Toxicological Analysis of the Neonicotinoid Insecticide Imidacloprid to Honey Bees, *Apis mellifera*, of Different Colonies

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ABSTRACT

The honey bee, *Apis mellifera*, provides about $15 billion USD in crop value each year in the U.S. alone in the form of pollination services. Since 2006, commercial beekeepers have reported an average annual overwintering loss of about 28.6% of all managed colonies. There are many factors that are thought to contribute to colony loss including bee-specific pests (e.g. the *Varroa destructor* mite), bee-specific pathogens (e.g. *Nosema* fungus), modern beekeeping practices, diminished genetic variability, poor queens, climate change, and exposure to agricultural pesticides. While not the single cause of colony loss, the neonicotinoid insecticides elicit sublethal effects to honey bees that could increase their sensitivities to other stressors that affect colony health. Previous studies found that honey bees have differential sensitivities to the neonicotinoid insecticide imidacloprid, which suggest a mechanism of tolerance to the insecticide in certain colonies. In this study, I examined the imidacloprid sensitivity of honey bees collected from different colonies. After determining a range of LC_{50} values in the tested colonies, I examined the metabolic detoxification activities of honey bees collected from two colonies that represented the highest and lowest LC_{50} values, between which there was a 36-fold difference in their LC_{50} values. I discovered that of the three main families of metabolic detoxification enzymes, general esterases, cytochrome P450 monooxygenases, and glutathione *S*-transferases (GSTs), a reduction of GST activity with diethyl maleate (DEM) significantly increased imidacloprid-mediated mortality to the honey bees. A comparative analysis of GST kinetic activity from imidacloprid-susceptible and -insensitive honey bees revealed a lower

bimolecular inhibition rate constant (k_i) for imidacloprid-insensitive individuals (5.07 ± 0.098 nmol/min/mg protein) compared to the imidacloprid-sensitive honey bees (17.23 ± 1.235 nmol/min/mg protein). The IC_{50} of DEM estimated for bees from each colony showed that the imidacloprid-susceptible honey bees possess a higher IC_{50} (10 μM) than that of the tolerant honey bees (3 μM). These data suggest that the GSTs in the imidacloprid-tolerant honey bees might be a more efficient detoxification mechanism for the conjugation and elimination of imidacloprid, or imidacloprid metabolites, compared to that of imidacloprid-susceptible honey bees. Therefore, I hypothesize that the differences in metabolic detoxification enzyme activities of honey bees collected from different colonies can result in the differential toxicities of honey bees exposed to neonicotinoid insecticides, such as imidacloprid. However, a thorough examination of imidacloprid detoxification in honey bees is warranted to confirm this hypothesis.

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GENERAL AUDIENCE ABSTRACT

Honey bees are the most important crop pollinator known to humans. The domestication and use of these insects constitutes a multi-billion dollar industry. Their pollination services alone are a necessary part of modern day agriculture. One of the concerns raised today with regard to honey bee health is their exposure to insecticides used widely in modern agriculture to manage crop pests and protect our food supply from devastating crop loss. One insecticide family that has gained much attention lately are the neonicotinoids. These insecticides are reported to elicit sublethal effects to honey bees that can affect colony health. Some of the more widely used neonicotinoids include, but are not limited to, imidacloprid, thiacloprid, and acetamiprid. The goal of this study was to examine the acute toxicity of imidacloprid to honey bees collected from different colonies and to compare the metabolic detoxification enzyme activities of the honey bees to understand the mechanism(s) of imidacloprid sensitivity in the honey bees. Here, I report a 36-fold difference in the acute toxicity of imidacloprid to the honey bees collected from different colonies. A comparison of glutathione *S*-transferases activities in imidacloprid-susceptible and -tolerant honey bees suggest that these metabolic detoxification enzymes may assist in the conjugation of imidacloprid, or associated metabolites, and thus facilitate the removal of the insecticide from the honey bees.

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INTRODUCTION

1.1 HONEY BEE BIOLOGY

The honey bee, *Apis mellifera* is a eusocial, colony-oriented organism biologically divided into three castes: the queen, the workers, and the drones (Stone, 2005; Winston, 1991). The queen is the only fertile female in a colony and as such is the colony's primary reproductive unit, laying up to 2,000 eggs per day (Stone, 2005; Winston, 1991). There is typically only one queen per colony, and her presence and fecundity are important determining factors of the health of a colony. The workers are altruistic sterile female bees who make up the main work force of a colony. They collect the pollen and nectar, defend the colony from intruders, clean debris from inside the colony, tend to the young, and attend the queen. A strong, healthy colony will have about 50,000 to 60,000 workers at various life stages performing all the necessary colony functions (Stone, 2005; Winston, 1991). Drones, the haploid male caste, are typically only present in the colony during the spring and early summer months when they go on mating flights to mate with virgin queens. There are only between 500 and 1,000 drones in the colony during this time, depending on the size and health of the colony (Stone, 2005; Winston, 1991). A drone serves no other purpose than mating and will either die after copulation or be ejected from the colony by his sisters in the autumn.

Honey bees are a model organism for aging, social evolution, phenotypic plasticity, and meiotic recombination. They have been domesticated since at least 3000 BCE for the collection of honey and wax, and since the 1600s have migrated with human settlers all around the world (vanEngelsdorp & Meixner, 2010).

1.2 IMPORTANCE OF HONEY BEES AS POLLINATORS

Honey bees are the most important crop pollinator known to man with an annual net value of over $15 billion in crop revenue in the United States alone, and over $200 billion worldwide (Fairbrother et al., 2014; Morse & Calderone, 2000; vanEngelsdorp & Meixner, 2010). It is estimated that 35% of global food production relies upon animal pollination and 52 of the 115 leading global food commodities rely on pollination by honey bees (Klein et al., 2007; vanEngelsdorp & Meixner, 2010). Many crops, such as almonds, are heavily dependent on pollination by managed honey bee colonies, and a drop in almond production has been linked to a decrease in the number of available colonies that can be shipped to California's almond fields (Allen-Wardell et al., 1998). Other crops, like soy beans, canola, and sunflowers see enhanced seed production as a result of pollination services (Aizen et al., 2008). Indeed, over the past 53 years, there has been an increasing shift globally toward the cultivation of pollinator-dependent crops (Aizen et al., 2008; Potts et al., 2010), which has increased our dependence on managed honey bees as a corner stone of modern agriculture since about 90% of commercial pollination is provided by managed honey bees (Genersch, 2010). Honey bees also contribute to biodiversity by pollinating wild plants and benefiting wild ecosystems (Potts et al., 2010).

1.3 COLONY DECLINE

General pollinator decline was first noticed in the 1990s. Between 1990 and 1996 there was a measured 25% decline in managed and feral honey bee colonies (Allen-Wardell et al., 1998). This decline was not known to follow one specific set of symptoms or causes, and more research has followed. More specific set of common symptoms was identified in the winter of 2006-2007 after a massive colony die off occurred in managed honey bee colonies in the United

States, and again in the winter of 2007-2008 (vanEngelsdorp et al., 2009). A post-hoc analysis of mortality revealed a common set of symptoms: the rapid loss of adult workers bees with excess brood populations, a lack of dead worker bees in or surrounding the colony, and delayed invasion by both colony pests and neighboring honey bees (vanEngelsdorp et al., 2009; vanEngelsdorp & Meixner, 2010). This was the first characterization of what is now known as colony collapse disorder (CCD). A trend in pollinator decline that goes beyond the symptoms of CCD, and can be attributed to general pollinator decline has since been noted on a global scale (Allen-Wardell et al., 1998; Potts et al., 2010). According to the most recent survey by the Bee Informed Partnership, about 28.1% of managed colonies were lost during the 2015-2016 winter, and an average of 28.6% overwintering losses recorded for the last ten years (Steinhauer et al., 2016). Losses over summer have also been dramatic. When combined with overwintering losses, a total of 44.1% of managed colonies died off between April 2015 and March 2016 (Steinhauer et al., 2016).

Although activists have attempted to label neonicotinoids as the sole cause of colony decline, peer reviewed research consistently demonstrates that this is not the case, listing a number of factors that act in combination to weaken bees, rather than a single cause, as the most likely explanation for the decline (Blacquière, Smagghe, van Gestel, & Mommaerts, 2012; Cresswell, 2011; Cresswell, Desneux, & VanEngelsdorp, 2012; Fairbrother et al., 2014; vanEngelsdorp & Meixner, 2010). Such factors include bee-specific pests like the varroa mite and hive beetles, pathogens that affect bees such as deformed wing virus and *Nosema* fungus, modern beekeeping practices, diminished genetic variability, poor queens, climate change, and exposure to agricultural pesticides (Cresswell, 2011; Cresswell, Desneux, et al., 2012; vanEngelsdorp et al., 2009; vanEngelsdorp & Meixner, 2010). In spite of the fact that no single

cause is responsible for colony decline, alleviating the harm of one possible contributor could reduce the harm done to bees by some of the other causes.

1.4 FACTORS CONTRIBUTING TO COLONY DECLINE

1.4.1 Abiotic and Biotic

A variety of stressors exist in the honey bees' environment that contribute to the general decline in the number of colonies, some of which will be covered in greater detail in the sections below. More notable abiotic stressors include climate change and pollutants in the environment such as insecticides (Blacquière et al., 2012; Decourtye & Devillers, 2010; Pettis, Vanengelsdorp, Johnson, & Dively, 2012). Some of the biotic stressors include parasitization by mites, disease pathogens, predacious animals, and habitat loss and poor nutrition due to human activity (Blacquière et al., 2012; Decourtye & Devillers, 2010; Pettis et al., 2012). These factors often potentiate each other, for example exposure to pesticides can increase susceptibility to disease pathogens (Di Prisco et al., 2013; Pettis et al., 2012).

1.4.2 Varroa Mites

One of the most important contributors to colony decline is the ectoparasitic mite, *Varroa destructor*. It is considered to be the most destructive parasite to bees and one of the main causes of bee death worldwide (Boncristiani et al., 2012; Khongphinitbunjong et al., 2015; Morse & Calderone, 2000; Rosenkranz et al., 2010). Female varroa mites enter a hive and crawl into an open cell in which bee brood is located and hides under the food for the larva (Rosenkranz et al., 2010). Once the cell is capped the female mite emerges from her hiding spot, feeds on the developing bee larva's hemolymph, and lays a haploid male egg, which then hatches and mates

with his mother, who then lays diploid female eggs (Rosenkranz et al., 2010). The mites feed on the developing larva until it emerges from its cell a fully grown bee, at which point a few mites will stay on the adult bee and continue to feed on it while most of the rest go to other cells and continue the process of reproduction (Rosenkranz et al., 2010).

One of the most dangerous aspects of varroa mites is their ability to vector diseases to bees while feeding on their hemolymph, much like how mosquitoes vector diseases to mammals when feeding on their blood. Presence of these mites has been associated with the spread of pathogens like *Nosema*, deformed wing virus, and acute bee paralysis virus (G. Di Prisco et al., 2011; Gisder, Aumeier, & Genersch, 2009; Khongphinitbunjong et al., 2015; Rosenkranz et al., 2010; vanEngelsdorp et al., 2009). If unchecked, a varroa mite infestation can kill a colony in weeks (Morse & Calderone, 2000).

1.4.3 Pathogens Affecting Bees

There is a multitude of bacterial, viral, and fungal pathogens that affect honey bee health, some of which are vectored by varroa mites. A brief overview is provided below for the most infectious and harmful bee pathogens that may contribute to colony decline.

Two of the most detrimental viruses that affect honey bees and contribute to decline are the deformed wing virus (DWV) and acute bee paralysis virus (ABPV) (Genersch, 2010; Khongphinitbunjong et al., 2015; vanEngelsdorp & Meixner, 2010). These are single stranded RNA viruses in the order *Picornavirales* (Highfield et al., 2009). DWV, as its name suggests, is known to cause wing deformities in developing honey bees, which make them incapable of flight and as a result leads to their deaths (Highfield et al., 2009). The ABPV causes paralysis and ultimately death in infected bees and is more virulent than DWV (Highfield et al., 2009).

The most devastating bacterium to honey bees is *Paenibacillus* larvae, the cause of American foul brood (Fünfhaus, Ashiralieva, Borriss, & Genersch, 2009; Hansen & Brødsgaard, 1999). This bacterium is found around the world and most heavily throughout the North American and European continents, and is highly infectious (Fünfhaus et al., 2009; Hansen & Brødsgaard, 1999). Bee larvae less than three days old that ingest the spores in their food will turn black and rot after being capped, giving off a characteristic foul odor (Hansen & Brødsgaard, 1999). Workers that clean out a cell of an infected larva spread the spores around, infecting other larvae. This can wipe out the brood in a hive and lead to the collapse of a colony (Fünfhaus et al., 2009; Hansen & Brødsgaard, 1999).

One of the most deadly fungal pathogens for bees are those belonging to the genus *Nosema*, particularly *N. apis* and *N. ceranae* (Genersch, 2010). These are fungal parasites that can only exist outside a host cell in spore form. While *N. apis* is only detected in the midgut and cause dysentery, *N. ceranae* has been found in the midgut, malpighian tubules, and hypopharyngeal glands, and causes death without outward visible symptoms (Genersch, 2010).

1.4.4 Insecticides

Major families of insecticides include, but are not limited to: organophosphates, pyrethroids, carbamates, chlorinated hydrocarbons (also called organochlorines), and neonicotinoids (Casida & Quistad, 1998; Jeschke & Nauen, 2008). Insecticides are largely neurotoxicants that target either voltage-gated sodium channels, GABA-gated chloride channels, acetylcholinesterase (AChE), or nicotinic acetylcholine receptors (nAChR) with the result of either producing a prolonged depolarization of the nerve cell, or the inhibition of signaling depending on the compound (Casida & Quistad, 1998). Organochlorines and pyrethroids target

voltage-gated sodium channels, organophosphates and carbamates target AChE, chlorinated hydrocarbons target GABA-gated chloride channels, and neonicotinoids target nAChRs (Casida & Quistad, 1998; Matsuda et al., 2001).

Though used as a means of preventing crop loss caused by pests, there has been a significant amount of concern about the harm insecticides can do to non-target species like honey bees (Brown et al., 1996; Klein et al., 2007; vanEngelsdorp et al., 2009). Besides being killed via incidental exposure to residues of insecticides that are sprayed on crops they were pollinating, honey bees are known to suffer a number of sub-lethal effects. The sub-lethal effects might include, but are not limited to, delayed larval development, delayed adult emergence, shortened adult longevity, increased susceptibility to pathogens, increased susceptibility to varroa mites, and premature shifts in worker roles including a premature shift to foraging (Boncristiani et al., 2012; Pettis et al., 2012; Wu, Anelli, & Sheppard, 2011). These effects, particularly the increased susceptibility to pathogens and parasites, can work in conjunction with other factors of colony decline to potentiate their effects (Perry, Søvik, Myerscough, & Barron, 2015). Of all the insecticide families, the one that has been of particular public concern lately has been neonicotinoids (Fairbrother et al., 2014).

1.5 NEONICOTINOIDS AND IMIDACLOPRID

Neonicotinoids are similar in chemical structure to nicotine, which target the nicotinic acetylcholine receptor (nAChR) (Iwasa, Motoyama, Ambrose, & Roe, 2004; Kagabu & Matsuno, 1997; Matsuda et al., 2001; Steeve Hervé Thany, 2010). The first neonicotinoid, tetrahydro-2-(nitromethylene)-2H- 1,3-thiazine, or nithiazine, was synthesized in the 1970s (Jeschke & Nauen, 2008). Nithiazine, a potent toxicant against adult house flies and the larvae

of corn earworms, is a photosensitive chemical that breaks down when exposed to sunlight, and thus, resulted in a commercially inviable product (Jeschke & Nauen, 2008). Further alterations of the chemical structure, namely the replacement of a six-member ring with a five-member ring, eventually lead to the development of the photo-stable N-{1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl}nitramide, otherwise known as imidacloprid (Jeschke & Nauen, 2008; Kagabu & Matsuno, 1997). Imidacloprid proved not only to be more stable than nithiazine, but had increased insecticidal activity compared to that of nithiazine and nicotine. There are many more variations of neonicotinoids that exist on the commercial market, such as thiacloprid, acetamiprid, and clothianidin. Imidacloprid, introduced into the commercial market in 1991, has been one of the most successful selling insecticides, and the most successful neonicotinoid, with annual sales of over $1 billion (Fairbrother et al., 2014; Iwasa et al., 2004; Jeschke & Nauen, 2008; Jones, Raymond-Delpech, Thany, Gauthier, & Sattelle, 2006; Karunker et al., 2009; Matsuda et al., 2001; Simon-Delso et al., 2014; Steeve Hervé Thany, 2010).

The neonicotinoid insecticides have many benefits as pesticides, such as higher selectivity for insect nAChRs over vertebrate nAChRs (Tomizawa & Casida, 2003), low application dose (field application concentrations range from 0.7-10 μg/L) (Cresswell, 2011; Laycock, Lenthall, Barratt, & Cresswell, 2012), act on a broad spectrum of insects due to acting on a variety of nAChR types (Buckingham, Lapied, Corronc, & Sattelle, 1997), and are lipophilic (Kagabu & Matsuno, 1997; Simon-Delso et al., 2014). Their selectivity for insect nAChRs over vertebrate nAChRs is due to the positively charged cationic residues in insect nAChR binding sites versus largely anionic binding sites on vertebrate nAChRs.

In honey bees, imidacloprid and other neonicotinoids are known to be more toxic when administered orally than on contact (Blacquière et al., 2012). Neonicotinoids can induce lethality

in honey bees at concentrations that are known to vary from colony to colony (Blacquière et al., 2012; Fairbrother et al., 2014; Iwasa et al., 2004; Mommaerts et al., 2010). For example, a review by Fairbrother et al. (2014) reported that for imidacloprid the contact LD_{50} values ranged from 7.8 – 242 ng/bee, and the oral LD_{50} values from 3.8 – 81 ng/bee in honey bees. However, the more widespread concern is over the variety of sub-lethal effects reported in response to neonicotinoid consumption. Radio-frequency identification (RFID) tracking of bees has demonstrated a reduction in foraging and longer foraging flights within the first three hours after feeding on a source of carbohydrates contaminated with neonicotinoids (Schneider, Tautz, Grünewald, & Fuchs, 2012). Such behavior may reflect the reported sub-lethal effects of reduction in appetite and decreased learning (Cresswell, 2011; Decourtye et al., 2004; Van der Sluijs et al., 2013). There are concerns that neonicotinoid-induced delay in larval development potentiates the development and spread of varroa mites (Van der Sluijs et al., 2013; Wu et al., 2011). There have also been studies that report neonicotinoid exposure weakens the honey bee immune system and, thus, increases susceptibility to pathogens like deformed wing virus and *Nosema* fungus (Di Prisco et al., 2013; Pettis et al., 2012; Van der Sluijs et al., 2013).

1.6 FACTORS THAT AFFECT IMIDACLOPRID TOXICITY

1.6.1 Target Site Affinity

First characterized in the electric eel, *Electrophorus electricus*, and the elasmobranch fish, *Torpedo californica* (Stroud & Finer-Moore, 1985), the nAChR is a five-sided molecule consisting of five homologous subunits around a central ion channel (Karlin, 2002; Sattelle, 1980; Stroud & Finer-Moore, 1985; Unwin, 2005). There are typically two α subunits, and one subunit each of β, δ, and ɣ subunits in a nAChR (Karlin, 2002); however, in the *Apis mellifera*

genome, there are no genes for δ or ɣ subunits. The honey bee genome codes for nine different variants of α subunits and two variants of β subunits (Jones et al., 2006). The α subunit is the subunit on which the ligand binding site for the receptor is located, having two cysteine residues right next to each other on the C terminal loop that are vital for acetylcholine (ACh) binding (Jones et al., 2006; Karlin, 2002; Sattelle, 1980; Unwin, 2005). In addition to binding to an α subunit, some agonists have been reported to bind at junctions between α and non-α subunits (Millar & Lansdell, 2010). In all cases, the α subunit is the key subunit for agonist binding. The nAChR occupies the post-synaptic clefts of neural junctions and serves to bring in sodium and potassium cations to generate an electric gradient that propagates a signal along the neural pathway. The interior of these transmembrane ion channels are lined with negatively charged groups of amino acids, providing an electrostatically stable environment for cations to pass through (Karlin, 2002; Unwin, 2005).

When a ligand, such as ACh, binds to a nAChR it triggers a conformational change in the α subunit towards a non- α form that pushes the other subunits of the nAChR into an open conformation, which in turn allows the free flow of cations at a rate of about 104 ions per millisecond per receptor (Karlin, 2002; Stroud & Finer-Moore, 1985; Unwin, 2005). Acetylcholine is subsequently metabolized by acetylcholinesterase, and removed from the active binding site on the α subunit. This subunit and those connected to it return to their normal configurations, closing the transmembrane pore. This mechanism of action is what makes ACh so useful for rapid signal transmission in the nervous system. Nicotine and neonicotinoids are not efficiently removed from the active binding site of nAChRs, and can leave the pore open longer than is physiologically normal (Steeve Hervé Thany, 2010). Repolarization of a nerve cell is necessary for the nerve cell to fire new action potentials, and prolonged depolarization can

lead to desensitization of a nerve cell, causing it to no longer respond to agonist binding (Stroud & Finer-Moore, 1985). Physiological responses to desensitization range from intoxication to death depending on severity.

It has been demonstrated in other insect species that differences in nAChR subunit composition have an effect on neonicotinoid binding affinity and sensitivity (Bodereau-Dubois et al., 2012; Jian Li et al., 2010; Liu et al., 2006; Yixi et al., 2009). In the rice brown plant hopper, *Nilaparvata lugens*, a point mutation at Y151S in the B loop of the α1 and α3 subunits reduced nAChR binding affinity for a range of neonicotinoids and, thus, increase rice brown plant hopper resistance to neonicotinoids (Liu et al., 2006; Yixi et al., 2009). It is yet to be observed whether an α subunit mutation or variant that imparts neonicotinoid tolerance exists naturally in honey bee populations.

In the honey bee, the α subunits that have been shown to be most prevalent in the brain are α2, α3, and α7-1 and α7-2 (Thany et al., 2003; Thany & Crozatier, 2005). Apisα3 is largely expressed in adult bees in the optic lobes, mushroom bodies, antennal lobes, and dorsal lobes (Thany et al., 2003), Apisα2 is expressed in the mushroom bodies, dorsal and optic lobes of adult honey bees, Apisα7-1 in mushroom bodies, dorsal and antennal lobes, and Apisα7-2 is in mushroom bodies, dorsal, optic, and antennal lobes (Thany & Crozatier, 2005). This suggests that these subunits play a key role in sensory processing and memory. Disruption of these target subunits by neonicotinoids would therefore be likely to induce the sublethal effects reported in bees on learning and memory (Blacquière et al., 2012; Cresswell, 2011; Mommaerts et al., 2010; Schneider et al., 2012). At this time, it is unknown whether certain strains of honey bees possess an α subunit architecture that might have lower binding affinity for imidacloprid than other strains. Such architecture could confer additional tolerance to imidacloprid.

1.6.2 Metabolic Detoxification

The super families of metabolic enzymes that could contribute to imidacloprid tolerance include cytochrome P450 monooxygenases (P450s), glutathione *S*-transferases (GSTs), and general esterases (ESTs). One previous study suggested that the sensitivity of *A. mellifera* to pesticides might be related to the fact that they have fewer overall genes for these super families of metabolic enzymes than other insects (Claudianos et al., 2006).

The P450s are a super family of phase 1 detoxification enzymes that act on a very broad range of compounds and are found in almost all organisms across all domains of life (Denisov et al., 2005). The P450 super family has about 20% conserved gene homology and a common protein folding and topology across all life forms that have them (Denisov et al., 2005). Their primary chemical mechanism of action involves a heme-oxygen complex that performs redox reactions on almost all xenobiotics (Denisov et al., 2005). A previous study has shown that imidacloprid is rapidly broken down by P450s to 5-hydroxyimidacloprid, olefin, and other residues such that little to no imidacloprid is found within honey bees after 6 and 24 hour exposures (Suchail, Debrauwer, & Belzunces, 2004). The whitefly, *Bemisia tabaci*, and the fruit fly, *Drosophila melanogaster*, have P450 variants that are directly correlated with imidacloprid metabolism and resistance (Cheesman et al., 2013; Karunker et al., 2009). A recent study was able to show that up regulation of four different clades of P450s were associated with exposure to the neonicotinoid thiacloprid and may be involved in its detoxification (Alptekin et al., 2016). These data suggest a strong possibility that P450s could play a key role in imidacloprid detoxification in honey bees.

A vital secondary detoxification enzyme that is found in all aerobic organisms are GSTs, a super family of metabolic enzymes consisting of about 25 families (Enayati, Ranson, &

Hemingway, 2005; Hemingway, 2000). GSTs mainly facilitate secondary metabolite detoxification via conjugation of electrophilic compounds to the thiol group on reduced glutathione (GSH), neutralizing the electrophilic sites of an originally lipophilic substrate and resulting in a thioester compound that is water soluble and more readily excreted (Alptekin et al., 2016; Claudianos et al., 2006; du Rand et al., 2015; Enayati et al., 2005; Hemingway, 2000). It is by this method of GSH conjugation that GSTs have been known to provide a broad range of insecticide resistance in conjunction with P450. The step 1 metabolic products of imidacloprid, some of which have been shown to have their own toxic effects on bees, possess chemical structures that make them susceptible to GSH conjugation (Suchail et al., 2004).

General esterases are a family of enzymes that act mainly by hydrolyzing the ester bonds of both endogenous molecules and xenobiotics (Montella, Schama, & Valle, 2012). However, esterases are incapable of acting on neonicotinoids in this fashion as neonicotinoids do not have ester bonds (Iwasa et al., 2004). Other than hydrolysis of ester bonds, though, esterases are capable of inactively binding to a xenobiotic, slowing their turnover rate there by providing a broad range of insecticide resistance (Alptekin et al., 2016; Hemingway, 2000; Montella et al., 2012). *Apis mellifera* is known to have only eight genes that code for carboxyl/cholinesterases that are involved in insecticide metabolism, so it is possible that their contribution to neonicotinoid tolerance may be minimal (Claudianos et al., 2006).

1.6.3 Aims

There are two overarching goals for this study in regard to the above literature review. My first aim was to assess local honey bee populations for sensitivity to imidacloprid. Second was to examine the metabolic profiles of the most and least imidacloprid-susceptible colonies.

The first aim allowed me to identify two bee colonies with extreme differences in acute toxicities represented by significantly different LC_{50} values. The second aim was to identify differences in the metabolic detoxification activities of the two colonies at the extreme ends of the acute toxicity range that might be informative of a possible mechanism of imidacloprid tolerance.

CHAPTER 2

Toxicological Analysis of the Neonicotinoid Insecticide Imidacloprid to Honey Bees, *Apis mellifera*, of Different Colonies

2.1 INTRODUCTION

The honey bee, *Apis mellifera*, is the most economically important crop pollinator with a net value of over $15 billion in crop revenue (Fairbrother et al., 2014). The increase in annual colony losses in recent decades has been a cause for some economic and ecological concern, and has spurred a wave of research into colony decline. Research into colony decline has indicated a number of factors that may contribute to the decline in managed bee colonies, which include, but are not limited to, bee-specific parasites, modern beekeeping techniques, and the spread of bee-specific pathogens (Allen-Wardell et al., 1998; Wu et al., 2011). Among those concerns is the wide-spread use of pesticides in modern agriculture (Allen-Wardell et al., 1998; Brown et al., 1996; Fairbrother et al., 2014; Johansen, 1977).

One family of insecticides that is used in agriculture is neonicotinoids. As the name implies, this family of insecticides is similar in structure to nicotine, and acts as an agonist of the insect nicotinic acetylcholine receptor (nAChR) (Bai et al., 1991; Bodereau-Dubois et al., 2012; Kagabu & Matsuno, 1997; Mathé-Allainmat et al., 2013). One of the first neonicotinoids to be developed, and the most commercially available, is imidacloprid, which works by binding to nAChRs with high affinity and causing slow and prolonged depolarizations of the neurons (Bai et al., 1991). In laboratory studies, exposure of honey bees to neonicotinoid insecticides is reported to have acute lethal effects in addition to sublethal effects on colony health, such as reduced adult longevity, increased brood mortality and development time (Wu et al., 2011).

However, the acute and chronic toxicity of imidacloprid in honey bees has been found to be variable between studies (Fairbrother et al., 2014; Iwasa et al., 2004; Mommaerts et al., 2010). The variability of honey bee sensitivity to imidacloprid may be due to a colony-based sensitivity to the insecticide. In a previous study investigating induced insensitivity to the neonicotinoid insecticide, thiacloprid, honey bees were shown to have increased metabolic detoxification gene expression in response to a sublethal exposure to the insecticide (Alptekin et al., 2016). It has also been reported that dietary intake of imidacloprid has a more pronounced effect on bumble bee workers than on honey bee workers (Cresswell, Page, et al., 2012; Cresswell, Robert, Florance, & Smirnoff, 2014). This is due to the ability of individual honey bees to metabolically clear dietary imidacloprid from their bodies more quickly than bumble bee workers. Therefore, it is possible that differences in imidacloprid sensitivity between different colonies could be metabolically related.

The goal of this study was to examine the acute toxicity of imidacloprid to honey bees collected from different colonies and to compare the metabolic detoxification enzyme activities of the honey bees to better understand the mechanism(s) of imidacloprid susceptibility in these honey bees.

2.2 MATERIALS AND METHODS

2.2.1 Acute Toxicity Bioassays with Imidacloprid

Acute toxicity bioassays were conducted using honey bee workers collected from pesticide untreated hives at apiaries maintained at Virginia Tech Price's Fork, Kentland Farm, and Moore Farm apiaries (Blacksburg, VA). Prices Fork is a dedicated research apiary, the Kentland Farm apiary is located in a agricultural research farm, and the Moore Farm apiary is

located near cattle grazing pastures. Approximately 400 honey bees were sampled from the center of brood nest frames from 19 different colonies and brought back to the laboratory for the analysis of imidacloprid toxicity. Honey bees were placed into experimental cages in an environmental chamber maintained at 32°C with 60% relative humidity. The honey bees were fed *ad libitum* with a 50% sucrose solution (w/v) overnight. If higher than 10% mortality was observed overnight in these honey bees, the collection and caging of honey bees was repeated, otherwise treatment with imidacloprid was applied. The honey bees were exposed to six nominal concentrations of imidacloprid that provide a range of 10-90% mortality after a 24-hour (h) exposure period. The same procedure was used for honey bees without imidacloprid as a control treatment. The honey bees were placed in experiment cages in an environmental chamber maintained at 32°C with 60% relative humidity and fed *ad libitum* with a 50% sucrose solution (w/v) 24 h. The bioassays were repeated four times for each imidacloprid concentration and control treatment. The lethal endpoint for each bioassay was measured as a lethal concentration (LC) for those individuals incapable of righting themselves following the imidacloprid exposure period.

2.2.2 Determination of General Esterase Activity

The general esterase activity of honey bees was measured according to the method described by Rakotondravelo et al. (2006) with some modifications. The head and thorax of each honey bee sample (n = 3) was homogenized in ice-cold 0.1 molar (M) sodium phosphate (pH 7.8) containing 0.3% (v/v) Triton X-100 (Sigma Aldrich, St. Louis, MO) at the rate of one honey bee per 1 milliliter (mL) of sodium phosphate. The individual homogenates were centrifuged at 10,000 times gravity (x g) for 10 min at 4°C and the supernatant was used as the

enzyme source for measuring general esterase activity with 0.3 mM α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA) as substrates. The hydrolysis of α-NA and β-NA to the products α-naphthol and β-naphthol, respectively, was measured using a microplate absorbance reader at 600 nanometer (nm) and 560 nm for α-NA and β-NA, respectively.

To assess the effect of metabolic synergists triphenylphosphate (TPP) and S,S,S-tributyl phosphorotrithioate (DEF) on general esterase activity, honey bees from each colony were exposed to TPP and DEF at 10 parts per million (ppm), 100 ppm, and 10,000 ppm, both *ad libitum* and topically for periods of 2 h and 4 h (n = 10 per test group). General esterase activity assays were run both in the presence and absence of TPP and DEF. The synergists were optimized for time and concentration with the purpose of obtaining maximal inhibition of the desired enzymes without any honey bee mortality before being used in acute toxicity bioassays.

2.2.3 Determination of Cytochrome P450 Monooxygenase Activity

The cytochrome P450 monooxygenase (P450) activity of the honey bees was measured according to the method described by Anderson and Zhu (2004) with some modifications. The abdomen of each honey bee sample (n = 3), with the stinger and venom gland removed, was homogenized in ice-cold 0.1 M sodium phosphate (pH 7.8) at the rate of 1 honey bee per 1 mL sodium phosphate. The individual homogenates were centrifuged at 10,000 x g for 5 min at 4°C and the supernatant was used as the enzyme source. The individual supernatants were transferred to the wells of a 96-well microplate containing 50 millimolar (mM) sodium phosphate (pH 7.2), 0.4 mM 7-ethoxycoumarin, 62.5 mM β-NADPH, 100 mM oxidized glutathione, and 1 enzyme unit (U) glutathione reductase. The honey bee supernatant was incubated at 37°C for 4 h followed by the addition of 50% (v/v) acetonitrile and Trizma base (pH

10). The P450-mediated deethylation of 7-ethoxycoumarin to the product 7-hydroxycoumarin was measured using a microplate fluorescence reader at 480 nm while exciting at 380 nm.

To assess the effect of metabolic synergist piperonyl butoxide (PBO) on P450 activity, honey bees from each colony were exposed to PBO at 10 ppm, 100 ppm, and 10,000 ppm, both *ad libitum* and topically for periods of 2 h and 4 h (n = 10 per test group). P450 monooxygenases activity assays were run both in the presence and absence of PBO. The synergist was optimized for time and concentration with the purpose of obtaining maximal inhibition of the desired enzymes without any honey bee mortality before being used in acute toxicity bioassays.

2.2.4 Determination of Glutathione S-Transferase Activity

The glutathione *S*-transferase (GST) activity of the honey bees was determined according to Jin-Clark et al. (2008) with some modifications. The head and thorax of each honey bee sample (n = 3) was homogenized in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% (v/v) Triton X-100 (Sigma Aldrich) at the rate of 1 honey bee per 1 mL sodium phosphate. The individual homogenates were centrifuged at 10,000 x g for 10 min at 4 ˚C and the supernatant was used as the enzyme source for measuring GST activity using 1-chloro-2,4- dinitrobenzene (CDNB) as a substrate. The GST-mediated conjugation of glutathione towards CDNB was determined by recording the change in absorbance at 340 nm for 20 min at 10-sec intervals using a microplate absorbance reader.

To assess the effect of metabolic synergist diethyl maleate (DEM) on GST activity, honey bees from each colony were exposed to DEM at 10 ppm, 100 ppm, and 10,000 ppm, both *ad libitum* and topically for periods of 2 h and 4 h (n = 10 per test group). Glutathione *S*-

transferase activity assays were run both in the presence and absence of DEM. The synergist was optimized for time and concentration with the purpose of obtaining maximal inhibition of the desired enzymes without any honey bee mortality before being used in acute toxicity bioassays.

2.2.5 Measurement of Total Protein

The total protein in each enzyme sample preparation was determined using a bicinchoninic acid assay as described by Smith et al. (1985) with bovine serum albumin (Sigma Aldrich) as a standard. Each honey bee sample (n = 3 per test group) was homogenized in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% (v/v) Triton X-100 (Sigma Aldrich) at the rate of one honey bee per 1 mL sodium phosphate. The individual homogenates were centrifuged at 10,000 x g for 10 min at 4 ˚C and used for measuring the amount of protein in each sample. Ten microliters of the supernatants were added to the individual wells of a 96-well microplate containing 10 µL of 0.1 M sodium phosphate (pH 7.8) and 90 µL bicinchoninic acid with 4% (v/v) copper sulfate. The protein samples were incubated for 30 min. at 37 °C and cooled to room temperature for 5 min. The total protein content in the honey bee samples was measured at 560 nm using a Molecular Devices SpectraMax M2® multimode microplate reader (Sunnyvale, CA). The optical densities of the protein samples were compared to those measured for the protein standard of bovine serum albumin.

2.2.6 Percent Mortality Bioassays with Imidacloprid and Metabolic Synergist

To assess the combined effect of imidacloprid with the synergists PBO, TPP, DEF, and DEM, the honey bees were exposed to a LC_{50} concentration of imidacloprid alone and in

combination with PBO, TPP, DEF, and DEM at 100 ppm. The synergists were optimized for time and concentration with the purpose of obtaining maximal inhibition of the desired enzymes without any honey bee mortality in each of the subsequent metabolic assays. The imidacloprid exposure methods are the same as described in section 2.2.1; however, the honey bees were provided a synergist treatment prior to imidacloprid exposure. DEF and TPP treatments were applied *ad libitum* for 4 h prior to imidacloprid exposure. PBO treatments were 2 µL topical treatment of each synergist for 4 h prior to imidacloprid exposure. DEM treatments were 2 µL topical treatment of each synergist for 2 h prior to imidacloprid exposure. DEF and TPP were applied *ad libitum* because topical administration precipitated on the cuticle. PBO and DEM were applied topically because the reduction of metabolic activity was greater with topical application than oral.

2.2.7 Determination of Glutathione S-Transferase Kinetic Activity

The kinetic activity for glutathione *S*-transferase of the imidacloprid-susceptible and -tolerant colonies was determined by the same method in section 2.2.4 with some modifications. The supernatants of the homogenate of three individual bees were pooled into one tube, and counted as a single biological replicate. Three of these replicates were used per colony per assay. The pooled supernatants were used as the enzyme source for measuring glutathione *S*-transferase activity using CDNB at 8 mM, 4 mM, 0.8 mM, 0.4 mM, 0.08 mM, and 0.04 mM, alone and in the presence of DEM at 0.029 mM, 0.0145 mM, 0.0073 mM, 0.0036 mM, and 0.0018 mM. For the determination of IC_{50} (concentration of inhibitor that achieves 50% inhibition) of DEM in samples from each colony, the above assay was run using 8 mM CDNB in

the presence and absence of the following range of DEM concentrations: 0.3 mM, 0.03 mM, 0.003 mM, 0.0003 mM, 0.00003 mM, and 0.000003 mM.

2.2.8 Statistical Analysis

The acute toxicity bioassays were analyzed using both probit analysis and analysis of variance (ANOVA) analysis. Probit analysis was performed using Polo Plus© Probit and Logit Analysis Software. ANOVA analysis and student's t-tests were performed using JMP® Pro statistical software to explore the significance of the relationships between the arcsine square root of the percent mortality and colony, apiary location, and imidacloprid concentration (μM), in a factorial manner. The effect of metabolic synergists on mortality when employed in acute toxicity bioassays was analyzed by one way ANOVA and for each paired comparison a student's t-test using JMP® Pro statistical software. Michaelis-Menten kinetic analysis of the GSTs in the imidacloprid-susceptible and -tolerant honey bees were calculated using GraphPad Prism® software.

2.3 RESULTS

2.3.1 Acute Toxicity Bioassays with Imidacloprid

The acute toxicities of imidacloprid to honey bees collected from different colonies at three apiaries are shown as LC_{50} values in Table 1. The acute toxicity of imidacloprid to honey bees ranged from ~8 to ~191 μM, with a median of ~58 μM, at Kentland Farm where as the acute toxicity of imidacloprid for honey bees at Moore Farm ranged from ~5 to ~99 μM, with a median of ~16 μM (Table 1). The acute toxicity of imidacloprid to honey bees ranged from ~11 to ~146 μM, with a median of ~47 μM, at Price's Fork (Table 1). Collectively, the acute toxicity

of imidacloprid to honey bees ranged from ~5 to ~191 μM, with a median of ~24 μM, at Kentland Farm, Moore Farm, and Price's Fork (Table 1). The LC_{50} values for honey bees collected from colonies 56 and 28 are ~5 μM and ~167 μM, respectively (Table 1). Honey bees from these colonies will be referred to as imidacloprid-susceptible and –tolerant, respectively, in the following bioassays based on their 36-fold difference in imidacloprid toxicity. The honey bees collected from colony 90 had a LC_{50} of ~191 μM, which is not significantly different from that estimated for colony 28, based on a student's t-test and overlapping 95% confidence intervals (Table 1). The honey bee colony from hive 90 did not survive the wintering period following the acute toxicity bioassays with imidacloprid and, thus, was not used for this study.

The ANOVA analysis of the acute toxicity bioassays represents the combined percent mortality data (normalized by taking the arcsine of the data), the colony identifier, apiary location, and imidacloprid concentration to determine a significant interaction between the combinations. A significant effect between the actual mortality and the predicted mortality indicated that the data was a good fit for the overall model ($p < 0.05$). There was a significant difference in the percent imidacloprid mortality for honey bees collected from the different colonies in each apiary ($p < 0.05$) with the imidacloprid-susceptible (colony 56) bees being most associated with mortality and the imidacloprid-tolerant (colony 28) bees from being least associated. In addition, the percent imidacloprid mortality for honey bees was significantly affected by the location of the apiary ($p < 0.05$), with Moore Farm being significantly different from Kentland Farm and Price's Fork. There was a significant difference in percent mortality between imidacloprid concentrations ($p < 0.05$); however, no single imidacloprid concentration was more highly associated with the percent imidacloprid mortality for honey bees at any one apiary location versus the other apiary locations ($p > 0.05$).

2.3.2 Determination of General Esterase Activity

The specific activity for general esterase of imidacloprid-susceptible and -tolerant honey bees was analyzed with and without the synergists TPP and DEF (100 ppm) applied *ad libitum* for 4 h. The general esterase specific activity for the imidacloprid-susceptible honey bees, without TPP and DEF exposure, was 14.72 ± 0.70 and 22.11 ± 1.82 nmol/min/mg protein, using α- and β-naphthyl acetate as substrates, respectively. The general esterase specific activity for the imidacloprid-tolerant honey bees, without TPP and DEF exposure, was 14.5 ± 1.18 and 19.11 ± 2.58 nmol/min/mg protein, using α- and β-naphthyl acetate as substrates, respectively. The general esterase specific activity of the imidacloprid-susceptible and -tolerant honey bees treated with TPP was 9.07 ± 0.96 and 10.36 ± 0.68 nmol/min/mg protein, respectively, using α-naphthyl acetate as a substrate. The general esterase specific activity of the imidacloprid-susceptible and -tolerant honey bees treated with TPP was 11.77 ± 1.51 and 14.07 ± 1.05 nmol/min/mg protein, respectively, using β-naphthyl acetate as a substrate.

The general esterase specific activity of the imidacloprid-susceptible and -tolerant honey bees treated with DEF was 11.08 ± 1.28 and 10.06 ± 1.05 nmol/min/mg protein, respectively, using α-naphthyl acetate as a substrate. The general esterase specific activity of the imidacloprid-susceptible and -tolerant honey bees treated with DEF was 16.17 ± 1.92 and 13.19 ± 1.90 nmol/min/mg protein, respectively, using β-naphthyl acetate as a substrate. Both TPP and DEF significantly reduced the general esterase activity of imidacloprid-susceptible and -tolerant honey bees using α-naphthyl acetate as a substrate (student's t-test, $p < 0.05$).

2.3.3 Determination of Cytochrome P450 Monooxygenase Activity

The cytochrome P450 monooxygenase (P450) activity of imidacloprid-susceptible and -tolerant honey bees was analyzed with the synergist PBO (100 ppm) applied topically for 4 h. The P450 activity of the imidacloprid-susceptible and -tolerant honey bees, without PBO treatment, was 344 ± 45 and 138 ± 30 RFU/mg protein, respectively. The P450 activity of the imidacloprid-susceptible and -tolerant honey bees, with PBO treatment, was 319 ± 115 and 62 ± 33 RFU/mg protein, respectively. The P450 activities of imidacloprid-susceptible and -tolerant honey bees, with and without PBO treatment, were not significantly different.

2.3.4 Determination of Glutathione S-Transferase Activity

The glutathione *S*-transferase (GST) activity of imidacloprid-susceptible and -tolerant honey bees was analyzed with the synergist DEM (100 ppm) applied topically for 2 h. The GST activity of the imidacloprid-susceptible and -tolerant honey bees, without DEM treatment, was 160.00 ± 6.84 and 183.20 ± 10.54 nmol/min/mg protein, respectively. The GST activity of the imidacloprid-susceptible and -tolerant honey bees, with DEM treatment, was 150.59 ± 21.21 and 140.86 ± 23.56 nmol/min/mg protein, respectively. The GST activities of imidacloprid-susceptible and -tolerant honey bees, with and without DEM treatment, were not significantly different.

2.3.5 Percent Mortality Bioassays with Imidacloprid and Metabolic Synergist

The percent mortality of the susceptible and tolerant honey bees to imidacloprid was examined with and without pre-exposure to the synergists TPP, DEF, PBO, and DEM. There was no significant increase in percent mortality for honey bees exposed to imidacloprid in

combination with TPP, DEF, or PBO compared to imidacloprid-treated individuals without the synergists (Fig. 1-2). However, there was a significant increase in percent mortality for honey bees exposed to imidacloprid in combination with DEM when compared to imidacloprid only treatments (Fig. 3). The percent mortality of the imidacloprid-susceptible honey bees treated with DEM increased by 37.56% ± 0.03 ($p = 0.0168$) compared to the honey bees without DEM treatment. The percent mortality of the imidacloprid-tolerant honey bees treated with DEM increased by 16.44 % ± 0.01 ($p = 0.0107$) compared to the honey bees without DEM treatment.

2.3.6 Determination of Glutathione S-Transferase Kinetic Activity

The glutathione *S*-transferase kinetic activity was examined in imidacloprid-susceptible and -tolerant honey bees. The Michaelis-Menten (Km) values for imidacloprid-susceptible and -tolerant honey bee GSTs are 0.66 ± 0.22 and 0.14 ± 0.06 mM, respectively (Fig. 4). The maximal velocity (Vmax) values for imidacloprid-susceptible and -tolerant honey bee GSTs are 59.07 ± 5.23 and 74.63 ± 6.36 nmol/min/mg protein, respectively (Fig. 4). The bimolecular rate constant (k_i) for imidacloprid-susceptible and -tolerant honey bee GSTs treated with the GST inhibitor DEM were 17.23 ± 1.235 and 5.07 ± 0.098, respectively (Fig. 5).

The glutathione *S*-transferase (GST) activity of imidacloprid-susceptible and -tolerant honey bees was measured against six concentrations of the GST inhibitor DEM for 10 min with 8 mM CDNB as a substrate. The DEM IC_{50} for the imidacloprid-susceptible honey bee GST was 10 μM (5 - 19 95% CI, 0.9 ± 0.2 Hill Slope, R^2 0.90). The DEM IC_{50} for the imidacloprid-tolerant honey bee GST was 3 μM (1 - 7 95% CI, 0.7 ± 0.2 Hill Slope, R^2 0.92).

2.4 DISCUSSION

The purpose of this study was to examine the cause of colony-to-colony variability in imidacloprid sensitivity. To this end I performed acute toxicity bioassays to establish a range of susceptibility. The activity of critical metabolic enzymes was then suppressed with synergists, and tested if enzyme activity reduction has a significant effect on imidacloprid-associated mortality. Finally I assessed the GST kinetic profiles of my most imidacloprid-tolerant and -susceptible colonies.

Both the probit analysis and the fit model analysis of the acute toxicity bioassays revealed that there was indeed a significant difference in acute toxicities between colonies with colony 56 being the most susceptible to imidacloprid and colony 28 being most tolerant. These colonies have a 36-fold difference in LC_{50} scores and were determined to be significantly different from each other in both a probit and a fit model analysis. The apiary location most associated with mortality was Moore Farm and was significantly different from Kentland Farm or the Prices Fork apiaries. While there was a significant difference in mortality between imidacloprid concentrations and between apiary locations, the effect of imidacloprid concentration on mortality was not significantly different between apiary locations. Previous studies have shown that the LD_{50} values for neonicotinoids can vary greatly in honey bees in both oral and contact exposure routes (Blacquière et al., 2012; Fairbrother et al., 2014; Iwasa et al., 2004; Mommaerts et al., 2010). The results of the acute toxicity bioassays in this study are in line with these previous findings.

Neither TPP, DEF, nor PBO in conjunction with imidacloprid administration produced a significant difference in mortality compared to imidacloprid administration alone in either the imidacloprid-susceptible or the imidacloprid-tolerant colony. However, treatment with DEM did

significantly increase mortality. This effect could be explained as follows. Even though ESTs are incapable of metabolizing imidacloprid due to its lack of an ester bond (Iwasa et al., 2004), ESTs have also been reported to provide broad-range insecticide resistance by sequestration (Hemingway, 2000). Certain esterases have been shown to be able to sequester imidacloprid in other insects, for example the esterase FE4 is known to be able to sequester imidacloprid in the aphid *Myzus persicae* (Philippou, Field, & Moores, 2010). The *Apis mellifera* genome does not code for FE-4, but a probable FE4-like esterase, CEst04 (Boncristiani et al., 2012). The results of my tests did not show that EST reduction increases susceptibility to imidacloprid. Several studies in the past have shown that P450 is both heavily correlated with and has a direct mechanism for the detoxification of imidacloprid, mainly in other insect species, but also as an important detoxification enzyme for neonicotinoids in honey bees (Alptekin et al., 2016; Cheesman et al., 2013; Crossthwaite, Rendine, Stenta, & Slater, 2014; Karunker et al., 2009; Suchail et al., 2004). The results of my tests were not in line with these previous studies. This does not mean that P450s play no role in imidacloprid detoxification, only that the reduction of P450 activity did not produce a significant increase in imidacloprid-associated mortality in this study.

An analysis of GST kinetic activity in the imidacloprid-susceptible and -tolerant colonies revealed that the susceptible colony had a k_i of 17.23 ± 1.235 nmol/min/mg protein and an IC_{50} of 10 μM (5 - 19 95% CI, 0.9 ± 0.2 Hill Slope, R^2 0.90) and the tolerant colony has a k_i of 5.07 ± 0.098 nmol/min/mg protein and an IC_{50} of 3 μM (1 - 7 95% CI, 0.7 ± 0.2 Hill Slope, R^2 0.92). The lower k_i and IC_{50} values of the tolerant colony indicate that GSTs might have a higher ligand binding affinity than the susceptible colony. Higher binding affinity, in turn, indicates that the GSTs of the tolerant colony more efficiently bind to and recruit GSH for conjugation to stage

one metabolites of imidacloprid. GSTs are known to provide broad-range insecticide resistance often as a secondary resistance mechanism in concert with esterases and P450s (Hemingway, 2000). GSTs have been reported in the past to be critical in the detoxification of insecticides such as organophosphates and organochlorines, and play a role in insecticide resistance both in honey bees and in other insects (Claudianos et al., 2006; du Rand et al., 2015; Enayati et al., 2005; Grant & Matsumura, 1989). Both the increased mortality of bees treated with DEM and imidacloprid in acute toxicity bioassays, and the higher ligand binding affinity of the tolerant colony suggest an important role of GSTs that are supported by previous studies.

There are some challenges to this kind of study, extraneous factors that could have an effect on imidacloprid toxicity in honey bees that were not accounted for. There are two extraneous factors in sampling the cohort; workers were collected without controlling for worker age, and only workers were collected. Worker bees perform different tasks in the colony as they age, progressing from nurse to forager, referred to as age polyethism, and these behavioral changes may reflect metabolic changes in the worker (Crailsheim, 1990; Margotta et al., 2012; Page & Peng, 2001; Rueppell, Christine, Mulcrone, & Groves, 2007). Workers were collected in this study without regard to age or role of labor in the colony in order to assay overall colony tolerance. While different castes are known to have different metabolic profiles (Begna, Fang, Feng, & Li, 2011; Begna, Han, Feng, Fang, & Li, 2012; Jianke Li et al., 2010), workers are both the most numerous caste, the caste that has the most contact with pesticides, and the caste that brings pesticides back to the colony. The effect of landscape was also not controlled or accounted for, and unknown and uncontrolled variables could enter into each apiary location, such as nutrition, that could have an effect on colony health and detoxification capabilities (Klein et al., 2007; Potts et al., 2010).

Besides metabolic detoxification there is the possibility that target site resistance could play a role in imidacloprid tolerance. Other insects such as *Nilaparvata lugens* have been shown to have increased resistance to imidacloprid due to point mutations in the genes coding for the α subunits of their nAChRs (Liu et al., 2006; Yixi et al., 2009). Though the possibility of such a target site tolerance in honey bees is not explored in this study, metabolic detoxification alone might not be the sole cause of such extreme differences in imidacloprid tolerance as was reported here. Future avenues of research could involve examining the role of landscape with common garden experiments, testing for effects of nAChR architecture on imidacloprid susceptibility in different honey bee populations with radioligand binding assays, or the mapping of qualitative trait loci (QTL) to assess informative differences in the genetic architecture of bee populations with different sensitivities to imidacloprid.

In an effort to maintain maximum crop yields farmers must strike a balance between pest management and harming crop-pollinating insects. To this end, there is a demand for pesticides with high specificity for target organisms. With such specificity there is the drawback that the target organism could eventually evolve a tolerance to the pesticide. Additionally, a new pest could enter the environment that is unaffected by such a highly specific chemical. If an important crop pollinator, like the honey bee can be made tolerant towards a family of pesticides with a broad application range, then it could help farmers to more easily maintain that balance of managing insects that are harmful to their crops while sparing insects that are beneficial.

Table1. Imidacloprid Toxicity Data Across Multiple Hives

Colony Location [c]	Colony #	Cohort Size	LC_{50} (μM) [a]	Slope	Standard Error	Chi-square [b]	95% lower
Moore	56	318	4.505	1.527	0.164	78.518	1.97
Kentland	44	319	7.666	0.974	0.112	37.236	3.848
Moore	59	313	7.915	1.169	0.130	37.352	4.377
Moore	62	315	10.476	1.149	0.127	16.344	6.957
Prices Fork	39	307	11.114	0.970	0.113	39.317	5.468
Prices Fork	5	307	15.987	1.302	0.145	30.759	9.671
Prices Fork	14	311	17.688	0.853	0.108	51.3	7.537
Kentland	30(2)	312	20.855	1.086	0.126	42.267	10.919
Moore	63	320	21.824	0.971	0.115	57.554	9.654
Moore	51	319	23.901	0.953	0.118	54.541	10.691
Kentland	29	316	27.632	0.801	0.106	50.921	11.359
Prices Fork	8	319	46.959	1.169	0.146	29.735	27.769
Prices Fork	52	294	60.579	0.800	0.119	38.407	26.241
Prices Fork	4	314	76.916	0.755	0.113	30.813	35.046
Kentland	30(1)	311	87.962	0.732	0.111	28.565	39.434
Moore	61	317	99.334	1.211	0.171	22.557	60.91

[a] The oral toxicity data are presented as LC_{50} and their 95 % confidence intervals in micro molar (μM), the concentrations at which 50 % of tested bees experienced mortality in a 24-hour bioassay. Log-probit analysis was used to estimate the endpoint concentration for imidacloprid.

[b] Chi-square goodness of fit test. Chi-square values displayed. Degrees of freedom = 21 for these tests, meaning that if chi-square < 32.671, then $p > 0.05$. The probability of $p > 0.05$ indicates that the observed regression model is not significantly different from the expected model.

[c] Colony Location represents the apiary in which a given colony is located.

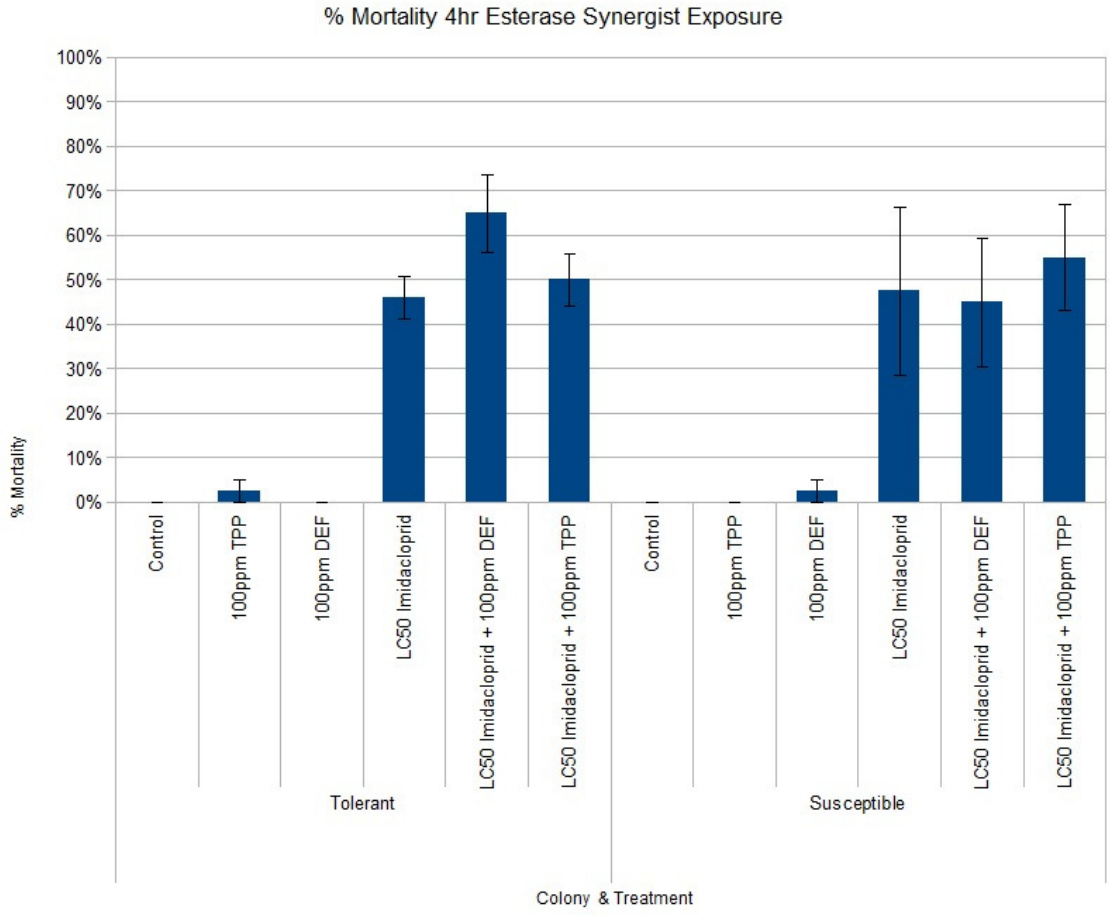

Figure 1. Bar graph of percent mortality of test groups in response to treatment with colony-specific LC_{50} of imidacloprid, both with and without the esterase inhibitors TPP and DEF. Control is 50 % sucrose solution with 0.3 % acetone to control for solvent effects. 100 ppm TPP and 100 ppm DEF treatments are 50 % sucrose solutions that contain a final concentration of 100 ppm of either TPP or DEF to control for synergist effects. LC_{50} of tolerant colony is 167 µM imidacloprid. LC_{50} of susceptible colony is 4.5 µM imidacloprid. Error bars represent standard error.

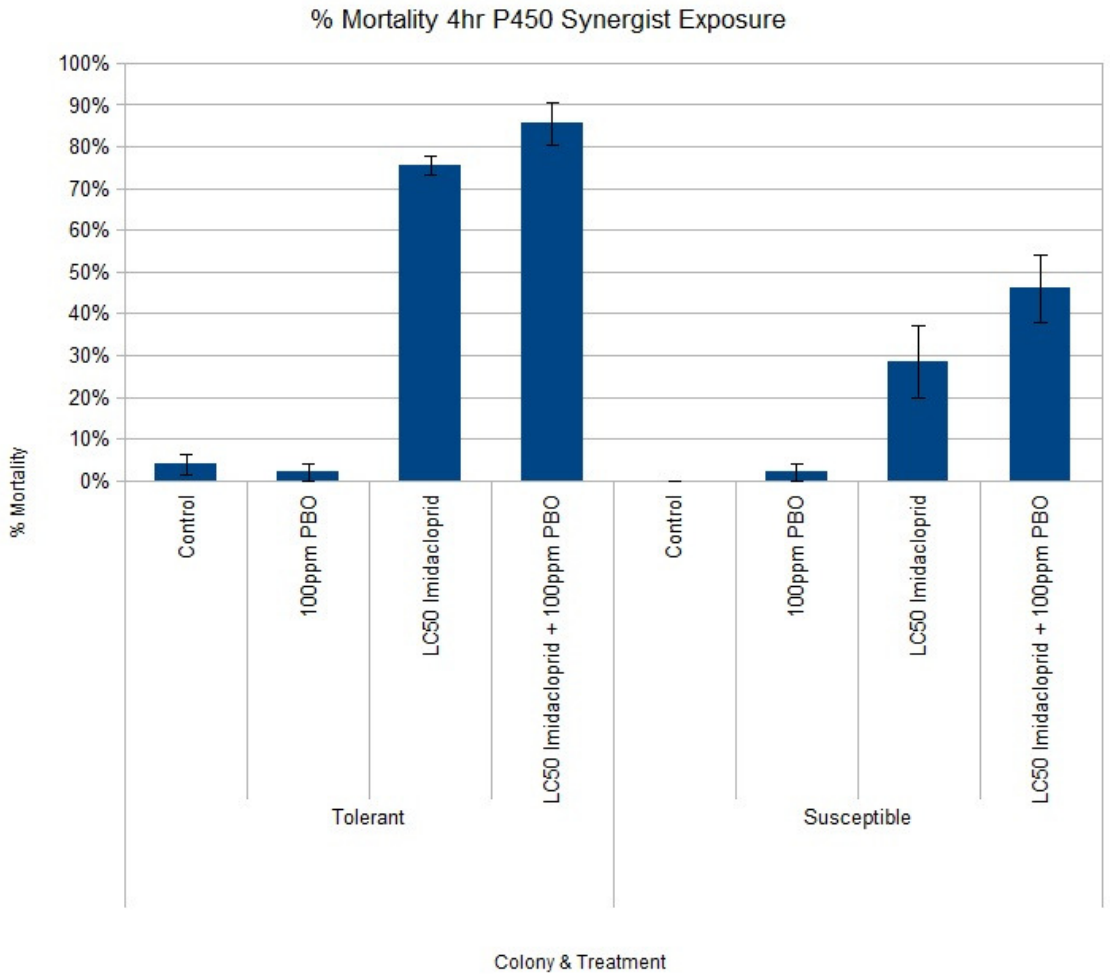

Figure 2. Bar graph of percent mortality of test groups in response to treatment with colony-specific LC_{50} of imidacloprid, both with and without the P450 inhibitor PBO. Control is 50% sucrose solution with 0.3% acetone to control for solvent effects. 100 ppm PBO treatment is 2 µL AE containing 100 ppm DEM applied topically on abdomen to control for synergist effects. LC_{50} of tolerant colony is 167 µM imidacloprid. LC_{50} of susceptible colony is 4.5 µM imidacloprid. Error bars represent standard error.

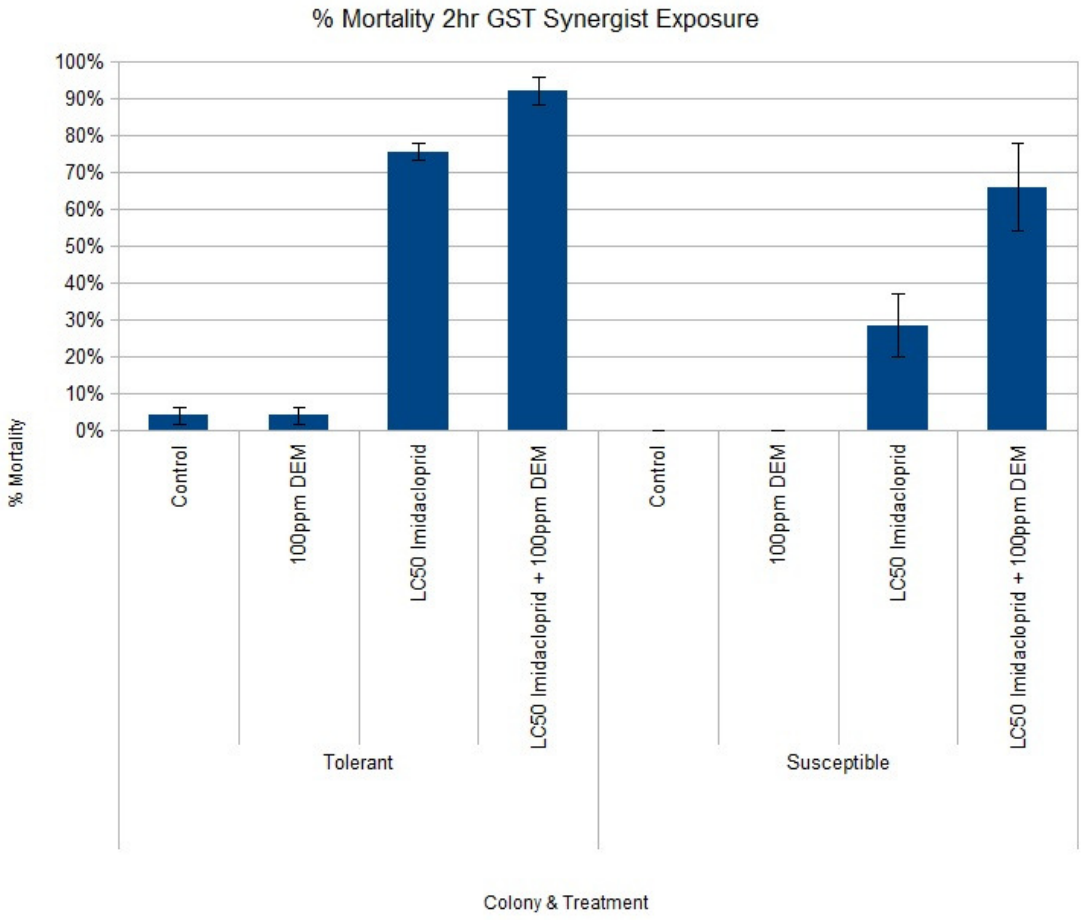

Figure 3. Bar graph of percent mortality of test groups in response to treatment with colony-specific LC_{50} of IMI, both with and without the GST inhibitor DEM. Control is 50% sucrose solution with 0.3% acetone to control for solvent effects. 100 ppm DEM treatment is 2 µL AE containing 100 ppm DEM applied topically on abdomen to control for synergist effects. LC_{50} of tolerant colony is 167 µM imidacloprid. LC_{50} of susceptible colony is 4.5 µM imidacloprid. Error bars represent standard error.

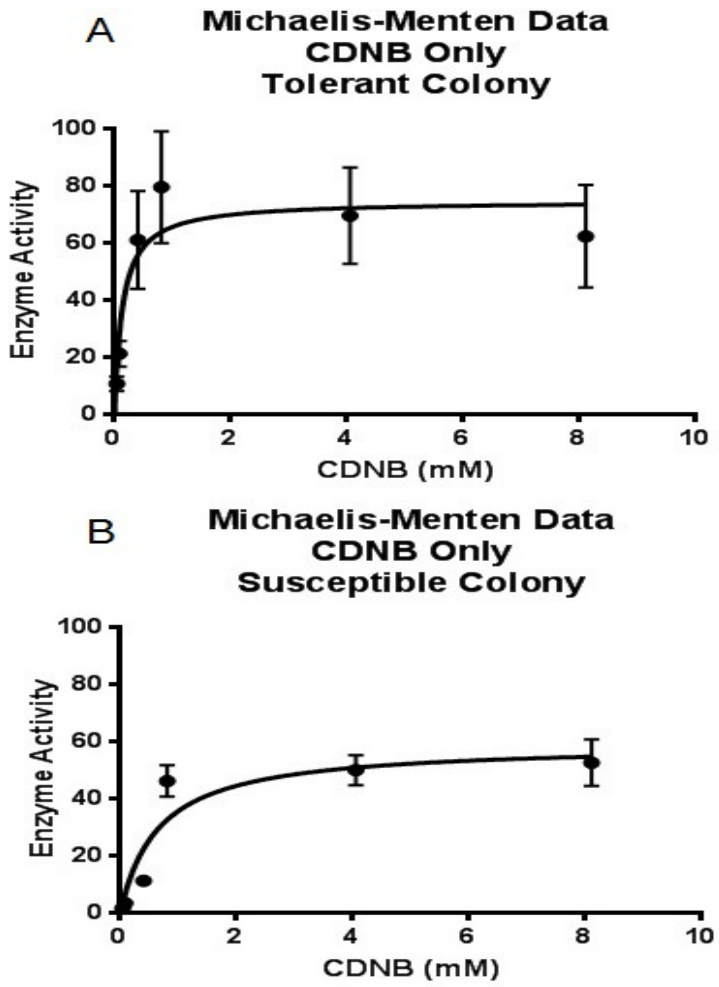

Figure 4. Michaelis-Menten plots of GST kinetic data at 8 minutes and 42 seconds. Plots display the relationship between the enzyme activity (nmol/min/mg protein) and the substrate (CDNB) concentration in milimolar (mM). A) Michaelis-Menten plot of GST kinetic activity in the imidacloprid tolerant colony. Km = 0.14 ± 0.06 mM, Vmax = 74.63 ± 6.36 nmol/min/mg protein. B) Michaelis-Menten plot of GST kinetic activity in the imidacloprid susceptible colony. Km = 0.66 ± 0.22 mM, Vmax = 59.07 ± 5.23 nmol/min/mg protein.

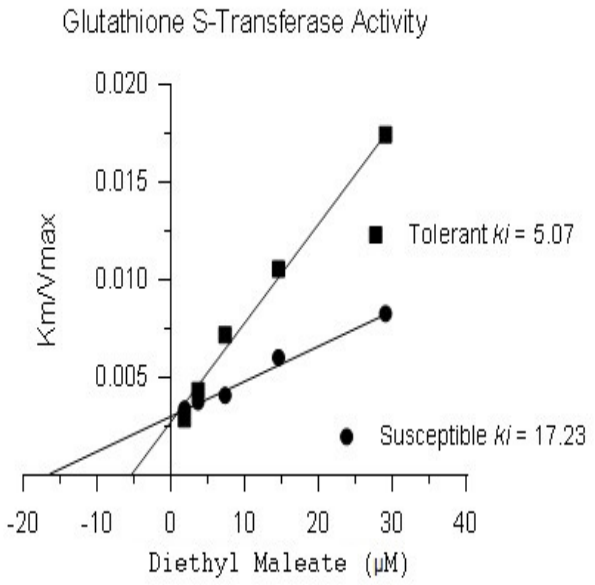

Figure 5. Linear regression curve of the relationship between the concentration of DEM in micro molar (µM) and the Km/Vmax in both the imidacloprid-tolerant and -susceptible colonies. Linear regression used to extrapolate X intercept values for each colony. Colony 28 has X intercept of -5.07. Colony 56 has X intercept of -17.23.

CHAPTER 3

SUMMARY

Several edible crops rely upon honey bee pollination for their existence. An estimated 35% of all food we eat is dependent upon or at least benefits from pollination services provided by honey bees (Klein et al., 2007; vanEngelsdorp & Meixner, 2010). Recent surveys by the Bee Informed Partnership estimate an average of 28.6 % overwintering losses recorded for the last ten years (Steinhauer et al., 2016). The evidence produced by various studies indicates that general colony decline is probably caused by several factors that work together in a non-additive way that increases the severity of each factor's effects when taken together (Allen-Wardell et al., 1998; Potts et al., 2010; vanEngelsdorp et al., 2009; Wu et al., 2011). This study examined the pesticide aspect of this web of factors and attempted to identify potential mechanisms of insecticide resistance in honey bees towards neonicotinoids.

The first objective of this research was to examine local honey bee colonies for differences in imidacloprid susceptibility. I performed acute toxicity bioassays on a number of colonies and established a range of acute toxicities to identify colonies at the upper and lower ends of this toxicity range from which to compare and contrast metabolic differences that might contribute to those extreme differences. Further analysis of the association of mortality with colony, apiary location, and imidacloprid concentration, were calculated and it was found that both individual colony and apiary location had a significant association with imidacloprid associated mortality.

The second objective was to compare the metabolic profiles of the most and least imidacloprid-susceptible colonies to test for differences that might impart greater imidacloprid tolerance to honey bees. Metabolic assays were run on bees both in the presence and absence of

synergists that are known to inhibit the actions of ESTs, P450s, and GSTs. After experimentally reducing metabolic enzyme activities bees from the tolerant and susceptible colonies were subjected to further acute toxicity bioassays both in the presence and absence of the metabolic synergists TPP, DEF, PBO, and DEM. A significant increase in imidacloprid-associated mortality was found with DEM treatment.

Therefore I focused on the enzyme kinetics of GSTs in the imidacloprid-tolerant and -susceptible colonies. After obtaining Km and Vmax values for each colony both in the absence and presence of a range of DEM concentrations, the Km/Vmax values were plotted against the DEM concentrations in a linear regression analysis to calculate the inhibition constant [k_i] values. A range of DEM concentrations was also run in conjunction with CDNB to calculate the concentration of DEM at which 50% inhibition of GST activity was achieved (IC_{50}). The tolerant colony was found to have a lower k_i and lower IC_{50} than the susceptible colony. These data suggest that the GSTs of the tolerant colony might be more efficient at conjugating the stage 1 metabolites of imidacloprid to reduced glutathione than the susceptible colony.

The goal of this study was to identify a possible detoxification mechanism that could account for differences in imidacloprid susceptibility between different honey bee populations. These data suggest that GSTs might play a role in imidacloprid tolerance in honey bees. Detoxification plays a role in insecticide tolerance in multiple species of insects, but can be influenced by a number of other factors both biotic and abiotic, and detoxification may not be the only factor contributing to insecticide tolerance. This study only points to one of the many possible elements that could have an effect on imidacloprid susceptibility in honey bees.

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