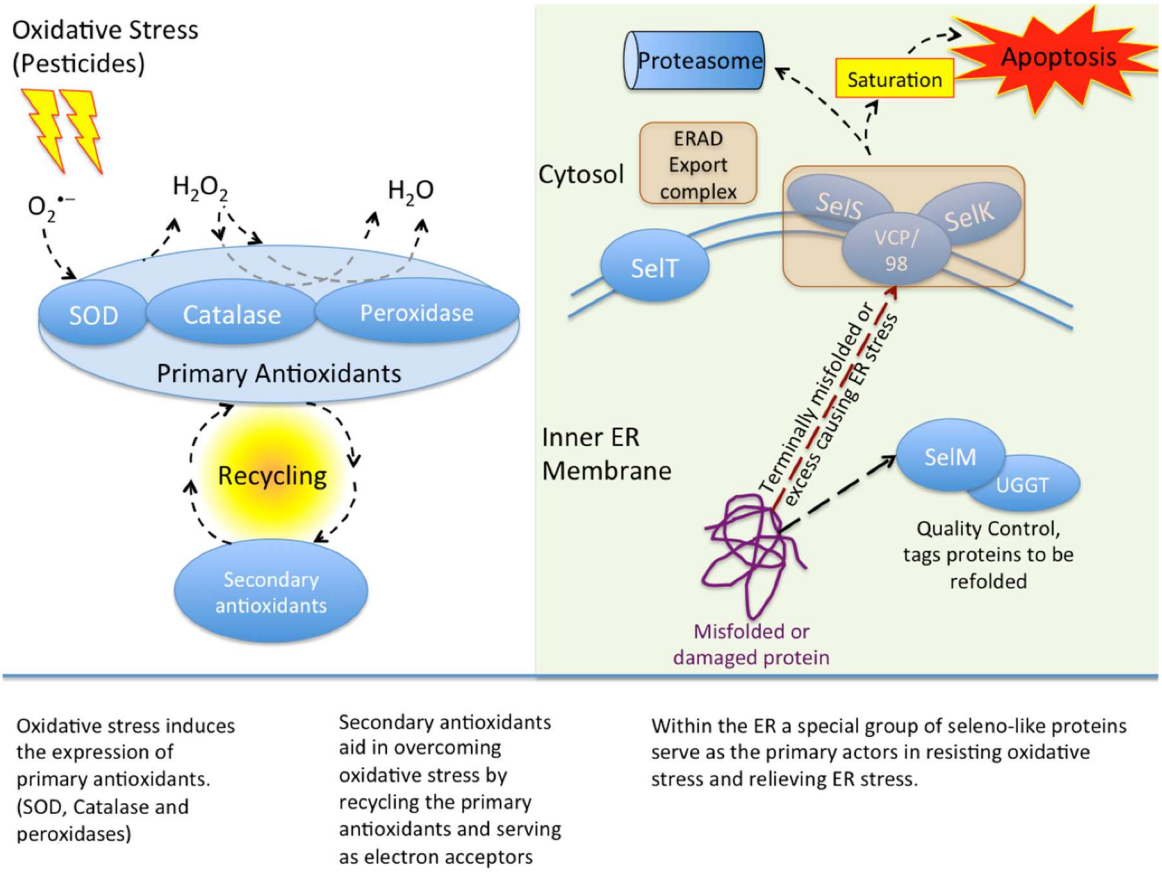

Figure 11: Illustration of the primary and secondary antioxidants activity in event of oxidative stress such as exposure to pesticides. Details are given on the role of seleno-like proteins as regulators in reducing oxidative stress in the endoplasmic reticulum ER and maintaining protein quality

Imidacloprid levels of 20 PPB and above have been shown to results in decreased foraging, communication and learning as well as other altered behavior in honey bees (Decourtye and Devillers, 2010; Meikle *et al.* 2016; Pisa *et al.,* 2015). These findings accompanied with the decreased food intake found here, Fig. 9, seems that imidacloprid levels result in alteration of the bees metabolic rate. The implication could be key to understanding and modulating the metabolic pathways in the honey bee. If imidacloprid is impeding the binding of acetylcholine, ACh, to its receptors, AChRs, resulting in a

decreased basil metabolic rate before blocking essential nAChRs essential for proper neurological it is possible that imidacloprid has a stronger affinity for the nAChRs based on the composition of certain subunits which governing metabolic processes within the bee. Thus at low concentrations, here 5 PPM, imidacloprid would bind and increase the metabolic process they modulate. This would agree with the finds honey bees have increased foraging activity after exposure to imidacloprid at 5 PPM (Decourtye and Devillers, 2010). At higher concentrations, here 20 PPM, imidacloprid will flood the receptors resulting in extended excitation and desensitization eventually causing pharmacological chaperoning and eventually loss of function (Christen *et al.,* 2016; LaLone *et al.,* 2017). The excess imidacloprid would then bind AChRs bearing subunits which it has a lower affinity imposing neurological defects and resulting in the slowing or dysregulation of other processes governed by the CNS. In honey bees, exposure to neionics like imidacloprid has been shown to greatly increase the expression of nAChRs in the brain (Christen *et al.,* 2016; LaLone *et al.,* 2017) which may further support the notion.

Two stargates could be employed to reduce the impact of coumaphos on the honey bee. The first would be the use of other substances to control the infestation of mites. With the well documented toxicity of coumaphos to honey bees a new variety of treatment for *V. destructor* could be accomplished by the use of branched amphiphilic peptide capsules, BAPCs. BAPCs are nanoparticles that have been shown to be a new and successful method of RNAi delivery (Avilla *et al.,* 2018). The use of these allows the passive introduction of lethal RNAi specifically tailored to target *Varroa destructor* and could be administered through food supplemented to the honey bee. Like the distribution

of coumaphos, these nanoparticles would be distributed among nest mates via Trophallaxis and could provide upon infestation be lethal to the mite thus eliminating the need for coumaphos.

The second strategy would involve the introduction or alteration of honey bees from a genomic standpoint to reduce the toxic effects imposed on the bee. Efforts have been made to selectively breed for a strain of honey bee that is less susceptible to *Varroa* infestation. Most of these have involved strategies have aimed to increase the grooming behavior and thickness of the cuticle as well as the bees ability to detect the mite (Spivak and Gilliam 2015; Rinderer *et al.,* 2010). These strategies were successful but were accompanied by unanticipated social changes such as decreased brood care and agitation. The aforementioned findings accompanied by the limited reports of coumaphos resistance in the mite may justify further research aimed toward the development of a transgenic bee that focuses on the molecular processing of pesticides as opposed to or even in conjunction to altering social interactions. This could be accomplished by using organisms that have naturally developed resistance to pesticides naturally as a model. Naturally acquired resistance to pesticides has been documented in numerous species. The mechanism attributed to this acquired resistance has an underlying theme emerging from the activity of cytochrome P450 as well as nAChR subunits and AChE duplications (Shang *et al*., 2012; Guerrero *et al*. 2012; Yang *et al*. 2013; Zhang *et al.* 2018).

CONCLUSIONS:

Transcripts coding for uncharacterized genes in *Apis mellifera* that contain a high degree of sequence similarity to known selenium containing genes in other organisms are expressed and are differentially regulated through the developmental stages of the bee. When the honey bee is exposed to pesticides commonly used for pest control and to prevent infestation by the parasitic mite *Varroa destructor* these transcripts show similar expression patterns to well characterized antioxidants suggesting they play a role in managing oxidative stress within the bee. We also showed that coumaphos, a pesticide commonly added to the hive by beekeepers, when encountered alone is more lethal to bees than when exposed to it along with imidacloprid, the most common pesticide used in consumer pesticides and prevent insect pests in crops.

APPENDIX A – Mite infestation

The effect of *Varroa* parasitism on honeybee antioxidant levels was evaluated by the subjecting bees to *Varroa* mite infestation for 9 days at a concentration of 40 mites per 15 bees. At least three biological replicates were collected for all samples. Upon collection samples were immediately submerged in RNA later. Each sample collected will be manually homogenized via pestle in lysis solution. The homogenized product will then quickly spun down and extracted using a 20g needle to remove cellular debris. All proceeding steps will done in accordance with the manufacturers provided protocol (inventogen RNA extraction). RNA concentration and purity was assessed using nanodrop. Isolated mRNA was set to a concentration of 500 ng before being used to generate cDNA (iscript kit, BioRad). Technical triplicates from two biological replicates were used for each treatment group. 7.5 ng of cDNA was used to asses transcriptional

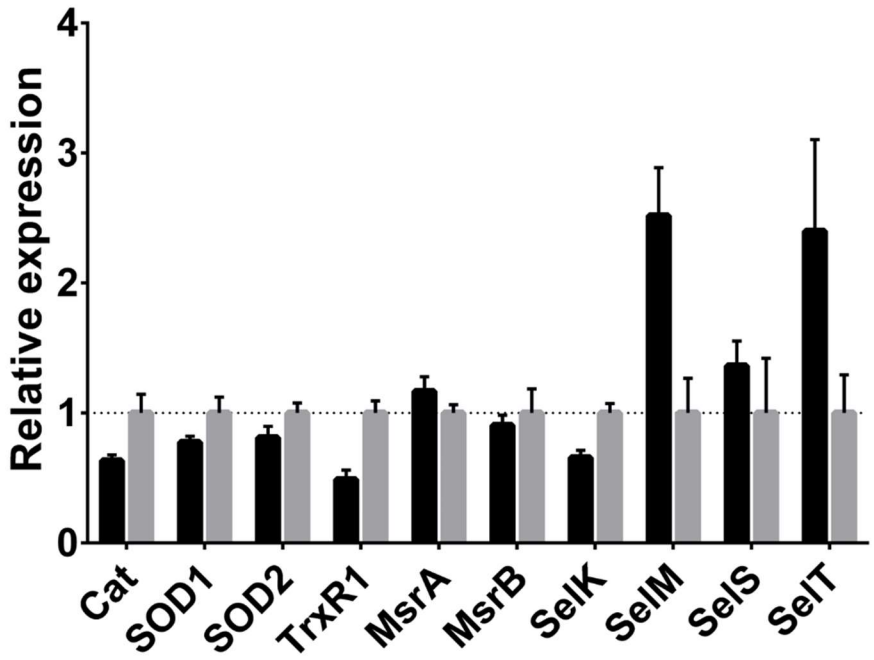

Figure S1: The relative expression of antioxidant genes in *Apis mellifera* after infestation for 9 days. The black bars represent the *Varroa* infested bees and the grey bars represent the control bees.

expression by SYBR green using qRT-PCR. The results were then normalized against four reference genes, see table 1, to find the relative gene expression.

APPENDIX B – IRB Approval Letter

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL REVIEW BOARD
118 College Drive #5147 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | Fax: 601.266.4377 | www.usm.edu/research/institutional.review.board

NOTICE OF COMMITTEE ACTION

The project has been reviewed by The University of Southern Mississippi Institutional Review Board in accordance with Federal Drug Administration regulations (21 CFR 26, 111), Department of Health and Human Services (45 CFR Part 46), and university guidelines to ensure adherence to the following criteria:

- The risks to subjects are minimized.
- The risks to subjects are reasonable in relation to the anticipated benefits.
- The selection of subjects is equitable.
- Informed consent is adequate and appropriately documented.
- Where appropriate, the research plan makes adequate provisions for monitoring the data collected to ensure the safety of the subjects.
- Where appropriate, there are adequate provisions to protect the privacy of subjects and to maintain the confidentiality of all data.
- Appropriate additional safeguards have been included to protect vulnerable subjects.
- Any unanticipated, serious, or continuing problems encountered regarding risks to subjects must be reported immediately, but not later than 10 days following the event. This should be reported to the IRB Office via the "Adverse Effect Report Form".
- If approved, the maximum period of approval is limited to twelve months.
  Projects that exceed this period must submit an application for renewal or continuation.

PROTOCOL NUMBER: 12345678
PROJECT TITLE: How to Achieve IRB Approval at USM
PROJECT TYPE: New Project
RESEARCHER(S): Jonas Doe
COLLEGE/DIVISION: College of Education and Psychology
DEPARTMENT: Psychology
FUNDING AGENCY/SPONSOR: N/A
IRB COMMITTEE ACTION: Expedited Review Approval
PERIOD OF APPROVAL: 01/02/2015 to 01/01/2016
Lawrence A. Hosman, Ph.D.
Institutional Review Board

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