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Effects of Truck Based Ultra-Low Volume Mosquito Adulticides on Honey Bees (*Apis mellifera*) in a Real World Scenario

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EFFECTS OF TRUCK BASED ULTRA LOW VOLUME MOSQUITO ADULTICIDES ON
HONEY BEES (*APIS MELLIFERA*) IN A REAL WORLD SCENARIO

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements of the degree of
Master of Science

In

The Department of Entomology

by
Vivek Pokhrel
B.S., Tribhuvan University, 2011

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ABSTRACT

Honey bees are crucial pollinators for many economically important fruit crops. The recent honey bee colony decline in the United States and other regions of the world has caused concern among commercial beekeepers, research groups, the government, and the general public. The role of pesticides in recent honey bee declines has not been fully determined. In Louisiana, it is a common practice to spray truck based ultra-low volume mosquito adulticides in Integrated Mosquito Management Programs to control mosquitoes and minimize the risk of vector borne viruses such as West Nile, chikungunya, and Zika. This study measured the effects of truck based ultra-low volume (ULV) mosquito adulticides on honey bees in a real world scenario. We looked at mortality, colony health (number of adult bees, brood quantity and quality), and detoxification enzymes (esterase and glutathione *S*-transferase) on honey bees from sentinel bee hives in Baton Rouge, Louisiana over a seven week period. The mosquito adulticides used by mosquito control programs during this study were Scourge, Duet and Deltagard. We did not find significant differences in honey bee mortality, colony health (frames of bees and brood quality) or detoxification enzymes among our control and treatment sites over the seven weeks. We found differences in brood quantity between control and treatment; however only two colonies at one of our treatment sites skewed the result in brood quantity. Although the findings of this study suggests that there is no effect of truck based ultra-low volume mosquito adulticides on bee mortality, colony health, and detoxification enzymes on honey bees, there might be deleterious effects if mosquito adulticides are used inappropriately.

CHAPTER 1. INTRODUCTION

1.1 Honey Bee Biology and Taxonomy

The Western honey bee (*Apis mellifera*) has some of the most interesting biology and behaviors in the insect world. It is a eusocial insect with the characters of reproductive division of labor, cooperative brood care, and overlapping generations. Honey bees have a haplodiploidy system of sex determination in which males develop from unfertilized eggs and are haploid, whereas females develop from fertilized eggs and are diploid. Hamilton (1963; 1964) calculated the mathematical ratio of relatedness between sisters in the honey bee colony and suggested that “kin selection”, whereby an individual can pass its genes not only in a direct way (i.e., reproducing itself) but also indirectly, (i.e., by favoring the reproduction of their siblings), makes the honey bee a eusocial insect. The honey bee has three castes in a colony: a queen, workers and drones. A single, mated queen is the main reproductive unit and produces thousands of eggs in her life time. She also manipulates the colony reproduction with her pheromones. The colony is dominated numerically by unmated female workers. Workers clean the colony, take care for the brood, and forage for pollen and nectar. Male bees (drones) are fewer in number than females and are involved only in mating.

There are a number of subspecies of honey bee with different characteristics. Western honey bees, *Apis mellifera* Linnaeus, are in the order Hymenoptera, family Apidae, and subfamily Apinae. Italian honey bees (*Apis mellifera ligustica*), are the most widely managed type in the United States. They are generally yellow in color, gentle, and good honey producers. Carniolan bees (*Apis mellifera carnica*) are very gentle, cold tolerant bees. Caucasian bees (*Apis mellifera caucasica*) are gentle, brown in color, and are good for honey production. German bees (European Dark Bees); (*Apis mellifera mellifera*) are dark or blackish in color. In addition to

these subspecies, there are some hybrid strains of bees that have been bred for enhanced vigor or honey production. For example, the Russian strain of bees were imported to the United States through a USDA research project to develop a honey bee strain that is resistant to Varroa mites (*Varroa destructor*) (Harris et al., 2002).

1.2 History of Beekeeping

Beekeeping is ancient in origin and has been practiced in many different ways throughout the ages. Originally (around 13,000 B.C.), hunters searched for honey in beehives in the forest or high in the mountains. Later (around 597 B.C.), humans attempted to domesticate wild bees in artificial hives made from hollow logs, pottery vessels, and wooden baskets (Engel et al., 2009). The first hive beekeeping occurred in Egypt, where people started sharing the knowledge and experience of beekeeping (Gupta et al., 2014).

In the United States, the growth of beekeeping was accelerated with the inventions of tools and accessories for beekeeping. Beekeeping in the United States began when honey bees were brought by human assisted migration from Europe during the year 1622 on the coast of Virginia (Delaplane, K.S., 2007). Populations of honey bees expanded from eastern North America over most parts of the United States (Sheppard, 1989). Beekeeping was made easier when, in 1851, Langstroth developed a rectangular bee box with removable wooden frames in which bees could be inspected easily by beekeepers (Oertel, 1980). By maintaining a space between frames in the hives, the business of beekeeping was revolutionized in North America (Johansson & Johansson, 1967). Similarly, the invention of wax-comb foundation in the frames of the hives made the consistent production of high quality combs of predominantly worker cells

(Pellett, 1938). With tools and inventories required for beekeeping, beekeeping became a popular business in the United States and slowly shifted from honey production to pollination.

1.3 Impact of Honey Bees in the United States

Advances in beekeeping equipment and technology facilitated a shift in beekeeping from small scale honey production to large scale pollination services. Although there are host native pollinators in the United States, modern commercial agriculture is dependent on easily managed pollinators. Fifty two out of 115 important global foods depend on honey bees for pollination directly or indirectly (Klein *et al.*, 2007). Not all crops are dependent on honey bees for pollination; however, honey bees have remained the most important pollinator in parts of the world where monocultures are present, such as in the United States (McGregor, 1976). There are up to 2.59 million honey bee colonies across the country (USDA-NASS, 2016). In addition, total value of crops pollinated by insect pollinators in the United States is estimated to be \$16.4 billion, of which \$12.4 billion is contributed by honey bees (Calderone, 2012). Crops like apples, almonds, blueberries, cherries, broccoli (seed), watermelons, cucumber, strawberry, alfalfa (seed) are pollinated by honey bees in the United States. The number of bee colonies transported to California for pollination of almonds is about 60-75% of all U.S. commercial hives (Horn, 2005). More than five commodities would have 90% yield reductions without honey bees (Klein *et al.*, 2007). About 15-30% of the human diet depend on honey bee pollination in the United States (Losey & Vaughan, 2006).

The biology of honey bees has contributed to making them successful pollinators. Honey bees can pollinate over large areas, travelling up to 4.5 km on average (Seeley, 2014). Similarly, their ability to communicate with nest members to relay information regarding location of food

sources make them good pollinators (Seeley, 2014). The overlapping generations within a colony, which is unlike the life history of most other native pollinators, helps pollination effectiveness.

1.4 Honey Bee Decline in the United States

Three periods of declining numbers of honey bee colonies have been observed in the United States: from 1947-72, from 1989-96 and the most recent declines from 2006-present (Figure 1.01; Committee on the Status of Pollinators in North America & National Academic Press (US), 2007). The total number of colonies dropped dramatically from 5.9 million in 1947 to 2.3 million colonies in 2008 (vanEngelsdorp and Meixner, 2009). There are no established reasons for the decline in bee populations from 1947-72, but it has been suggested that the widespread use of synthetic insecticides such as carbaryl, parathion, malathion and diazinon was at least partially responsible (Johansen & Mayer, 1990). The sharp decline in bee populations from 1989-96 is likely due to the introduction of Varroa mites in the colonies of European honey bee (DeJong, 1997). The reasons for most recent declines (from 2006-present; Figure 1.01B) of managed bee colonies are unknown.

Colony Collapse Disorder (CCD) is a fairly recent phenomenon that is not fully understood. In mid-November, 2006, Pennsylvania beekeeper Dave Hackenberg first reported the problem of CCD (Elizabeth, 2012), in hives where forager bees do not return to the nest, leaving only the queen and brood in the colony; ultimately, such colonies cannot perform well and collapse. Several possible factors are being investigated as possible causes for CCD.

Several losses in honey bee colonies have been observed from the survey data in the recent years. The Bee Informed Project (BIP), an organization that surveys the number of

managed colonies every year across the United States, found the average loss of managed bee

colonies was 44.1%

between April 2015

and March 2016

(Figure 1.01B).

During this period

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(Steinhauer *et al.*,

2016). It is common

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acceptable. During

a survey conducted

by BIP and other

institutions, survey

participants considered 15% as an acceptable loss (VanEngelsdorp *et al.*, 2013). During the past

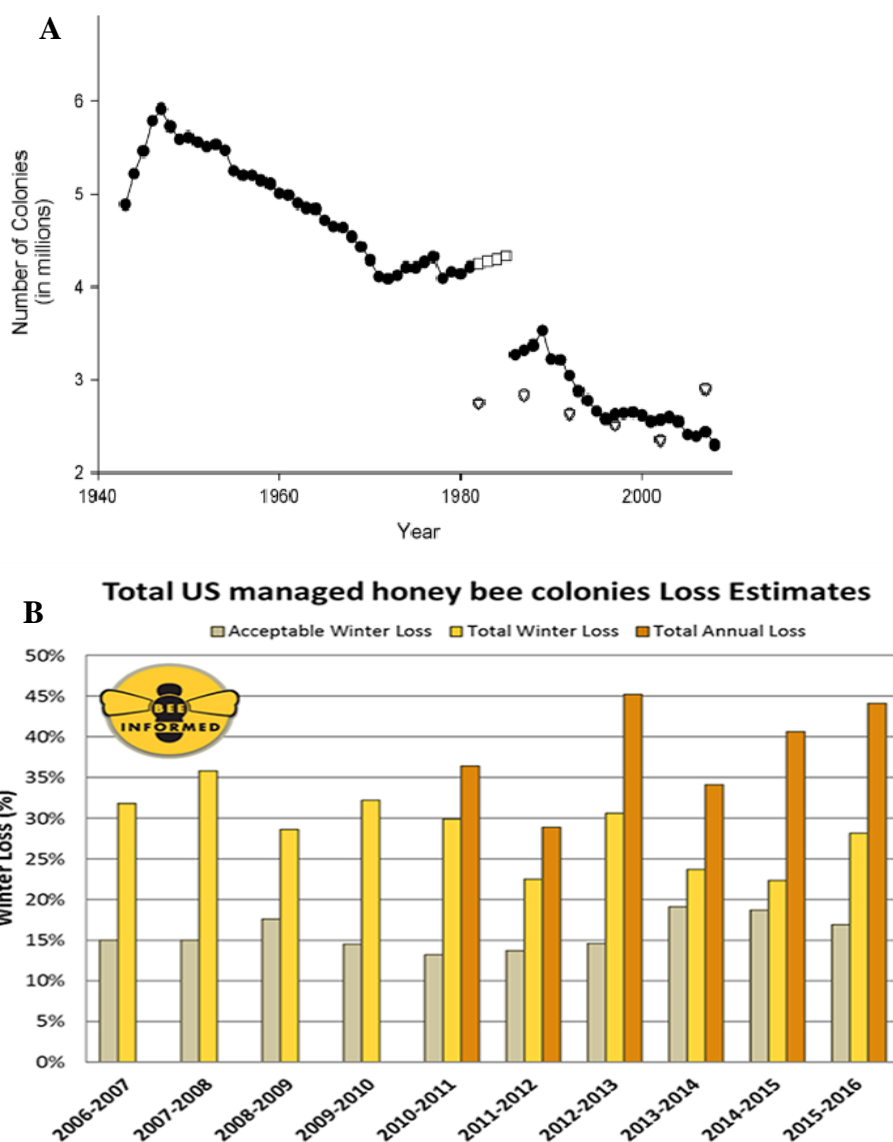


Figure 1.01. (A) Total honey bee colonies loss in the United States from 1947 to 2000 (white square box represents the predicted value). (B) Total colonies lost across nine years (from 2006 to 2016) in the United States including total winter loss and acceptable loss (Steinhauer *et al.*, 2016).

years, the overwinter loss has gone far beyond the acceptable loss ranging between 22% (2014) and 36% (2007) (Steinhauer *et al.*, 2016).

Several losses in honey bee colonies have been observed from the survey data in the recent years. The Bee Informed Project (BIP), an organization that surveys the number of managed colonies every year across the United States, found the average loss of managed bee colonies was 44.1% between April 2015 and March 2016. During this period of time, there was not only winter loss but also a higher percentage of summer loss (Steinhauer *et al.*, 2016). It is common to have a winter loss of honey bees that beekeepers deem to be acceptable. During a survey conducted by BIP and other institutions, survey participants considered 15% as an acceptable loss (VanEngelsdorp *et al.*, 2013). During the past years, the overwinter loss has gone far beyond the acceptable loss ranging between 22% (2014) and 36% (2007) (Steinhauer *et al.*, 2016). The problem of recent decline in the number of honey bee colonies appears to be regional. The United States, Canada and most countries in Europe are facing this problem. The Food and Agriculture Organization estimates that managed bee colonies decreased by 49.5% in United States and by 26.5% in Europe between the period of 1961 and 2007. However, other regions of the world fared better: the same study shows increases in colonies in Asia (42.6%), Africa (130%) and South America (86 %) (FAO, 2009). Asian bees, *Apis cerana* are not infested with Varroa mites like in *Apis mellifera* species, and use of insecticides is not dominant. Similarly, in Africa there is *Apis mellifera* and the use of insecticides is limited. In Australia, people do not use honey bees for pollination service to the extent as in the United States and Canada and there are no Varroa mites introduced. Despite the ban of neonicotinoid insecticides in most of the European countries, there has been same level of bee declining. Looking over the spatial pattern of

bee declining, it seems complex and suggests that there might be several other stresses involved in it.

There are several possible causes of recent declines of honey bees. Some of the likely causes are pests and diseases (*Varroa*, *Nosema* and others), stress (migration), climate change, lack of genetic diversity, poor nutrition, and pesticides (Eccleston, 2007; Ellis, 2007; Johnson, 2007).

1.5 Agricultural Pesticide Issue and Bee Decline

The use of insecticides for suppressing insect pests also might affect non-target insects including pollinators. Pimentel (2005) estimated the cost of pollination losses due to pesticide exposure at about \$210 million. Some of the chemicals introduced into the hives by the bees themselves during foraging, is in the form of contaminated pollen and nectar (Rortais *et al.*, 2005; Chauzat *et al.*, 2006; Chauzat & Faucon, 2007). In addition, there are other chemicals introduced into the hives by the beekeeper for the treatment of diseases and pests of honey bees (Watanabe, 2008). For example, fluvalinate and coumaphos are two commercial acaricides that are used extensively in the hive for the treatment of mites (Sammataro & Avitabile, 2005, Pettis & Jadcak, 2005). Use of commercial acaricides in South Carolina and Georgia decreases brood viability, homing ability of adult bees, and foraging rates compared to untreated hives (Berry *et al.*, 2013). Similarly, chemicals (both contact and systemic) used for agricultural crops affect bees. Honey bees exposed to low doses of insecticides have sublethal symptoms that may affect colony survival (De Wail *et al.*, 1995, Kadar & Faucon, 2006; Morandin *et al.*, 2005, Dai *et al.* 2009). In addition, a mix of the chemicals to maximize efficacy and reduce cost is common in commercial agriculture, and might have synergistic effects on bees. For example, bees exposed

to prochloraz, a common agricultural fungicide, were 72 times more susceptible to coumaphos and over 1,000 times more susceptible to taufluvalinate (Johnson *et al.*, 2013). Similarly, it was found that *Nosema* microsporidians and the insecticide, imidacloprid caused greater mortality to adult bees than either did alone (Alaux *et al.*, 2010).

1.6 Controversy Over Neonicotinoids and Bee Loss

There is currently a controversy regarding the role played by neonicotinoids in CCD. This neurotoxic class of insecticides ranks first among the insecticides applied in agriculture, both in the USA as well as rest of the world. Being systemic insecticides, they are expressed throughout the plant, including pollen and nectar, and bees may be exposed to the insecticides by multiple routes. For example, an extremely high level of active ingredient has been recorded on planter dust during planting of neonicotinoid treated corn seeds, and residues are found in soil samples even in unplanted fields and surface water (Krupke *et al.*, 2012). Moreover, sublethal doses of neonicotinoids may have negative impact on bee colony health. For example, the homing ability of honey bees is impaired by non-lethal exposure to thiamethoxam (Henry *et al.*, 2012). Further, sublethal levels of neonicotinoids can disrupt honey bee learning and behavior as they cause mushroom body neuronal inactivation (Palmer *et al.*, 2013). The impaired olfactory learning and memory can be assessed by decreased likelihood of conditioned proboscis extension towards an odor associated with reward (Williamson & Wright, 2013). In one study, a sublethal dose of neonicotinoids effected winter survival of honey bees and subsequently lead to CCD (Lu *et al.*, 2014).

Other studies cast doubt on neonicotinoid exposure as a cause of CCD. In a field experiments in Ontario, Canada, in which honey bee colonies were placed in clothianidin seed-

treated canola (*Brassica napus*) but colony weight gain, honey production, and bee mortality were not different from the control honey bee colonies (Cutler *et al.*, 2014). Similarly, another field study found a low risk to honey bees from systemic residues in nectar and pollen following use of thiamethoxam as a seed treatment on oilseed rape and maize (Pilling *et al.*, 2013).

1.7 Mosquito Control

Beside agricultural pesticides, other insecticides are used for public health. Mosquitoes are primary vectors of viruses of serious diseases such as West Nile, chikungunya, dengue, Eastern Equine encephalitis, and Saint Louis encephalitis. Approximately 500 to 700 million human cases of mosquito borne diseases with 3 million deaths occur every year (AMCA, 2010). Since the mosquitoes have major impact on people's lives, humans have been developing methods to control the mosquito population. Integrated Mosquito Management is a new approach to control mosquito populations in which, several methods or combination of methods are used that give maximum control of mosquito populations with minimal impact on non-target organisms and the environment (Rose, 2001). The components of this strategy include mosquito sampling and surveillance, physical, chemical, and biological control, disease surveillance, public education, and mosquito susceptibility monitoring (AMCA, 2010). Surveillance helps to provide information regarding the species, density, and demographics in order to direct and evaluate control measures. Physical control methods reduce the source population by eliminating breeding sites through management of standing water. Chemical control involves the use of insecticides for adult (adulticide) or larval (larvicide) populations. Biological control methods are in various stage of development and could play an important role in mosquito control (AMCA, 2010) Diseases surveillance helps to identify different vector borne pathogens transmitted by mosquitoes in the laboratory, which will facilitate appropriate control measures to reduce risk of

human disease caused by pathogens. Public education increases awareness within communities through outreach activities. Mosquito susceptibility monitoring includes testing susceptibilities of different species of mosquitoes against different classes of chemicals, and measuring resistance development in mosquito populations. Larvicides are the most common chemicals used for mosquito control in the United States due to their minimal impact on non-targets and the environment. Although there are various management techniques available for suppressing mosquito populations, the use of mosquito adulticide is used as an ultimate control measure to suppress adult mosquito populations when there is more threatening of vector borne diseases such as Zika and West Nile. Application of both larvicides and adulticides occur either by ground or by air. This current research is focused on adulticides. Ground and aerial application of adulticides normally take place in the evening and night to minimize effects on other, non-target insects.

Advancement in technologies have made the adulticide application more efficient. In the past, portable hand-held sprayers and sophisticated Ultra Low Volume (ULV) cold-fog generators were used for spraying adulticides. Presently, mosquito control districts in urban areas rely on truck-mounted ULV sprayers as the primary method of controlling populations of adult mosquitoes. Adulticides are sprayed from aircraft for treatment of large areas that are inaccessible to trucks. However, there are some conditions when aerial spraying will not be a good option for treatment such as; bad weather, high wind speed, or low visibility (WHO, 2003). In addition to air sprays, truck mounted ULV ground aerosol sprayers are a common and efficient method used by mosquito professionals. Sprayers have specialized nozzles that atomize undiluted insecticides into droplets small enough to create an aerosol that will drift through the target zone (WHO, 2003). Additionally, the incorporation of Global Information Systems and

Global Positioning Systems have increased the efficiency of ground application of mosquito adulticides (Bonds, 2012; Nawrocki, 2004). This current project is focused on ground application of truck mounted ULV mosquito adulticides.

There are certain classes of chemicals used as mosquito adulticides. The chemicals generally used as mosquito adulticides are organophosphates (malathion, naled, and temephos) and pyrethroids (permethrin, resmethrin, sumithrin mixed with piperonyl butoxide) (Rose, 2001). To combat the rise of mosquito populations, mosquito control professionals routinely use insecticides such as Naled (an organophosphate) or resmethrin (a pyrethroid) in their regions (Peterson *et al.*, 2013; Nasci, 2104). Organophosphates inhibit acetylcholinesterase in nerve cells and make the insects toxic (Fukuto, 1990). Similarly, pyrethroids insecticides act on the sodium channel of nerve fibers and are toxic to insects (Vijverberg and vanden Bercken, 2008).

1.8 Impact of Mosquito Adulticides on Non-Target Organisms

Although mosquito adulticides are effective chemicals to control mosquito populations, there are concerns that they can impact non-target organisms. For example, there was huge impact on abundance and diversity of 34 different families of insects from a single ULV application of pyrethrins (Kwan *et al.*, 2009). Similarly, naled, malathion, and non-synergized permethrin were highly toxic to five species of butterflies, including both larval and adult stages (Salvato, 2001). Further, mosquito adulticides have been identified as a likely factor for decline of several rare species of lepidopterans in Florida (Calhoun *et al.*, 2002; Salvato, 2001). Similarly, adult house cricket, *Acheta domesticus*, was found as a good surrogate species for estimating potential impacts of pyrethroid on non-target terrestrial insects (Antwi & Peterson, 2008).

Other research have found no effect on non-target organisms by mosquito adulticides. In a field study, a truck-mounted application of synergized or unsynergized permethrin or naled were nontoxic to caged crickets (Schleier & Peterson, 2010). Similarly, there was no overall effect on most aquatic and terrestrial arthropods after multiple applications of a permethrin (Davis & Peterson, 2008). Ultra-low volume application of pyrethrin, permethrin, and malathion had no abundance and biomass on populations of aquatic macroinvertebrates such as snails, mussels, and nymphs of dragonflies (Jensen *et al.*, 1999). Finally, there was no effect of pyrethrin mixed with piperonyl butoxide, sprayed in ultralow volumes, on mortality, diversity and abundance of dragonflies, spiders, butterflies, or honey bees (Boyce *et al.*, 2007).

1.9 Lethal Impacts of Mosquito Adulticides on Bees

Of special concerns are effects of mosquito adulticides on bees. Coldburn and Langford (1970) found significant bee mortality, when caged bees were sprayed with mosquito adulticides such as naled, malathion, and pyrethrum. Similarly, caged honey bees experienced significant mortality from drift of malathion sprays (Pankiw & Jay, 1992). In addition there was significantly higher bee mortality at closer distance when the caged bees ways were sprayed at several distances by ULV malathion from the spray truck (Caron, 1979). Similarly, significant bee mortality was observed both in open areas and forest areas from the sprays of ground ULV malathion (Hester *et al.*, 2001). Finally, in a field study Zhong *et al.*, (2003) found higher bee mortality with higher deposition of naled residues around the hives.

1.10 Sub Lethal Impacts of Mosquito Adulticides on Honey Bees

Detoxifying enzymes, such as esterase and glutathione *S*- transferase (GST), have been evaluated as biomarkers of toxicity caused by organophosphate and pyrethroid insecticides

(Conyers *et al.*, 1998; Vontas *et al.*, 2001). Esterases are a large, multi-gene family of enzymes that hydrolyze a vast array of ester containing compounds including insecticides such as organophosphates and pyrethroids (Dauterman, 1976). Similarly, GSTs are a family of detoxification enzymes that catalyze addition of the tripeptide, glutathione, to different electrophilic centers within substrates including insecticides (Cahng *et al.*, 1981; Ottea & Hammock, 1986; Konno & Shishido, 1992; Yang *et al.*, 2001; Enayati *et al.*, 2005). GSTs plays a role in the detoxification of organophosphates by two ways: O-dealkylation or O-dearylation. In O-dealkylation the glutathione (GSH) is conjugated with the alkyl portion of the insecticide, whereas in O-dearylation, the GSH reacts with the leaving group (Daute, 1998). The role of GST in detoxification of pyrethroids relates to its capacity to reduce peroxidative damage induced by pyrethroids, mainly by detoxifying lipid peroxidases formed during the process of pyrethroid metabolism (Vontas *et al.*, 2001). Similarly, GSTs act as an antioxidant when there is oxidative stress by the exposure of insecticides. GST helps in preventing and repairing the damage of secondary products generated by reactive oxygen species (Yunchuan *et al.*, 2005).

Some studies examined the effects of organophosphates and pyrethroids on esterase and GST enzymes in insects. Esterase mediated metabolic resistance to organophosphates and pyrethroids has been found in different insects (Holwerda & Morton, 1983; Prabhakar *et al.*, 1988; Chiang & Sun, 1996; Conyers *et al.*, 1998; Bass & Field, 2011). GSTs have been identified as a resistance mechanism in the house fly to organophosphates (Wei *et al.*, 2001). Similarly, GST was associated with in pyrethroid resistance on planthopper (Vontas *et al.*, 2001). In honey bees, esterases and GSTs have been selected as biomarkers to study the exposure of pyrethroid and organophosphate insecticides (Yu *et al.*, 1984; Bendahous *et al.*, 1999). There are few studies in which the role of esterases and GST on detoxification of

mosquito adulticides by honey bees have been examined. Selecting these two enzymes will be helpful to understand the role of esterase and GST on detoxification of mosquito adulticides.

1.11 Rationale of the Study

Despite the many studies on the effects of agricultural insecticides on bees, few have examined the effects of mosquito adulticides on bees. Most have focused on immediate exposure of caged bees to the mosquito adulticides (Coldburn & Langford 1970; Womeldorf *et al.* 1974; Pankiw & Jay, 1992; Boyce *et al.* 2007). However those studies do not consider the realistic scenario where bees are in their hives during the night. The studies done in the past regarding the effect of mosquito adulticides on bees were more lab based or semi field studies. In current field study we attempted to examine effects of truck based mosquito adulticides sprays on honey bees in a realistic scenario. Acute mortality, colony health indicators (measured by number of bees, brood quantity, and brood quality) and levels of detoxifying enzymes (esterase and GST) were measured. The following objectives were set in this study.

1. To compare the effects of mosquito adulticides on bee mortality and colony health in sentinel bee hives located in exposed and unexposed areas of mosquito adulticide spray zones.
2. To compare the effect of mosquito adulticides on detoxifying enzymes (esterase and GST) in honey bees from sentinel bee hives located in exposed and unexposed area of mosquito adulticide spray zone.

1.12 References

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CHAPTER 2. DESIGN OF DEAD BEE TRAP: A TOOL TO MEASURE HONEY BEE MORTALITY IN COLONIES

2.1 Introduction

The impact of pesticides on honey bees has become controversial in recent years, while negative effects due to exposure of neonicotinoids have been demonstrated (Henry *et al.*, 2012; Palmer *et al.*, 2013; Lu *et al.*, 2014; Traynor *et al.*, 2016). Although some studies showed no effect on honey bees due to pesticides exposure (Pilling *et al.*, 2013; Cutler *et al.*, 2014), it is still a great concern among beekeepers, government institutions, regulatory agencies, and researchers where the number of bee colonies across the country continuous to decline (Steinhauer *et al.*, 2016). For this reason, it is important to have tools that allow researchers to better assess daily or weekly mortality in honey bee colonies.

Measuring bee mortality in the hive is an important tool for assessing the impacts of pesticide exposure on honey bees in the field, and several studies examined bee mortality as one indicator of pesticides exposure (Pankiw & Jay, 1992; Zhong *et al.*, 2003; Krupke *et al.*, 2012). In earlier studies, mortality of bees in the hive was monitored with the help of cleared bare ground areas covered with white colored hardware cloth in front of the hives. A shortcoming of this technique is there was a risk of predators, runaway bees. Thus data collected were not accurate (Atkins *et al.*, 1970). The first prototype dead bee trap was introduced by Todd (Atkins *et al.*, 1970) and so many several modifications collections of bees was facilitated and accuracy of data has increased (Illies *et al.*, 2002).

Within a bee colony, nurse bees (undertaker bees) will remove dead bees from inside a hive to the outside of the nest (Visscher, 1993). A dead bee trap prevents dead bees from being completely removed by trapping undertaker bees, using a fine mesh on the top of the trap. While

a number of different dead bee traps have been designed and used to measure the mortality of honey bees in the hive, our goal was to develop a trap that was inexpensive, and did not affect the overall health of the hive. In the current study, we have designed a dead bee trap that is more efficient, less expensive, and has minimal effect on normal hive behavior. This trap was modified from that of Hendrkisma and Hartel (2010) and which was designed for small test hives (nuclei), but we modified it to fit into large 10 frame, Langstroth hives. In addition, wooden frames were added to make the trap more stable on the hives.

2.2 Materials and Methods

Our Modified dead bee trap consists of a white, plastic box (Sterlite; 34.6 cm× 21 cm× 12.4 cm), white plastic lid, which was cut from all four sides leaving only a 2 cm margin . Both sides of the plastic lid were fixed with wooden frames of similar length as of the lid with two screws on each side. The rectangular shape of the trap was formed by joining two parallel wooden frames of 11 inches with another wooden frame of 15 inches as shown in the figure 2.01. The lid of the box with wooden frames on it was fitted into the plastic box, which makes the collection container. The top portion of the trap was fixed with a fine wire mesh (1cm) as shown in

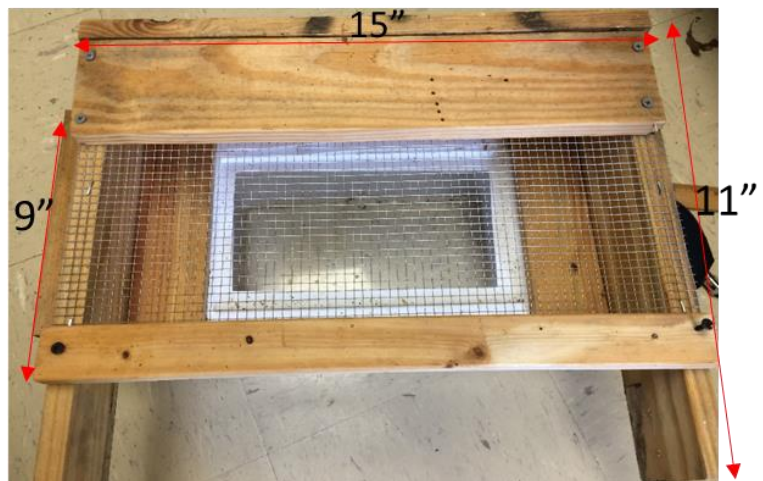


Figure 2.01. Cross section view of dead bee trap

the figure 2.01. A separate flat wooden frame of 15 inches in length (figure 2.01) was attached with the fine wire mesh on the top of the traps to give it a closed structure. The trap was attached to the hives using screws as shown in figure 2.02. and 2.03. Breadth of the dead bee trap was

designed as similar of the breadth of 10 deep frames hive. Small holes were made on the bottom of the traps for drainage.

In a trial, five dead bee traps were fixed into five different hives at the USDA Honey Bee Breeding Genetics and Physiology Research Unit (Baton Rouge, LA) and bees were allowed to acclimatize the traps. After a week, 100 bees from each of the five hives were collected and killed by freezing. All dead bees were marked on the thorax with yellow enamel paint. The one hundred marked bees from each of the respective hives were dropped into their own hive after cleaning the dead bee traps. After five days, the numbers of marked bees in each dead bee trap were recorded and used for calculating the efficiency of traps. The efficiency was calculated as the percentage of marked dead bees that were recovered in the dead bee trap.



Figure 2.02. Dead bee trap fixed to the hive



Figure 2.03. Front view of dead bee trap

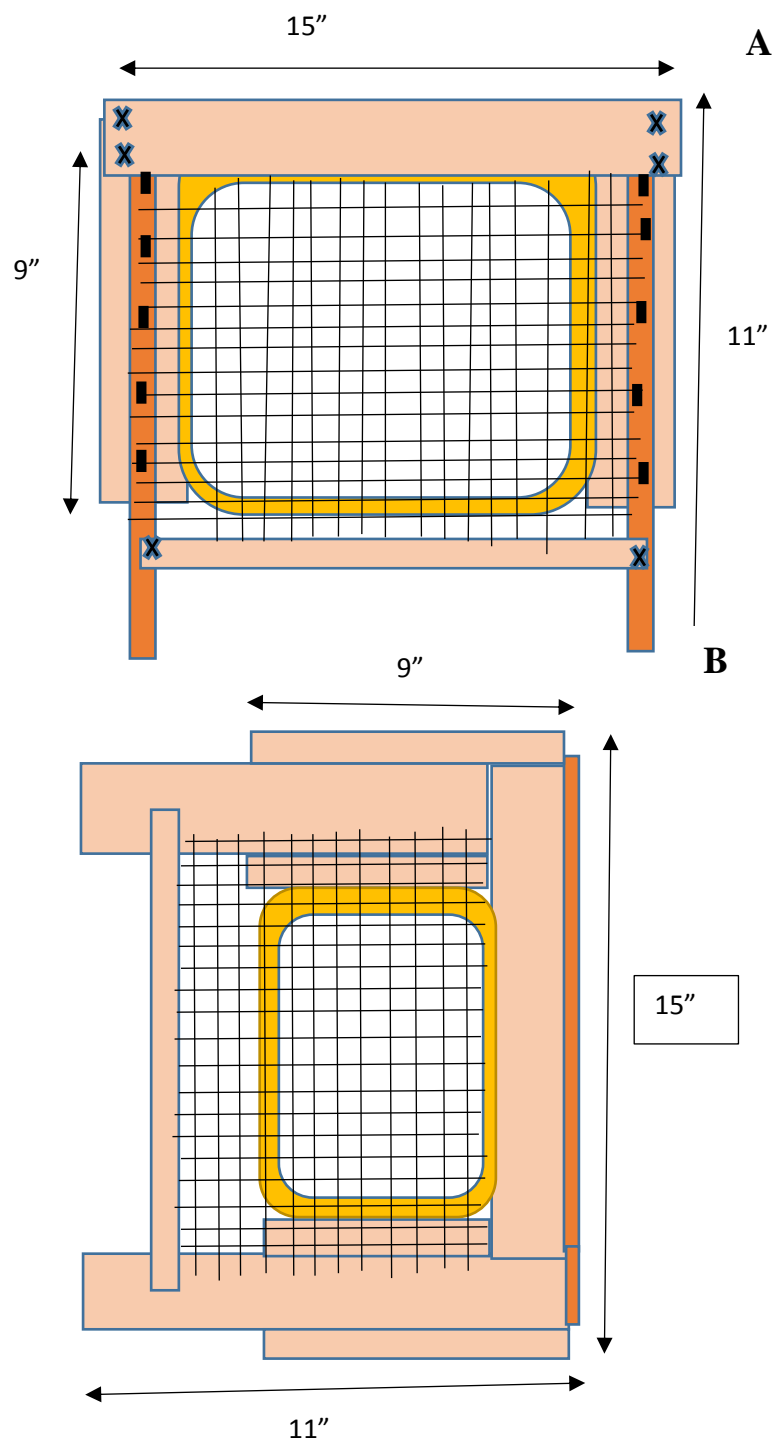
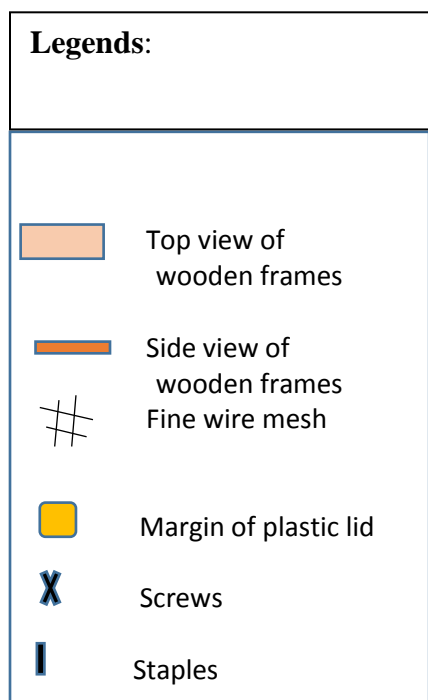


Fig 2.04. Drawings of Dead bee traps; (A) Front view of dead bee trap (B) Side view of dead bee trap

2.3 Results and Discussion

Table 2.01. Different dead bee traps with their efficiency and limitations				
Types of Traps	Efficiency	Sample size	Limitations	References
Gary Trap	84%	N= 15	Increased artificial mortality, modifies the bee behavior	(Gary, 1960; Illies <i>et al.</i> , 2002)
Todd Trap	90%	Not reported	Difficult to clean the debris and expensive	(Atkins <i>et al.</i> , 1970)
Munster Dead Bee Trap	76.4%	N=12	Low efficiency	(Illies <i>et al.</i> , 2002)
Under Basket Trap	71%	N=12	Risk of predators	(Accorti <i>et al.</i> , 1991; Porrini <i>et al.</i> , 2003)
Traps for Small Hives	93% \pm 2.7%	N = 9	Used for only small hives	(Hendriksma and Hartel, 2010)
Modified Hedriksma and Hartel trap	94.8% \pm 3.12%	N= 5	Must be drilled into the bottom frame	This study

We found that an average of $94.8\% \pm 3.12$ marked dead bees were recovered from the dead bee traps after five days. The range of dead bees collected from the dead bee traps was 83 to 100. The higher recovery rate, along with minimal interference with the bee hive compared to the other types of dead bee traps (Table 2.01) could make this trap more efficient in future research in monitoring the dead bees in the field by the exposure of pesticides. Compared with earlier traps, this trap has higher efficiency (Gary, 1960; Atkins *et al.*, 1970; Illies *et al.*, 2002). Additionally, we made the design in such a way that it is easy to collect dead bees and clean the

traps. The bottom part of the trap, plastic box/container for holding dead bee traps can be pulled out separately from the whole trap. Similarly, the use of plastic box made the trap cheaper compared to the traps that were made with wooden frames on all sides. We use the wooden frames on only some part of the traps in order to fit and stabilize attachment to the hive. Similarly, the use of fine wire mesh on the top of the traps allowed for ventilation to the bees inside the hive.

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CHAPTER 3. EFFECT OF TRUCK BASED ULTRA LOW VOLUME (ULV) MOSQUITO ADULTICIDES ON HONEY BEE (*APIS MELLIFERA*) MORTALITY AND COLONY HEALTH

3.1 Introduction

The recent decline in the number of managed honey bees, *Apis mellifera* Linnaeus, has raised concerns, regarding potential causes. While researchers cannot pinpoint a single cause, many studies have focused on evaluating the effects of stressors, such as mites, disease, nutrition, pesticides, and habitat loss. While there are some consistencies in our understanding on many of these stressors, there are confounding results regarding the effect of agricultural pesticides on honey bee declines (Kessler *et al.*, 2015; Dively *et al.*, 2015). In addition, while concerns regarding agricultural pesticides continue, there have been few studies to evaluate non-target effects of public health pesticides on honey bees.

Integrated Mosquito Management (IMM) is the use of multiple control strategies to reduce populations of biting mosquitoes. While much of the focus of IMM is on source reduction and larviciding (killing immature mosquitoes with biorational products), the use of mosquito adulticides to kill adult flying mosquitoes is often warranted in times of high mosquito nuisance and virus activity. The use of various mosquito adulticide products are often conducted through truck mounted Ultra Low Volume (ULV) equipment. This is often more common than aerial applications in urban areas because of its high efficiency in controlling adult mosquitoes (Nawrocki, 2004; Bonds, 2012).

Previous studies have shown possible impacts of mosquito adulticiding on several non-target organisms including honey bees (Coldburn and Langford, 1970; Pankiw & Jay, 1992; Zhong *et al.*, 2003), and other insects (Jensen *et al.*, 1999; Zhong *et al.*, 2004; Macedo *et al.*,

2010). However, Studies regarding the effects of mosquito adulticides on honey bees have often focused on acute mortality of bees and from immediate exposure of caged bees to the insecticides (Womeldorf *et al.*, 1974; Boyce *et al.*, 2007). While acute mortality is an important indicator of pesticide exposure, there might be other chronic effects on colony health. In addition, most studies have not utilized realistic scenarios such as where bees return to their hives during night (Seeley, 1996). The purpose of this study is to measure the effect of mosquito adulticides in a realistic scenario on bee mortality and colony health. Colony health was measured by number of honey bees in a colony and brood status.

3.2 Materials and Methods

3.2.1 Experimental Sites

The 7-week period of the study was 7 August to 25 September 2015. This season of the year was selected due to the high mosquito-control spraying frequency during the active season of mosquitoes. The experimental sites were selected with the coordination of the Louisiana Beekeeper Association, Capitol Area Beekeepers and the East Baton Rouge Mosquito and Rodent Control Unit, Baton Rouge, LA. Local beekeepers who showed interest to volunteer for this study allowed use of their hives as sentinels for this study. Treatment and control sites were fixed with the help of East Baton Rouge Mosquito and Rodent Control Unit, Baton Rouge, LA. Five sites that were sprayed with mosquito adulticides were treatment sites and four sites that were not sprayed were control sites. We selected three colonies at each of our experimental sites, for a total of 12 control and 15 treatment colonies. Colony health was assessed before the start of the experiment. All the information about experimental sites can be found in appendix

3.2.3 Dead Bee Collection

One week before the start of experiment, dead bee traps (described in chapter 2) that were designed for this study were fixed onto all the experimental hives of control and treatment sites.

The purpose of fixing dead bee traps prior to the experiment was to acclimatize the bees with the dead bee traps. Experimental sites were assigned with the number from 1 to 9 in order to make consistency and easy during the sample collections throughout the experimental period. Sites with the number 1,5,6 and 9 were our control sites and sites with the number 2,3,4,7 and 8 were treatment sites. Throughout the experiment period, sample

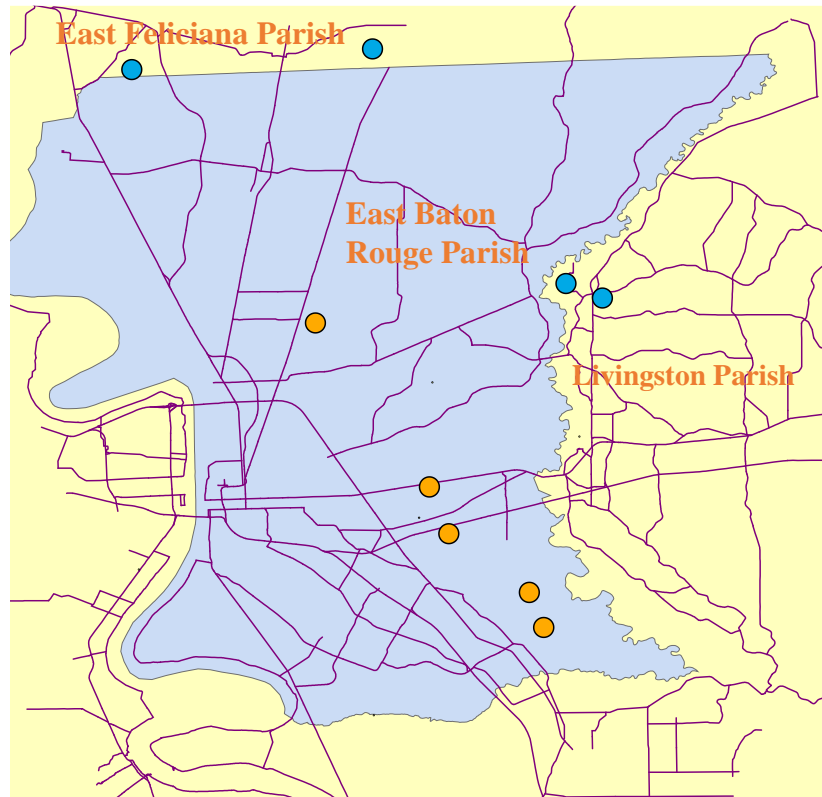


Figure 3.01. Experimental sites used for study. Blue circles represent control sites and orange circles represent treatment sites.

collection was done starting at number 1 and ending at number 9. Collection of dead bees from the dead bee traps was done on every Friday throughout the 7 weeks of the experimental period. Dead bee traps were cleaned each time after the collection of dead bees. During the dead bee collection a light smoke of pine needle was given at the entrance of bee hive in order to calm the bees. Only the plastic box of a dead bee trap was pulled out from its lid during the collection. This design made the traps efficient to handle during collection. The collected dead bees were packed in plastic bags that were pre-labelled with the experimental site number, hive number and

date of collection. Dead bees were kept in a cooler with ice packs in the field and counted later in the laboratory after collecting the dead bees from all the sites.

3.2.4 Colony Strength(Frames of Bees, Brood Quantity and Brood Quality)

Parameters used to measure colony strength were number of bees, brood quantity and brood quality. Pre- and-post assesment of all experimental hives were done in order to find the percentage change in all of the parameters used for measuring colony strength. Pre assesments were done at the beginning of experimental period and post assesments were done at the end of experiment. The methods used for measuring all the parameters are discussed below.

3.2.5 Frames of Bees

Number of bees were calculated by measuring the percentage area of frames covered by bees. Individual frames of bees (both sides) were observed separately for all the frames in a colony. Separate readings were taken from deep, medium and shallow frames of a colony. After recording the total frames of bees from a colony, it was converted into number of bees within a single colony using values mentioned by Burgett & Burikam (1985). Burgett and Burikam calculated the total number of bees in deep, medium and shallow frames of Langstroth hive (i.e., Deep= 2,430, Medium=1570, and shallow=1280). Observation of frames of bees was done by the same person during the entire period of experiment in order to reduce personal bias. Before opening the colony, two to three puffs of light smoke from pine needles was given into the entrance of hives in order to calm the bees.

3.2.6 Brood Quantity

Brood quantity was measured by visual observation of the surface area covered by capped brood on both sides of a frame (Woykr, 1984). Bees on the frames were slowly removed

with the help of a smoker and bee brush in order to read the brood on the frame. Each frame (both sides) within a colony was observed for brood quantity. Readings were taken in percentages (percentage area covered by capped brood) of the frames and later were converted into square centimeters, multiplying average area covered by different size of frames. The value for the area covered by deep , medium and shallow frames of a colony was adopted from Burgett & Burikam (1985). According to the Burgett and Burikam, the total areas covered by deep, medium and shallow frames of Langstroth hive are 1,759, 1,129, and 922 cm² respectively.

3.2.7 Brood Quality

A rhombus shaped plastic grid (measured as 10 by 10 honey bee cells) was used for measuring brood quality. The grid was placed on selected frames with good patches of capped brood and the empty cells or uncapped cells within that area was recorded. Three readings were taken from three frames, and they were averaged later. While looking for brood quality, bees on the frames were gently removed with the help of smoke and a bee brush in order to visualize the brood on the frame.

3.2.8 Mosquito Adulticides

Mosquito Adulticides were sprayed by East Baton Rouge Mosquito and Rodent Control Unit, Baton Rouge, LA at all of our treatment sites according to their routine. Mosquito adulticides were sprayed by truck in a ultra low volume(ulv) just after the sunset. All of the adulticides used were pyrethroid insecticides.

Table 3.01. Spray history at treated sites			
Spray date	Site	Chemicals used	Application Rate
Aug. 20, 2015	3,4, and 7	Duet™	a) Deltagard™ = 0.00045 lb/acre of deltamethrin
Aug. 31, 2015	3 and 7	Scourge™ and Deltagard™	b) Scourge™ = 0.002 lb/acre of resmethrin and 0.0059 lb/acre piperonyl butoxide (PBO) c) Duet™ = 0.00036 lb/acre prallethrin, 0.0018 lb/acre sumithrin, and 0.0018 lb/acre piperonyl butoxide (PBO)
Sep. 1, 2015	2	Scourge™	
Sep.11, 2015	2	Scourge™	
Sep. 14, 2015	8	Scourge™	
Sep. 15,2015	3,4, and 7	Duet™, Scourge™, and Deltagard™	
Sep. 21, 2015	2	Scourge™	

3.2.9 Insecticide Droplet Collection

The droplets of the mosquito adulticides were collected from the treated sites during the sprays. Droplets were collected on Teflon coated slides (Leading Edge, Fletcher, NC) that were set on the spinners. Two slides were set on a spinner(Leading Edge, Fletcher, NC) that was set one foot above the ground. Slides were labelled with the information about site number, distance from the road, and direction of the slides (left or right). Spinners were mounted above the ground with the help of an iron rod and which was later covered by 0.025



Figure 3.02. Spinners with Teflon coated slides

meter PVC pipe. Spinners were set at 15.24 and 30.48 meter from the road, as well as directly in front of hive entrance. One or two spinners were set near the experimental hives depending upon how far are they from the road. Sites that had all the hives equidistant from the road had one spinner near the hives. But the sites that had the hives scattered from each other and not equidistant from the road had two spinners around the hives.. In this way, total 3 to 4 spinners were set on the sites depending upon the distance from the road. Spinners were fixed at least 2 hours before the spray event. All the slides were picked up from the spinners in early morning of the day after a spray. Control spinners and slides were fixed on non spray event at treatment sites considering sites as paired.

Insecticide droplets were read at East Baton Rouge Mosquito and Rodent Control Unit, Baton Rouge, LA. Drop Vision software (Version 2.4) was used for measuring droplets. Thirty shots per slide was fixed while measuring each slide. Ten shots were captured on the top, 10 on the middle and 10 on the bottom of the slides. Volume Mean diameter (VMD) was measured to one micron by the software. All the droplet data (treatment and control), were corrected to a standard area (200 sq. cm.). Frequency of treatment droplets was then calculated by subtracting the number of control droplets for each droplet diameter. The frequency was then multiplied by the diameter size to determine a volume. This was then divided by the total volume on the corrected treatment slide. The VMD was then calculated as the droplet diameter in which 50% of the cumulative volume was reached.

3.2.10 Data Analysis

Sites were considered as a random variable in this experiment. The number of dead bees collected was converted into percentage mortality with the formula ($\% \text{ mortality} = (\# \text{ dead})$

bees/total bees)* 100. PROC MIXED two way analysis of variance in SAS 9.4 (SAS Institute, Cary, NC, 2013) was used to compare the percentage of dead bees per colony between treatment and control colonies. Means were compared at $\alpha < 0.05$ by Tukey's Honest Significant difference test. Similarly, the percentage change in the number of adult bees was found with the formula (% change in adult bees= (initial number of bees - final number of bees)/initial number of bees *100). Proc t-test (SAS institute, Cary, NC, 2013) was used to compare the percentage change in number of adult bees between control and treatment colonies during the experimental periods. In the same way, percentage change in brood quantity and brood quality was calculated. Due to non normal distribution of brood quantity data, Mann Whitney's test was used to compare the percentage change in brood quantity between control and treatment colonies. PROC t-test was used to compare the difference in change in brood quality between treatment and control colonies.

3.3 Results

3.3.1 Bee Mortality

Total percentage mortality of bees collected from dead bee traps was not significantly different ($F_{1, 6.8} = 0.51$; $P = 0.498$) between control and treatment colonies (Figure 3.03). Total dead bees in control colonies was 0.33% and total dead bee in treatment colonies was 0.221%. When comparing the mortality among experimental sites, we did not see the significant difference in any sites (Figure 3.04). Similarly, there was no significant difference ($F_{6, 133} = 1.71$; $P = 0.124$). between control and treatment colonies during any week across the seven weeks of the study (Figure 3.05).

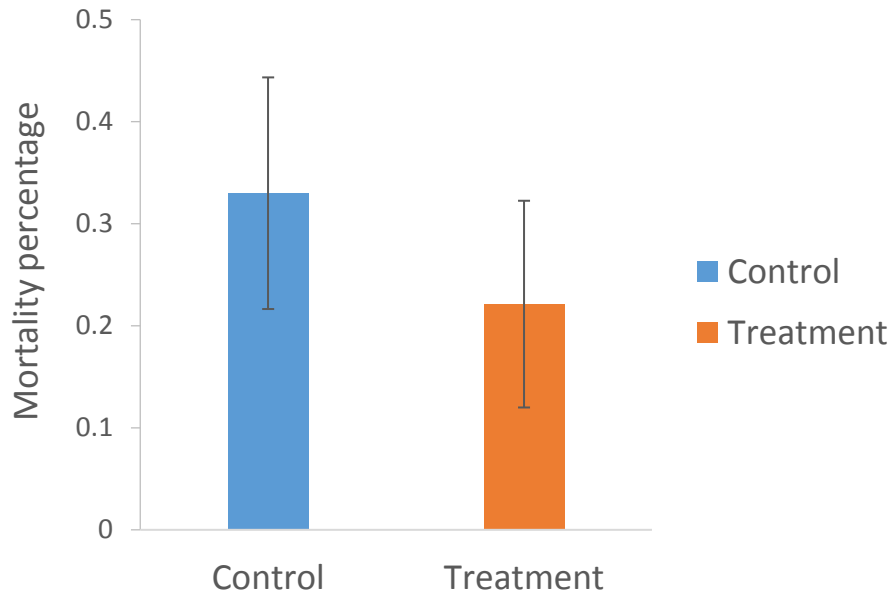


Figure 3.03. Total percentage of bee mortality collected from insecticide sprayed (treatment with orange bar) or unsprayed (control with blue bar) sites over seven weeks periods. Bars represent the mean activity (\pm SEM).

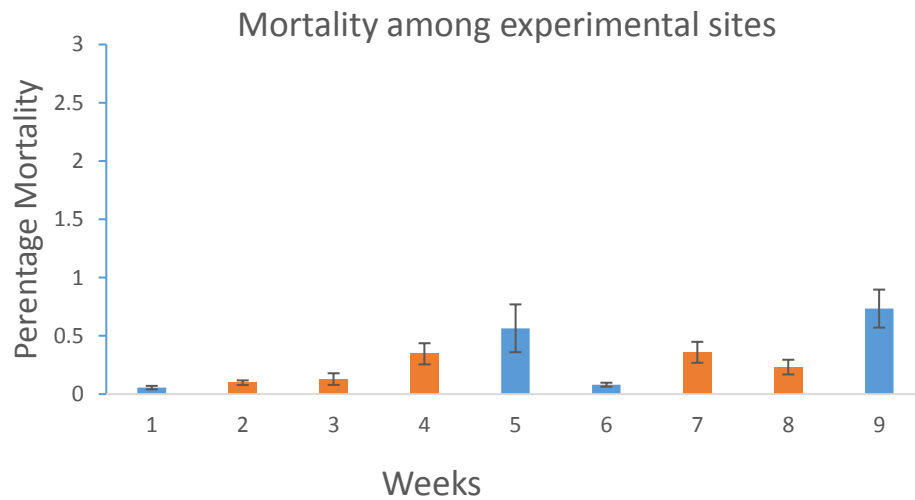


Figure 3.04. Mortality of bees collected from individual experimental sites that were sprayed (orange bar) or unsprayed (blue bar). Bars represent the mean activity (\pm SEM).

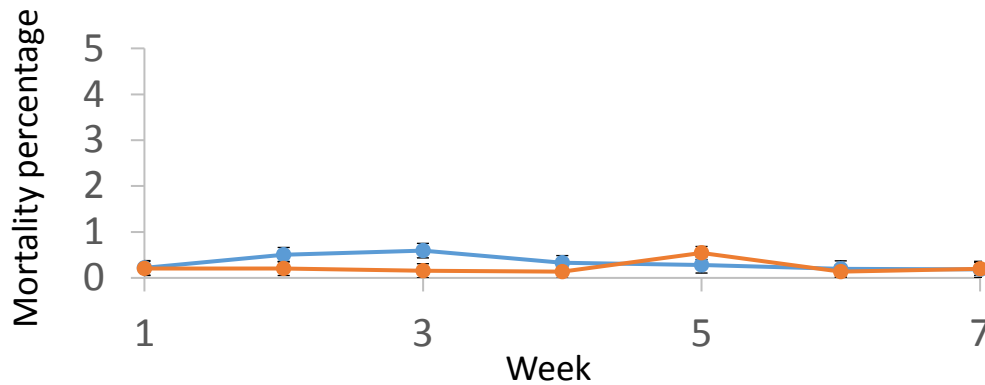


Figure 3.05. Mortality of bees collected from experimental sites; that were sprayed (treatment sites with orange line) or unsprayed (control with blue line) over seven weeks period of experiment. Points represent the mean activity (\pm SEM).

3.3.2 Frames of Bees

The bee populations did not differ between control and treatment colonies ($T_{20} = -0.14$; $P = 0.886$). There were decreasing numbers of bees in both treatment and control colonies during the study. There was a 7.36% reduction in number of bees in control colonies and a 4.18% reduction in number of bees in treatment colonies (Figure 3.05)

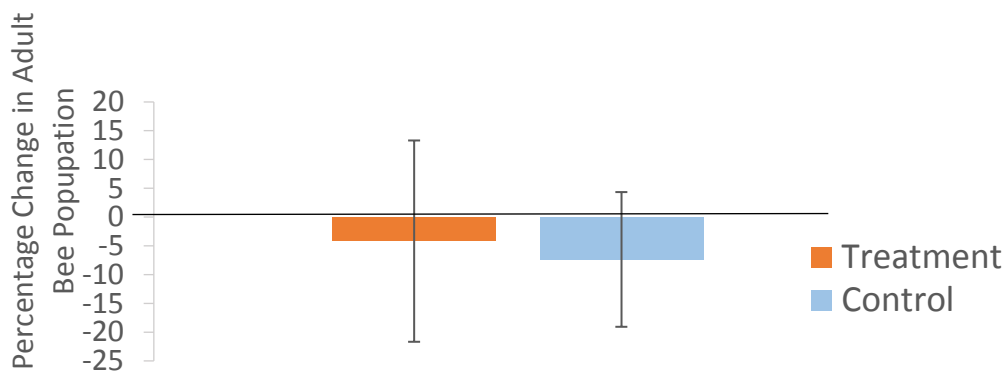


Figure 3.06. Total percentage change in number of bees in experimental sites that were sprayed (treatment sites with orange bar) or unsprayed (control sites with blue bar). Bars represent the mean activity (\pm SEM).

3.3.3 Brood Quantity and Brood Quality

There was a significant difference ($U_{75,135}=20$; $P=0.0344$) in the percentage change in brood quantity between control and treatment colonies (Figure 3.07). There was no significant difference ($F_{9,9}=1.66$; $P=0.4617$) in the percentage change in brood quality between control and treatment colonies (Figure 3.06). The average percentage change in brood quality was -21.67% for the treatment colonies and -21.59% for the control colonies.

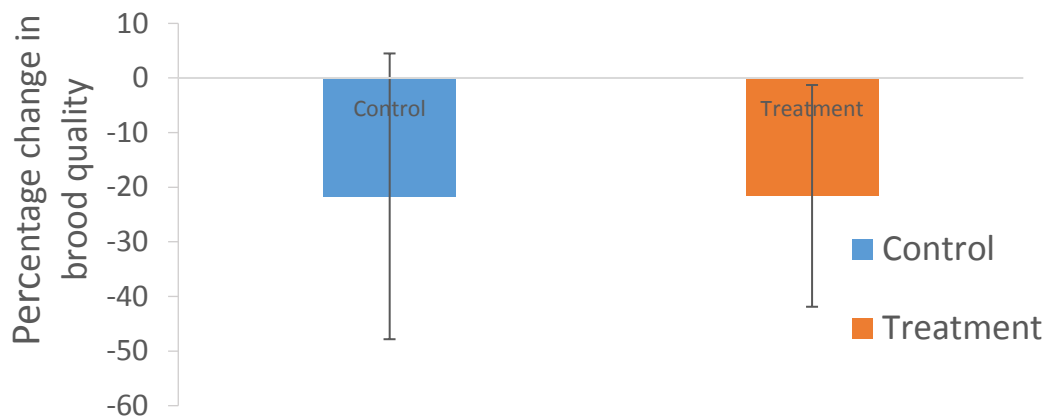


Figure 3.07. Total percentage change in brood quality in experimental sites; that were sprayed (treatment sites with orange bar) or unsprayed (control sites with blue bar). Bars represent the mean activity (\pm SEM). Percentage change in brood quality between control and treatment colonies.

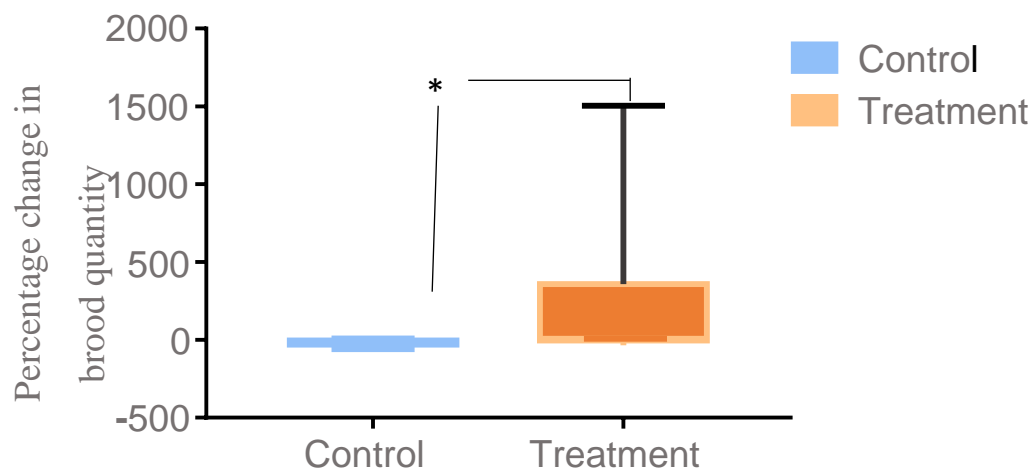


Figure 3.08. Total percentage change in brood quantity in experimental sites; that were sprayed (treatment sites with orange bar) or unsprayed (control sites with blue bar). Bars represent the mean activity (\pm SEM). * sign denotes significant difference.

3.3.4 Insecticide Droplets

Similarly, when looking at the average insecticide droplet result, it was found that DV50 values (mean diameter in volume) was between 10.33 to 12.59 microns. The products Scourge, Deltaguard, and Duet had average DV50 values of 12.59, 10.33, and 11.81 microns, respectively (Figure 3.08). Adulticide droplets had similar DV50 values near the hives, at 50 m and 100 m distance from the road (Figure 3.09).

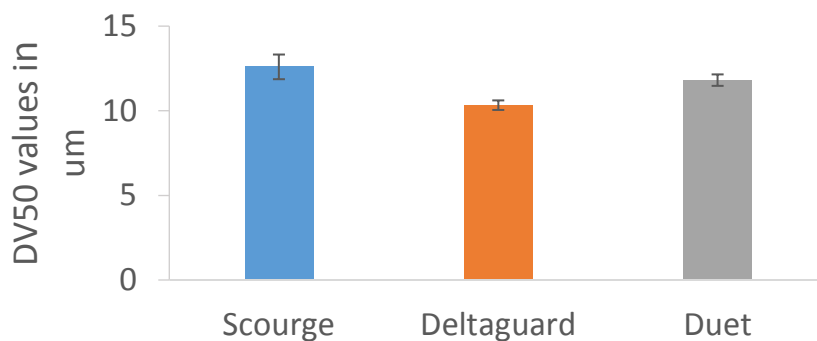


Figure 3.09. Average droplet size of insecticides collected from the treated sites. Bars represent the mean activity (\pm SEM).

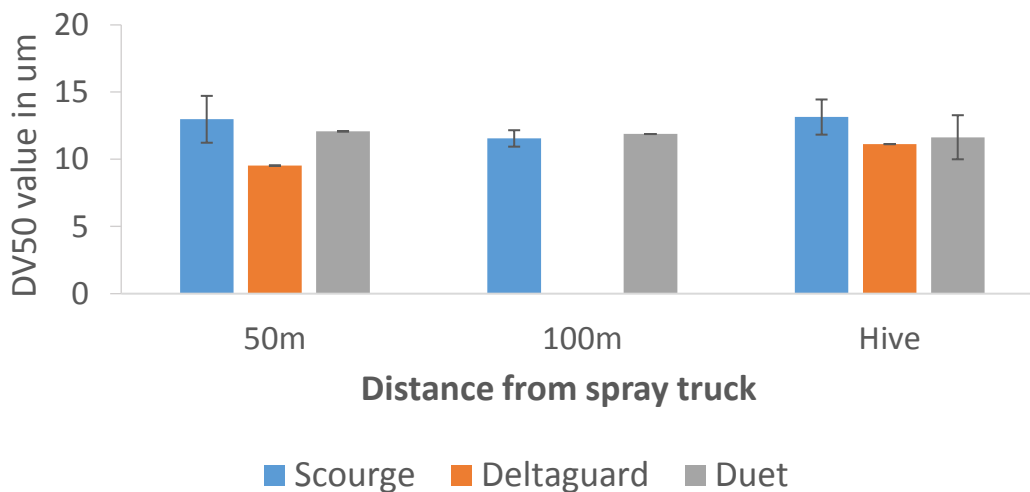


Figure 3.10. Insecticide droplets size collected from treated sites at different distance from the road. Bars represent the mean activity (\pm SEM).

3.4 Discussion

There was no significant difference in the percentage of dead bees in colonies located in the adulticides exposed sites and the unexposed sites. The dead bees seen in all the experimental sites except on few sites were considered as natural bee mortality, as up to 100 dead bees per day is considered as natural mortality (Tew, 1998). Although total dead bee counts was found higher in control than treatment, it was not significantly different. Higher bee mortality in control than treatment was due to the two of the control sites, site 5 and site 9 (Figure 3.04), which had higher bee mortality compared to the others. Two of the weaker colonies in site 5 were infested with varroa mites and additionally a queen failure in one of these colonies might have resulted in higher mortality of bees. Similarly, due to infestation of varroa mites in all the colonies of site 9 (information from beekeeper through personal communication) might have resulted in higher mortality. There was fewer adult bees, and less worker brood in 5 out of 6 colonies that were infested with *Varroa destructor* compared to normal and healthy colony found in a study done in Canada (Downey and Winston, 2001).

Similarly, earlier research showed no significant effect of mosquito adulticide on honey bees mortality. Boyce (2007) found no effect of adulticide spraying in California on non-target sentinel species including honey bees. By contrast, their study found higher diversity and numbers of non-target arthropods on the traps set in sprayed areas compared to unsprayed areas (Boyce, 2007). Previous researchers have observed higher bee mortality during day application of ground based ULV malathion, with night applications having no measurable bee mortality (Caron, 1979). During a night time application of mosquito adulticides, bees are considered to be inside the hive, which might reduce risk of acute mortality (Seeley, 1996). Additionally, the use of modern equipment (nozzle system) in spraying might have low contamination in the

environment as the change in high pressure nozzle system from flat-fan nozzle system decreases the mortality of bees by more than half (Zhong *et al.*, 2004). These factors might have minimized exposure of honey bees to mosquito adulticides.

There was no significant difference in populations of bees between control and treatment colonies. We observed decreasing numbers of bees in colonies at both sprayed and unsprayed sites, however the decline was not significantly different between sites. Poor resources available in August and early September, might have resulted in decreasing bee populations in our treatment and control sites. Another factor might be the lack of nutritious food (pollens with proteins, lipids, vitamins, and minerals) during these seasons, which can result in the poor health of bees affecting their survival and development (Brodschneider and Crailsheim, 2010). Similarly, studies have found that there was no significant difference in cluster counts of bees between pre and post treatment of mosquito adulticides in both open or forest area (Hester *et. al*, 2001).

Brood quantity and quality are considered important parameters of colony strength. While assessing the brood quantity and brood quality before and after the study, we found no significant difference in brood quality. However brood quantity was significantly greater in treatment colonies than in control colonies ($P=0.0344$). While looking at our raw data, we observed that difference between control and treatment was due to only two of the colonies of the treatment site 2. Brood quantity data showed that there was 11 and 16 fold increased in brood quantity from our initial reading to final reading, which in overall influence the result. During our initial reading these two colonies were apparently poor, observed from poor brood quantity and poor brood quality. Thus, we predict that difference in brood quantity between control and treatment was due to the two outliers data which mostly skewed our results. Similarly, there

was no effect of ground based ULV mosquito adulticides (deltamethrin and d-phenothrin) on the bee colonies including adult bee population and bee brood (Chaskopoulou *et al.*, 2014)

Although our insecticide droplet readings show that there was exposure to mosquito adulticides (Figure 3.10), we did not see significant differences in mortality and colony health (measured by frames of bees, brood quantity, and brood quality) in honey bee colonies at treatment and control sites. Generally, adulticides of droplet size, 5 to 25 μm is most efficient to deliver a toxic dose to adult mosquitoes (Haile *et al.*, 1982) and our droplet size (Figure 3.10) fall within this range. Additionally, our droplet readings show that there was a same level of exposure at 50 meter distance from the road and at the hives, where we can assume that all of our treatment hives were exposed with mosquito adulticides. Thus our droplet reading study also support that there was minimal effect of mosquito adulticides on honey bees.

This study found no effects of truck based ULV mosquito adulticides on bee mortality and colony strength in sentinel hives. Minimal exposure of adulticides might have caused the low impact on bee mortality. There are also other factors in the real environment like temperature, humidity, wind speed, wind direction, orientation of hives, protective vegetation and buildings around the hives which can influence the result. For example, during warm weather, bees may cluster outside the entrance of hives during night and they might get exposed to mosquito adulticides (Atkins *et al.*, 1981). Thus, it would be better to take account of these factors while doing further studies.

3.5 References

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CHAPTER 4. EFFECT OF TRUCK BASED ULTRA-LOW VOLUME (ULV) MOSQUITO ADULTICIDE ON DETOXIFICATION ENZYMES (ESTERASE AND GLUTATHIONE S-TRANSFERASE) IN HONEY BEES (APIS MELLIFERA).

4.1 Introduction

Declining health of the honey bee (*Apis mellifera*) has been an issue from several decades earlier to the present in the United States, where the total number of honey producing colonies has dropped dramatically from 5.9 million in 1947 to 2.3 million colonies in 2008 (vanEngelsdor *et al.*, 2010). Unacceptable losses in numbers of bee colonies in recent years (Steinhauer *et al.*, 2016) has raised concerns and questions regarding their possible causes. Most studies evaluating the effect of agricultural pesticides on honey bees have had confounding results (Henry *et al.*; 2012, Palmer *et al.*; 2013; Pilling *et al.*, 2013, Cutler *et al.*, 2014). Thus, the cause of the recent decline of honey bee colonies is unclear and has been a great concern among commercial beekeepers, research groups, and regulatory agencies.

The role of public health insecticides on declining honey bee health is unclear. The use of truck mounted ultra low volume (ULV) mosquito adulticides is common in urban areas to control populations of adult mosquitoes (Bonds, 2012; Nawrocki, 2004) in order to minimize the risk of vector borne viruses, such as those causing West Nile and Zika. Studies examining effects of mosquito adulticides on non-targets (Jones & Ottea, 2013) or beneficial organisms including bees (Pankiw & Jay, 1992; Zhong *et al.*, 2004; Macedo *et al.*, 2010), have mostly focused on acute mortality (Coldburn & Langford, 1970; Caron, 1979; Zhong *et al.*, 2003). However, studies are needed to evaluate if honey bees are responding physiologically to additional stress they encounter in the environment.

Detoxification enzymes have been used as biomarkers for insecticide exposure in many insects, including honey bees (Yu *et al.*, 1984; Papadopoulos *et al.*, 2004; Claudianos *et al.*, 2006; Mao *et al.*, 2011). However, few studies have examined the effect of public health insecticides on detoxification enzymes in honey bees. In additions, many studies were laboratory focused where it is difficult to predict the real dose of insecticides that bees encounter in a natural environment (Johnson *et al.*, 2006; Badiou-Beneteau *et al.*, 2012).

Esterases and glutathione *S*-transferase (GST) are detoxification enzymes that may be used to evaluate exposure of honey bees to mosquito adulticides (Achalek *et al.*, 2009; Bisset *et al.*, 2013; Carvalho *et al.*, 2013). Esterases detoxify many organophosphate or pyrethroid insecticides (some of which are used as mosquito adulticides) by hydrolyzing the ester moieties and making products that are more hydrophilic and less toxic (Dauterman, 1976). Similarly, GSTs detoxify xenobiotics (including some insecticides) by accelerating the reaction between reduced glutathione and electrophilic centers, making products that are more water soluble and generally less toxic (Booth *et al.*, 1961; Boyer, 1989). GSTs also play a role in antioxidant defense and ameliorate effects of oxidative stress from exposure to insecticides by preventing damage of secondary targets by reactive oxygen species (Yunchuan *et al.*, 2005). Some studies have used these enzymes to evaluate exposure of honey bees to mosquito adulticides (Bendahou *et al.*, 1999; Badiou-Beneteau *et al.*, 2012), and found decreased enzyme activities following exposure of insecticides.

In this study, we examined the effect of application of truck based, ULV mosquito adulticides on esterases and GST enzymes in honey bees in a field setting. Knowledge gained from this study will help validate the role of these enzymes as biomarkers for exposure to mosquito adulticides.

4.2 Materials and Methods

4.2.1 Chemicals

Sodium phosphate (monobasic monohydrate ($\geq 98\%$), sodium phosphate dibasic heptahydrate (98%)), Brilliant Blue G-250 (ultra pure), dimethyl sulfoxide ($\geq 99\%$), Fast Blue B salt (approx. 95%), L-glutathione, reduced ($\geq 98\%$), 1-naphthyl acetate (α NA) ($\geq 98\%$), and 1-chloro 2,4-dinitrobenzene (CDNB, 98%) were purchased from Sigma Aldrich (St. Louis, MO). Phosphoric acid (85%), hydrochloric acid (99.7%), sodium dodecyl sulfate (SDS) (99%), and sodium hydroxide (ACS grade) were purchased from Fisher Scientific (Kansas City, MO). Bovine serum albumin (biotechnology grade) and acetone (ACS grade) were purchased from Amresco (Solon, OH). Ethyl alcohol (absolute; ACS/USP grade) was purchased from Pharmco-Aaper (Brookfield, CT).

4.2.2 Experimental Sites

Nine sites were used for experiments during the 7-week period from August 7 to September 25, 2015. The experimental sites were selected with the assistance of the Louisiana Beekeeper Association, Capitol Area Beekeepers and the East Baton Rouge Mosquito and Rodent Control Unit, Baton

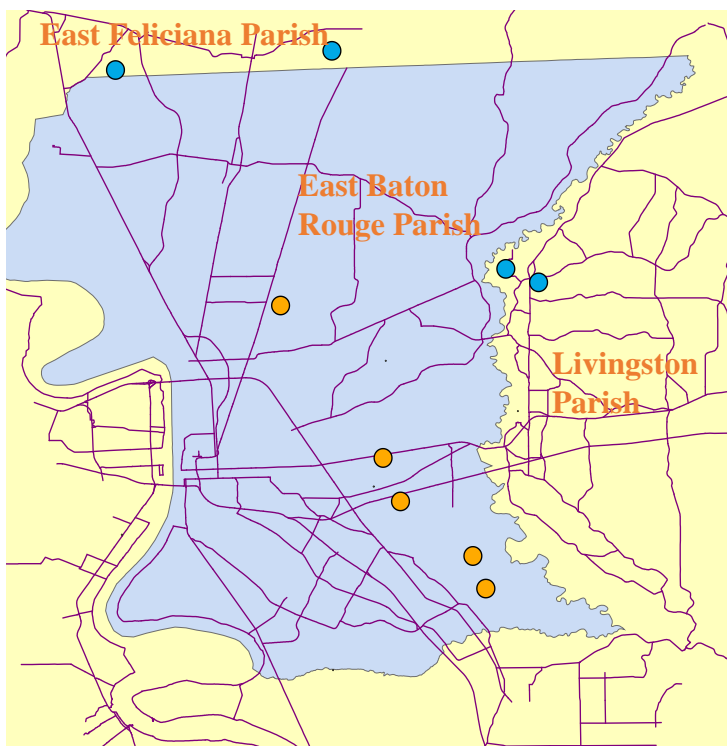


Figure 4.01. Experimental sites used for study. Blue circles represent control sites and orange circles represent treatment sites.

Rouge, LA. Local beekeepers volunteered and allowed use of their hives as sentinels for this study. Treatment and control sites were delimited in coordination with the East Baton Rouge Mosquito and Rodent Control Unit. Five sites received treatment with mosquito adulticides (treatment sites) and four sites not sprayed (control sites; (Figure 4.01). Three colonies were selected for study at each of our experimental sites, for a total of 12 control and 15 treatment colonies. Colony health (i.e., number of adult bees, brood quantity, and brood quality) was assessed before the start of the experiment. Additional details of experimental sites can be found in the appendix.

An average of ten forager bees (*Apis mellifera ligustica*) were collected randomly from the experimental hives weekly from August 7th to September 26th, 2015 transferred to the laboratory in an ice-filled cooler, and kept in a -80°C freezer until enzyme assay. Pyrethroid insecticide were sprayed by East Baton Rouge Mosquito and Rodent Control Unit in response to local needs at each of the treatment sites by truck as ULV sprays just after sunset (Table 4.01).

Table 4.01. Spray history at treated sites			
Spray date	Site	Chemicals used	Application Rate
Aug. 20, 2015	3,4, and 7	Duet TM	a) Deltagard TM = 0.00045 lb/acre of deltamethrin b) Scourge TM = 0.002 lb/acre of resmethrin and 0.0059 lb/acre piperonyl butoxide (PBO) c) Duet TM = 0.00036 lb/acre prallethrin, 0.0018 lb/acre sumithrin, and 0.0018 lb/acre piperonyl butoxide (PBO)
Aug. 31, 2015	3 and 7	Scourge TM and Deltagard TM	
Sep. 1, 2015	2	Scourge TM	
Sep.11, 2015	2	Scourge TM	
Sep. 14, 2015	8	Scourge TM	
Sep. 15,2015	3,4, and 7	Duet TM , Scourge TM , and Deltagard TM	
Sep. 21, 2015	2	Scourge TM	

4.2.3 Tissue Preparation

Abdomens were removed from frozen bees and homogenized in 1.15% KCL (1 bee/ ml) using 10 strokes of an all glass mortar and pestle. For the pH optimization assay, buffers at varying pH were used for homogenization. Homogenates were centrifuged at 4°C for 10 min at 14,600 rpm. Resulting supernatants were held in ice and diluted with buffer to adjust protein for enzyme assays.

4.2.4 Biochemical Assays

Activities of esterase towards α NA were measured using the method of Gomori (1953) as modified by van Asperen (1962) and Grant *et al.* (1989). The assay was performed in polystyrene 96-well flat bottom microplates (Costar, Cambridge, MA). All the microplates were prewashed with 2.5% Tween 20 (v/v in water). A stock solution of a α NA (30mM) in acetone was diluted in buffer to a concentration of 0.3 mM. Reactions, containing 20 μ l of either enzyme homogenate (0.02 insect equivalent; 0.0044 mg protein), or buffer, were started by adding 200 μ l of α NA (0.22 mM, final concentration). After 10 mins at 27°C, reactions were terminated by addition of 50 μ l of Fast Blue B dye (0.15 gm Fast Blue B salt + 14 ml distilled water + 30 ml 5% SDS solution; 2.18 mM final concentration). Reactions with buffer were used as control. Optical density of reactions, measured at 570 nm using a Thermomax microplate reader (Molecular Devices, Palo Alto, CA) , was converted to μ mol/min using an experimentally derived extinction coefficient of $0.0235 \mu\text{M}^{-1} \text{ 250 } \mu\text{l}$ for alpha naphthol.

Activities of GST towards CDNB were measured following the method of Booth *et al.* (1960) and Jakoby (1978), as modified by Grant *et al.* (1989). A stock solution of CDNB (50mM in DMSO) was diluted in buffer to a concentration of 0.66 mM. Glutathione (65 mM) was prepared in double distilled water (for pH optimization assays) or in buffer of optimal pH (for

routine assays). A typical reaction mixture consisted of 20 μ l of enzyme homogenates (0.02 insect equivalent; 0.0044 mg protein) or buffer (control), 30 μ l of glutathione (7.8 mM final concentration) and 200 μ l of CDNB (0.53 mM final concentration). Rate of change in optical density was measured for 10 mins at 340 nm using a Thermomax microplate reader (Molecular Devices, Palo Alto, CA) and first order reaction rates were converted to pmol/min using the experimentally derived extinction coefficient of 8.39 mM^{-1} 250 μ l for conjugated CDNB (Grant *et al.*, 1989).

4.2.5 Data Analysis

Sites were considered as random variables in this experiment. PROC GLIMMIX Repeated ANOVA (SAS Institute, Cary, NC, 2013) was used to analyze differences in enzyme activities between two treatments. Tukey-Kramer ($P < 0.05$) method was used to compare means between sites, weeks and treatments. Similarly, pair wise Students T-test was used to compare enzyme activities between pre- and post-spray collections

4.3 Results

4.3.1 pH Optima and Protein Linearization

One peak of esterase activity was found in pH optimization experiments with sodium phosphate buffer at pH 7.4 (Figure 4.02). In contrast, two peaks of GST activity were found in pH optimization experiments: the first at pH 7.4 with sodium phosphate buffer and a second, higher peak of activity using Tris-HCl buffer at pH 9 (Figure 4.03). For subsequent assays, esterase and GST activities were measured in buffers with pH values of 7.4 and 9.0 respectively.

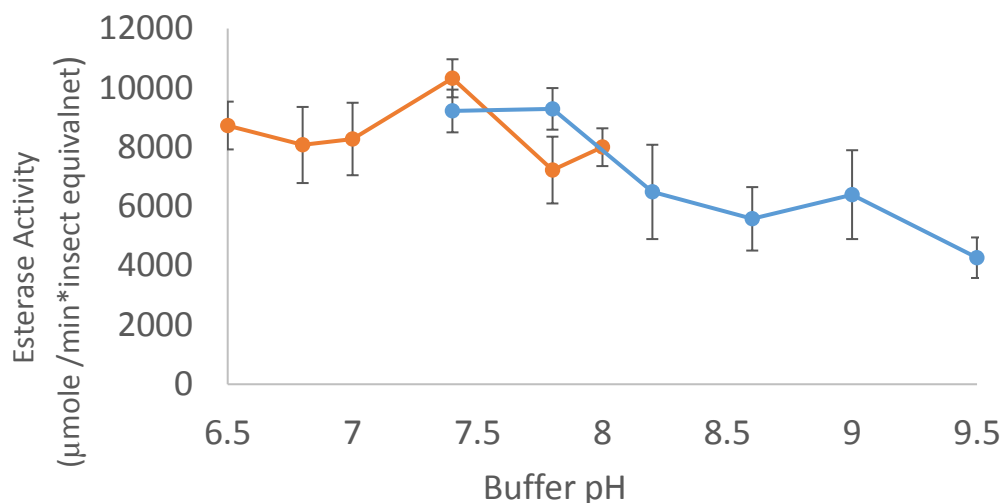


Figure 4.02. Optimization of pH for measurement of esterase activity towards α NA with 0.1M sodium phosphate (orange line) and 50 mM Tris- HCl (blue line) buffers. Points represent mean activity (\pm SEM) based on 3 determinations with different homogenates on different days.

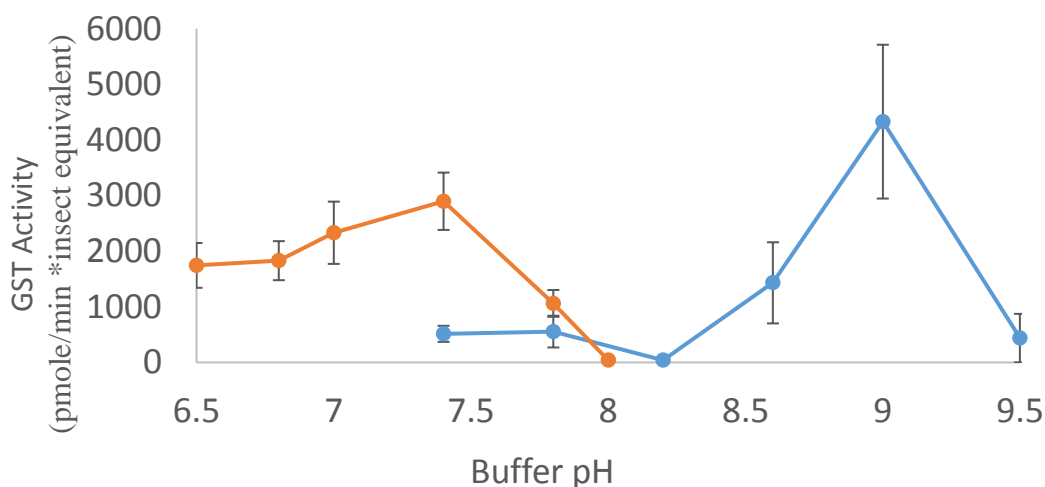


Figure 4.03. Optimization of pH for measurement of GST activities towards CDNB with 0.1M sodium phosphate (orange line) and 50 mM Tris- HCl (blue line) buffers. Points represent mean activity (\pm SEM) based on 3 determinations with different homogenates on different days.

Linear relationships between protein and enzyme activity were measured for both esterases and GSTs (Figure 4.04). Esterase activities were linear up to 4.44 μ g protein/assay whereas GST activities were linear upto 6.67 μ g protein/assay activity.

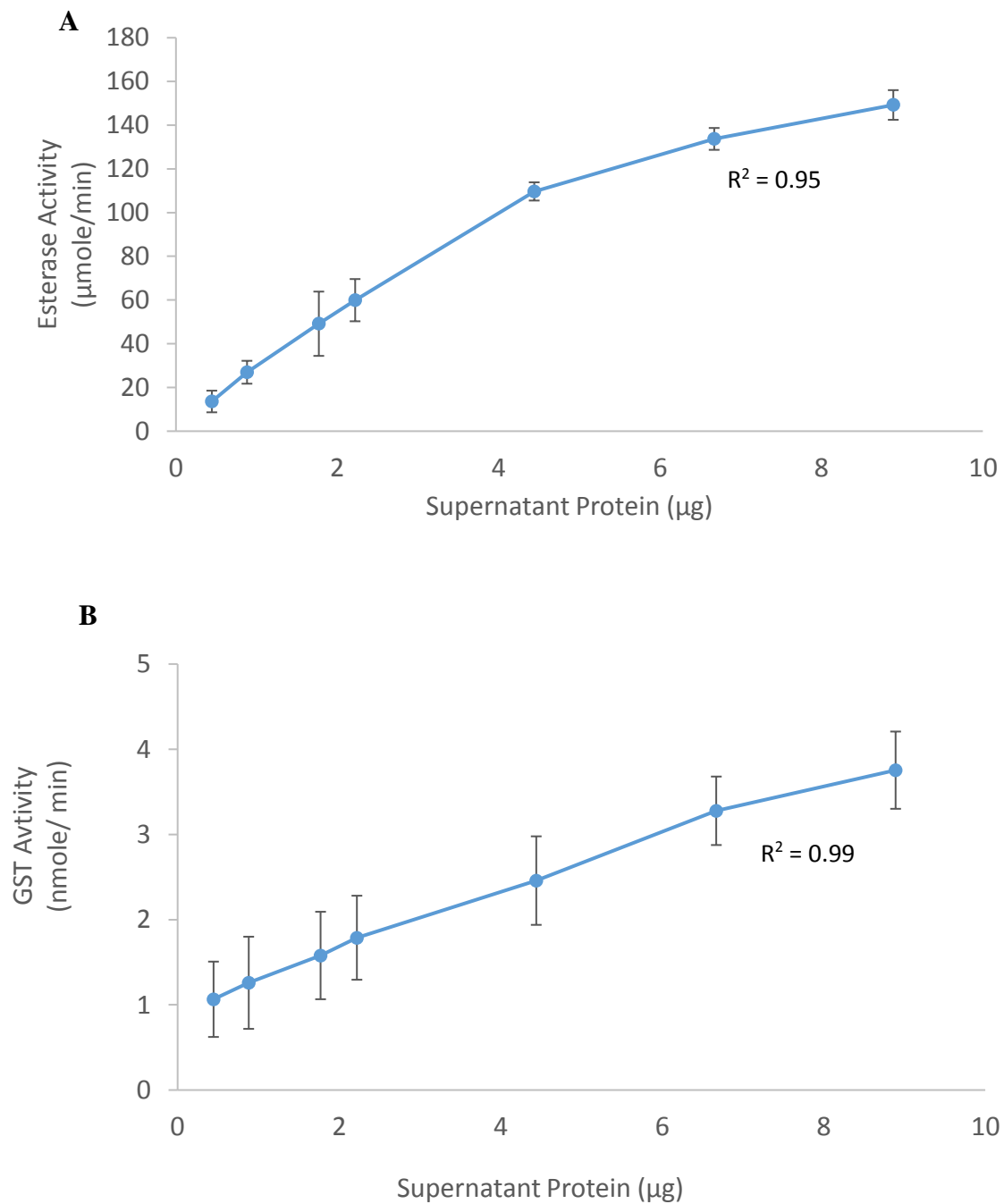


Figure 4.04. Relationship between protein and activities of esterase towards α NA (A) or GST towards CDNB (B). Points represent mean activity (\pm SEM) based on 3 determinations with different homogenates on different days .

4.3.2 Comparison of Enzyme Activities Among Experimental Sites

Effects of insecticide sprays on enzyme activities were minimal. On average, there were no significant differences in either esterase or GST activities between control and treatment sites ($F_{1,7} = 0.08$ and $P = 0.7902$ for esterase activity; $F_{1,7} = 0.05$ and $P = 0.8309$ for GST; Figure 4.05).

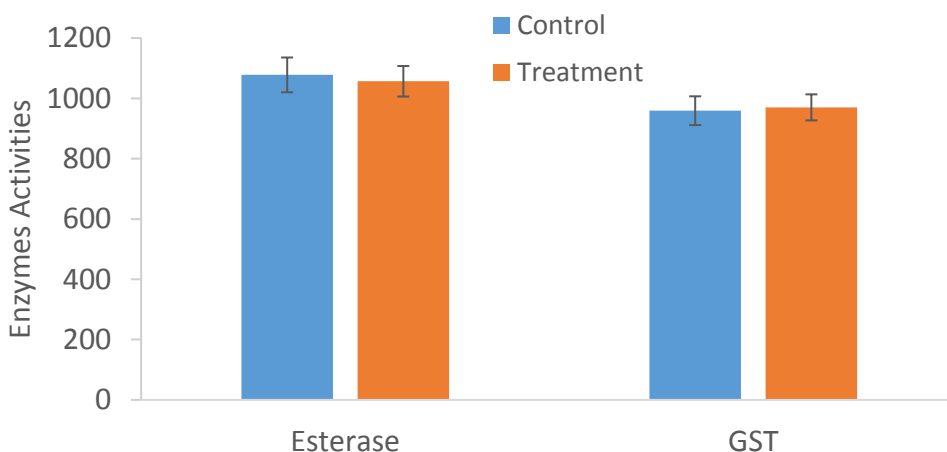


Figure 4.05. Enzyme activities from bees collected from experimental sites that were sprayed with insecticide (orange bars) or unsprayed (blue bars). Activities as expressed as $\mu\text{mole}/\text{min} \cdot \text{mg}$ protein (for esterases) or $\text{pmole}/\text{min} \cdot \text{mg}$ protein (for GSTs). Bars represent mean activity (\pm SEM) based on determinations made from 1,890 homogenates, each prepared on different days.

In addition, when sites were examined individually, enzyme activities were similar among control and insecticide-treated sites (Figure 4.06 and Figure 4.07). There were no significant differences in esterase activities among experimental sites except for site 1 ($T_{1568} = 2.29$ and $P = 0.022$) and site 4 ($T_{1568} = -2.18$ and $P = 0.0291$). Site 4 was a treated site with esterase activity of 913.01 $\mu\text{mole}/\text{min} \cdot \text{mg}$ protein. Site 1 was a control site with esterase activity of 1299.83 $\mu\text{mole}/\text{min} \cdot \text{mg}$ protein. Similarly, there were no significant differences in GST activities among individual sites except for site 9 ($T_{1568} = 2.11$ and $P = 0.035$).

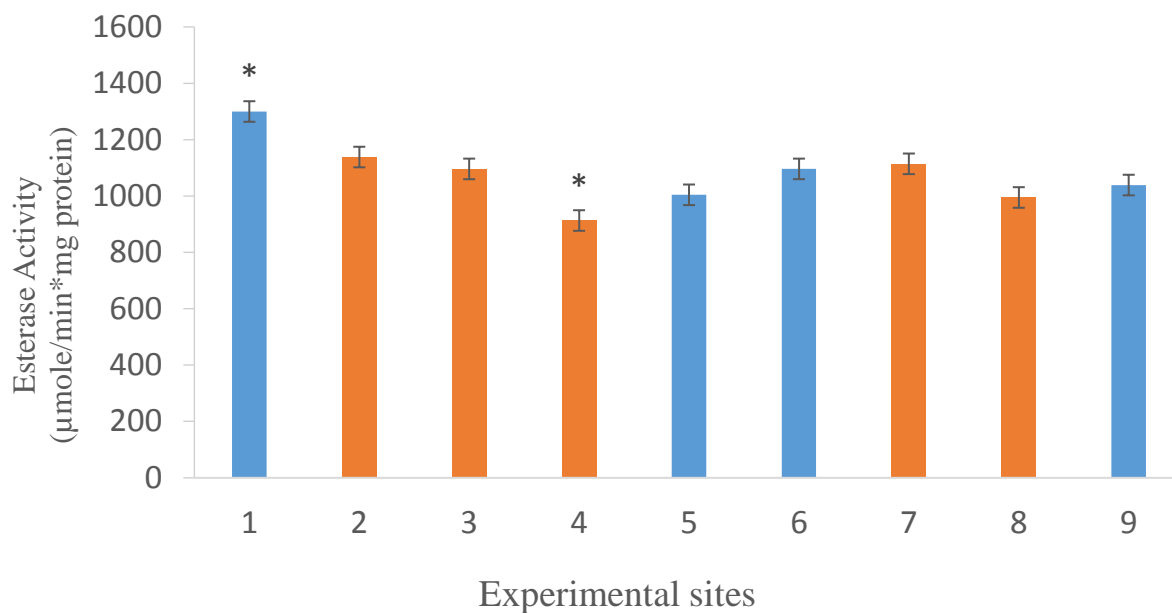


Figure 4.06. Esterase activity ($\mu\text{mole}/\text{min} \cdot \text{mg protein}$) of bees collected from individual experimental sites that were sprayed (orange bars) or unsprayed (blue bars) with insecticide. Bars represent mean activity (\pm SEM) based on determinations made from 210 homogenates, each prepared on different days .

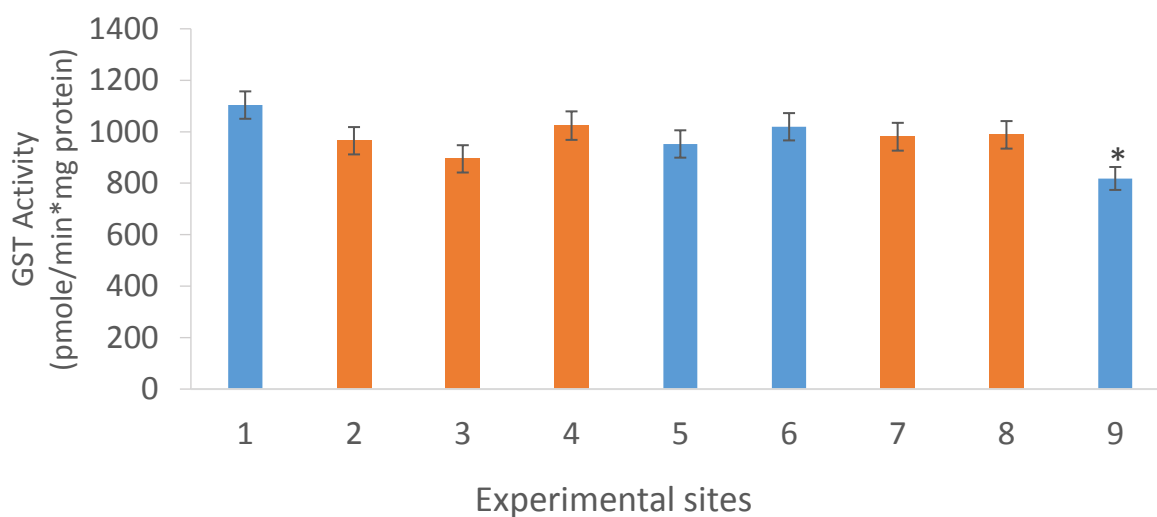


Figure 4.07. GST activity ($\text{pmole}/\text{min} \cdot \text{mg protein}$) of bees collected from individual experimental sites that were sprayed (orange bar) or unsprayed (blue bar) with insecticide. Bars represent mean activity (\pm SEM) based on determinations made from 210 homogenates, each prepared on different days .

Finally, there were no differences in enzyme activities at the treatment sites ($|T|_{190} = -0.21$ and $P = 0.3075$ for esterase activity; $|T|_{190} = 0.7$; $P = 0.4827$ for GST activity) measured prior to and following insecticide spray (Figure 4.08).

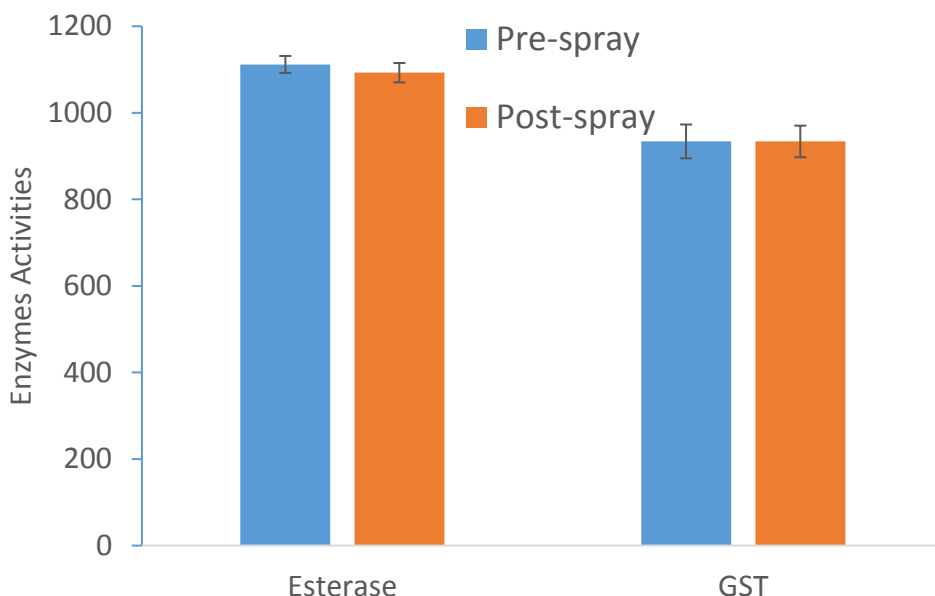


Figure 4.08. Effect of insecticide sprays on activities of GSTs (pmole/min*mg protein) or esterase (μ mole/min*mg protein). Bars represents mean activity (\pm SEM) based on determinations made from 204 homogenates, each prepared on different days .

4.3.4 Comparison of Enzyme Activities Over Time

For both esterases and GSTs, activities were similar from control and treated sites during the seven weeks period of the study. Esterase activities peaked at week 2, decreased to week 5, then remained relatively constant through the seventh week (Figure 4.09). GST activity also peaked at week 2, and declined up to 5th week, and then remained constant through week 7 (Figure 4.10).

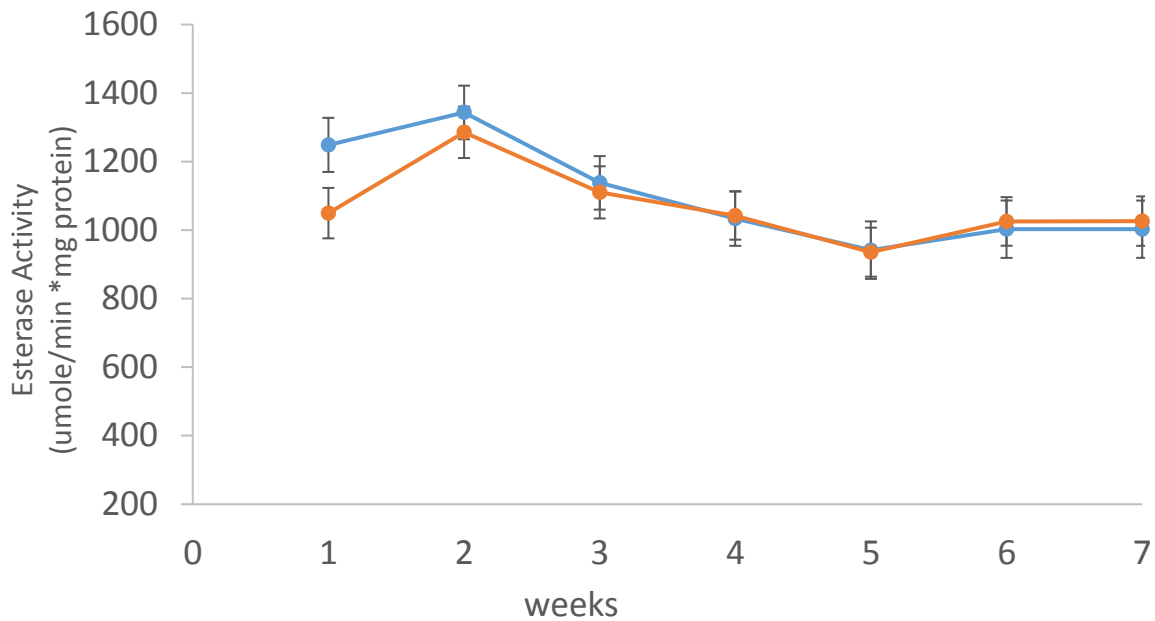


Figure 4.09. Esterase activity ($\mu\text{mole}/\text{min} \cdot \text{mg protein}$) towards αNA of bees collected from experimental sites. Blue line represents activities from untreated (control) sites whereas the orange line represents activity from treated (treatment) sites. Points represent mean activity (\pm SEM) based on determinations made from 204 homogenates, each prepared on different days .

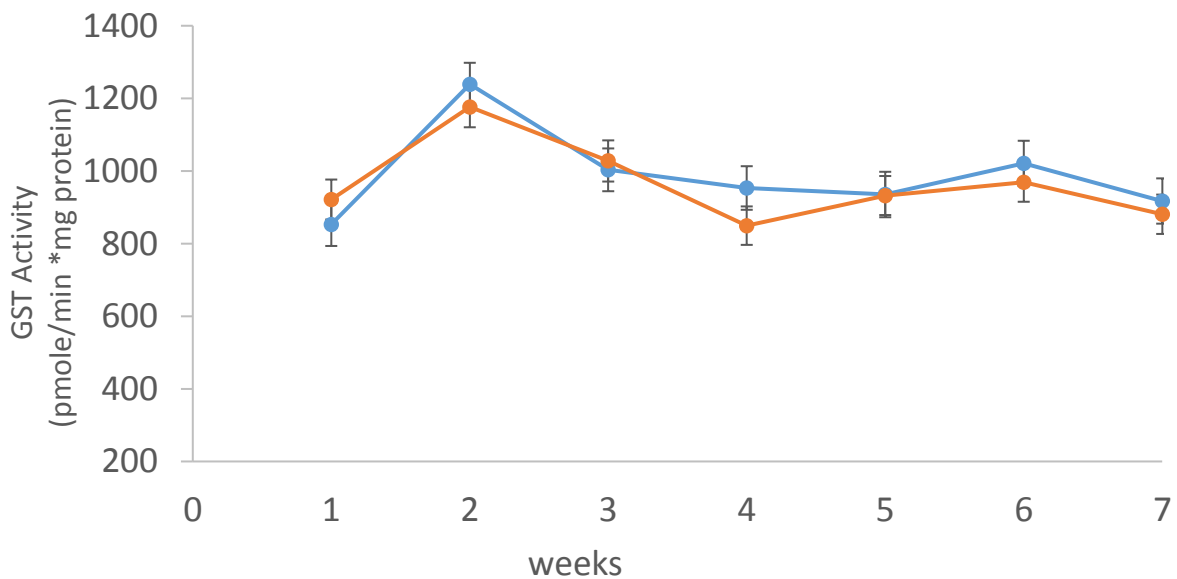


Figure 4.10. GST activity ($\text{pmole}/\text{min} \cdot \text{mg protein}$) towards CDNB of bees collected from experimental sites. Blue line represents activities from untreated (control) site whereas the orange line represents activity from treated (treatment) sites. Points represent mean activity (\pm SEM) based on determinations made from 204 homogenates, each prepared on different days .

4.4 Discussion

Exposure to insecticides, including mosquito adulticides, may be a factor in bee decline. Thus, methods are needed to detect exposure of bees to insecticides, especially at sublethal concentrations. Moreover, additional studies are required to determine safety of domesticated bees to ULV sprays using mosquito adulticides. In our study, there were no obvious, negative effects of mosquito adulticides during a seven week period of active spraying. In addition, there was no effect of putative exposure to adulticides on esterase or GST activities in honey bees at our experimental sites.

There is no question that insecticide application kills honey bees. There are number of reports in which improper application (e.g., spraying when bees are foraging) of insecticides has killed bees (Hester *et al.*, 2001; Zhong *et al.*, 2003). In addition, lab studies have shown that bees are highly susceptible to many insecticides (Decourtye *et al.*, 2005; Wu *et al.*, 2011; Rinkevich *et al.*, 2015). However, few studies have examined the effects of routine application of mosquito adulticides on honeybees (Zhong *et al.*, 2004). Our study found extremely low bee mortality (< 2%; data not shown) at the sprayed sites, suggesting that there is minimal impact on acute mortality of backyard bees when insecticide is sprayed at the recommended rates and time (i.e., after sunset when bees are not foraging inside the hive). These findings are similar to those from earlier studies (Coldburn & Langford, 1970; Zhong *et al.*, 2004) that showed minimal effect on honey bees of exposure to mosquito adulticides.

Several studies have demonstrated that activities of detoxifying enzymes may serve as indicators of insecticide exposure (Gomes *et al.*, 1999; Fulton & Key, 2001; Marks *et al.*, 2010). However, results from our study showed no effect of putative exposure to insecticides

on esterase or GST activities. These disparate results are a likely result of different methodologies. Result from our study reflect a real world sceanrio, but insecticide exposure was not controlled, or known. Earlier studies were from laboratory experiment, in which individual bees were exposed to known (often, very high) concentrations of insecticides. The insect of known exposure in field scenario is being evaluated further.

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CHAPTER 5. SUMMARY AND CONCLUSION

The honey bee plays a major role in pollination of the angiosperm plants in the world. In the United States, it has a crucial role in pollination of economically important food crops like apples, almonds, blueberries, cherries, broccoli and many other crops.

Declines in the number of honey bee colonies has been a serious concern in the past in the United States due to factors like widespread use of synthetic insecticides and introduction of new pests. However, causes of the recent decline in honey bees (i.e. from 2006 to the present) is unknown. Most of the research groups, governmental institutions, public sector, and beekeepers are concerned about the effects of pesticides on honey bees. Recent studies have shown the confounding results about the effect of agricultural pesticides on honey bees. Beside the agricultural pesticides there are other classes of insecticides in the environment which are used for public health safety. Integrated Mosquito Management (IMM) is the use of multiple control strategies to reduce populations of biting mosquitoes. While much of the focus of IMM is on source reduction and larviciding (killing immature mosquitoes with biorational products), the use of mosquito adulticides to kill adult flying mosquitoes is often warranted in times of high mosquito nuisance and virus activity. This study examined the effect of truck based mosquito adulticide sprays on the mortality, colony health and detoxifying enzymes (glutathione s-transferase and esterase) in honey bees in sentinel bee hives in a real world setting. Many of the earlier studies involved caged bees, which do not provide the natural situation of the real environment where bees are usually are in the hive at night during the spray of mosquito adulticides. Similarly, most of the studies were more focused on acute mortality of honey bees caused by mosquito adulticides.

During the seven weeks of exposure, we did not see a significant difference in bee mortality between our control and treatment sites. We did not observe significant differences in honey bee mortality between individual sites. For colony health, we measured frames of bees, brood quantity, and brood quality. We found that there were no significant differences in the percentages of change in number of bees and in brood quality between colonies in our control and treatment sites. Although there was a significant difference in the percentage change in brood quantity, it was mostly due the two outliers in data, which skewed the result. The two colonies at one of the treatment sites, which were apparently poor make the difference in brood quantity between control and treatment. Similarly, we measured the enzymes activities (glutathione *S*-transferase and esterase) of forager bees and did not see any significant differences in enzyme activities between control and treatment sites.

Thus we observed no effect of mosquito adulticides on honey bee mortality, colony health, and detoxifying enzymes (i.e. glutathione *s*-transferase and esterase). Although we did not see an effect of truck based mosquito adulticides on the honey bee mortality, colony health, esterase and glutathione *s*-transferase activities, there might be other deleterious sublethal effects. There might be several effects on honey bee if the use of mosquito adulticides is done in inappropriate ways: for example higher dose than recommended dose and bad spraying time (i.e. before sunset). There are several other possible causes which might effect on the health of honey bees like diseases and pests, poor nutrition, lack of genetic diversity and changing landscape and interaction of these factors with the xenobiotics.

APPENDIX

Site 1: This site is on the East Feliciana Parish, north of Baton Rouge where there was no mosquito adulticides spray. This was one of our control sites. Bee colonies were approximately 278 feet (85 meter) distance from the road. They were just behind the house building. The hives were facing towards the road. The bee colonies on this sites were strong with more bee populations. According to beekeeper no in hive chemicals were used. All of the bee colonies were of Italian race.

Site 2: It is our treatment site which is located in north of East Baton Rouge Parish near to the Baton Rouge metropolitan airport. All the hives were at a distance of 322 ft. (98 m) from the road. Hives were facing toward the road. There was building between the hives and road. Two of the colonies were weaker with less developed brood and few number of bee populations. Miticide was used in three of the colonies for varroa mite treatment All of the bee colonies were of Italian race.

Site 3: It is another treatment site which is located in East Baton Rouge Parish. The distance between the hives and road was 498 ft. (152 m). Hives were facing towards the road. House building was between the hives and road. Bee colonies were healthy form the beginning with high number of bee populations, good brood and no sign of infestation of mite. No in hive chemicals were used in the colonies according to the beekeepers. All the bee colonies were of Italian race

Site 4: It is next treatment site which is located in East Baton Rouge Parish. The distance between road and the hives was 152 ft. (46 m).Two hives were facing towards the road and third hive was facing against the road. House building was between the road and the hives. Acaricides

(Taufluvanite) was used for Varroa mite treatments in the hives. All of the bee colonies were of Italian race.

Site 5: This is another control site. This site was also on the East Feliciana Parish, north of Baton Rouge. The distance from the road to the bee colonies was approximately 3,231 ft. (985 meter). The colonies were on the open field with tall trees behind it. All the

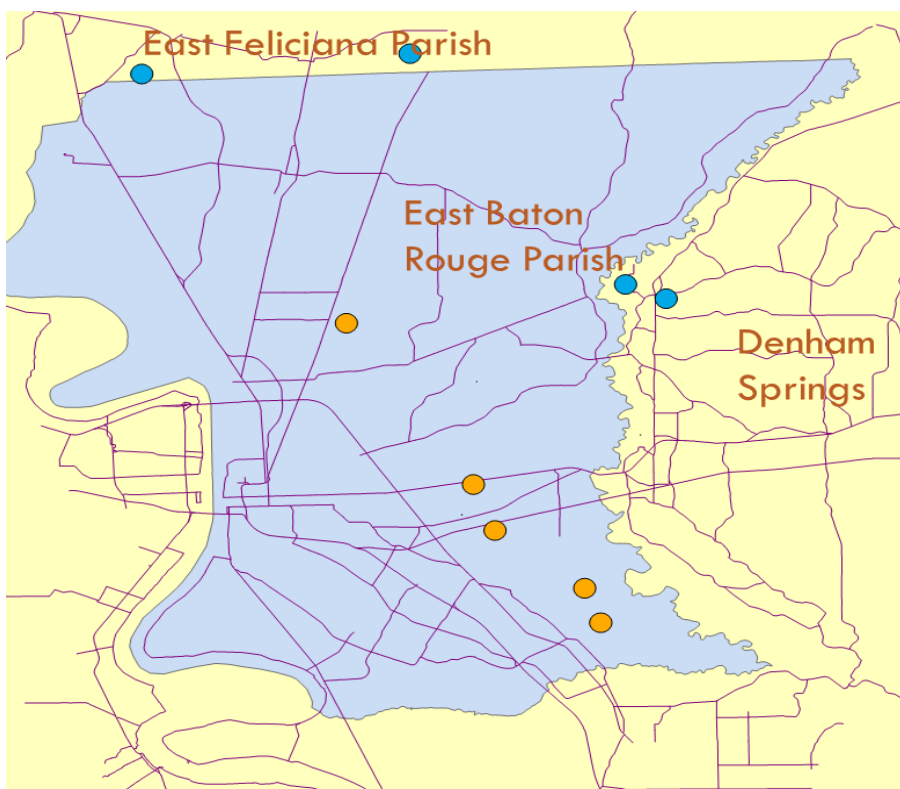


Figure A. Experimental sites used for study. Blue circles represent control sites and orange circles represent treatment sites.

hives were facing towards the road. There was absence of queen in one of the colonies during the mid of our experiment. New queen was replaced during the end period of our study. Bee colonies were treated with acaricide against varroa mites. The bee colonies were of Italian race.

Site 6: It is another control site which is in Denham Spring Parish. Two hives were at a distance of 180 ft. (146 m) from the road and one hive was at a distance of 250 ft. (76 meter) from the road. All the hives were on the open field with grass and trees were behind the hives. All the experimental hives were facing parallel to the road. All the bee colonies looked healthy and strong with high number of bee populations. Acaricide was used for the treatment of varroa mite

treatment. These sites have the colony of Italian race and mixed races which were brought from swarming.

Site 7: This treatment site is located in East Baton Rouge Parish. The distance between the road and hives was 184 ft. (56 m). All the hives were facing towards the road. Between the hives and road there were no any buildings and dense vegetation. There were few small bushes scattered on the ground. No acaricides were used in the colonies. Many small hive beetles were seen in two of the colonies. All of the bee colonies were of Italian race.

Site 8: It is the last treatment site which is located in East Baton Rouge Parish. The distance between the road and the hives was approximately 191 ft. (58 m). All the hives were facing towards the road. House building was between the road and the hives. All the bee colonies were brought from USDA Honey Bee Breeding, Genetics and Physiological Research Unit, Baton Rouge, LA. All the bee colonies were strong with high number of bee populations and good brood. All the colonies were of strong with Italian race.

Site 9: It is the control site which is also in Denham Spring Parish. Hives were at a distance of 310.69 ft. (94.70 m) from the road. Hives were facing against the road. All the experimental hives were of Italian races. All the colonies were newer bee colonies with less number of bee populations. Beekeeper started using acaricide from the second week of our experiment when he noticed more bee mortality in his bee colonies. Between the road and hives there were cluster of large trees.

VITA

Vivek Pokhrel was born and raised in Dang, Nepal. He received his bachelor's degree in agriculture with a major in plant pathology in 2011 from the Institute of Agriculture and Animal Sciences, Tribhuvan University, Nepal. After getting his bachelor's degree, he was employed as an agriculture instructor for diploma level students in Mid-West, Nepal. In the fall of 2014, he started his master's degree in entomology at Louisiana State University (LSU), Baton Rouge, Louisiana under the supervision of Dr. Kristen Healy. His research was focused on the effects of truck based ultra-low volume mosquito adulticides on honey bees.