ABSTRACT

Title of Thesis:	EFFECT OF NRF2 INDUCERS ON HONEY BEE GENE EXPRESSION AND PESTICIDE-RELATED MORTALITY
	Elizabeth A. Brandt, Master of Science, 2019
Thesis Directed By:	Dr. David Hawthorne, Department of Entomology

Honey bees are vitally important as pollinators to ecosystems and agricultural economy, yet they are threatened by the presence of pesticides and the wide array of xenobiotics they encounter while foraging. To better understand their metabolic detoxification of these compounds, it is important to elucidate the gene expression pathways involved in their response to toxin exposure. I investigated the potential detoxification role in honey bees of the Nrf2/Keap1 regulatory pathway, one of the most well-researched cellular toxin response mechanisms in vertebrates. I analyzed the effect of inducers on the toxicity of three different pesticides when exposed to bees, and the effects of Sulforaphane on select detoxification gene expression. Inducer consumption effects on pesticide toxicity ranged from synergistic to abrogative depending on the pesticide tested. PCR analysis of gene expression did not reveal significant effects of inducer consumption on expression of detoxification genes. This study and its results lay important groundwork for future research of this regulatory pathway in honey bees.

EFFECT OF NRF2 INDUCERS ON HONEY BEE GENE EXPRESSION AND PESTICIDE-RELATED MORTALITY

by

Elizabeth A. Brandt

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Advisory Committee:
Dr. David Hawthorne, Chair, Associate Professor
Dr. Leslie Pick, Full Professor
Dr. Jay Evans, Research Leader, USDA ARS

Dedication

I dedicate this thesis to my father, Lawrence Brandt, whom I believe would have been proud of my work and whose passion for science and learning will always inspire me. I also dedicate it to my mother, sister, and partner, all of whom have supported and been there for me every step of the way. I could not have gotten where I am without them and their belief in me.

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Chapter 1: The Nrf2/Keap1 Pathway in Insects, and its role in Honey Bees: An Overview

Introduction

Organisms respond to toxin exposure through molecular cascading pathways. These pathways are species-specific in their downstream results, but research on genetically conserved upstream responses has demonstrated similarities among a diverse array of organisms. One such upstream response is the Nrf2/Keap1 regulatory pathway, by which a toxic electrophilic compound is sensed by the Nrf2/Keap1 protein complex, ultimately leading to change in detoxification gene expression. This pathway has been heavily researched in mammalian and vertebrate systems, yet consensus on its role and conservation in insect systems remains to be achieved. Experimental data is available for eight insect species. By taking advantage of the conserved nature of the pathway, we gain indicators of which vertebrate Nrf2 pathway components may be important in the pathway's insect counterpart (called Cap-n-collar isoform C, CncC). Of the eight insect species, there is yet to be a representative of the order Hymenoptera to have its CncC characterized. This study is the first to present research and preliminary investigative data on the utilization of CncC in a Hymenopteran, using the European honey bee *Apis mellifera*. This thesis will begin with an overview of the Nrf2/Keap1 regulatory mechanism, the current available research of this mechanism in insects, and outline our reasoning for our proposal of *A. mellifera* as an appropriate next insect model through which to study this mechanism.

Structure and basal mechanism of the Nrf2/Keap1 proteins

The transcription factor nuclear factor erythroid 2 p45-related factor 2 (Nrf2) and its negative inhibitor, Kelch-like ECH-associated protein 1 (Keap1), make up a conserved

mechanism that has been examined in vertebrate systems for the many beneficial physiological effects that are associated with its induction. The effects of Nrf2 target gene expression and upregulation have included anti-stress, anti-aging, and anti-cancer (Kobayashi *et al.*, 2009; Sykiotis & Bohmann, 2008). This project, however, will focus on one role in particular as a key component in xenobiotic recognition and metabolizing pathways. This ability makes the Nrf2/Keap1 system crucially important to vertebrates' abilities to detoxify the toxins regularly encountered through environmental interactions (Motohashi & Yamamoto, 2004).

Nrf2 is a member of the Cap'n'collar (CNC) bZIP leucine zipper family of proteins, which also includes the proteins Nrf1, Nrf3, Bach1, and Bach2. These proteins are transcription factors defined by a specific CNC domain in their structure, and are known for having multiple roles in gene expression, tissue development and differentiation. Its structure contains six Nrf2-ECH homology (Neh) domains, of which perhaps the most important for the system is the N-terminal Neh2, which contains the proposed binding site for Keap1. The now widely-accepted model for this binding is termed the "hinge-and-latch" mechanism, wherein amino acid motifs ETGE and DLG function respectively as the strong-affinity binding site "hinge" and the lower-affinity "latch" (Tong *et al.*, 2006)

In basal (unstressed) conditions, Nrf2 is cytoplasmically sequestered by Keap1 binding and then facilitating Nrf2's rapid degradation by forming a bridge with a Cullin3-based E3-ligase ubiquitination complex (Bertrand *et al.*, 2015). In the presence of an electrophilic xenobiotic, the multiple cysteine residues of Keap1 can be disturbed (oxidized or conjugated) (Pandey *et al.*, 2017). This allows the release of Nrf2, allowing it to accumulate in the cytoplasm and translocate to the nucleus. This role of the cysteine residues has led to them being referred to as electrophilic compound "sensors". Once in the nucleus, Nrf2 forms a heterodimer with nuclear transcription factors, such as small Maf proteins, and

targets antioxidant response element (ARE) sequences for upregulation of cytoprotective protein expression (Katsuoka *et al.*, 2005; Kensler *et al.*, 2007; Magesh *et al.*, 2012; Sykiotis & Bohmann, 2008).

In mammalian and vertebrate systems, these ARE sequences are frequently found in the cis-regulatory elements of gene families associated with both phase II (conjugation) and phase III (transportation/excretion) of the xenobiotic detoxification/metabolism enzymatic pathway (phase I being oxidation, reduction, and hydrolysis of xenobiotic compounds). Some of the most well-researched of these detoxification targets in vertebrates include the enzyme NAD(P)H:quinone oxidoreductase 1 (NOQ1) and the conjugating enzyme families of glutathione-s-transferases (GSTs), UDP-glucurosyltransferases (UGTs), and epoxide hydrolases (EHs) (Motohashi & Yamamoto, 2004).The cytochrome P450 super-family of monooxygenases (phase I) have limited representation in vertebrate Nrf2 literature as target genes, in contrast to the insect Cnc literature. Insect cytochrome P450 genes, particularly those in the CYP3 superfamily, are generally associated with the evolutionary development of insecticide resistance (Feyereisen, 1999; Ranson *et al.*, 2002).

Structure/Function of CncC in *Drosophila* and other insect models

In 2008, a functional homologue of Nrf2 in the vinegar fly *Drosophila melanogaster* was identified in a transcript variant encoding the segmentation protein cap'n'collar (Cnc) that contains an N-terminal ETGE/DLG motifs, similar to the Neh2 domain of Nrf2, and was named as isoform C (CncC) (Figure 1). Additional isoforms (CncA, CncB) have also been identified that do not have the ETGE/DLG motifs and do not bind Keap1 (Sykiotis &Bohmann, 2008; Deng *et al.* 2014). Assuming the "hinge-and-latch" model, the presence of the N-terminal motifs allows Keap1 binding and sequestration, and indeed Keap1 knockout analysis led to CncC overexpression (Sykiotis & Bohmann, 2008; Tong *et al.*, 2006). Further *Drosophila* CncC studies soon confirmed it to have a role in xenobiotic detoxification, as it

was upregulated in insecticide-resistant lines (Deng, 2014; Misra *et al.*, 2011). Following this model, recent studies have proposed a detoxification role for CncC in additional species, including the malaria vector *Anopheles gambiae*, the flour beetle *Tribolium castaneum*, the Colorado potato beetle *Leptinotarsa decemlineata*, the cotton aphid *Aphis gossypii*, the silkworm *Bombyx mori*, and the Noctuid moths *Spodoptera exigua* and *Spodoptera litura* (Chen *et al.*, 2018; Hu *et al.*, 2019; Hu *et al.*, 2018; Ingham *et al.*, 2017; Kalsi & Palli, 2017; Peng *et al.*, 2016) , all with a focus on expression in insecticide-resistant lines.

Both CncC and Keap1 (called dKeap1 in *D. melanogaster*) were found to have mRNA expression in the alimentary canal (a xenobiotic-encountering location) and the Malpighian tubules (an excretion organ) (Sykiotis & Bohmann, 2008). Interestingly, CncC and dKeap1 in the alimentary canal were also shown by Hochmouth (2011) to indirectly control intestinal stem cell proliferation in the midgut epithelium by maintaining low levels of intracellular reactive oxygen species (ROS, agents of oxidative damage) (Hochmuth *et al.*, 2011). High turnover of instestinal stem cells in the midgut can be a mechanism of insecticide resistance, as a method of healing xenobiotic injury (Castagnola & Jurat-Fuentes, 2016). Sykiotis and Bohmann also proposed an insect CncC model similar to the mammalian model, in which the CncC-dKeap1 complex is restricted to the cytoplasm (Sykiotis & Bohmann, 2008). Chromatin visualization of bound loci done by Deng & Kerppola (2014), however, predicts that CncC-Keap1 complex is predominantly found localized to nuclei in *D. melanogaster* tissues, the cooperative complex can also act to bind chromatin to activate select gene expression when induced by phenobarbital feeding (Deng *et al.*, 2014). Additional research is needed to clarify this conflict.

Value of CncC Insect Research

Utilizing an upstream stage in xenobiotic detoxification pathways, rather than looking at downstream detoxification target genes, would be beneficial in assessing and

predicting the metabolic defensive capabilities of different insect species. Functional analysis of toxin-challenged insects would also be improved with the option of a more conserved upstream regulator, as it is difficult to predict in a novel organism which stress gene orthologs might be most transcribed after toxin exposure. This is particularly true in the case of P450s, the diversity of which is not consistently conserved across insect species (Ranson *et al.*, 2002). In the CYP3 class in particular, orthologies are not clearly precise (Oakeshott *et al.* 2010). Different P450s are also researched for different insects because the enzyme family can be expansive, with some species such as *L. decemlineata* estimated as having up to 221 P450 genes (Kumar, 2014). Even insect species considered to be "depauperate" (having relatively low numbers of detoxification genes), such as the European honey bee *Apis mellifera*, can be estimated to have up to 48 putative P450s (Claudianos *et al.*, 2006). This presents the problem of inefficiency when selecting detoxification genes of interest.

The work already performed in insect CncC systems suggests that insecticide-resistant pest species have higher CncC expression levels (and therefore detoxification abilities) than susceptible strains (Ingham *et al.*. 2017, Kalsi and Palli, 2017, Peng *et al.*. 2016, Hu *et al.* 2018, Chen *et al.* 2018). Insecticide resistance may be decreased with the introduction of a Nrf2 activity inhibitor, as work on chemoresistant cancerous cells demonstrated that their Nrf2 overactivation could be counteracted by an inhibitor (No *et al.* 2014). The consumption of the multidrug resistance transporter inhibitor Verapamil increased the toxicity of several insecticides in honey bees (Hawthorne & Dively, 2011).

Honey Bees as a model for CncC system study

As of the publication of this thesis, research on the CncC-Keap1 system in insects has been limited to the context of pest species and their resistance to insecticides. However, this system should also be investigated in beneficial pollinator species, as knowledge on their xenobiotic metabolic defenses may aid in evaluating potential toxic threats to their

physiology. Evaluating the expression of an upstream detoxification gene-targeting transcription factor may be a technique used to test the physiological response of beneficial insects to toxic challenges, such as pesticides. The honey bee is an ideal candidate for this research, as the value of produced crops directly dependent on pollination by the *A. mellifera* alone reached a value of $11.68 billion in 2009, and it has the most prominently annotated pollinator genome currently available in literature (Calderone, 2012). Thorough understanding of their complex upstream genetic response towards pesticide exposure is therefore necessary, beyond just the expression of downstream target genes, in order to make accurate predictions of toxin response genes. Honey bees are also considered to be quantitatively lacking identified P450s and GSTs in comparison to other well-researched insect genomes (i.e. *D. melanogaster, A. gambiae,* and *T. castaneum*) (Claudianos *et al.* 2006). This and other physiological factors have led many to speculate whether honey bees are particularly sensitive to synergistic toxin exposure (Berenbaum & Johnson, 2015). Upstream regulatory mechanisms may be more vital to honey bees in order to compensate, and therefore important to elucidate.

CncC and Keap1 may be an important factor in these upstream regulatory mechanisms. I performed BLAST and ClustalO sequence alignment to identify the CncC and Keap1 homologues in the honey bee (Figure 1, 2). At least one transcript variant of the identified honey bee *Cnc* encodes the two motifs (DLG/ETGE) needed for the "hinge-and-latch" mechanism (Figure 1). Honeybees also consume and interact with compounds known to be Nrf2 inducers in vertebrate systems, as components of propolis (caffeic acid phenethyl ester, C.A.P.E.), pollen and nectar (quercetin) (Campos *et al.*, 1997; Granado-Serrano *et al.*, 2012; Lee *et al.*, 2010). There is therefore evidence to hypothesize that the CncC/Keap1 system as a detoxification mechanism may be used in honey bee metabolism, but this has yet to be characterized through functional studies. Honey bee research would therefore benefit

from quantitative analysis determining the effect of the consumption of Nrf2-inducing compounds on bee detoxification gene expression.

Chapter 2: Effects of Sulforaphane, an Nrf2/Keap1 Inducer, on Honey Bee Pesticide-Induced Mortality and Detoxification Gene Expression

Introduction

The Nrf2/Keap1 regulatory pathway is a genetic response mechanism to toxin exposure, by which electrophilic compounds disturb the cytoplasmic sequestration of the transcription factor Nrf2 by Keap1, and allow Nrf2 to translocate to the nucleus and upregulate detoxification genes (Bertrand *et al.*, 2015). This pathway has been studied in mammalian and vertebrate systems, and in recent years been identified and researched in several insect models, with Nrf2 being renamed for insects as Cap'n'collar isoform C (Cncc) (Ingham *et al.*. 2017, Kalsi and Palli 2017, Peng *et al.*. 2016, Hu *et al.* 2018, Chen *et al.* 2018). By taking advantage of the pathway's conservation in eukaryotes, we may gain indicators of which vertebrate Nrf2 inducers may yield either detoxification gene expression or attenuation of toxin exposure mortality in insects. In this study, I aimed to test the effect of the Nrf2 inducer treatment on both these potential effects of induction. This was, to myknowledge, the first study to investigate the role of CncC in the European honey bee *Apis mellifera*. I did this through the treating of bees with Nrf inducers and subsequently either exposing them to semi-lethal doses of pesticides, or extracting RNA to semi-quantify the expression of CncC and chosen target genes relative to untreated bees. Before expanding on our experimental objectives, this introduction will review the mechanism of Nrf2/CncC induction and discuss known and potential target genes of CncC in insect models.

Xenobiotic Induction of the CncC/Keap1 Pathway

"Inducing" in the context of the CncC/Keap1 system can be referring to several proposed models in which a compound leads to CncC-mediated gene upregulation. Electrophilic and redox compounds may disturb (oxidize) the sensitive cysteine residues of Keap1, leading it to release CncC to translocate to the nucleus; or molecules may act as inhibitors of Keap1, binding to it in and disrupting the CncC-Keap1 protein-protein interaction (Bertrand *et al.*, 2015). In vertebrate systems, Nrf2 can also be phosphorylated as a result of several cellular kinase pathways, including glycogen synthase kinase-3 (GSK-3) and protein kinase C (PKC) (Battino *et al.*, 2018). However, these pathways are not clearly direct results of xenobiotic exposure.

Because manipulation of this pathway may provide medical benefits, many naturally-derived and synthetic inducers of this mechanism have been investigated in human cellular model systems (Bertrand *et al.*, 2015). Naturally-derived chemicals known to increase the expression of antioxidant and antixenobiotic genes via the transcription factor Nrf2 include isothiocyanates (ITCs), terpenoids, and polyphenolic compounds (Kobayashi *et al.*, 2009) . All three of these chemical classes are found in plant secondary metabolites, and investigated for, among many things, being potentially anti-cancer (chemopreventative agents) - a benefit which is also often attributed to Nrf2 induction (Bertrand *et al.*, 2015). In this project, we focused on the CncC induction potential of two Nrf2 inducers: sulforaphane, an ITC, and Oltipraz, a synthetic dithiolethione.

ITCs are a breakdown product of glucosinolates, organosulfur defense compounds found in cruciferous vegetables (e.g. broccoli, kale, and Brussels sprouts). They are the hydrolysis products of the plant's enzyme myrosinase reacting with stored glucosinolates when the plant tissue sustains chewing damage. Sulforaphane is one of the most well-researched Nrf2-inducing ITCs in vertebrates, and can be produced through aqueous

extraction of broccoli sprouts (Bertrand *et al.* 2015). The dithiolethione organosulfur compounds include the synthetic Oltipraz.

The current state of knowledge of CncC inducers in insects is limited in comparison to vertebrate knowledge. Kalsi and Palli (2017) used extract of potato leaves as a treatment of Colorado potato beetle cells, and confirmed that four CYP3 genes were reduced in expression in CncC knockdown cells compared to controls. This design does not define the specific causal compounds in the leaf extract, however. Potatoes are in the family Solanaceae, associated with alkaloid defensive compounds such as nicotine, a common p450 inducer in many insect species (Du Rand *et al.*, 2015). Caffeine, another alkaloid found in plants, was used as an inducer by Misra *et al.* (2011) and found to increase CncC expression in *Drosophila melanogaster* (Misra *et al.*, 2011). Oltipraz-fed *Drosophila* were shown to have detoxification gene upregulation, the effect of which was abrogated with CncC knockdown (Sykiotis and Bohmann, 2008). Sulforaphane has been studied in several *Drosophila* models of Parkinsons disease as suppressing neuron loss (Tarozzi *et al.*, 2013; Trinh *et al.*, 2008).

Previous research in multiple species, including the malaria vector *Anopheles gambiae*, the flour beetle *Tribolium castaneum*, the Colorado potato beetle *Leptinotarsa decemlineata*, the cotton aphid *Aphis gossypii*, and the Noctuid moths *Spodoptera exigua* and *Spodoptera litura*, has found a correlation between resistance to certain insecticides and increased CncC expression (Ingham *et al.*. 2017, Kalsi and Palli 2017, Peng *et al.*. 2016, Hu *et al.* 2018). Overexpression of CncC in *Drosophila* was found to significantly improve survival of flies 16 hours post-exposure to a semi-lethal dose of the herbicide Paraquat (Sykiotis and Bohmann, 2008). This would suggest that activity of the gene's expression may decrease an insecticide's toxicity. The toxicity of the pyrethroid pesticide τ-fluvalinate to bees has been shown to significantly decrease after feeding on the detoxification gene inducer

quercertin -a compound which has also been shown to have Nrf2 induction properties in human cells (Granado-Serrano *et al.*, 2012; Johnson *et al.*, 2012).

Target Detoxification Genes and Tissue-specific Expression of CncC

In order to further elucidate the role of CncC in honey bee xenobiotic detoxification, it is necessary to identify its potential target genes. CncC studies on insect species include research in cytochrome p450 monooxygenases (phase I of detoxification) and glutathione-S-transferases (GSTs, phase II) as target genes.

. In insects, members of the CYP3 superfamily of genes (including the CYP6 and CYP9 genes) are associated with the development of insecticide resistance in pest insects to (Ranson *et al.*. 2002, Feyereisen 1999). Recent insect studies have used RNAi to suppress CncC transcripts and observe the response of CYP6 and CYP9 expression (Peng *et al.*, 2016; Kalsi and Palli 2015; Kalsi and Palli 2017; Ingham *et al.* 2017). CYP3 genes may be inconsistently regulated by CncC; they may be up or downregulated depending on the CncC inducer, and some do not contain the conserved Nrf2 binding site in their promoter (Giudice *et al.*, 2010; Ingham *et al.*, 2017). Kalsi and Palli identified four CYP3 genes as being regulated by CncC in *L. decemlineata,* and one in *T. castaneum* (Kalsi and Palli 2015, Kalsi and Palli 2017).

GSTs have also been investigated in several insect models as potential target genes of CncC. These enzymes can be associated with insecticide resistance to organochlorines such as DDT through a dehydrochlorination reaction, (Clark & Shamaan, 1984; Ranson *et al.*, 2001) as well as organophosphates through glutathione conjugation (Ranson et. al. 2001). Indeed, the canonical consensus ARE binding sequence of the CncC-Maf-S complex in insects was first identified upstream of the *Drosophila* gene *GSTD1* (Sykiotis & Bohmann, 2008). Specifically, *GSTD1* homologues were identified as target genes of the CncC-Maf-S complex by both Misra *et al.* (2011) and Ingham *et al.* (2017) in *Drosophila* and *Anopheles*

gambiae, respectively. *GSTD* was similarly shown to be regulated by CncC in the silkworm and the beet armyworm (Hu *et al.* 2018, Hu *et al.* 2019). *GSTD* is a member of the Delta class of GSTs, a class which is unique to insects and, similar to CYP3 genes, is commonly associated with conferring insecticide resistance (Ranson *et al.*, 2002). Genes of the CYP3 and GST enzyme families are therefore the good CncC target gene candidates to investigate when choosing genes for induced gene expression analysis.

Experimental Objectives

To further expand upon the knowledge of the role of insect CncC in metabolic xenobiotic detoxification, we chose to use the model of the European honey bee *Apis mellifera*, a member of the order Hymenoptera. Much of the research discussed on insect CncC has broadly focused on the comparison of CncC expression between insecticide-resistant and susceptible lines in the chosen species, which is a comparison not available in honey bees. We aimed to determine the effect that the treatment of the Nrf2 inducers sulforaphane and Oltipraz, known to induce a upregulatory metabolic detoxification pathway in vertebrate systems, would have on honey bees. We hypothesized that the consumption of these compounds would have a significant effect on both honey bees' mortality caused by pesticide exposure, as well as their expression of genes associated with detoxification. We tested this through two approaches: a bioassay study, in which inducer treatment was followed by exposure to a pesticide and mortality was compared to uninduced bees, and a genetic expression study, in which RNA was extracted and reverse transcribed in order to perform PCR and gel band analysis to quantify expression levels.

Whole Insect Bioassay Study

As mentioned, much of the body of research on insect CncC utilized the comparison of insecticide-resistant and susceptible lines of species, an experimental design not available in honey bees. Evidence suggests that certain phytochemical compounds, such as quercetin

(an NRF2 inducer) and the phytohormone abscisic acid, are capable of attenuating the toxicity of certain insecticides (Johnson *et al.*, 2012; Negri *et al.*, 2015). In honey bees, increased transcripts of certain CYP3 genes (a class of genes shown to be regulated by CncC activation) have been demonstrated to mediate pesticide detoxification (Mao *et al.* 2011, Peng *et al.*, 2016; Kalsi and Palli 2015; Kalsi and Palli 2017; Ingham *et al.* 2017). We therefore chose an *in vivo* induction approach of feeding potential CncC inducers to bees for 48h pre-exposure to a semi-lethal dose of pesticide, and asking if the potential modulation of detoxification genes shifted bees' mortality in comparison to non-induced bees. The inducers sulforaphane and Oltipraz were chosen for investigation, and the pesticides chosen were the organophosphates coumaphos and chlorpyrifos, as well as the synthetic pyrethroid τ-fluvalinate. Coumaphos and τ-fluvalinate are acaricides used in honey bee colony management to treat colony *Varroa* mite infestation, and chlorpyrifos is a widely used insecticide in crop management. These three pesticides are some of the most frequently detected toxins in honey bee colony wax (Mullin *et al.*, 2010).

Gene Expression Study

To determine the effect of inducer treatment consumption on gene expression, I dissected and isolated the midgut and Malpighian tubules of honey bees. Many genes belonging to both CYP3 and GST enzyme families (families with genes targeted by CncC in other insect models) have been identified in *A. mellifera* as being utilized in bees' gene expression responses to pesticide exposure (Li *et al.*, 2018). Therefore, in addition to the honey bee CncC gene, we amplified CYP306A1 as a potential CYP3 candidate target gene of the CncC complex, and GSTD1 as a potential GST. We chose to isolate the bees' midgut and Malpighian tubules post-induction treatment as a potential location of CncC and tissue-specific detoxification genes expression. Both CncC and Keap1 (called dKeap1 in *D. melanogaster*) were found to have mRNA expression in similar staining patterns in the

alimentary canal (a xenobiotic-encountering location) and the Malpighian tubules (detoxification organs) of *D. melanogaster* and the silkworm *Bombyx mori* (Sykiotis & Bohman, 2008). The midgut epithelium is an important location of ingested xenobiotic interaction within the honey bee (Forkpah *et al.*, 2014). Multiple time points of feeding lengths were chosen, as induced CncC mRNA levels can peak at different hours post-treatment depending on the inducer treatment (Chen *et al.* 2018). Sulforaphane was again chosen as an inducer, not only for the large body of research supporting it as a potent inducer of Nrf2 in vertebrates (as mentioned in the Introduction), but recent work also demonstrated another glucosinolate (indole-3-carbinole) as inducing CncC and detoxification gene expression in *Spodoptera litura* midguts (Chen *et al.* 2018). Glyceraldehyde 3-phosphate dehydrogenase is an enzyme that has been previously determined to be a suitable reference gene for pesticide effects on honey bee gene expression as it is stable in bee tissues, and was therefore chosen as a control gene (Alburaki *et al.*, 2017). Nicotine was chosen as a positive control as a demonstrated inducer of honey bee GSTD1 protein upregulation (du Rand *et al.* 2015).

Materials and Methods

Whole-Insect Bioassay

Insects

Honey bees were sourced from the University of Maryland apiaries from May-September in 2017 and 2018. Source colonies did not receive any chemical treatments for disease or parasites. Frames with capped cells of late-stage worker brood were removed from colonies and kept in dark incubators at 34°C. Newly emerged bees were removed by hand from the frame in 24 h intervals and caged in groups of 19-26. Cages consisted of wax-coated paper 207ml drink cups (Solo Cup Co., Lake Forest IL) covered in squares of fine mosquito netting secured with a rubber band (Figure 1). Bees were supplied with a diet of 50% sucrose

water, contained in 2 ml centrifuge tubes with feeding holes punctured by sterile 21 G syringe needles (Johnson *et al.*, 2012).

Experimental Diet and Toxin Concentrations

Doses of both inducers and toxins were chosen based on preliminary dosage trials performed in summer of 2017 and rechecked in summer of 2018. Stock solutions (100 mM) of Sulforaphane (Sigma Aldrich) and Oltipraz (Sigma Aldrich) were made. All inducers were dissolved in DMSO (Sigma Aldrich) then diluted in sugar water (1:1 sucrose in distilled water) to a final concentration of 1% DMSO in the feeding tubes (0.1, 0.3, or 1m6+M sulforaphane; 1mM of Oltipraz). This concentration of DMSO, as well as all inducer concentrations used, were determined through preliminary trials to yield 0% mortality when fed to bees over 7 days of observation. Bees were exposed to the insecticidal toxins either topically (coumaphos and τ-fluvalinate) or orally (chlorpyrifos). Toxins τ-fluvalinate and coumaphos (Sigma Aldrich) were both diluted in acetone, with τ-fluvalinate at a final concentration of 15mg/ml and coumaphos to the two concentrations 30mg/ml and 75mg/ml. Chlorpyifos was diluted first in DMSO, then in sugar water to a final concentration to be fed to bees of 10ng/µl in 5µl amounts (a total of 50ng). Additional inducers and toxins were tested, including inducers C.A.P.E, dimethyl fumarate (D.M.F.), bardoxolone methyl (CDDO-Me), and curcumin, and toxins ivermectin (an avermectin) and imidacloprid (a neonicotinoid) (Appendix Figure A-H).

Toxin Exposure

Bees were exposed to toxins at 3 days post-eclosion. Before exposure, bees were anesthetized by gentle chilling before application of the toxin. Coumaphos and τ-fluvalinate in acetone were topically applied to the thorax of bees (1 ul) using a Hamilton repeating dispenser (Hamilton). Acetone alone was determined to yield 0% mortality. Toxin droplets were allowed to dry before the bees were replaced into a clean solo cup with feeding tubes.

Chlorpyrifos was hand-fed using a micropipettor to bees similarly anesthetized. For chlorpyrifos and τ-fluvalinate trials, all bees received only sucrose solution post-exposure. For the coumaphos trial, equivalent numbers of bees for each sulforaphane concentration were either pre-treated alone ("Pre-fed") or received the experimental diet again post-exposure ("Continuous"). The numbers of dead bees per cup were recorded at 24-hour intervals. Bees were scored as dead when immobile on the cup floor and unresponsive when their container was jostled. Four cup replicates of bees were used for each treatment. All cups were maintained in a dark incubator at 34 C before and after toxin exposure.

Statistical Analysis

To determine significant inducer diet effects on bee mortality, statistical comparisons between treatments and uninduced controls were performed using one-way analysis of variance with inducer treatment as a fixed factor (Program R). Tests for differences among treatment means were performed using a Bonferroni multiple comparisons correction. Mean honey bee mortality between treatment replicates and their respective standard error of the means were calculated and graphed in Microsoft Excel (Microsoft Office '16).

Gene Expression Analysis

Insects

Honey bees were retrieved, stored, and fed according to methods outlined in the Bioassays Materials and Methods.

Inducer Treatments

Five different treatments were incorporated in the 50% sucrose syrup fed to bees: sulforaphane (10µM, 0.1mM, and 1mM), 0.1%DMSO, and 300µM nicotine. The final DMSO concentration in each sulforaphane-containing bee food tube was 0.1%. Nicotine was water-soluble and no additional DMSO dilution step was necessary. Bees aged 3-5 days old were caged in groups of ten. Caged bees were starved for one hour before being fed

treatments for lengths of 3, 6, 12, 24, and 48 hours, then similarly chilled as with toxin application in Chapter 1 and dissected. Nicotine-fed bees were tested only at 48h. Five replicates of each treatment and feeding length combination were performed.

Gut dissection and RNA extraction

Midguts and Malphigian tubules were dissected from anesthetized honey bees and pooled in groups of 10 into 200µl of Trizol (Invitrogen). Pools were of bees from the same treatment and cup. Pooled midguts were homogenized using a pestle, and immediately frozen at -80° C. Samples were later thawed and total RNA was isolated using Trizol following the manufacturer's protocol. Qubit 4 fluorometric analysis (Invitrogen) and agarose gels were used to quantify and visually assess RNA quality.

cDNA synthesis and PCR amplification

Reverse transcription of 1 µg of RNA from each sample was individually performed using QuantiNova kits, including a gDNA removal treatment (Qiagen). cDNA was then used as template in PCR reactions. Four genes were independently amplified: *Cap'n'collar*, *GSTD1*, *CYP306A1*, and the control gene glyceraldehyde 3-phosphate dehydrogenase 2 (*GAPD2*) (Table 1). Primers original to this study were designed using gene sequences retrieved from the National Center for Biotechnology Information (NCBI) and the primer design software Primer3 (Version 0.4.0). PCR amplification reactions were performed using a PTC-200 Thermal Cycler (MJ Research). All reactions contained 1 ul cDNA sample, 2.5 ul of 10X Thermopol buffer (New England Biolabs), 1.25 ul of 2.5mM dNTPS (New England Biolabs), 0.75 ul of each 10mM primer (Integrated DNA Technologies, excepting *CYP306A1* which was Invitrogen), 0.2 ul (1U) standard Taq polymerase (New England BioLabs) and 18.55 ul H_2O for a total 25 ul reaction. Thermocycler amplification programs were set as follows: 94° C for 4 min, followed by cycles of 30 s at 94° C, 25 s at 49° C, and 30 s of 72° C, ending with a final extension of 5 min at 72° C. The annealing temperature was chosen as

the lowest calculated Tm of all tested primers. In order to further clarify potential differences in PCR product concentration, time courses of cycles were performed to determine the smallest number of cycles needed before a product was visible for each specific target gene (using gel protocol described below, data not shown). The number of cycles used for each gene are included in Table 1. The total of treatmeant-timepoint combinations yield a total of 25 separate cDNA template samples. *GAPD2* reactions were performed in separate wells than the target gene reactions using the same template and cycle number as the target gene, within the same 96-well plate. PCR products were purified using the Monarch PCR & DNA Cleanup Kit (New England Biolabs) and sequenced (Genewiz).

Gel electrophoresis and band image analysis

PCR products (3 ul) and a 100 bp ladder (New England Biolabs) were run on 2% agarose gels cast in 1X TBE and GelRed (Biotium), for 120 minutes at 95V. Gel images were captured at manual exposure with zero saturation (UVP GelDoc-It Imager, Analytikjena). Band intensity of amplified products was quantified using gel image analysis software ImageJ (Abràmoff *et al.*, 2004). Fold-change in expression of target genes in treated bees relative to bees fed control DMSO treatment was quantified by first normalizing the band area with that of the mean *GAPD2* area for the treatment and then calculating the inducer-treated to untreated ratio.

Results

Bioassays

Being fed experimental inducer diet significantly affected bees' mortality when exposed to all three toxins, though the effect itself differed between pesticides (Figures 4-7). For the coumaphos trial, three timepoints (24, 48, and 68h) were analyzed, as the mortality increased past 24h. No treatments had a significant effect on coumaphos toxicity at 24h post-

exposure. The three "continuous" sulforaphane treatments (cups with experimental diet returned to bees after toxin exposure) had no significant effect on bee mortality in comparison to uninduced bees at the additional time points of 48 and 68h. Of the bees that were pre-fed sulforaphane treatment and had sucrose solution post-toxin exposure, 0.1mM sulforaphane-fed bees also did not have significantly different mortality from uninduced bees at 48 and 68h. At 48h, however, both 0.3 and 1mM sulforaphane pre-fed bees did show significantly decreased mortality relative to uninduced bees. For 0.3 mM sulforaphane-fed bees, this relatively lower mortality was no longer present by 68h, but remained for the 1mM sulforaphane pre-fed bees (Figure 4). This particular treatment was further tested with the higher coumaphos concentration of 75mg/ml, and the effect of 1mM sulforaphane again significantly attenuated coumaphos toxicity at 19h post-exposure, and was very close to significant at 24h (Figure 5). The timepoint of 48h was not included for the 75mg/ml coumaphos trial as mortality for both treatment and control was near 100% (data not shown). Pretreatment with 1mM sulforaphane or 1mM Oltipraz significantly increased the toxicity of both τ-fluvalinate and chlorpyrifos at 24h, though for τ-fluvalinate exposure there was no significant difference between the two inducers' effects (Figure 6, 7). Mortality was not analyzed for later time points for these trials as they were determined to not significantly change past 24h (data not shown). In conclusion, inducer treatment can significantly affect pesticide-related mortality in honey bees.

Gene Expression Analysis

Band quantification of PCR amplification products using target genes *CncC*, *CYP306A1*, and *GSTD1* did not yield significant differences in expression between and within treatments or between time points for sulforaphane treatments (Figure 8, 9). Our positive control treatment-target gene reaction, *GSTD1* amplified on nicotine-treated bees, did not significantly express relative to untreated bees (Figure 10). Additional target genes

amplified for nicotine-treated bees *CncC* and *Cyp306A1* were not significantly expressed differentially from controls (Figure 10). Sequencing results of PCR products yielded high similarity with target amplicon sequences, though the product sequences were incomplete (Appendix Figure I-L). Attempts to perform multiplex reactions with *GAPD2* as an internal control yielded inconsistent product results, leading to the choice to perform control and target gene reactions separately. Three replicates of sulforaphane-treated bees were analyzed for the target gene *CncC*, but two replicates were analyzed for the target genes *GSTD1* and *Cyp306A1* as gel band quality for the third replicate PCR reactions was too low to analyze. There were limited observable trends in the nominal means of relative band density for each target gene, such as *CncC* expression being lower at 48h than at 3h, and *GSTD1* expression increasing from 3h to 48h. Additionally, band density relative to DMSO-treated bees of all target genes was largely under 1.0, indicating possible decreased expression. The standard deviation and standard error of the means demonstrate extreme variance in band density values, however, making these trend observations statistically insignificant (Figure 8, 9). According to this analysis therefore, inducer treatment may have an insignificant effect on select detoxification gene expression.

Discussion

Our investigation has provided additional new information towards elucidating the role of CncC in Insecta as a whole, by investigating a member of the order Hymenoptera. By utilizing a known Nrf2 inducer's effect on pesticide-induced mortality, we also have outlined a method of characterizing inducer effects beyond gene expression. Our results also indicate some promise in the potential of an inducer (sulforaphane) to attenuate pesticide toxicity, validating our approach (Figure 4, 5). The inducer-feeding studies mentioned in our introduction (Kalsi and Palli 2017, Misra *et al.* 2011, Chen *et al.* 2018) are a very small representative of what could be a large body of future literature on how the CncC system of

pollinating and herbivorous insects responds to consumption of the inducer-containing plant tissues of their hosts. A recent study of CncC in the silkworm utilized the organophosphate phoxim as an inducer, but did not consider the secondary metabolites of the mulberry leaves the silkworm was provided as food (Hu *et al.* 2018). If many of these inducer compounds are secondary metabolites with a defensive role against herbivores, insects may have adaptive molecular mechanisms of detoxification specific to these compounds.

There exist several examples of plant-insect interactions wherein an insect is consuming known vertebrate system Nrf2 inducers during the course of its feeding. Examples in Figure 1 include the diamondback moth *Plutella xylostella*, an economically important pest of cruciferous vegetables (glucosinolate producers). The existence of these relationships begs inclusion of this potential upstream regulatory system into analysis when investigating the broader scope of insect adapted molecular defense mechanisms. Experimental research in insect CncC should therefore strive to additionally include the potential Nrf2 induction role of the compounds the insect naturally consumes, in order to determine the CncC system's role in detoxification.

The response of an organism to xenobiotic exposure can be difficult to predict, given the complex metabolic pathways involved in detoxification. This is particularly relevant in the study of honey bees, which have detoxification mechanisms that are not yet fully elucidated, despite their importance as vital contributors to U.S. agriculture (Calderone 2012). We hypothesized that the consumption of compounds known to induce a upregulatory metabolic detoxification pathway in vertebrate systems would have a significant effect on both honey bees' mortality caused by pesticide exposure, and their genetic expression of detoxification genes.

Bioassays

Our *in vivo* inducer feeding approach demonstrated a promising attenuation effect with certain doses of a Nrf2 inducer in conjunction with pesticide exposure. Within the scope of our tested inducer-pesticide combinations tested, the pesticide used was the determining factor in the inducers' effect upon the pesticides' toxicity. This is not unsurprising, given that honey bees are affected by different pesticides to different degrees, and may employ different genetic detoxification responses depending on the toxic xenobiotic challenge (Li *et al.* 2018). Yet at least one of our inducer-toxin combinations yielded the effect of toxicity attenuation (Sulforaphane-Coumaphos, Figure 4 & 5). The toxicity of acaricides coumaphos and τ-fluvalinate, both often used to control parasitic mites in honey bee colonies, were affected by inducers in opposing manners. Though the inducer synergistically increased toxicity of τ-fluvalinate, some inducer doses decreased the toxicity of coumaphos (Figure 4, 6). This may reflect the fact that these two acaricides represent different chemical classes, as τ-fluvalinate is a pyrethroid and coumaphos is organophosphate-based. Chlorpyrifos, however, is an organophosphate as well, and demonstrated contrasting mortality effects to the coumaphos trials, with the inducer behaving synergistically with the toxin similarly to the τ-fluvalinate (Figure 5). The method of toxin exposure differed between chlorpyrifos and coumaphos due to chlorpyrifos evaporating too quickly within the needle dispenser, necessitating the handfeeding method, though preliminary data supported the synergistic chlorpyrifos-inducer relationship with cuticular exposure as well (data not shown). It may be that structural differences between the two organophosphates lead to differences in interaction with the inducer. Consuming the inducer while also metabolizing the toxic exposure ("continuous" induction) did not significantly decrease coumaphos toxicity, though mean mortality was still lower than uninduced bees. The concentration of inducer may also have a significant role, as the toxicity of coumaphos demonstrated an inverse relationship with sulforaphane concentration. It is surprising that sulforaphane synergistically increased toxicity of τ-

fluvalinate, as previous research has demonstrated that the Nrf2 inducer quercetin had a contrasting effect of decreasing toxicity in honey bees exposed to this insecticide, though this may be attributed to the two inducers' differing chemical structure (Granado-Serrano 2012, Johnson *et al.* 2012). It is also worthy of note that preliminary dosage-determination trials determined concentrations of 1mM for both sulforaphane and Oltipraz, a high concentration, was well tolerated by bees and yielded no mortality over 7 days of observation. It may be interesting to incorporate these two inducers in a longevity assay, as previous research has indicated the addition of dietary phytochemicals (including caffeine, which is known to induce CncC activity in *D. melanogaster*) can significantly increase worker bee lifespan (Bernklau *et al.*, 2019).

Gene Expression Analysis

Differences in select detoxification gene expression between control treatment bees and inducer-fed bees were not significant (Figure 8-10). Though results of our semi-quantification of inducer-treated gene expression proved insignificant, we have gained insight into appropriate and future experimental design in investigating the detoxification gene expression effects resulting from inducer treatment. Semi-quantification of gene expression utilizing gel band analysis of PCR products may be a relatively insensitive technique compared to other expression quantification methods such as RNAseq and qPCR. Broad trends in our resulting relative band densities, however, indicate that future quantitative analysis studies may not need to extensively account for multiple time points of inducer feeding lengths, as our results showed no significant differences over time (Figure 8, 9). A quantitative expression analysis method such as RNAseq may also be more appropriate in future studies, as a more broad array of analyzed target genes may be necessary to observe expressive differences between induced and control bees. As mentioned, honey bees have been demonstrated as expressing different detoxication genes depending on the toxin treatment (Li *et al.* 2018). Though the

two candidate target genes chosen for analysis in this study yielded no significant expression differences when treated with sulforaphane versus untreated bees, it could be that the expression of other candidate genes are still affected. The target genes chosen (*CncC*, *CYP306A1*, and *GSTD1*) were successfully amplified as expressing in the midgut, therefore their inclusion in future expression analysis may still prove insightful. This study is the first to design and amplify *CncC* primers in *A. mellifera*, providing important experimental information for future study of the gene and its role in the honey bee. Experimental challenges such as the number of replicates analyzed, and the inability to co-amplify housekeeping and target genes within a reaction, affected analysis in obfuscating differences in treatment effects. There are currently six predicted transcript variants of *Cap-n-collar* for *A. mellifera* available on NCBI, the shared sequence of which contain the Keap1 bnding motifs DLG and ETGE (alignment not shown). Our designed *CncC* primers were designed to amplify all six transcript variants, which may have obfuscated expression results such that a single isoform's potential increase or decrease of expression may not have been possible to identify. Increasing replicate number and optimizing gene primers such that co-amplification of housekeeping and target genes were made possible might have elucidated more significant and informative results.

The results of this study provide important foundation pieces to add to the larger picture of understanding the transcription factor CncC's role in honey bee detoxification. The Nrf2 inducer sulforaphane showed promising results as a potential attenuator of pesticide toxicity in honey bees. Though we have investigated several inducer-toxin combinations beyond what is discussed in this chapter (Appendix Figure A-H) there exist still inducers and toxin combinations to be tested, as honey bees may metabolize different toxins by different enzymatic processes. Though semi-quantification of potential CncC target gene expression post-inducer consumption demonstrated insignificant effects in this study, further study

utilizing more sensitive quantification methods and a broader array of known honey bee detoxification genes may still indicate inducer effect. Not only is knowledge of this mechanism's role in honey bees crucial to the full understanding of honey bee detoxification abilities, but will add to the broader understanding of this conserved mechanism's role in Insecta as whole.

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Tables

Gene Name	Gene Accession ID	Primer Sequence (5'-3')	Product size	Number of PCR Cycles	Reference
CncC	GB42329	F: TGGAAGCAACGAAGAGAAGG R: CGAGATCAATGGTGTAAGGGAG	86 bp	25	This study
GSTD1	BE844335.1	F: GGGGAAAACTATGTGGCAGGG R: AAAGTGGAGACAGTGGATACGATGC	71 bp	23	Alburaki *et al.*, 2017
CYP306A1	GB12311. 5	F: CGTCGATGGGAAGGATAAAA R: TCGGTGAAATATCCCGATTC	162 bp	33	Gregorc *et al.*, 2012
GAPD2	GB14798	F: GATGCACCCATGTTTGTTTG R: TTTGCAGAAGGTGCATCAAC	203 bp		Thompson *et al.*, 2007

Table 1. Genes amplified using cDNA reverse-transcribed from extracted inducer-treated honey bee midgut RNA. Gene name, accession ID, primer sequences, amplicon sizes, PCR cycles tested, and primer sequence references are included. Genes include honey bee *CncC*, potential CncC target genes *GSTD1* and *CYP306A1*, and housekeeping gene *GAPD2*. Sequences retrieved from NCBI (https://www.ncbi.nlm.nih.gov/)

Figures

Figure 1. **Multiple Sequence Alignment of Conserved Motif ETGE in Predicted Cnc Transcripts in Multiple Insects.** ClustalO (1.2.4) sequence alignment of the cap'n'collar segmentation gene mRNA transcript for 10 different insects, with the tetrapeptide motif ETGE boxed in red. Common names for insects, from top to bottom: vinegar fly, navel orangeworm, diamondback moth, pea aphid, brown marmorated stinkbug, bed bug, flour beetle, red harvester ant, honey bee, and Eastern bumble bee. All sequences retrieved from NCBI (https://www.ncbi.nlm.nih.gov/)

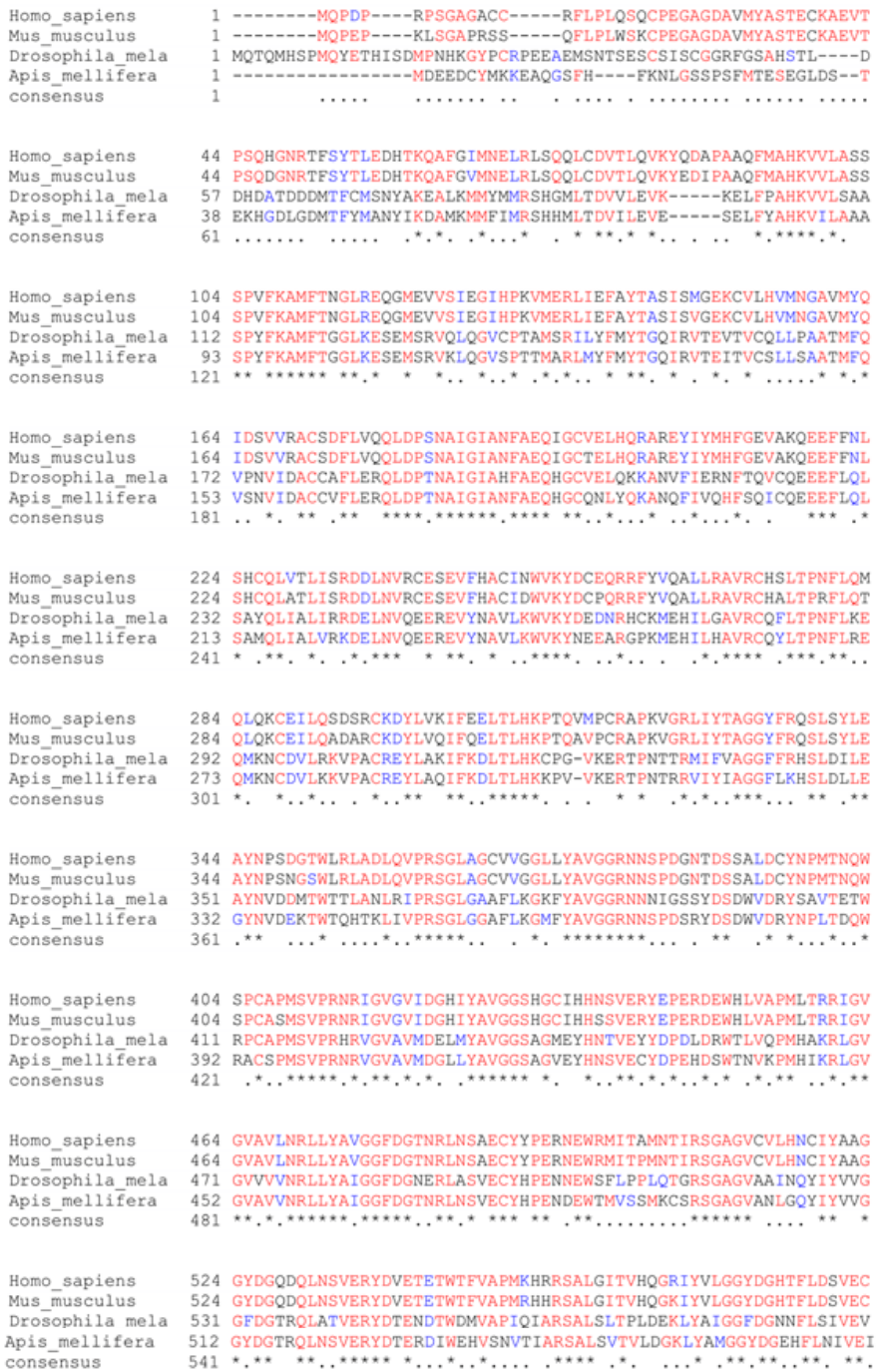

Homo_sapiens 1 --------MQPDP----RPSGAGACC-----RFLPLQSQCPEGAGDAVMYASTECKAEVT
Mus_musculus 1 --------MQPEP----KLSGAPRSS-----QFLPLWSKCPEGAGDAVMYASTECKAEVT
Drosophila_mela 1 MQTQMHSPMQYETHISDMPNHKGYPCRPEEAEMSNTSESCSISCGGRFGSAHSTL----D
Apis_mellifera 1 ----------------MDEEDCYMKKEAQGSFH----FKNLGSSPSFMTESEGLDS--T
consensus 1
Homo_sapiens 44 PSQHGNRTFSYTLEDHTKQAFGIMNELRLSQQLCDVTLQVKYQDAPAAQFMAHKVVLASS
Mus_musculus 44 PSQDGNRTFSYTLEDHTKQAFGVMNELRLSQQLCDVTLQVKYEDIPAAQFMAHKVVLASS
Drosophila_mela 57 DHDATDDDMTFCMSNYAKEALKMMYMMRSHGMLTDVVLEVK-----KELFPAHKVVLSAA
Apis_mellifera 38 EKHGDLGDMTFYMANYIKDAMKMMFIMRSHHMLTDVILEVE-----SELFYAHKVILAAA
consensus 61
Homo_sapiens 104 SPVFKAMFTNGLREQGMEVVSIEGIHPKVMERLIEFAYTASISMGEKCVLHVMNGAVMYQ
Mus_musculus 104 SPVFKAMFTNGLREQGMEVVSIEGIHPKVMERLIEFAYTASISVGEKCVLHVMNGAVMYQ
Drosophila_mela 112 SPYFKAMFTGGLKESEMSRVQLQGVCPTAMSRILYFMYTGQIRVTEVTVCQLLPAATMFQ
Apis_mellifera 93 SPYFKAMFTGGLKESEMSRVKLQGVSPTTMARLMYFMYTGQIRVTEITVCSLLSAATMFQ
consensus 121
Homo_sapiens 164 IDSVVRACSDFLVQQLDPSNAIGIANFAEQIGCVELHQRAREYIYMHFGEVAKQEEFFNL
Mus_musculus 164 IDSVVRACSDFLVQQLDPSNAIGIANFAEQIGCTELHQRAREYIYMHFGEVAKQEEFFNL
Drosophila_mela 172 VPNVIDACCAFLERQLDPTNAIGIAHFAEQHGCVELQKKANVFIERNFTQVCQEEEFLQL
Apis_mellifera 153 VSNVIDACCVFLERQLDPTNAIGIANFAEQHGCQNLYQKANQFIVQHFSQICQEEEFLQL
consensus 181
Homo_sapiens 224 SHCQLVTLISRDDLNVRCESEVFHACINWVKYDCEQRRFYVQALLRAVRCHSLTPNFLQM
Mus_musculus 224 SHCQLATLISRDDLNVRCESEVFHACIDWVKYDCPQRRFYVQALLRAVRCHALTPRFLQT
Drosophila_mela 232 SAYQLIALIRRDELNVQEEREVYNAVLKWVKYDEDNRHCKMEHILGAVRCQFLTPNFLKE
Apis_mellifera 213 SAMQLIALVRKDELNVQEEREVYNAVLKWVKYNEEARGPKMEHILHAVRCQYLTPNFLRE
consensus 241
Homo_sapiens 284 QLQKCEILQSDSRCKDYLVKIFEELTLHKPTQVMPCRAPKVGRLIYTAGGYFRQSLSYLE
Mus_musculus 284 QLQKCEILQADARCKDYLVQIFQELTLHKPTQAVPCRAPKVGRLIYTAGGYFRQSLSYLE
Drosophila_mela 292 QMKNCDVLRKVPACREYLAKIFKDLTLHKCPG-VKERTPNTTRMIFVAGGFFRHSLDILE
Apis_mellifera 273 QMKNCDVLKKVPACREYLAQIFKDLTLHKKPV-VKERTPNTRRVIYIAGGFLKHSLDLLE
consensus 301
Homo_sapiens 344 AYNPSDGTWLRLADLQVPRSGLAGCVVGGLLYAVGGRNNSPDGNTDSSALDCYNPMTNQW
Mus_musculus 344 AYNPSNGSWLRLADLQVPRSGLAGCVVGGLLYAVGGRNNSPDGNTDSSALDCYNPMTNQW
Drosophila_mela 351 AYNVDDMTWTTLANLRIPRSGLGAAFLKGKFYAVGGRNNIGSSYDSDWVDRYSAVTETW
Apis_mellifera 332 GYNVDEKTWTQHTKLIVPRSGLGGAFLKGMFYAVGGRNNSPDSRYDSDWVDRYNPLTDQW
consensus 361
Homo_sapiens 404 SPCAPMSVPRNRIGVGVIDGHIYAVGGSHGCIHHNSVERYEPERDEWHLVAPMLTRRIGV
Mus_musculus 404 SPCASMSVPRNRIGVGVIDGHIYAVGGSHGCIHHSSVERYEPERDEWHLVAPMLTRRIGV
Drosophila_mela 411 RPCAPMSVPRHRVGVAVMDELMYAVGGSAGMEYHNTVEYYDPDLDRWTLVQPMHAKRLGV
Apis_mellifera 392 RACSPMSVPRNRVGVAVMDGLLYAVGGSAGVEYHNSVECYDPEHDSWTNVKPMHIKRLGV
consensus 421
Homo_sapiens 464 GVAVLNRLLYAVGGFDGTNRLNSAECYYPERNEWRMITAMNTIRSGAGVCVLHNCIYAAG
Mus_musculus 464 GVAVLNRLLYAVGGFDGTNRLNSAECYYPERNEWRMITPMNTIRSGAGVCVLHNCIYAAG
Drosophila_mela 471 GVVVVNRLLYAIGGFDGNERLASVECYHPENNEWSFLPPLQTGRSGAGVAAINQYIYVVG
Apis_mellifera 452 GVAVVNRLLYAIGGFDGTNRLNSVECYHPENDEWTMVSSMKCSRSGAGVANLGQYIYVVG
consensus 481
Homo_sapiens 524 GYDGQDQLNSVERYDVETETWTFVAPMKHRRSALGITVHQGRIYVLGGYDGHTFLDSVEC
Mus_musculus 524 GYDGQDQLNSVERYDVETETWTFVAPMRHHRSALGITVHQGKIYVLGGYDGHTFLDSVEC
Drosophila_mela 531 GFDGTRQLATVERYDTENDTWDMVAPIQIARSALSLTPLDEKLYAIGGFDGNNFLSIVEV
Apis_mellifera 512 GYDGTRQLNSVERYDTERDIWEHVSNVTIARSALSVTVLDGKLYAMGGYDGEHFLNIVEI
consensus 541

Figure 2. ***Keap1*** **Multiple Sequence Alignment for Human, Mouse, Vinegar Fly, and Honey Bee Sequences.** Keap1 ClustalO(1.2.4) alignment using Boxshade for *Homo sapiens, Mus musculus, Drosophila melanogaster, and Apis mellifera.* Keap1 is well-conserved across species. All sequences retrieved from NCBI (https://www.ncbi.nlm.nih.gov/)

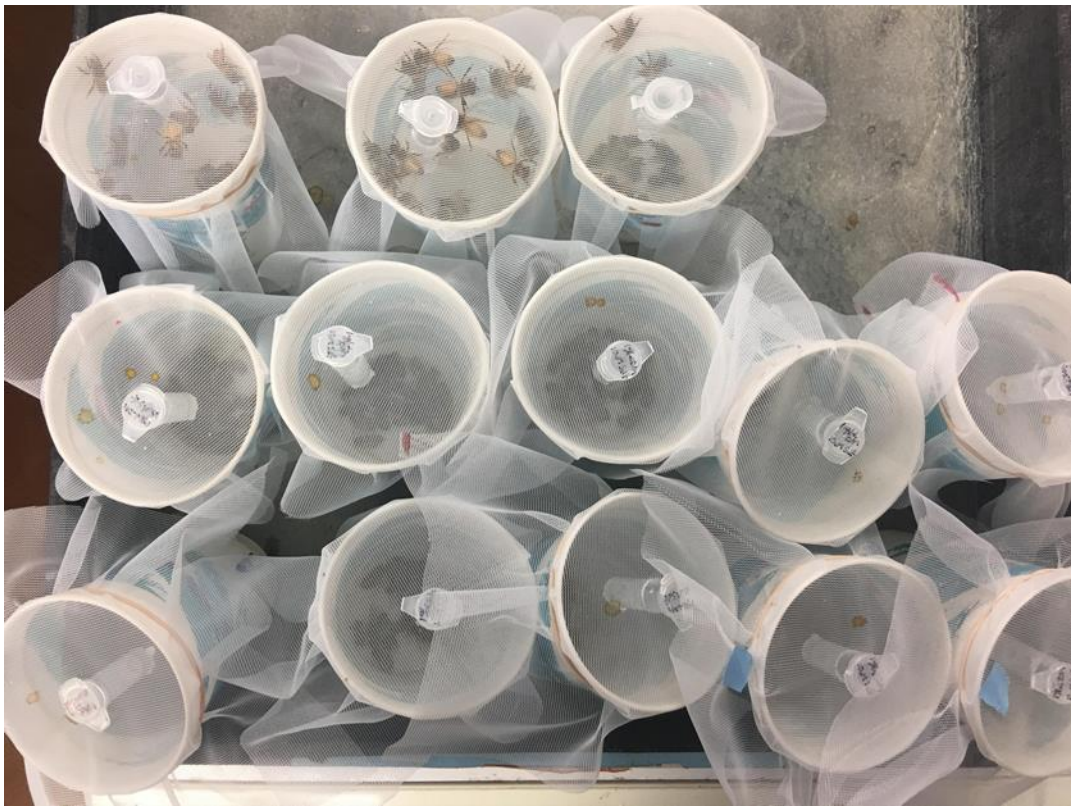


Figure 3. **Honey Bee Cage Design.** Bee cage design for newly emerged bees, to be used in bioassay and gene expression analysis. Feeding tubes suspended in mesh top are visible.

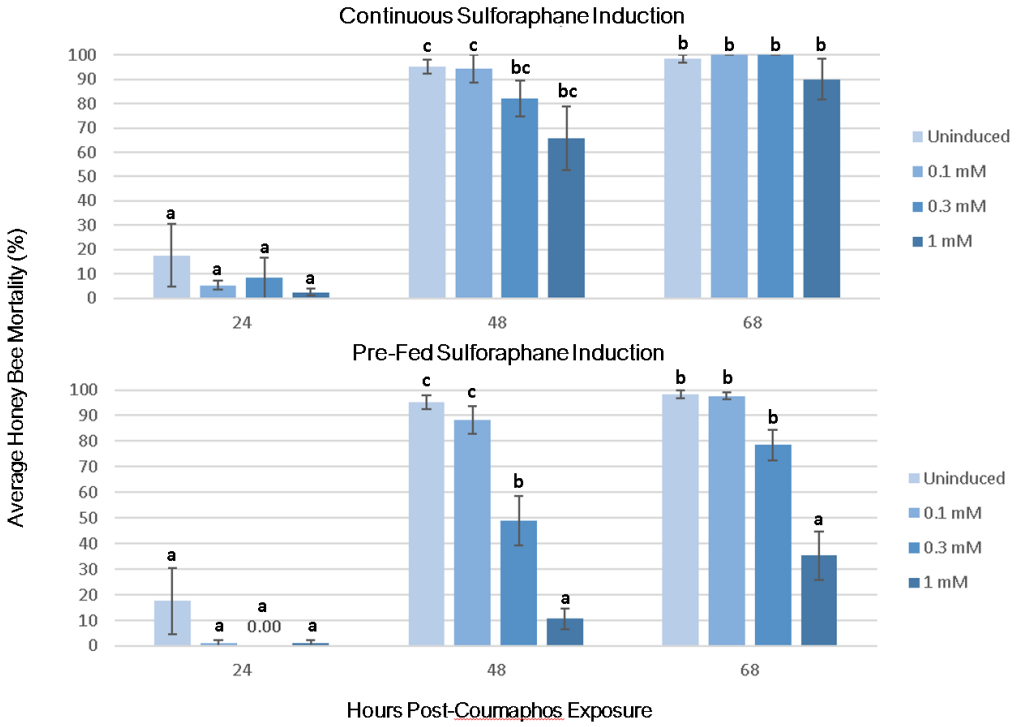


Figure 4. **Effect of Continuous and Pre-feeding Sulforaphane Treatment on Coumaphos (30mg/ml) Toxicity.** Mean mortality (±SEM) of honey bees exposed to topical application of 1ul of coumaphos (30mg/ml) after being pre-fed for 48 hours (with experimental treatment diet returned to feed *ad libitum* post-exposure (Top), or pre-fed for 48 hours with experimental treatment and given control sucrose (50%) solution post-exposure (Bottom). Bees were fed 0.1, 0.3, or 1mM sulforaphane in sucrose (50%) solution, or sucrose solution alone (Uninduced). Mortalities were recorded at 24, 48, and 68 hours post-exposure. Different letters indicate significant differences among treatment means within a time point ($p<0.05$).

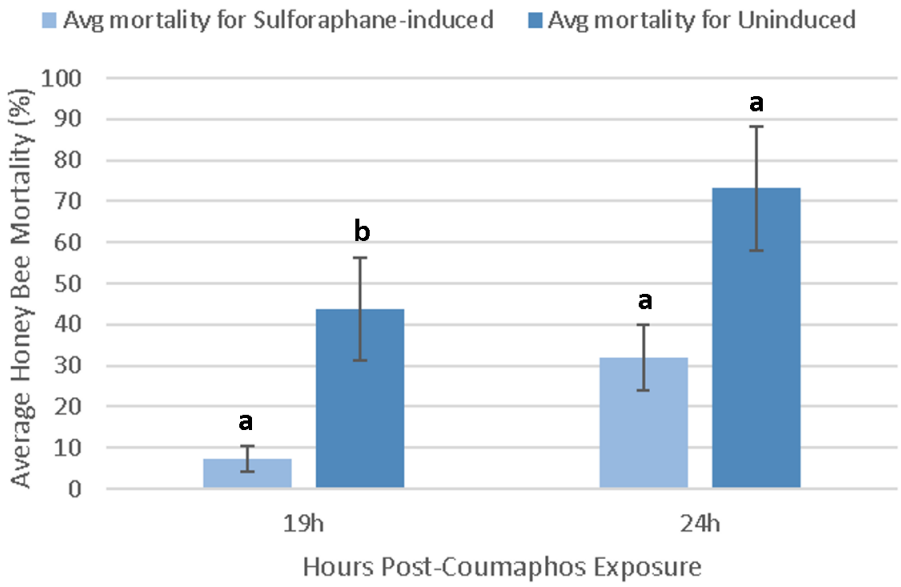


Figure 5. **Effect of pre-fed Sulforaphane on Coumaphos (75mg/ml) Toxicity.** Mean mortality (±SEM) of honey bees exposed to topical application of 1ul of coumaphos (75mg/ml) after being pre-fed for 1mM sulforaphane 48 hours and given control sucrose (50%) solution post-exposure or fed sucrose solution alone (Uninduced). Mortalities were recorded at 19 and 24h post-exposure. Different letters indicate significant differences among treatment means within a time point ($p<0.05$)(19h time point p value: 0.03, 24h time point p value: 0.053).

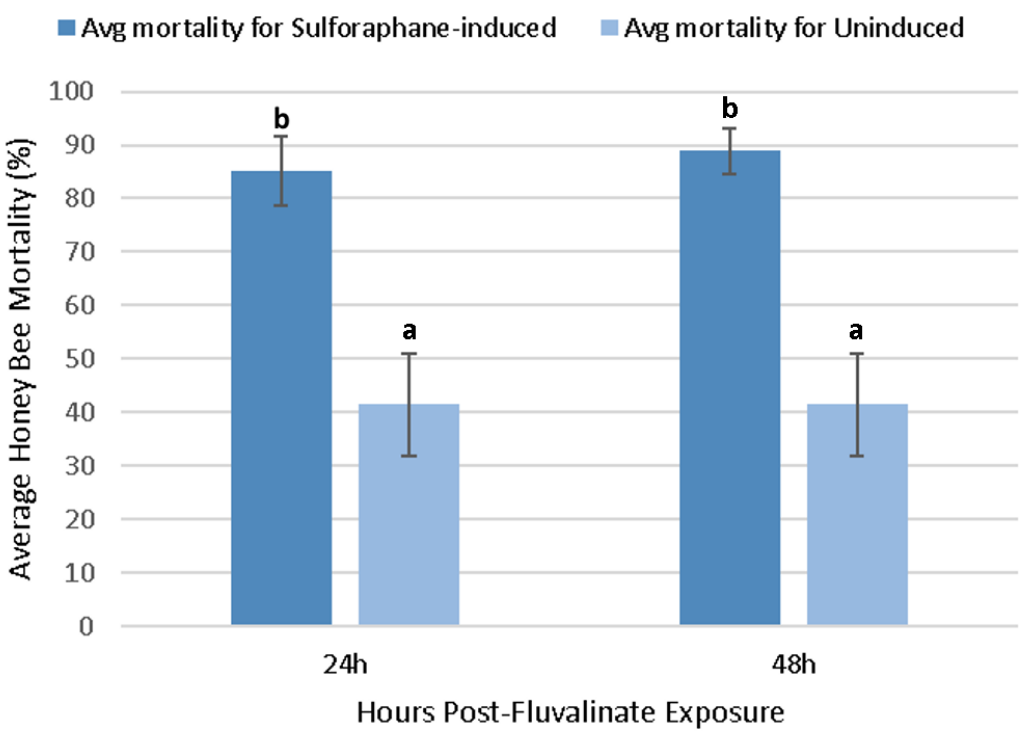


Figure 6. **Effect of Sulforaphane Treatment on τ-fluvalinate Toxicity.** Mean mortality (±SEM) of honey bees exposed to topical application of 1ul of τ-fluvalinate (15mg/ml) after being pre-fed with either 1mM Sulforaphane in sucrose (50%) solution, or sucrose solution alone (Uninduced). Different letters indicate significant differences among treatment means within a time point (p<0.05).

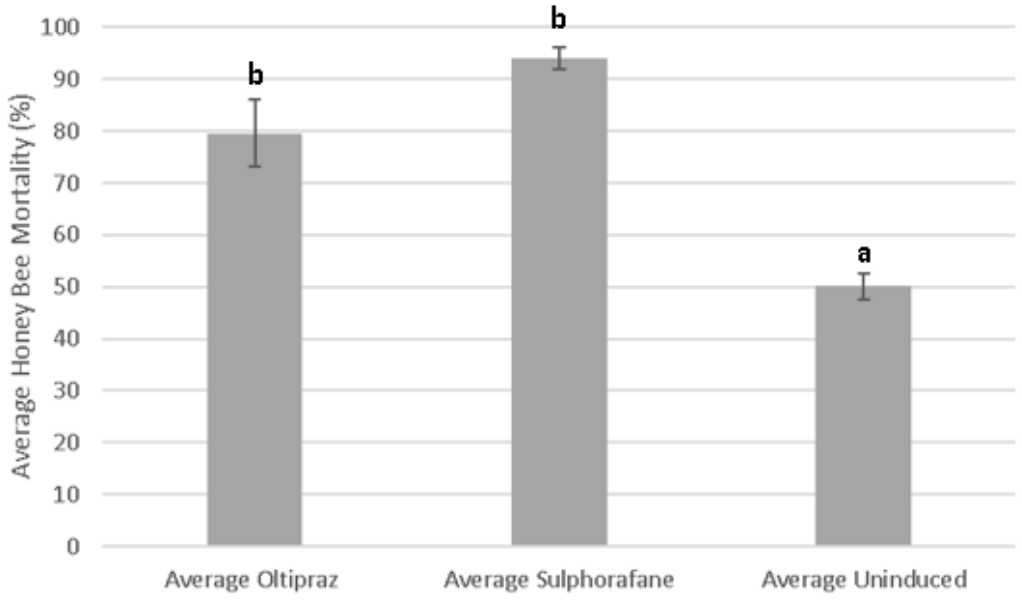


Figure 7. **Effect of Oltipraz or Sulforaphane Induction on Chlorpyrifos Toxicity.** Mean mortality (±SEM) of honey bees 24h after being exposed to oral application of 5ul of chlorpyrifos (10ng/ul) after being pre-fed for 48h with either 1mM Sulforaphane or 1mM Oltipraz in sucrose (50%) solution, or sucrose solution alone (Uninduced). Different letters indicate significant differences among treatment means within a time point ($p<0.05$).

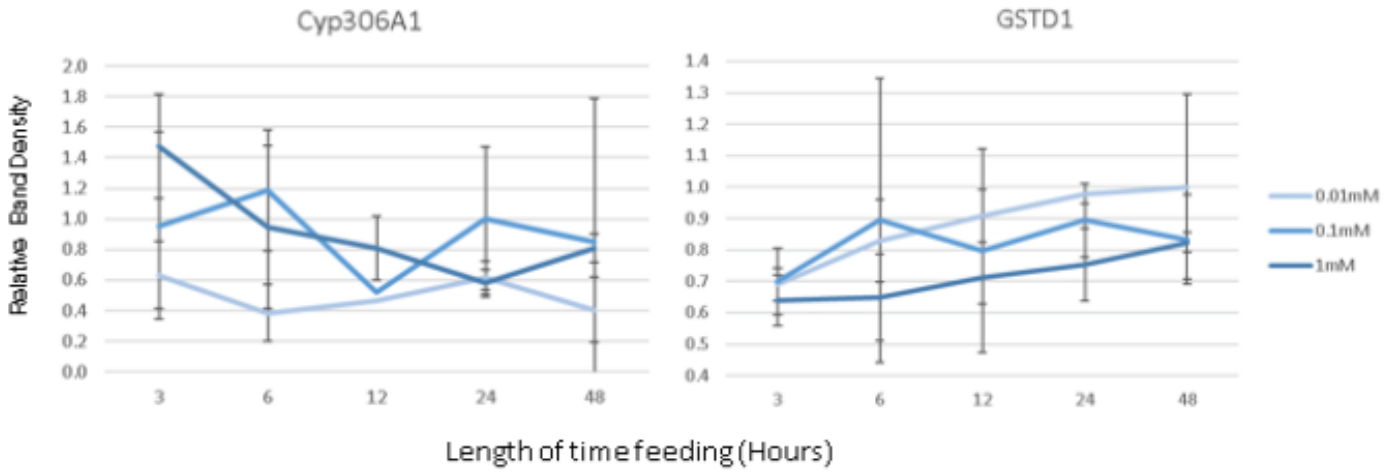


Figure 8. **Effect of Sulforaphane Treatment on *CYP306A1* and *GSTD1* Expression**. Mean relative PCR product band density (±Standard Deviation) of two target genes *CYP306A1* (left) and *GSTD1* (right). Genes were amplified with cDNA reverse transcribed from midgut RNA extracted from two replicates of bees fed three different doses of sulforaphane (0.01mM, 0.1mM, and 1mM) for five different lengths of time (3, 6, 12, 24, and 48 hours). Band density (unitless) is calculated relative to band density expression of control-treated bees. A relative band density of 1 indicates no fold change in expression relative to control bees.

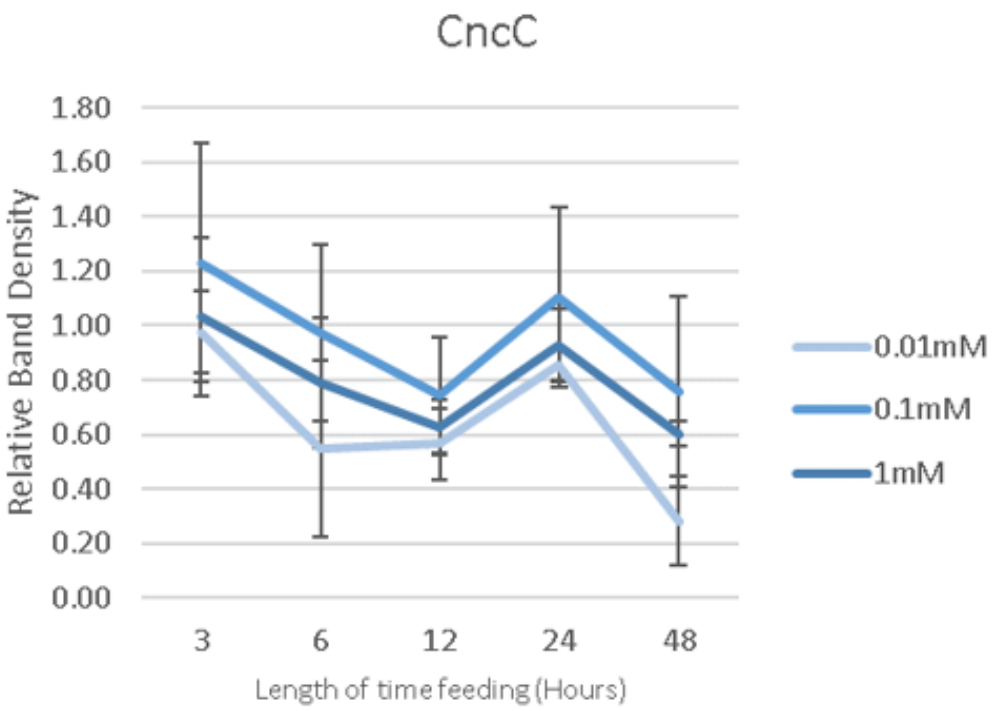


Figure 9. **Effect of Sulforaphane Treatment on *CncC* expression.** Mean relative PCR product band density (±SEM) of the target gene *CncC* as a representation of gene expression. Genes were amplified with cDNA reverse transcribed from RNA extracted from the midguts of three replicates of bees fed multiple doses of sulforaphane (0.01mM, 0.1mM, and 1mM) for multiple lengths of time (3, 6, 12, 24, and 48 hours). Band density (unitless) is calculated relative to band density expression of control-treated (0.1% DMSO-fed) bees. A relative band density of 1 indicates no fold change in expression relative to control bees.

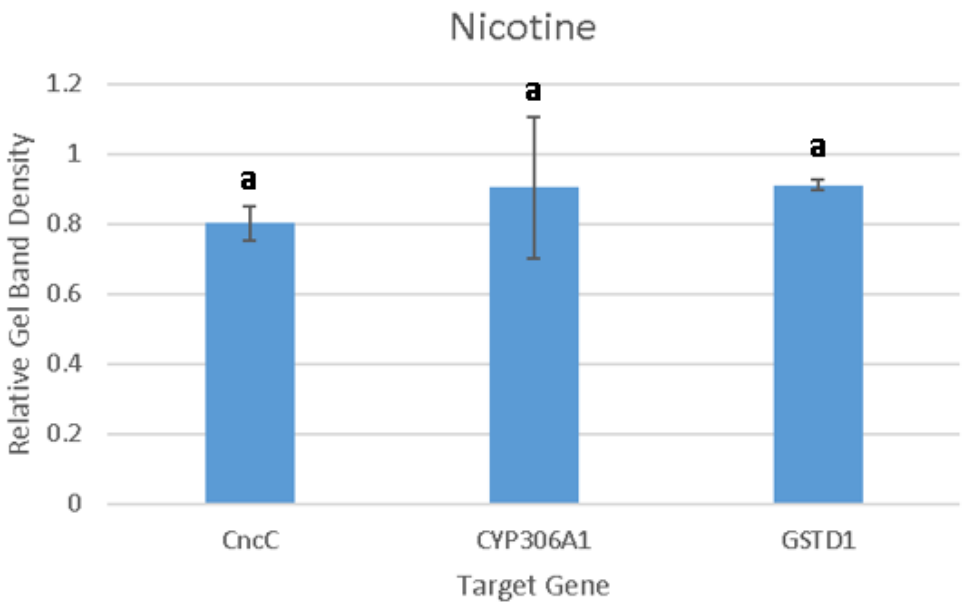
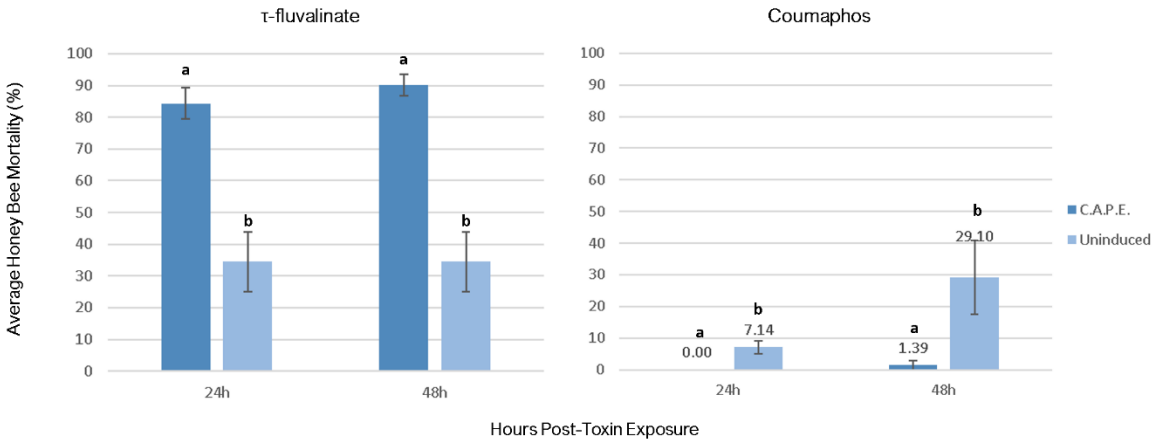


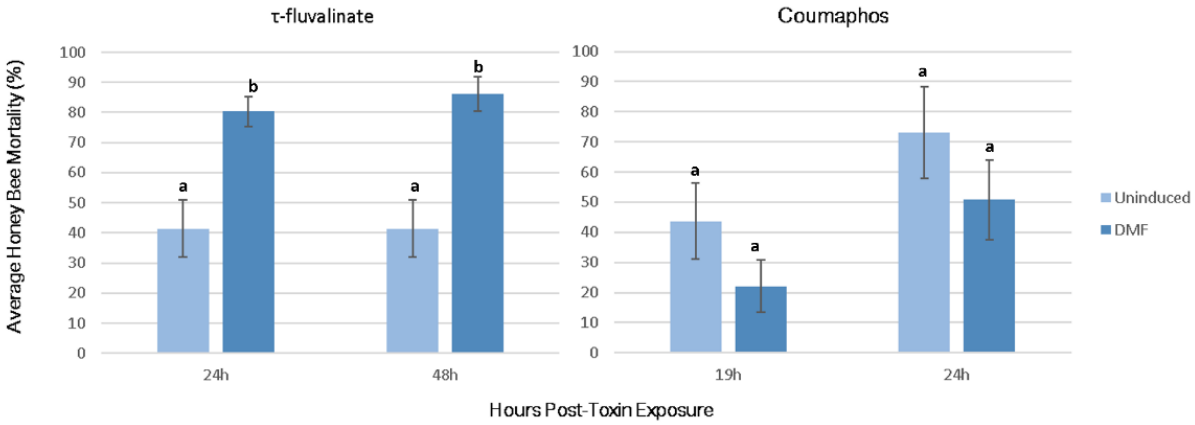
Figure 10. **Effect of Nicotine Treatment on *CncC, CYP306A1,* and *GSTD1* expression.** Mean relative PCR product band density (±SEM) of all three target genes *CncC, Cyp306A1,* and *GSTD1* for nicotine-treated bees. Genes were amplified with cDNA reverse transcribed from RNA extracted from the midguts of three replicates of bees fed 300µM for 48 hours. Band density (unitless) is calculated relative to band density expression of control-treated (sucrose solution) bees. A relative band density of 1 indicates no fold change in expression relative to control bees. Different letters indicate significantly different treatments ($p<0.05$).

Appendix



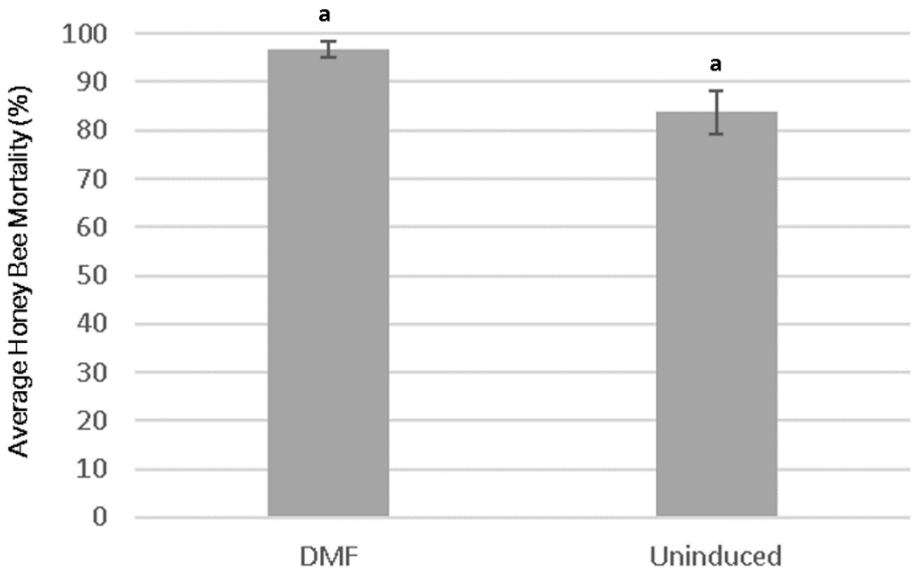
Appendix Figure A. Effect of Caffeic Acid Phenethyl Ester (C.A.P.E.) Treatment on τ-fluvalinate and Coumaphos toxicity.

C.A.P.E. treatment significantly decreases coumaphos toxicity, and increases τ-fluvalinate toxicity. Mean mortality (±SEM) of C.A.P.E.-induced honey bees exposed to topical application of 1µl of τ-fluvalinate (10mg/ml) (Left) or coumaphos (30mg/ml) (Right). Honey bees were fed 1mM C.A.P.E. for 48h pre-exposure, or fed sucrose solution (Uninduced). Dilution and application of toxins were as described in bioassay materials and methods (Chapter 2). C.A.P.E was dissolved in DMSO, then diluted to a final concentration in sucrose solution of 1mM, with a final DMSO concentration of 1%. Application of C.A.P.E. was as described for inducers in Chapter 2 bioassay materials and methods. Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point (p<0.05).



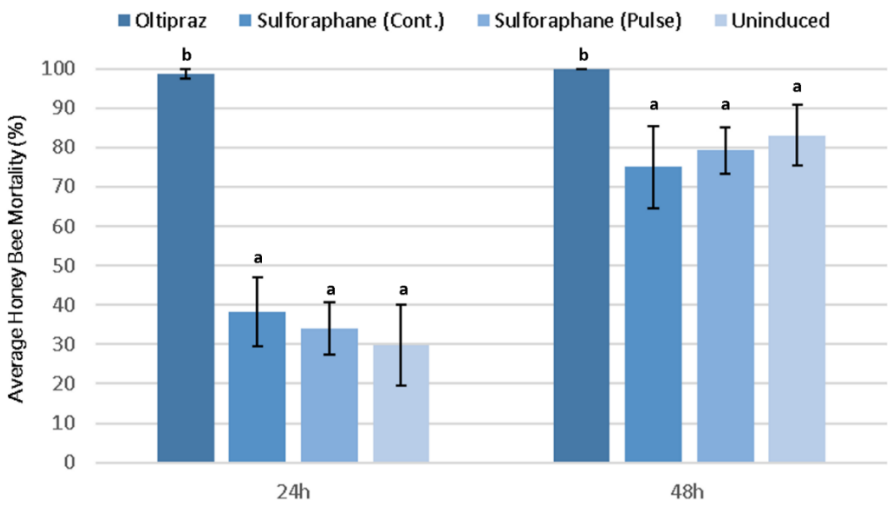
Appendix Figure B. Effect of Dimethyl Fumarate (D.M.F) Treatment on either τ-fluvalinate or coumaphos toxicity.

D.M.F. significantly increases τ-fluvalinate toxicity, and does not significantly decrease coumaphos toxicity despite decreasing mortality nominal means. Mean mortality (±SEM) of D.M.F.-induced honey bees exposed to topical application of 1µl of τ-fluvalinate (10mg/ml)(Left) or coumaphos (75mg/ml)(Right). Honey bees were fed 0.3mM D.M.F. for 48h pre-exposure, or fed sucrose solution (Uninduced). Dilution and application of toxins were as described in bioassay materials and methods (Chapter 2). DMF was dissolved in DMSO, then diluted to a final concentration in sucrose solution of 0.3mM, with a final DMSO concentration of 1%. Application of DMF was as described for inducers in Chapter 2 bioassay materials and methods. Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point (p<0.05).



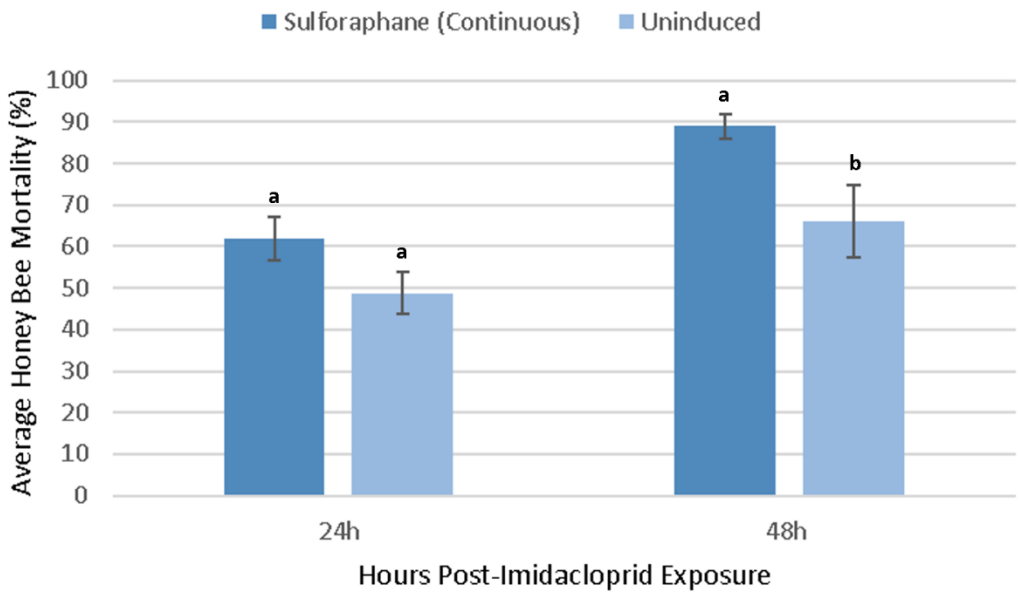
Appendix Figure C. Effect of Dimethyl Fumarate (D.M.F) Treatment on Chlorpyrifos Toxicity.

The nominal mean of D.M.F-induced bee mortality was higher than uninduced bees, though this effect was not statistically significant. Mean mortality (±SEM) of D.M.F.-induced honey bees exposed to oral application of 5µl of chlorpyrifos (10ng/µl). Honey bees were fed 0.3mM D.M.F. for 48h pre-exposure, or fed sucrose solution (Uninduced). Dilution and application of toxin were as described in bioassay materials and methods (Chapter 2). DMF was first dissolved in DMSO and diluted to a final concentration in sucrose solution of 0.3mM DMF and 1% DMSO. Application of DMF was as described for inducers in Chapter 2 bioassay materials and methods Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point ($p<0.05$) ($p= 0.053$).



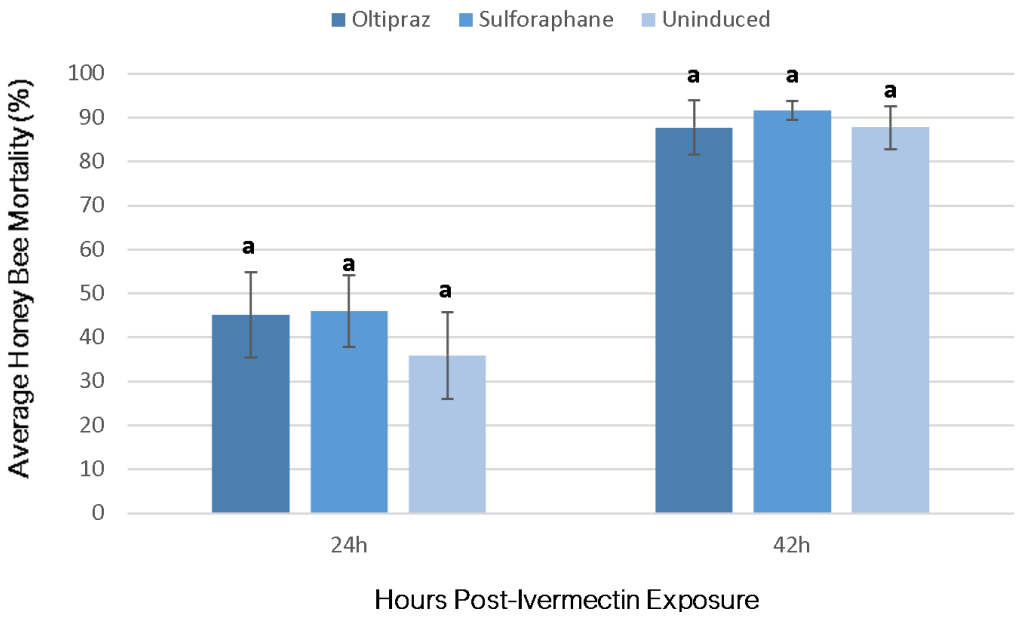
Appendix Figure D. Effect of Oltipraz and Continuous and Pre-fed Sulforaphane Treatment on Imidacloprid Toxicity.

Oltipraz treatment significantly increased imidacloprid toxicity, and continuous and pre-fed sulforaphane treatments had no significant effect on toxicity in comparison to uninduced bees. Mean mortality (±SEM) of honey bees exposed to oral application of 5µl of imidacloprid (15ng/µl in sucrose solution) (Sigma Aldrich). Honey bees were fed 1mM Oltipraz or 1mM sulforaphane ("Continuous" [Cont.] or pre-fed only [Pulse]) for 48h pre-exposure, or fed sucrose solution (Uninduced). Dilution and application of both inducers were as described in bioassay materials and methods (Chapter 2). Imidacloprid was dissolved first in DMSO, then to 15ng/ µl in sucrose solution with a final DMSO concentration of 1%, and fed in 5µl to bees using a micropipettor in the same feeding method as chlorpyrifos (described in bioassay methods, chapter 2). Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point ($p<0.05$).



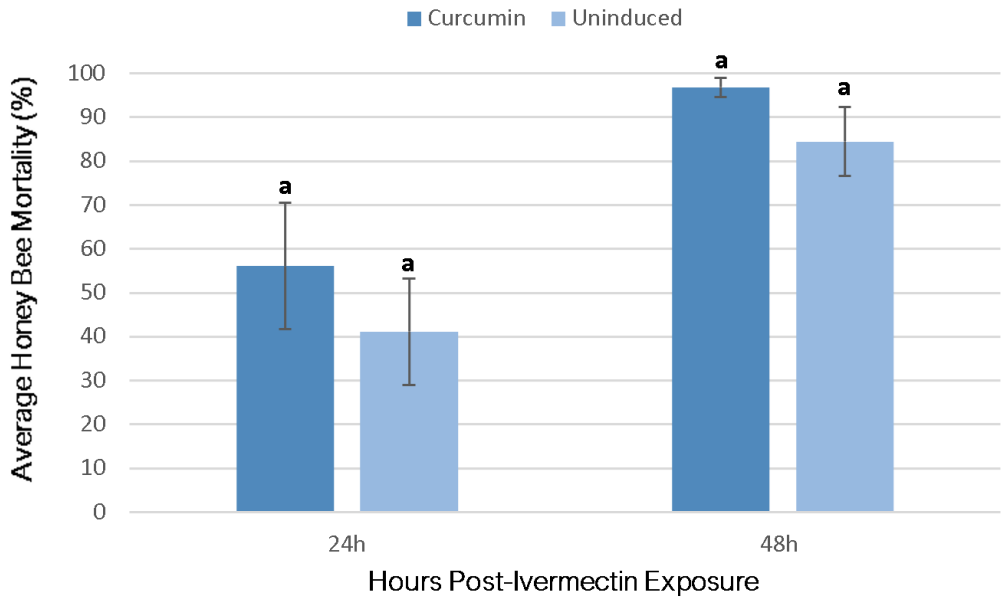
Appendix Figure E. Effect of Continuous Sulforaphane Treatment on Imidacloprid Toxicity.

Separate additional replicate trial of "continuous" sulforaphane treatment did not significantly affect imidacloprid toxicity at 24h, but significantly increased toxicity at 48h. Mean mortality (±SEM) of honey bees exposed to oral application of 5µl of imidacloprid (15ng/µl in sucrose solution) (Sigma Aldrich). Honey bees were fed 1mM sulforaphane ("continuous") for 48h pre-exposure, or fed sucrose solution (Uninduced). Sulforaphane was diluted and fed as described in bioassay materials and methods (Chapter 2). Imidacloprid was dissolved first in DMSO, then diluted in 50% sucrose solution to a final concentration of 15ng/µl toxin and 1% DMSO. Imidacloprid was fed in 5µl to bees using the same exposure method as chlorpyrifos (described in bioassay methods, chapter 2). Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point ($p<0.05$).



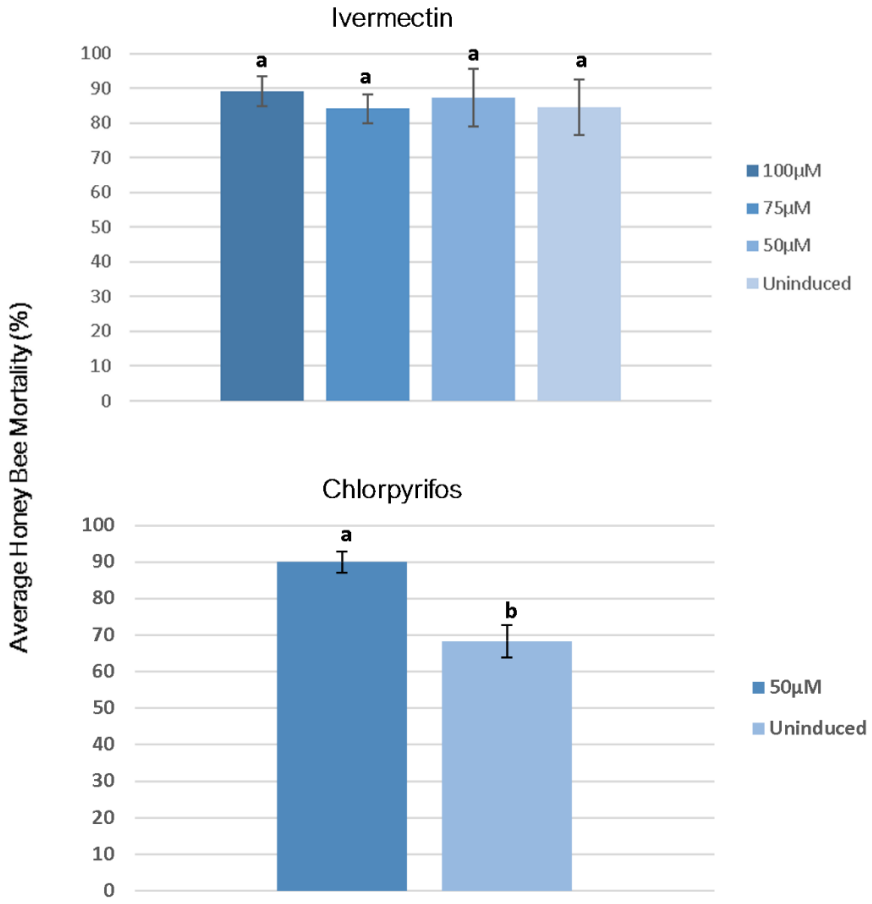
Appendix Figure E. Effect of Oltipraz and Sulforaphane Treatment on Ivermectin Toxicity.

Treatment with either Oltipraz or sulforaphane did not significantly affect ivermectin toxicity. Mean mortality (±SEM) of Oltipraz- and sulforaphane-treated honey bees exposed to oral exposure of ivermectin (1µg/ml). (Sigma Aldrich). Honey bees were fed 50µM sulforaphane or 50µM Oltipraz for 48h pre-exposure, or fed sucrose solution (Uninduced). Dilution and application of inducers were as described in bioassay materials and methods (Chapter 2). Ivermectin was diluted to 1µg/ml in 50% sucrose solution and fed to bees in feeding tubes over a 42h total period, after 48h inducer feeding period. Feeding tubes were the same design as those containing inducer diet. Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point ($p<0.05$).



Appendix Figure F. Effect of curcumin treatment on ivermectin toxicity.

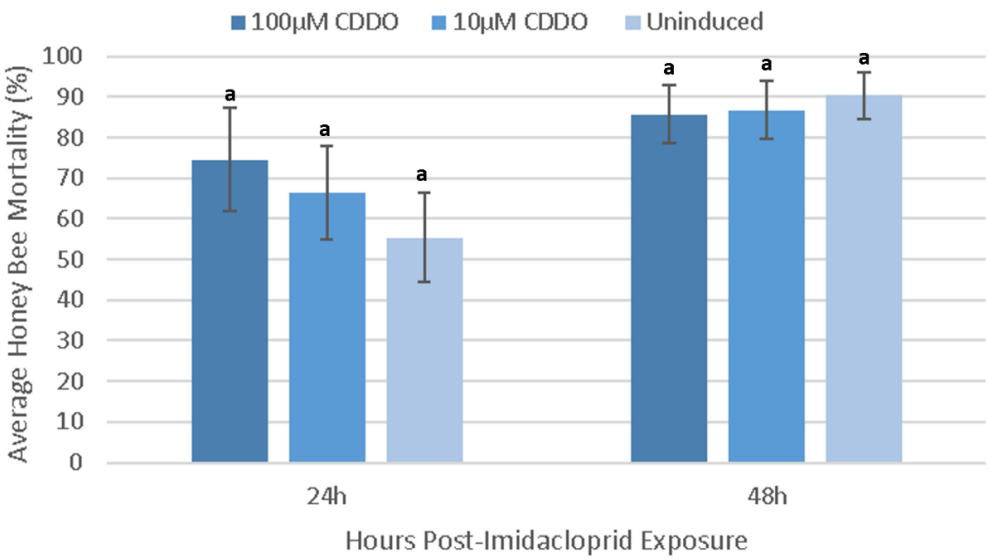
Curcumin treatment did not significantly affect ivermectin toxicity. Mean mortality (±SEM) of curcumin-treated honey bees exposed to oral exposure to ivermectin (1µg/ml). (Sigma Aldrich). Honey bees were fed 50µM curcumin for 48h pre-exposure, or fed sucrose solution (Uninduced). Curcumin was dissolved in DMSO, then diluted to a final concentration of 50µM in sucrose solution (final DMSO concentration of 1%). Application of curcumin was as described for inducers in bioassay materials and methods (Chapter 2). Ivermectin was diluted in 50% sucrose solution to 1µg/ml and fed to bees in feeding tubes over a 42h total period, after 48h inducer feeding period. Feeding tubes were the same design as those containing inducer diet. Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point (p<0.05).



Appendix Figure G. Effect of Bardoxolone Methyl (CDDO-Me) on Ivermectin and Chlorpyrifos Toxicity.

CDDO-Me treatment significantly increases chlorpyrifos toxicity, but does not significantly affect ivermectin toxicity. Mean mortality (±SEM) of CDDO-Me – induced honey bees 24h post-oral exposure to ivermectin (1µg/ml, tube-fed) or 5µl of chlorpyrifos (10ng/µl). Honey bees were fed CDDO-Me (100µM, 75µM, or 50µM) for 48h pre-exposure, or fed sucrose solution (Uninduced). CDDO-Me was diluted first in DMSO, then diluted to final concentrations of 100µM, 75µM, or 50µM in 50% sugar-water, with a final DMSO concentration of 1%. Application of CDDO-Me was as described for inducers in bioassay materials and methods (Chapter 2). Dilution and application of chlorpyrifos was as described in bioassay materials and methods (Chapter 2). Ivermectin (Sigma Aldrich) was diluted in

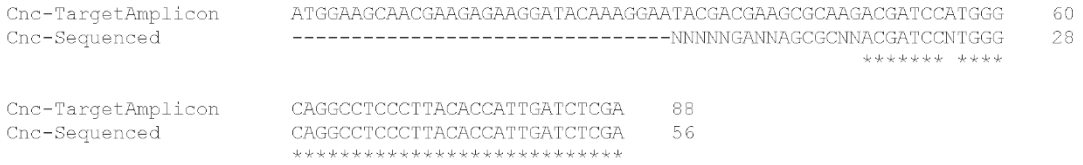
sucrose solution to 1µg/ml and fed to bees in feeding tubes over a 42h total period, post-induction. Feeding tubes were the same design as those containing inducer diet. Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point ($p<0.05$).



Appendix Figure H. Effect of Bardoxolone Methyl (CDDO-Me) on Imidacloprid Toxicity.

CDDO-Me treatment does not significantly affect imidacloprid toxicity.

Mean mortality (±SEM) of CDDO-Me – induced honey bees post-oral exposure to imidacloprid (5µl, 15ng/µl) (Sigma Aldrich). Honey bees were fed CDDO-Me (100µM, 75µM, or 50µM) for 48h pre-exposure, or fed sucrose solution (Uninduced). CDDO-Me was diluted first in DMSO, then diluted to final concentrations of 100µM or 10µM in sucrose solution, with a final DMSO concentration of 1%. Application of CDDO-Me was as described for inducers in bioassay materials and methods (Chapter 2). Imidacloprid was dissolved first in DMSO, then to 15ng/ µl in sucrose solution with a final DMSO concentration of 1%, and fed in 5µl to bees using a micropipettor in the same feeding method as chlorpyrifos (described in bioassay methods, chapter 2). Significance of treatment mean comparisons were analyzed with single-factor ANOVA. Different letters indicate significant differences among treatment means within a time point ($p<0.05$).



Appendix Figure I. Sequence alignment of *Cnc* target amplicon with sequenced PCR product. Sequenced base pairs display high fidelity to the target amplicon sequence, though PCR product sequencing was incomplete.

Appendix Figure J. Sequence alignment of *Cyp306A1* target amplicon with sequenced PCR product. PCR product sequence is incomplete, but sequenced base pairs exhibit high similarity to the target amplicon sequence.

```
GSTD1_Sequenced          ------------------------NNNNNNNNNNNNNNNGANNTAAGCNNCGTATCCACT  36
GSTD1-TargetAmplicon     GGGGAAAACTATGTGGCAGGGAAGAACATGACTCTTGCCGATTTAAGCATCGTATCCACT  60
                                                              **  *****  **********

GSTD1_Sequenced          GTCTCCACTTNANAG       51
GSTD1-TargetAmplicon     GTCTCCACTTT----       71
                         **********
```

Appendix Figure K. Sequence alignment of *GSTD1* target amplicon with sequenced PCR product. Sequenced base pairs exhibit high similarity to the target amplicon sequence, though PCR product sequence is incomplete with some low quality base calls.

```
GAPD2_Sequenced      -------------------------NNNNNTNNNNNNGNNNTNNNNNNGTTATACTATT       34
GAPD2-Target         GATGCACCCATGTTTGTTTGTGGTGTTAATTTGGATGCTTATGATCCAAGTTATACTATT       60
                                             *                ***********

GAPD2_Sequenced      GTTTCCAATGCTTCTTGTACTACTAATTGTTTAGCACCTCTTGCTAAAGTTATTCATGAT       94
GAPD2-Target         GTTTCCAATGCTTCTTGTACTACTAATTGTTTAGCACCTCTTGCTAAAGTTATTCATGAT       120
                     ************************************************************

GAPD2_Sequenced      AATTTTGAAATCGTTGAAGGTCTTATGACTACTGTTCATGCTGTTACTGCTACACAAAAA       154
GAPD2-Target         AATTTTGAAATCGTTGAAGGTCTTATGACTACTGTTCATGCTGTTACTGCTACACAAAAA       180
                     ************************************************************

GAPD2_Sequenced      GTTGTTGATGCACCTTCTGCAAAA   178
GAPD2-Target         GTTGTTGATGCACCTTCTGCAAA-   203
                     ***********************
```

Appendix Figure L. Sequence alignment of *GAPD2* target amplicon with sequenced PCR product. PCR product sequence is incomplete, but sequenced base pairs exhibit high fidelity to the target amplicon sequence.
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