12-2018

Toxicity of Formulated Insecticide Mixtures to Apis and Non-Apis Bees

Joseph Edward Belsky
University of Arkansas, Fayetteville

Toxicity of Formulated Insecticide Mixtures
to *Apis* and Non-*Apis* Bees

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Entomology

by

Joseph Edward Belsky
Cornell University
Bachelor of Science in Hotel Administration, 2010

December 2018
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

_____
Neelendra Joshi, Ph.D.
Thesis Director

_____
David Biddinger, Ph.D.
Committee Member

_____
Nicholas Seiter, Ph.D.
Committee Member

_____
Cammy Willett, Ph.D.
Committee Member

Abstract

This work investigates ecotoxicology of new insecticide mixtures to *Apis* and non-*Apis* bees. Previous studies have demonstrated the acute and sub-lethal toxicity of individual active ingredient insecticides to honey bees and to a lesser extent non-*Apis* bees. However, the contact toxicity of formulated premix insecticides containing multiple active ingredients to bees has not been thoroughly assessed. To address this void, laboratory studies simulating exposure to four premix insecticides under different field-realistic scenarios were conducted for two bee species.

Honey bee, *Apis mellifera* (Linnaeus) contact exposure was examined by simulating three scenarios: (1) when bees continuously forage in a field and are directly exposed to insecticides when they are being applied in that field; (2) when bees are exposed to insecticide sprays while passing through a field; and (3) when bees forage through a field in which insecticides were applied during the previous day. Insecticides comprised of neonicotinoid and pyrethroid chemistries were found to be the most toxic, and induced rapid onsets of mortality, while those comprised of diamide, molting hormone agonist and spinosyn chemistries were relatively less toxic, inducing a slower onset of mortality. At 96-hours, overall high mortality was observed for all treatments, thereby indicating the high acute toxicity of these insecticide mixtures to honey bee foragers.

Like honeybees, blue orchard bee, *Osmia lignaria* (Say), contact exposure was examined by simulating (1) when male and female bees are foraging in an orchard and come into spray-contact with premix insecticides, and (2) when male and female bees come into contact with individual active ingredient insecticides or their 1:1 binary combination. Similar to honey bees, insecticides comprised of neonicotinoid and pyrethroid active ingredients posed the highest toxicity and most rapid onset of mortality for blue orchard bees, and insecticides comprised of

diamide, molting hormone agonist and spinosyn chemistries had somewhat lower toxicity, but with more gradual mortality onset. High mortality resulted at 96-hours for all treatments, thereby indicating the high acute contact toxicity of these insecticides. These results will help to improve the current risk assessment framework for laboratory-based insecticide safety trials on bees.

Acknowledgements

There are a number of people who have provided me meaningful aid, advice and suggestions over these past two- and one-half years to whom I would like to express my gratitude. First, I would like to thank my faculty advisor and thesis director, Neelendra Joshi for sharing his wealth of knowledge with me, his willingness to engage in my research projects and support opportunities that have come my way, and for his consistent support and patience in considering all of my research ideas and proposed questions. Second, I would like to thank my committee members, David Biddinger, Nicholas Seiter and Cammy Willett for their insightful contributions from various perspectives of agricultural science and their time in helping me incorporate these contributions in crafting a dynamite master's thesis. Third, I would like to thank Nicholas Seiter and Jason Smith for all of their advice, aid and detailed conversations regarding the selection of appropriate statistical tests for analyzing my datasets. Fourth, I would like to thank Virgil Piazza for all of his help behind the scenes in constructing certain materials used for conducting the experiments in my research thesis chapters. Fifth, I would like to thank the other faculty members in the entomology department for taking the time to learn about my future goals and taking the time to offer their sage and seasoned advice regarding Ph.D. programs and research directions. Sixth, I would like to acknowledge my appreciation to all of my departmental peers for serving as a conduit and sounding board through which to channel many conversations about research and existential life. Seventh, I would like to extend a huge thank you to my parents for their constant unconditional love, advice on everything, constant enthusiasm and sincere interest in my research throughout my graduate school journey.

Table of Contents

List of Tables

List of Figures

List of Appendices

Chapter I: Introduction and Literature Review

I. Introduction

A. Bees Are Integral Pollinators

Pollination services provided by animals are integral for successful fertilization and resulting seed set of many angiosperm plants. During the process of foraging, these animals cross-pollinate plants of different cultivars by transferring pollen grains from the anthers of one flower to the stigma of another. Approximately 80% of wild plant species depend on animal pollinators to produce fruits and seeds (Biesmeijer et al. 2006; Potts et al. 2010), while approximately 35% of agricultural food and fiber crops depend on animal pollinators to yield harvestable fruits, vegetables, nuts, seeds and fibers (Klein et al. 2007). Bees provide the majority of pollination services and are considered to be the most important pollinators from agricultural crop yield and economic perspectives (Klein et al. 2007).

The bees (Hymenoptera: Apoidea: Anthophila) are a large and diverse clade comprising more than 17,500 described species and up to 20,000 species worldwide (Michener 2007). They are taxonomically classified as three distinct groups: honey bees (7 species), bumble bees (approximately 300 species) and solitary or wild bees (approximately 17,000 species, some of which are not solitary, but eusocial) (Michener 2007). Bees can also be classified biologically based upon their nesting behavior: approximately 70% of bees excavate tunnel-like nests in holes underground with specializations for soil, moisture, and salinity, 20% excavate nests above ground in pre-existing cavities in deadwood, dead stems where they have removed the pith, free standing structures or construct stand-alone nests and 10% are cleptoparasites that rob nectar and pollen provisions from the nests of other bee species (Cane 1991; Cane 2001; Wilson and Messinger Carril 2016).

Native to Africa, Europe and the Middle East, European honey bees [*Apis mellifera* (Hymenoptera: Apidae) (Linnaeus, 1758)] are domesticated for their pollination services and production of a variety of hive products (including honey) throughout most of the world. They are generally considered to be the most valuable pollinators of monoculture crops given their perennial biology, large workforce of foragers, ecology as non-specific pollinators, widescale availability, ease of transportation and economic affordability (Klein et al. 2007; vanEngelsdorp and Meixner 2010). Honey bees are also valuable for agriculture because their activity is controlled by ambient temperature throughout the autumn and winter in warmer climates, as opposed to other bee species that are only active during specific seasons and have an extended diapause (Wilson and Messinger Carril 2016). Therefore, honey bees are commercially managed for crop pollination events. They are especially valuable for pollinating crops that bloom during the winter and early spring such as almond [*Prunus dulcis* (Rosales: Rosaceae) (Mill. and Webb)] in California, which occurs before most solitary bees begin pupal eclosion in the late spring. In fact, given their widescale use as managed pollinators, honey bees account for nearly 80% of all crop pollination in the United States (Wilson and Messinger Carril 2016). From an economic perspective, in 2009 within the United States, honey bees accounted for $11.68 billion USD in production for crops directly dependent on bees for pollination, and $5.39 billion USD in production for crops indirectly dependent on bees for pollination (Calderone 2012).

Bumble bees [*Bombus* species (Hymenoptera: Apidae) (Latreille, 1802)] are considered to be a foundation for natural ecosystems given their generalist pollinator ecology and the complexity of flowering plants they forage in diverse habitats, particularly in mountainous regions where they are most abundant (Heinrich 1979; Memmott et al. 2004). They are also capable of providing pollination services in cooler ambient temperatures and throughout a wider

daily timescale than honey bees (Corbet et al. 1993; Willmer et al. 1994). Bumble bees are unique in their ability to pollinate flowers requiring high frequency sonication, commonly referred as buzz pollination (King and Buchmann 2003). While honey bees are incapable of buzz pollinating, several other species of bees beyond bumble bees are, including carpenter bees, [*Xylocopa* species (Hymenoptera: Apidae) (Latreille, 1802)] and several other Apidae species. A few species of bumble bees are commercially bred and have been shown to increase fruit yield of solanaceous (e.g. bell pepper, eggplant, potato and tomato) and ericaceous (e.g. blueberry, cranberry and huckleberry) cultivars that benefit from sonication for sufficient fertilization (Velthuis and van Doorn 2006; Drummond 2012). However, from a grower's perspective, they are more expensive than honey bees ($1.00-2.00 USD per bumble bee worker versus $0.01-0.02 USD per honey bee worker) (Calderone 2012). However, considering their pollination efficiency, bumble bees may not be that much more expensive. For example, if domesticated bumble bees are 80 times more efficient at pollinating a certain crop than honey bees, (Biddinger; pers. comm.) then the cost per each bee should be adjusted.

Solitary bees comprise the majority of the approximately 4,500 native bee species present in North America and are primarily divided into five taxonomic families: Andrenidae, Apidae, Colletidae, Halictidae and Megachilidae (Michener 2007). While the majority of solitary bees are generalists pollinating flowers from a diversity of plant species, several are specialists and will only pollinate one or a select few species of plants. For example, squash bees [*Peponapis* species (Hymenoptera: Apidae) (Robertson, 1902)] only pollinate squashes [Cucurbitaceae species (Cucurbitales) (Juss.)] and long-horned bees [*Melissodes* species (Hymenoptera: Apidae) (Latreille, 1829)] primarily specialize on sunflowers [*Helianthus* species (Asterales: Asteraceae) (Linnaeus)] (Wilson and Messinger Carril 2016). However, unlike honey bees and bumble bees,

most solitary bees only actively forage at specific annual time frames that are in sequence with the bloom of the flowers they pollinate. A handful of solitary bees are commercially managed for crop pollination services, for example mason bees [*Osmia* species (Hymenoptera: Megachilidae) (Panzer, 1806)] are used to pollinate pome, stone and nut fruits [(*Malus* species (Mill.), and *Prunus* species (Linnaeus)], while alfalfa leafcutter bees [*Megachile rotundata* (Hymenoptera: Megachilidae) (Fabricius, 1787)] are used to pollinate alfalfa [*Medicago sativa* (Fabales: Fabaceae) (Linnaeus)] and other forage crops (Bosch and Kemp 2001; Pitts-Singer and Cane 2011). Similar to bumble bees, commercially bred solitary bees are generally more expensive than honey bees (Bosch and Kemp 1999; Crown Bees; Mason Bees for Sale.com). However, considering their pollination efficiency, solitary bees may not be that much more expensive. For example, if Japanese orchard bees [*Osmia cornifrons* (Hymenoptera: Megachilidae) (Radoszkowski, 1887)] are 80 times more efficient at pollinating a certain crop than honey bees, (Biddinger, pers. comm.) then the cost per each bee should be adjusted. Solitary bees have been estimated to provide $3 billion USD in annual crop pollination services in the United States alone, and their pollination services to insect pollinated crops have been estimated to be over $3000 USD per hectare (Losey and Vaughn 2006; Kleijn et al. 2015). However, these values probably underestimate the true contribution of solitary bees to crop pollination.

B. Potential Causes of Bee Declines in Recent Years

Beginning in the winter of 2006-2007, honey bee colonies suffered significant losses marked by a specific set of symptoms 1.) rapid exodus of adult worker bees from colonies leaving massive quantities of intact brood behind, 2.) dearth of dead worker bees within and surrounding afflicted colonies and 3.) delay of hive pest infestation and cleptoparasitism by neighboring colonies (vanEngelsdorp et al. 2009). This phenomenon, described as Colony

Collapse Disorder (CCD), has persisted in affecting honey bees at varying levels over the last decade. For example, commercial beekeepers have experienced approximately 30% annual losses of overwintering colonies during this time frame, marked by a high of 45% in 2012-2013 (Steinhauer et al. 2016). In an epizootiological study of CCD-affected and non-affected apiaries, vanEngelsdorp et al. (2009) identified a group of interacting honey bee stressors and their interactions as being potential causes of CCD, without isolating a single variable as the principle causative agent. Bumble bees and solitary bees have also steadily declined in abundance and species richness over the past 50 years, with several species having major population decreases and a few becoming endangered or extinct (Goulson et al. 2015). At the same time, demand for bee pollination services has substantially increased given the upward trajectory in cultivation of obligatory-bee pollinated crops in both the developed and developing world during this same time period (Aizen et al. 2008). Additionally, the crucial role of solitary bees in providing crop pollination services within their native habitats has become increasingly understood through a myriad of relatively recent ecological studies that show previous estimates of their value was probably underestimated (Park et al. 2018). Therefore, concern has mounted that we are on the brink of a "pollination crisis" characterized by a surge in crop cultivation and an insufficient quantity of bees to provide pollination services (Holden 2006; Gross 2008). While no such crisis currently exists, data suggest that insufficient agricultural crop pollination has resulted in reduced yields at localized levels (Goulson et al. 2015).

These events have served as the impetus triggering a flurry of scientific research investigating the full spectrum of potential causes of bee declines over the last decade. Findings arising from these studies are debatable and incomplete owing to the various stakeholders producing them, combined with the shortage of monitoring surveys accurately tracking bee

(especially solitary bees) population changes overtime. However, these studies have consistently identified the following bee stressors that are hypothesized to individually and in combination interact (additively or synergistically) to affect bees and hence drive their population declines: 1.) Habitat destruction and fragmentation has decreased the availability of bee floral resources and nesting areas, which has been a long-term stressor spanning the 20th century in most of the developed world. 2.) Monoculture plantings have diminished the diversity of different flowering plant species within the radius of bee foraging distances. Therefore, these plantation-like agro-ecosystems are thought to inhibit bees from obtaining nectar and pollen from a diversity of floral sources to meet their nutrition requirements. 3.) Parasites, pathogens and pests that naturally afflict bees and foster the spread of bee diseases. 4.) Human-mediated bee movements have been linked to inducing bee stress and introducing foreign parasites to native bee populations. 5.) Increased competition amongst bees within the same nest or ecological niche for limited resources is also proposed to be a factor contributing to their population decreases. 6.) Climate change resulting in inconsistent weather and seasonal patterns affecting the time frame of floral blooming events has also been proposed to contribute to bee declines. 7.) Pesticide applications (and particulary active ingredients in seed treatments) have dramatically spiked as a result of crop pest resistance buildup and agricultural intensification, which has simultaneously exposed non-target organisms including bees to increasing levels of these toxic compounds. 8.) Interactions of multiple bee stressors occurring within the same environment at the same time are thought to create cocktail-like effects that further intensify the sharp population declines of bees observed over the last 10-15 years.

II. Bee Stressors Other Than Pesticides

A. Habitat Destruction and Fragmentation

It is widely agreed that the decrease and fragmentation of natural habitats as a result of land use and agricultural intensification is a major long-term stressor contributing to the decline of bees (Goulson et al. 2008; Potts et al. 2010). A meta-analysis of 54 studies on different factors impacting bee ecological communities found habitat destruction was the most important variable for wild bee abundance and species richness (Winfree et al. 2009). Similarly, a quantitative synthetic analysis of 23 studies on 17 crops in agro-ecosystems worldwide showed increased agricultural crop distance from surrounding natural habitats significantly decreases wild bee abundance and species richness (Ricketts et al. 2008). As these studies demonstrate, disrupted landscapes lacking diverse floral resources necessary for adequate bee nutrition within close proximity to where bees forage can substantially impair their ability to survive. Obtaining proper nutrition during forage is essential for 1.) adult bees to meet their food maintenance requirements and reproductive needs, and 2.) the ability of adult female bees to provision their nests with appropriate food resources ensuring healthy offspring development. The severity of habitat destruction and fragmentation as a bee stressor is maximized when one considers specialist solitary bees that unlike generalist pollinators, only forage a single species or group of closely related species of flowering plants (Cane 2001). For example, bee diversity in apple [*Malus* species (Rosales: Rosaceae) (Mill.)] orchards has been found to be driven by plant species diversity in surrounding habitats (Kammerer et al. 2016). The impacts of habitat destruction on bee biology and ecology are generally classified into 1.) factors effecting availability of floral resources for adult bees to forage; 2.) factors affacting the ability of adult female bees to properly

excavate and provision their nests; and 3.) the positive and negative impacts of urbanization on natural resources essential to bees.

Intensification of agricultural area has been a major driver of pollinator habitat loss and disturbance. For example, the loss of 97% of floral grasslands in the United Kingdom spanning the course of the 20th century has drastically constricted the range of bees associated with these ecological communities (Howard et al. 2003). Similar declines have occurred in North American grasslands beginning in the 19th century (Samson and Knopf 1994). Specific examples of historical land use manipulation impacting pollinators over time are as follows. Comparing the diversity of bees and hoverflies combined with the plants they pollinate (pre-versus post-1980), Biesmeijer et al. (2006) found a causal correlation in decline mostly marked by specialist pollinators and obligate-insect pollinated plant species. Surveying bumble bee abundance and species richness over the course of the 20th century in Illinois, Grixti et al. (2009) found major declines between 1940 and 1960 coinciding with large-scale agricultural intensification.

Disturbed and fragmented habitats can also decrease the ability of female bees to excavate and provision their nests; particularly for bee species reliant on a variety of ecological features for nest construction. Namely, Cane (2001) proposes that the adverse distributions of bee species seen in these studies might be explained by their nesting ecology instead of their floral ecology. For example, because *Hesperapis oraria* [(Hymenoptera: Melittidae) (Snelling and Stage, 1996)] is entirely dependent upon friable sands of vegetated backdunes for building nests, this species does not inhabit inland areas beyond coastal backdunes that also contain its only floral host (Cane et al. 1996; Cane 2001). This bee's highly specific habitat dependence directly controls its geographic range and occurrence throughout the northern Gulf of Mexico. Measuring the effects of a number of habitat disruption variables on the response of different bee

species, Williams et al. (2010) concluded that species specific nesting biology and life-history behavior are helpful to predict an outcome. For example, above ground nesting bees were more negatively affected by agriculture practices leading to habitat fragmentation and fire, while below ground nesting bees were more affected by soil tilling practices in agricultural environments. Carrié et al. (2017) also found positive associations between below ground nesting bees and increased access to grassland and hedgerows, while the opposite held true for above ground nesting bees in southwest France. Interestingly, this study found increased above ground nesting bee density linked to semi-natural habitat, while below ground nesting bee density increased in landscapes comprised of agricultural crops in southeastern Australia. Investigating differences in bee species functional-trait diversity in a mosaic landscape, Forrest et al. (2015) conclude that while bee communities on conventional and organic farms comprised smaller numbers of above ground nesting species and larger numbers of below ground nesting species, the opposite effect was observed in surrounding natural habitats. They attribute these differences to increased availability of suitable nesting substrates for above ground nesting bees (such as trees and shrubs) in natural habitats and larger areas of bare soil suitable for ground dwelling bees to excavate their nests on farms. These findings are further supported by statistical analyses demonstrating that bee community trait diversity is most strongly driven when the variable nesting location is included.

An analysis of bee species richness in response to habitat fragmentation found an interactive effect between bee species abundance and amount of forest cover at regional and local scales (Ferreira et al. 2015). A positive relationship was shown between above ground nesting bees and increased regional forest cover, while less regional forest coverage resulted in increased abundance of below ground nesting bees. Social bee impacts of local scale forest cover

were driven by regional forest composition. In assessing the effects of forest fragmentation on stingless bee [Meliponini species (Hymenoptera: Apidae) (Lepeletier, 1836)] nesting excavation, Lichtenberg et al. (2017) most commonly found ground nesting and inquiline nesting species in pastures compared to cavity nesting species, thereby indicating an evolutionary adaptation to surviving in heavily deforested areas. In agreement, Smith and Mayfield (2018) show that cavity nesting bees most frequently pollinated all fragment study plots, with the exception of soil nesting bee species dominating small fragments of *Acronychia acidula*, [(Sapindales: Rutaceae) (F. Muell.)] suggesting a similar bee species adaptation in ecological niches lacking large quantities of trees.

B. Monoculture Plantings

Agro-ecosystems have become increasingly intensified with large monocultures of row crops such as soybeans and maize and orchards of various pome, stone and nut fruits. Analyzing crop rotation patterns in the Central United States between 2003-2006 and 2007-2010, Plourde et al. (2013) show the quantity of maize grown in segments of four continuous years doubled between the two study periods, thereby approaching a monoculture cropping practice. California almond acreage increased from 430,000 acres (174,015 hectares) in 1996 to a projected 800,000 acres (323,749 hectares) in 2012 (Sumner and Boriss 2006). By 2016, almonds accounted for 1,240,000 acres (501,810 hectares) in California (National Agricultural Statistics Service 2017). In the wake of monoculture agricultural designs and increased cultivation of pollinator-dependent crops, the demand for bee pollination services has spiked. This is most evident in honey bee almond pollination where hive rental fees rose from $54 USD to $136 USD between 2004 to 2006 (Sumner and Boriss 2006), and steadily increased to $175.52 USD by 2015 (Ferrier

et al. 2018). Simultaneously, the quantity of pollinator-dependent agricultural crops planted worldwide over the last 50 years has exponentially increased (Aizen et al. 2008).

Bees require a variety of nutrients in their diets comprised of carbohydrates (in the form of sugar from nectar or honey), amino acids (in the form of protein from pollen), lipids, vitamins, minerals and water in the correct ratios for proper growth and development (Huang 2010; Vaudo et al. 2015). Monoculture floral blooming events are defined as short massive gluts of the same species of flower dominating an agricultural landscape that is temporally and spatially isolated from other species of blooming flowers including wildflowers (Goulson et al. 2015). It is hypothesized that exposure to massive spreads of monoculture environments has negatively impacted bees by depriving their access to the wide diversity of floral resources necessary to meet their nutritional requirements.

Research studies have shown the ability to obtain pollen from a diversity of plant species directly determines the overall lifespan of honey bees. For example, caged honey bees fed pollen from multiple plant species lived longer compared to their counterparts fed pollen from a single plant species (Schmidt 1984; Schmidt et al. 1987; Schmidt et al. 1995). Assessing honey bee susceptibility to *Nosema ceranae* mites [(Dissociodihaplophasida: Nosematidae) (Fries et al., 1996)] Eischen and Graham (2008) found that poorly nourished bees were more susceptible compared to well nourished bees by quantifying significantly more adult bees in pollen supplement-fed colonies. Similarly, Eischen et al. (2008) found lower *Varroa destructor* mite [(Parasitiformes: Varroidae) (Anderson and Trueman, 2000)] mite infestation levels in honey bee colonies fed pollen substitute powder (BeePro®) + 4% pollen. In an early experiment by Rinderer and Elliott (1977) and a more recent experiment by Huang (2012) caged honey bee workers exposed to poly-floral pollen have been shown to be more resistant to pathogenic stressors.

These studies demonstrated the effects of honey bee pollen nutrition on *Nosema apis* [(Dissociodihaplophasida: Nosematidae) (Zander, 1909)] mite resistance. Similarly, the work of Di Pasquale et al. (2013) demonstrates the effects of diverse and quality pollen nutrition on honey bee resistance to *N. ceranae*. Pollen nutrition leading to sufficient protein diets have also been found to increase honey bee immunity, as in DeGrandi-Hoffman et al. (2010) where honey bees fed protein-free sugar syrup diets had lower protein levels in their heads, smaller hypopharyngeal glands and higher deformed wing virus titers compared to honey bees fed with pollen or a protein supplement (MegaBee®).

Landscape design and resulting flower abundance can also affect the specific nutrition requirements of bumble bees at the individual species and colony levels. For example, in analyzing the impact of spatial and temporal variations in floral resources, Moquet et al. (2017) show peak bumble bee sensitivity during colony development compared to nest excavation or mating. Additionally, larger colonies were found to require larger landscapes of favorable floral resources, while the proportion of large colonies declined in areas dominated by spruce plantations, which was an unfavorable resource. Allocating *Bombus impatiens* [(Hymenoptera: Apidae) (Cresson, 1863)] colonies in grasslands differing in floral resource allocation, Spiesman et al. (2017) show that dominance by a few species of flowering plants, as opposed to diversity in floral species richness is the most important local-scale factor influencing colony growth and reproduction. Harmon-Threatt and Kremen (2015) found that feral bumble bees blend their pollen collection from native and exotic plants to meet their unique protein and essential amino acid nutritional needs. Placing *B. impatiens* colonies in three different ecological habitats, Vaudo et al. (2018) concluded that while colony growth and reproductive output was not strongly related to nutritional quality of collected pollen, colony acquisition of specific nutrients (protein,

lipid and sugar) was. These results suggest that abundance of floral resources with appropriate nutrients is imperative to bumble bee colony success. Examining foraging behaviors of *B. impatiens*, Vaudo et al. (2016) demonstrated a preference for flowers with high protein: lipid (P:L) ratios and more specifically, for nutritionally modified pollen with P:L ratios of 5:1 and 10:1, thereby indicating that foraging decisions are influenced by specific macronutrient ratios.

Solitary bees also have similar nutritional requirements, for which most studies suggest a departure from monoculture plantings is the best remedy. Assessing bee species dietary balance, diverse floral composition presenting nectar (carbohydrate) and pollen (protein) rewards in phenological succession throughout a day and season is shown to maximize proper nutrition of multiple bee species comprising an ecological guild (Vaudo et al. 2015). Estimating the number of flowers required to rear a single larva for 41 European bee species Müller et al. (2006) determined a range (7 to 1,100 flowers or 0.9 to 4.5 flower heads) depending on both the bee species and the host plant. Feeding larvae of the generalist pollinator *Osmia cornuta* [(Hymenoptera: Megachilidae) (Latreille, 1805)] experimental diets containing different quantities of unfavorable *Ranunculus acris* [(Ranunculales: Ranunculaceae) (Linnaeus)] pollen and favorable *Sinapis arvensis* [(Brassicales: Brassicaceae) (Linnaeus)] pollen, Eckhardt et al. (2014) found that an unfavorable pollen diet usually resulted in larval mortality, while mixed pollen diets had no effect on larval survival, development time and adult body mass. Simulating pollen quantity and quality manipulations to *Ceratina calcarata* [(Hymenoptera: Apidae) (Robertson, 1900)] in a laboratory setting, Lawson et al. (2017) showed that larvae provided additional pollen significantly consumed more pollen, had larger adult head widths and contained greater stored fats compared to those provided reduced pollen. Fischman et al. (2017)

similarly concluded that *M. rotundata* fed small quantity diets weighed less as adults, while those fed large quantity diets entered diapause more often.

C. Parasites, Pathogens, and Pests

Bees are naturally afflicted by a variety of parasites, pathogens and pests. The vast majority of research has focused on 1.) honey bees and to a lesser extent on 2.) bumble bees, therefore little information is currently known regarding 3.) solitary bees. Naturally occurring disease agents are known to control populations of their bee hosts within their native range, although most are not well understood.

Most honey bee viruses including deformed wing virus are vectored by *Varroa destructor* mites as directly injected virus particles followed by mite foreign salivary proteins into the bee hemocoel (Rosenkranz et al. 2010). Di Prisco et al. (2016) uncovered a mutualistic symbiosis between *Varroa* and deformed wing virus, affecting cellular immune responses with nuclear factor-$_{K}$B signaling, that enhances mite reproduction. Ryabov et al. (2017) used next generation sequencing to confirm Varroa destructor virus-1 presence in 603 United States apiaries, finding a 66% infection rate in honey bee pupae, compared to a 2.7% infection rate from a 2010 analysis of 75 colonies. *Nosema* species are obligate intra-cellular parasites affecting both individual honey bee adults and entire colonies after consumption. Roberts et al. (2015) found *Nosema* spores in the semen of infected honey bee males, and subsequent higher infection of *N. ceranae* in queens artificially inseminated with either spores or semen of infected males. In a molecular survey highlighting *Nosema bombi* [(Dissociodihaplophasida: Nosematidae) (Fantham and Porter, 1914)] spread to bumble bee queens, Tripodi et al. (2014) detected *N. bombi* in 27% of *B. pensylvanicus* and 13% of *B. auricomus*.

Several other pathogens and pests have been documented to afflict honey bees and bumble bees. These include tracheal mites [*Acarapis* species (Trombidiformes: Tarsonemidae) (Rennie 1921)], American foulbrood [*Paenibacillus larvae* (Bacillales: Paenibacillaceae) (Ash et al. 1994)], European foulbrood [*Melissococcus plutonius*, (Lactobacillales: Enterococcaceae)], chalkbrood [*Ascosphaera* species, (Onygenales: Ascosphaeraceae) (Olive and Spiltoir 1955)], small hive beetles [*Aethina tumida*, (Murray, 1867) (Coleoptera: Nitidulidae)], greater and lesser wax moths [*Galleria* species (Lepidoptera: Pyralidae) (Blom 1764)], and several trypanosomatids [*Crithidia* species (Trypanosomatida) (Léger 1902)] and [*Apicystis* species (Neogregarinorida: Lipotrophidae) (Liu et al. 1974)].

Several recent studies investigating pathogenic stressors of solitary bees have been conducted given increased interest in their use as alternative pollinators to honey bees. In a survey of six RNA viruses in a sample of honey bees and bumble bees, McMahon et al. (2015) concluded that while virus occurrence in honey bees significantly predicted infection in bumble bees, in some cases such as for acute bee paralysis virus, feral bumble bees contained substantially higher infection levels than domesticated honey bees. Using PCR to sequence *O. cornifrons* DNA for detection of pathogenic microorganisms, Hedtke et al. (2015) identified pathogenic and apathogenic fungi [*Ascosphaera and Aspergillus* species (Eurotiales: Trichocomaceae) (Micheli 1729)]), and a hypothesized apathogenic bacteria strain (within the *Paenibacillus* genus) in asymptomatic adult bees that might be disease carriers. Similarly, analyzing meta-transcriptomes of eight species of wild social and solitary bees, Schoonvaere et al. (2018) identified four viruses homologous to insect pathogens, 11 unclassified arthropod viruses, seven plant viruses and one fungal virus. This analysis also isolated three parasites in the wild bee samples as well as a yeast in the solitary bee samples, thereby demonstrating the

widescale diversity of viruses and microorganisms inhabiting feral bees. Screening honey bees and several species of solitary bees, Ravoet et al. (2014) found a variety of viruses and parasites in the majority of bees sampled. These results indicate either 1.) pathogen spillover between commercially managed bees and wild bees or vice-versa, 2.) pathogen transmission between bee species sharing ecological niches, 3.) pathogen transmission within a particular bee species such as social bees sharing the same nest, or 4.) all of these forms of pathogen spread amongst bees, all of which warrant further investigation. In reviewing literature on bee viruses, Manley et al. (2015) argue that the commercialization of managed bees for pollination events is a main driver of the rapid spread and increased prevalence of bee pathogens.

D. Human-Mediated Bee Movements

Anthropogenic actions related to the domestication of a handful of bee species for commercial pollination are indicated as contributing to bee stress by increasing disease spread amongst domesticated bees and pathogen spillover from domesticated bees to wild bees native to the region in which their colony was placed. For example, widescale transportation of commercial honey bee hives to pollination events is hypothesized to stress colonies by means of confinement and irregular disturbance, induced vibration, and exposure to extreme temperatures and high levels of carbon dioxide (Goulson et al. 2015). Placing large quantities of honey bee hives in close proximity during pollination events is hypothesized to increase disease and pest spread between colonies (Goodwin et al. 2006; Forfert et al. 2015) Poor husbandry practices involving rearing *Bombus terrestris* [(Hymenoptera: Apidae) (Linnaeus, 1758)] queens alongside honey bee workers have been implicated in the spread of deformed wing virus in bumble bees (Genersch et al. 2006). Global distribution of *B. terrestris* colonies has been associated with the spread of *N. bombi* leading to the failure of commercial *Bombus occidentalis* [(Hymenoptera:

Apidae) (Greene, 1858)] breeding programs in western North America (Brown 2011; Manley et al. 2015).

Introductions of honey bee species to non-native ranges have resulted in negative outcomes. For example, the importation of *A. mellifera scutellata* colonies to Brazil and subsequent escape of virgin queens led to interbreeding with feral *A. mellifera* species resulting in hybridization of highly aggressive "Africanized" honey bee strains (Byatt et al. 2016). Importation of western *A. mellifera* into the native range of eastern *Apis cerana* [(Hymenoptera: Apidae) (Fabricius, 1793)] has resulted in intermating between the two honey bee species rendering inviable offspring and possible queen thelytokous parthogenesis (Remnant et al. 2014). Reviewing human-mediated actions related to the spread of honey bee pathogens, Owen (2017) suggests that the massive transport of colonies to California for almond pollination is likely to increase pathogen and pest spread between colonies (Goodwin et al. 2006; Forfert et al. 2015). For example, deformed wing virus vectored by *Varroa* mites has spread worldwide following international honey bee trade (Wilfert et al. 2016), while migratory beekeeping in the United States and Australia have been proposed to favor the spread of honey bee pathogens such as chalkbrood (Gordon et al. 2014). A field experiment comparing commercial and experimental honey bee hives that were either stationary or migratory found significant decreases in lifespan and increased oxidative stress for bees exposed to migratory treatments (Simone-Finstrom et al. 2016). Additionally, transportation-induced stress has been suggested as a causative factor in honey bee declines in Hungary (Bakonyi et al. 2002).

Commercialization and subsequent global distribution of bumble bees has raised concerns regarding the spread of non-native species and non-local genotypes to intact ecological communities (Byatt et al. 2015; Evans 2017). For example, the wide environmental adaptability,

large colony size, generalist foraging and flexible nesting behavior of *B. terrestris* (Kraus et al. 2009; Dafni et al. 2010) indicates the high capacity for managed colonies of this species to naturalize and establish in non-native habitats (Evans 2017). Evidence of this has been demonstrated in Japan (Goka 1998) and Argentina (Torretta et al. 2006). In Japan, *B. terrestris* mating with the native species *Bombus hypocrita* [(Hymenoptera: Apidae) (Pérez, 1905)] and *Bombus ignitus* [(Hymenoptera: Apidae) (Smith, 1869)] has been shown to result in non-viable offspring (Kanbe et al. 2008). While multiple subspecies of *B. terrestris* have been classified, only one is commercially bred, thereby resulting in introduction of non-native subspecies in western Europe (Goulson 2010). Supporting this concern, evidence of non-native *B. terrestris* subspecies outcompeting native subspecies in foraging and reproduction has been demonstrated (Ings et al. 2006). Extracting *Nosema* species DNA from six species of bumble bee museum specimens collected between 1980-2011, Cameron et al. (2016) show significant increases in *N. bombi* infection rates during this period, which coincides with a period of massive *N. bombi* outbreaks in North American commercial bumble bee colonies. However, this study found no evidence of an exotic origin for *N. bombi*, and moreover concluded that the current *N. bombi* strain infecting declining bumble bee species was present in the United States before the initiation of commercial breeding and trade. Also, this strain of *N. bombi* is not detectably different from that found in western Europe. Screening adult *B. impatiens* workers with qPCR from commercialized colonies originating from 120 greenhouses in central Mexico, Sachman-Ruiz et al. (2015) detected one or more pathogens in bumble bees originating from 54 greenhouses (representing a 45% infection rate). The authors argue that more rigorous screening protocols of commercial bumble bee colonies such as the methodology utilized here should be

mandated to ensure maximized greenhouse pollination services and that pathogen spillover to native bee species does not occur.

PCR screening and DNA analysis of *O. cornifrons* detected *Ascosphaera* species fungal spores in asymptomatic adult bees, thereby indicating transport of these fungi within the bees themselves from their native habitat in Japan to their introduced regions in eastern North America (Hedtke et al. 2015). Confirmation of *O. cornifrons* exposure to these fungi, which had previously only been isolated in nests of other related bee species demonstrates the potential for their spread to native North American bee species. This is a result of the less stringent screening processes required by the USDA at the time of *O. cornifrons* introduction to the United States for pollination of mid-Atlantic orchards in the 1990's. Distribution of commercially bred Meliponini species bees throughout Brazil is of concern for endangered species (Francisco et al. 2014) such as *Melipona capixaba* [(Hymenoptera: Apidae) (Moure and Camargo, 1994)] which might be at risk for loss of genetic identity by means of hybridization (Nogueira et al. 2014), and which has been documented to interbreed with domesticated *Melipona scutellaris* [(Hymenoptera: Apidae) (Latreille, 1811)] (Nascimento et al. 2000). Although it is currently unknown if human-mediated movements of stingless bees in Australia is responsible for genomic extinction of rarer species through interbreeding (Halcroft et al. 2013), species including *Tetragonula carbonaria* [(Hymenoptera: Apidae) (Smith, 1854)] and *T. hockingsi* [(Hymenoptera: Apidae) (Cockerell, 1929)] are capable of rendering viable hybrid offspring in native and introduced habitats (Franck et al. 2004; Brito et al. 2014).

One other aspect is the effects of introducing non-bee species into various regions of the world that serve as predators of bees. For example, establishment of the Asian hornet [*Vespa velutina nigrithorax* (Hymenoptera: Vespidae) (Lepeletier, 1836)] in Japan has contributed to the

loss of honey bees (*A. cerana japonica* and introduced *A. mellifera*) because only a few adults can completely decimate a hive in a short time and subsequently feed on brood (Ueno 2014). Interestingly, *A. cerana* have evolved a defensive behavior against wasp attacks where honey bee workers use their body heat to kill an invading wasp by forming a ball-like shape around it (Ono et al. 1987; Ichino and Okada 1994; Abrol 2006). Use of dinotefuron for controlling spotted lanternfly [*Lycorma delicatula* (Hemiptera: Fulgoridae) (White, 1845)] in the mid-Atlantic (Dara 2018; Derato et al. 2018) is problematic for bees given the low contact LD_{50} of dinotefuran to honey bees (Iwasa et al. 2004). In Pennsylvania, widespread applications of dinotefuran for controlling spotted lanternfly on tree of heaven [*Ailanthus altissima* (Sapindales: Simaroubaceae) (Mill and Swingle)] have negatively impacted bees (Biddinger; pers. comm.). Application of broad-spectrum pesticides (especially pyrethroids) for controlling brown marmorated stink bug [*Halyomorpha hays* (Hemiptera: Pentatomidae) (Stål, 1855)] in apple orchards interfered with integrated pest management programs (Leskey et al. 2012). Pyrethroid application specifically resulted in outbreaks of secondary pests such as European red mites [*Panonychus ulmi* (Trombidiformes: Tetranychidae) (Koch, 1836)], wolly apple aphids [*Eriosoma lanigerum* (Hemiptera: Diaspididae) (Hausmann, 1802)] and San Jose scale [*Quadraspidiotus perniciosus* (Hemiptera: Diaspididae) (Comstock, 1881)] , that are normally controlled by natural enemies. Native bee populations nesting in orchard edges were also negatively impacted by pyrethroid applications made to control brown marmorated stink bugs in orchard edges (Biddinger et al. 2011). Maintaining surrounding native habitat should be emphazised in orchards given that pesticide impacts on bees can be reduced in these environments (Park et al. 2015). Finally, spotted wing drosophila [*Drosophila suzukii* (Diptera: Drosophilidae) (Matsumura, 1931)] infestations have forced growers to dramatically increase broad-spectrum pesticide usage in

small fruit (Beers et al. 2011; Van Timmeren and Isaacs 2013). Similar to brown marmorated stink bug, this has disrputed integrated pest management programs and affected the surrounding environment (including habitats where bees forage).

E. Increased Competition for Limited Resources

While ascertaining the impact of competition between bee species for limited floral and nesting resources on resulting bee species abundance and diversity is relatively difficult (Goulson et al. 2015), some evidence does point toward increased competition in ecological communities that is increased by introductions of non-native species, and particularly when these introduced species occur in high quantities (Goulson 2003). Most studies demonstrating significant effects of increased competition between bee species have found that the presence of honey bee apiaries negatively effects the diversity of feral bumble bees and other solitary bee species native to the specific ecological niche being studied (Huryn 1997; Goulson 2003).

High concentrations of managed honey bee colonies have been shown to displace native bumble bees from flowering plants they normally forage (Forup and Memmott 2005; Walther-Hellwig et al. 2006). Quantifying floral resource consumption in rosemary, [*Rosmarinus officinalis* (Lamiales: Lamiaceae) (Linnaeus)] and thyme, [*Thymus vulgaris* (Lamiales: Lamiaceae) (Linnaeus),] plots combined with floral resource density and bee diversity, Torné-Noguera et al. (2016) found that resource consumption was primarily driven by honey bee visitation and marginally by bumble bee (*B. terrestris*) visitation. This study also showed lower wild bee diversity in plots closer to apiaries, and concluded that as the major consumer of pollen and nectar from these two flowering plant species, honey bees at the studied density of 3.5 hives/km^2 affect wild bee diversity and abundance. Conversely, analysis of the interactions between Africanized honey bees and Meliponini species competing for a honey-water resource

demonstrated rare and mild *Apis* bee aggression towards Melponini bees, however *Apis* bees were found to display high aggression towards each other (Roubik and Villanueva-Gutiérrez 2017). Moreover, in foraging for a common food resource, *Apis* bees were found to be the most widely attacked by other Hymenopterans and often behaved evasively following an attack.

Applying 38 red mason bees, *O. bicornis* in treatment cages with 0, 100 or 300 honey bees (10-20% of which were of foraging age) containing flowering plants, Hudewenz and Klein (2015) found reduced *O. bicornis* flower visitation and reproduction in the presence of honey bees. Moreover, the wide range niche of *O. bicornis* was found to diminish as the quantity of honey bees increased, while the overlap of floral resources remained constant, thereby demonstrating the potential for temporary impacts on wild bees from competition with honey bees in isolated flower patches. Similarly, in calculating that a strong honey bee hive collects the same amount of pollen as is needed to produce 100,000 progeny of an average-sized solitary bee (*M. rotundata*), Cane and Tepedino (2017) predict that a typical apiary containing 40 honey bee hives collects the equivalent of 4 million solitary bee larval pollen provisions over an average 3-month summer, thereby directly competing with and negatively affecting native wild bees. Warzecha et al. (2016) found that increased competition for common food resources amongst four *Andrena* [(Hymenoptera: Andrenidae) (Fabricius, 1775)] species resulting from habitat fragmentation led to increased body sizes of the two medium-sized bees. In this context, pollen analysis demonstrated that larger individuals forage significantly smaller spectrums of flowering plants. This study shows that ecological landscape changes may alter bee pollination services without impacting bee species richness and diversity.

F. Climate Change

Climate change as defined by global warming combined with erratic weather conditions has been proposed to affect the symbiotic interactions of pollinating insects and the flowering plants they pollinate; however, the specific impacts of this variable are not well understood (Goulson et al. 2015). One primary concern is that mismatches between the timing of adult bee emergence and the onset of plant flowering will result. While evidence of temporal mismatches in plant-pollinator phenology have been documented in a few cases, most ecological data suggest that parallel shifts following linear trajectories have occurred between these mutually interacting organisms in response to warming temperatures and other environmental stimuli (Hegland et al. 2009; Forrest 2015). Another concern is that increased temperatures and resulting changes in the distribution of flowering plants have shifted the ranges of bees further north and to increased elevations, thereby curtailing their natural ranges at southern latitudes and lower elevations (Ploquin et al. 2013; Kerr et al. 2015; Miller-Struttmann et al. 2015). Extreme weather conditions such as storms, excessive flooding and drought have also been proposed, and in some cases have been shown to negatively impact the normal temporal interactions between flowering plants and the bees that pollinate them (Visscher et al. 1994; Goulson et al. 2015).

Bartomeus et al. (2011) demonstrate linear changes between bee and flowering plant phenology in the northeastern United States over a duration of 130-years, while Ovaskainen et al. (2013) found long-term consistency between early bumble bee foraging and spring flowering plants in Russia. Similarly, concurrent linear trends have been found for increased temperatures and earlier flowering dates (Sparks et al. 2000) and increased temperatures and earlier first appearances of honey bees (Gordo and Sanz 2005). Moreover, several studies analyzing plants have demonstrated linear patterns between temperature and pollinator phenology that are most

robust in the early spring season (Roy and Sparks 2000; Forister and Shapiro 2003; Menzel et al. 2006). Conversely, widening gaps between the spring appearance of honey bees and cabbage white butterflies [*Pieris rapae* (Lepidoptera: Pieridae) (Linnaeus, 1758)] and the initiation of flowering of several host plants utilized by these species have been observed and attributed to insect sensitivity to increased temperatures (Gordo and Sanz 2006). Earlier flowering of the bumble bee queen-dependent *Corydalis ambigua* [(Ranunculales: Papaveraceae) (Cham. and Schltdl.)] resulting from earlier snowmelt has been found to create a mismatch between flowering and bee appearance, which is not impacted by earlier snowmelt (Kudo and Ida 2013). Similarly, Kudo et al. (2004) found that warm spring temperatures resulted in advanced plant flowering while having no effect on bumble bee queen emergence, thereby leading to reduced seed-set in plants pollinated by bumble bees. Williams et al. (2012) found that timing of floral resource availability affects growth of *Bombus vosnesenskii* [(Hymenoptera: Apidae) (Radoszkowski, 1862)] colonies, however no impact on colony reproduction was seen. Simulating the effects of increasing temperatures on plant-pollinator interactions, Memmott et al. (2007) show that between 17-50% of all pollinator species assessed would be negatively impacted by food shortages as a result of temporal mismatches. Laboratory studies demonstrate that depending on accumulation of fat reserves, *Osmia* species males can survive for a week without food at 20°C (Bosch et al. 2010), therefore suggesting some flexibility in asynchrony between bee emergence and plant flowering. However, the dearth of data on how climatic variables impact the symbiosis of *Osmia* species with pollinating flowers (with a particular emphasis on data relating to the specific alimentation needs of these bees), warrants future work given the importance of these bees in commercial pollination of orchard, fruit and nut crops.

Analyzing changes in tongue length between specimens of *Bombus balteatus* [(Hymenoptera: Apidae) (Dahlbom, 1832)] and *Bombus sylvicola* [(Hymenoptera: Apidae) (Kirby, 1837)] collected in the Central Rocky Mountains over a 40-year timespan, Miller-Struttmann et al. (2015) found significant shortening of tongues overtime. This study attributes this finding to reduced flower density at lower elevations specifically characterized by decreased abundance of plant species containing deep corollas in their flowers that is being driven by an on average increase in summer temperatures. Similarly, assessing bumble bee species distributions over a 20-year duration, Ploquin et al. (2013) determined that an overall uphill shift in range emphasized retractions in the lower limits of species occurrence, combined with a simultaneous increase in temperature by approximately 0.9°C throughout the study period. Supporting these findings, Kerr et al. (2015) found changes in bumble bee distribution across Europe and North America over a 30-year duration characterized by an inability of species to follow warming temperatures over time at their northern range limits, retractions in the southern limits of species' natural ranges, and movements towards higher elevations for southern occurring species. Using various climatic models to investigate current and future trends in bee diversity across six locations, Hannah et al. (2017) projected that areas containing less temperature warming (found to be the southern-most locations), higher rainfall, lowest water deficit and wider forest cover currently have the highest bee diversity and will serve as the best reservoirs of bee diversity in the future.

Extreme and erratic weather patterns have also studied for their potential impacts on plant-pollinator interactions. For example, Visscher et al. (1994) found late emergence of *Calliopsis pugionis* [(Hymenoptera: Andrenidae) (Cockerell, 1925)] compared to flowering of the primary plant it pollinates, *Encelia farinosa*, [(Asterales: Asteraceae) (Torr. and A. Gray)]

following heavy rains. However, this mismatch was considered an isolated event because the bee nests in lowland pools that seasonally flood, while the plant grows in upland areas that remain drier, therefore these authors hypothesized that this is not a recurring asynchrony. Extreme winter values of the Pacific Decadal Oscillation index have been linked to population declines of the alpine butterfly *Parnassius smintheus*, [(Lepidoptera: Papilionidae) (Doubleday, 1847)] (Roland and Matter 2013), thereby potentially impacting the pollination of plants that this butterfly commonly utilizes for nectar resources. Simulating pollinator range shifts along a rain gradient, Devoto et al. (2007) show that changes in rain fall would result in relatively few species going extinct, with relative resilience in pollination systems to climatic changes. This outcome held true even when assuming that pollinating insects were specialists obligated to one species of flowering plant and that flowering plants were completely dependent on pollinating insects to successfully be fertilized and induce seed-set.

III. Pollinators and Agricultural Pesticides

A. Insecticide Toxicity to Honey Bees

The acute toxicity and chronic sub-lethal effects of pesticide exposure have been extensively researched in honey bees. For example, more than 150 pesticide residues have been found at various concentrations in honey bees and hive products (Johnson et al. 2010; Mullin et al. 2010). Pyrethroid, organophosphate and carbamate insecticides have been the subject of many scientific investigations of chemical toxicity to bees, and not surprising for chemicals designed to kill insects, many have been demonstrated to be highly toxic. As a newer class of insecticide designed to replace some of the older classes because of the lower mammalian toxicity, neonicotinoids over the last decade have come under increased scrutiny regarding their toxicity to bees because they are plant systemic and thus can be translocated to nectar and pollen. Within

neonicotinoid bee research, many scientific studies have investigated the imidacloprid, thiamethoxam and clothianidin that are the most heavily used products in seed treatments planted on huge acreages of field crops. Showing the toxic effects of these compounds, Sandrock et al. (2014) demonstrated that honey bee queen survival and colony weakness might be linked to neonicotinoid exposure. Specifically, this study chronically exposed honey bee colonies to pollen feeding inside hive settings containing field realistic concentrations of thiamethoxam and clothianidin. Colonies exposed to both neonicotinoids had decreased short-term performance over two brood cycles resulting in a significant 28% reduction of adult bees and a 13% reduction in brood. Also, compared to control colonies, honey production and pollen collection for treatment colonies was reduced by 29% and 19% respectively. While colonies recovered after 3.5 months and overwintered successfully, they had significantly decelerated growth that was associated with queen supersedure (observed in 60% of the neonicotinoid-treated colonies within a one-year period) beginning the following spring. While neonicotinoid exposure was linked with a reduction in swarming, the authors acknowledged that the effects of neonicotinoid exposure on colony performance were significantly correlated to the honey bee genetic background. Di Prisco et al. (2013) showed that clothianidin exposure inhibits nuclear factor-$_{\kappa}$B immune signaling linked to deformed wing virus, and Fischer et al. (2014) concluded that neonicotinoid exposure limited the ability of bees to turn correctly at a specific landmark and successfully return to their hive. Refuting this, however, is a four-year field experiment (Pilling et al. 2013) that determined long-term exposure to thiamethoxam residues posed only low risk to bees foraging systemically seed-treated flowering oil seed rape. In a separate field experiment exposing bees to clothianidin residues in seed-treated canola during the summer of 2012 and the following spring of 2013 Cutler et al. (2014) concluded that this treatment posed low risk to bee

health, development and colony overwintering survival. A number of colony endpoints including colony weight gain, honey production, bee mortality, pest incidence, number of adults and amount of sealed brood as a result of beehive placement in clothianidin treated versus control fields were measured in this study. The authors found no significant differences and concluded that honey bees are relatively unaffected by exposure to clothianidin residues in seed-treated canola.

Fipronil is one of the first of the modern insecticides to have been thoroughly tested for acute and chronic toxicity to honey bees. Therefore, several fipronil studies reviewed in this section were conducted in the previous decade or earlier. Aliouane et al. (2009) determined that both oral and topical exposure to fipronil at 0.1 ng/bee induced complete mortality one-week after the beginning of treatment, and that the lower oral and topical exposure to fipronil at 0.01 ng/bee resulted in 25% and 20% mortality respectively at the end of the 11-day treatment period. Decourtye et al. (2005) used the proboscis extension reflex bioassay to expose honey bees to three concentrations of sugar syrup contaminated with fipronil. The low dose at 2.2 µg /L induced 40.6% chronic mortality, while the high dose at 9 µg /L induced 91.1% chronic mortality, both of which were significantly different from the 6.6% mortality observed in the control group. Vidau et al. (2011) tested the effects of fipronil on honey bee mortality to bees previously infected with *Nosema ceranae* spores by feeding infected bees fipronil *ad libitum* in sugar syrup. The mortality of honey bees previously infected with *N. ceranae* was significantly higher when fed fipronil at a daily oral intake of about $1/100^{th}$ of the LD_{50} at 1 µg/L at the end of the 20-day infection and 10-day insecticide exposure period.

Neonicotinoids and related compounds comprise a large portion of the modern insecticides that are commonly applied in agro-ecosystems given their broad-spectrum coverage

of many pests and increased human safety over the products they replaced. Several recent studies have reported variations in toxicity of insecticides to honey bees due to differences in honey bee race, subspecies, physiology, etc. For instance, in a laboratory study, Laurino et al. (2013) conducted oral and indirect contact bioassays to determine the acute oral and indirect contact toxicity of imidacloprid, thiamethoxam and clothianidin administered in contaminated sugar solution to three subspecies of honey bees and found significant differences. Compiling data from residue surveys and pesticide toxicity analyses of honey and bumble bees Sánchez-Bayo and Goka (2014) found neonicotinoid and pyrethroid residues pose the greatest risk to both bee species by contact exposure with contaminated pollen. In this study, honey residue dietary toxicity was high for thiamethoxam and cypermethrin, which primarily affects nectar foragers and secondarily larvae. Imidacloprid and clothianidin also posed high mortality risk to foragers (6-23% and 3-22%, respectively) and moderate risk for larvae (0.2-1.2%). Dietary toxicity from pollen was found to be highest in nurse bees, which are the only stage of adult honey bees depending entirely on this type of food. Specifically, three neonicotinoids (imidacloprid, clothianidin, and thiamethoxam) and phosmet were found to pose moderate risks (1-5%) for both nurses and the larvae they feed. In a molecular survey, Di Prisco et al. (2013) found that topically treating *V. destructor* mite contaminated-newly emerged honey bees with increasing doses of either clothianidin or imidacloprid, increased replication of deformed wing virus 24-hours post treatment. This was attributed to the negative impact of clothianidin on nuclear factor-$_{K}$B molecular immune responses by upregulating leucine-rich repeat proteins. This in turn suppress nuclear factor-$_{K}$B expression in insects and thereby enables deformed wing virus to be undetected. As a result, deformed wing virus successfully infects honey bees until their death.

Exposure to pesticides may also cause sub-lethal behavioral changes in bees. Aliouane et al. (2009) found oral exposure to acetamiprid increased bee responsiveness to water but did not affect consumption. Oral exposure to thiamethoxam at 1 ng/bee decreased honey bee responsiveness to 3% and 10% sucrose concentrations, while topical exposure to thiamethoxam at 0.01 ng/bee induced a significant decrease of honey bee olfactory learning performance or retention 24-hours post treatment. However, both treatment and control performances were the same at 48-hours post treatment. Thiamethoxam topical application at 1 ng/bee resulted in a significant decrease in olfactory learning performance during the third and fourth proboscis extension reflex retention trials, however, acetamiprid exposure did not affect bee learning or memory performance. Tan et al. (2014) found that *A. cerana* exposed to feeders containing 40 µg/L of imidacloprid contaminated sugar solution had significantly decreased returning rates to feeders, compared to bees exposed to 10 µg/L imidacloprid contaminated sugar solution and controls. Similarly, Henry et al. (2012) found that honey bee foragers exposed to thiamethoxam at 1.34 ng/20µl sucrose solution had significantly increased homing failure. Subsequently, Guez (2013) critiqued the estimation of thiamethoxam exposure, thiamethoxam dose used, and quantification of the homing failure in this study. Repeating the bioassays of Cresswell et al. (2014), Sánchez-Bayo et al. (2017) confirmed that honey bees exposed to syrup containing 125 µ/L imidacloprid contained imidacloprid body residues of 2.7-5.7 ng/g displayed trembling and restless behaviors. In conclusion, these bees did not have complete clearance of imidacloprid over the 10-day exposure period.

B. Insecticide Toxicity to Bumble Bees

While most pollinator ecotoxicology studies have focused on *Apis*-bees, several studies have investigated *Bombus* spp. As with honey bees, the majority of recent pesticide toxicology

research concerning bumble bees has focused on the neonicotinoids imidacloprid, thiamethoxam and clothianidin. Much of this was done on the commercially managed species *B. terrestris* in Europe (Gill et al. 2012; Gill and Raine 2014; Stoner 2016) and *B. impatiens* in North America (Stoner 2016). Larson et al. (2013) showed that high level rate field applications of clothianidin (0.45 kg AI ha^{-1}) for controlling scarab grub to turf with blooming white clover were toxic to commercial colonies of *B. impatiens* placed in secluded foraging enclosures within the field setting two-days after treatment. Clothianidin exposure during the six-day period of the experiment resulted in reduced foraging activity, increased worker mortality, lack of queen production and a decrease in colony weight gain. Notably, Larson et al. (2013) also exposed commercial *B. impatiens* to the high-level rate of chlorantraniliprole (0.23 kg AI ha^{-1}) (the first anthranilic diamide labeled for control of lawn insect pests) under the same experimental conditions but did not observe any effects.

Laboratory and field studies assessing the field-realistic lethal doses of these pesticides to bees have generated mixed results that are overall inconclusive. Thompson et al. (2015) quantified that *B. terrestris* fed sucrose solution contaminated with four concentrations at 0, 1, 10 and 100 µg/L of different neonicotinoids (imidacloprid, thiamethoxam and clothianidin) exhibited anti-feeding effects. These effects were reversible when bees were subsequently fed non-contaminated sucrose solution. Feeding the bumble bees with 10 µg/L of clothianidin, 10 or 100 ug/L of imidacloprid resulted in a significantly lower intake of the contaminated sucrose solution compared to controls over the four-day test period. In a compilation of studies on bumble bees and honey bees, Sánchez-Bayo and Goka (2014) determined that bumble bee pollen residue contact toxicity was high for chlorpyrifos (8.3-12.9%), imidacloprid (31.8-49.0%) and clothianidin (2.5-13.3%). Due to the differential sensitivities between bee species, they conclude

that these insecticides were 2-3 times more toxic to bumble bees and posed a higher risk to them over honey bees. Exposing queen-less micro-colonies of *B. terrestris* to honey water and pollen paste containing thiamethoxam at 1 or 10 mg/kg for 28-days, Elston et al. (2013) showed that exposure to both thiamethoxam concentrations reduced *B. terrestris* consumption of honey water and decreased the number of wax cells produced.

Using radio frequency identification chips, Gill and Raine (2014) concluded that the exposure to either lambda-cyhalothrin, imidacloprid or a combination of the two active ingredients significantly impaired the foraging activity of *B. terrestris* micro-colonies. Fauser-Misslin et al. (2014) concluded that chronic dietary exposure of *B. terrestris* to thiamethoxam and clothianidin during early colony development negatively affected long-term colony performance. Additionally, when a parasite (*C. bombi*) was introduced to laboratory colonies exposed to these neonicotinoids, a negative interaction resulted in significantly impaired mother queen survival (Fauser-Misslin et al. 2014). In a semi-field study, Stanley et al. (2015) found that *B. terrestris* from colonies pre-exposed to sucrose solutions containing 10 ppb thiamethoxam provided statistically lower visitation to apple flowers and made statistically fewer foraging trips compared to controls.

C. Insecticide Toxicity to Solitary and other Bees

A few solitary bee species are managed in agricultural settings for pollination services, for example, alfalfa leafcutter bees (*Megachile routundata*), alkali bees [*Nomia melanderi* (Hymenoptera: Halictidae) (Cockerell, 1906)], Japanese orchard bees (*Osmia cornifrons*) and blue orchard bees [*Osmia lignaria* (Hymenoptera: Megachilidae) (Say, 1837)] (Pisa et al. 2015). However, the more than 20,000 species of unmanaged solitary bees are integral for pollinating certain crops such as tree fruits, berries and cucurbits (Bosch and Kemp 2001; Park et al. 2018)

and provide valuable ecosystem services (Losey and Vaughn 2006). These bees are generally smaller in size, have shorter foraging ranges, many are univoltine, and most nest underground, thereby potentially placing them closer to pesticide applications (Pisa et al. 2015). As a result, the impacts of pesticide exposure can be more pronounced for solitary bees than in colonial, semi-eusocial, and eusocial bees. Solitary bees are less resilient to pesticide insecticide exposure than honey bees because they do not have large social colonies (Pisa et al. 2015; Hopwood et al. 2016). They can also become more limited in their food resources and habitat than social bees. However, this would not necessarily be the case for solitary bees foraging crops from adjacent habitats; but might in part explain why solitary bees do not commonly nest in orchards (Biddinger pers. comm.). Moreover, a large meta-analysis of bee pesticide exposure (Arena and Sgolastra 2014) found that solitary bees are overall less sensitive than honey bees. All of these factors are known from existing accurate studies on the toxicity of pesticides to bees and highlight the need for future studies to investigate new untested pesticide compounds. Existing studies on pesticide toxicology to bees also indicate problems with using the acute and sub-lethal toxicity of honey bees as a surrogate model for determining the effects of pesticides to other species of bees (for example *Bombus* species and commercially managed solitary bee species).

Quantifying clothianidin oral acute toxicity to *A. mellifera*, *B. terrestris* and *Osmia bicornis* [(Hymenoptera: Megachilidae) (Linnaeus, 1758)] Spurgeon et al. (2016) found the following LD_{50} values at 48, 96, and 240-hours post treatment for each bee species. *A. mellifera* (14.6, 15.4 and 117 ng/bee), *B. terrestris* (26.6, 35.0 and 57.4 ng/bee) and for *O. bicornis* (8.4, 12.4 and 28.0 ng/bee). Therefore, this study demonstrated that while *B. terrestris* are more tolerant to clothianidin than honey bees at 24 and 48-hours, they are more sensitive at 240-hours. The increased sensitivity at 240-hours is surprising given that *B. terrestris* are larger than *A.*

mellifera. It was also found that *O. bicornis* are the more susceptible to clothianidin. Sgolastra et al. (2017) also quantified the relative acute toxicity of clothianidin to these same three species of bees at 24 and 96-hours post treatment, and their results agree with those of Spurgeon et al. (2016). Together, the findings of these two studies suggest that neonicotinoids are overall more toxic to smaller bodied bees; a position also pointed out by (Hopwood et al. 2016).

Several insecticide acute toxicology studies have been done on stingless bees (Meliponini species). Costa et al. (2015) found that imidacloprid posed higher toxicity to *M. scutellaris* compared to OECD contact LD_{50} values for *A. mellifera* by determining *M. scutellaris* topical LD_{50} at 2.41 ng/bee and 1.29 ng/bee at 24 and 48-hours post treatment respectively. This study also found *M. scutellaris* oral LC_{50} at 2.01 ng/µL and 0.81 ng/µL at 24 and 48-hours post treatment respectively, demonstrating the higher oral toxicity of imidacloprid to this species compared to oral LC_{50} values for *A. mellifera*. Similarly, Lourenço et al. (2012) showed that fipronil posed higher contact and oral toxicity to *M. scutellaris* at topical LD_{50} at 0.6 ng/bee and and oral LC_{50} at 0.011 ng/µL respectively at 48-hours post treatment compared to OECD values for *A. mellifera*. Field realistic dosages of thiamethoxam at 0.004 to 4.375 ng/larva were found to decrease *Scaptotrigona depilis* [(Hymenoptera: Apidae) (Moure, 1942)] larval survival in vitro (Rosa et al. 2016). Analyzing the effects of fipronil on *Scaptotrigona postica* [(Hymenoptera: Apidae) (Latreille, 1807)] brain physiology, Jacob et al. (2015) found a low topical dose at 0.27 ng/bee and a low oral dose at 0.24 ng/bee affected the brain mushroom bodies by means of apoptosis or necrosis, with a more pronounced effect than that of *A. mellifera*. Tomé et al. (2012) showed imidacloprid effected *Melipona quadrifasciata anthidioides* [(Hymenoptera: Apidae) (le Peletier, 1836)] mushroom bodies by impairing respiration and flight activity. Finally, Valdovinos-Núñez et al. (2009) found imidacloprid, thiamethoxam and thiacloprid were more

toxic than permethrin and diazinon to the stingless bees *Melipona beecheii* [(Hymenoptera: Apidae) (Bennett, 1831)], *Trigona nigra* [(Hymenoptera: Apidae) (Cresson, 1878)] and *Nannotrigona perilampoides* [(Hymenoptera: Apidae) (Cresson, 1878)]. This study specifically calculated LD_{50} values of each pesticide for all three species. Callow workers of all three species had statistically higher mortality than foragers. For *M. beecheii*, statistically more males died than females when exposed to similar pesticide doses. Also, *M. beecheii* gynes were statistically more susceptible than workers.

In the meta-analysis of Arena and Sgolastra (2014) *A. mellifera* was overall more sensitive to the 53 pesticides analyzed, however there were several instances (20 out of 53 analyzed pesticides) where these compounds were more toxic to other bee species. Across the six pesticide classes tested in this meta-analysis, only neonicotinoids were found to be more toxic to solitary bees than *A. mellifera*. For acute contact LD_{50}, only seven cases demonstrated a toxicity that was 10-fold higher for a solitary bee than *A. mellifera*. For instance, specifically compared to *A. mellifera*, acetamiprid had a 12-fold higher toxicity to *O. cornifrons* (Biddinger et al. 2013), while cyhalothrin had a 11-fold higher toxicity to *M. rotundata* (Devillers et al. 2003). Similarly, fipronil had a 14-fold higher toxicity to *M. scutellaris* and a 24-fold higher toxicity to *S. postica*, while thiacloprid had a 2,086-fold higher toxicity to *N. perilampoides*. Therefore, conclusions are hard to find because bee family makes a difference in susceptibility to pesticides and sometimes even the same species may react differently to various pesticides even within the same class.

Most work investigating the toxicity of pesticides to solitary bees have concerned *Osmia* and other genera within the Megachilidae because they are easy to manage and rear in large numbers necessary for bioassasy (Mader et al. 2011). A number of these studies have compared

the toxicity of pesticides to *Apis* and non-*Apis* bees including both *Bombus* species and solitary bees. For example, a recent report on neonicotinoids has concluded that bees can contact residues in pollen and nectar, residues can persist in soil and plants for months to years after application, and that residues from seed-treated crops can contaminate the surrounding environment (Hopwood et al. 2016). This report also found that home product applications can be made at higher levels than the maximum approved field rate and foliar contact with the most toxic neonicotinoids has resulted in bee mortality. Analyzing comparative responses of *A. mellifera* and *O. cornifrons* to two neonicotinoids (acetamiprid and imidacloprid) and two organophosphates (dimethoate and phosmet), Biddinger et al. (2013) found strikingly different results. Specifically, this study demonstrated (1) different bee species can have unique responses to insecticides within the same class, and (2) some neonicotinids are more toxic than others to bees. Similarly, numerous studies have been done comparing the toxicity of active ingredients to alfalfa leafcutter bees (*M. rotundata*) and honey bees. In early studies, using contact application to quantify toxicities for comparison, Torchio (1973) found that alfalfa leafcutter bees had the highest tolerance while honey bees had the lowest tolerance. Comparing the LD_{30} and LD_{95} of trichlorfon for honey bees and male and female alfalfa leafcutter bees, Ahmad and Johansen (1973) showed that trichlorfon was 18-34 times more toxic to honey bees than to female alfalfa leafcutter bees. This difference in trichlorfon toxicity was attributed to higher pH of body fluid in alfalfa leafcutter bees, which is 6.0 for honey bees and 6.8 for alfalfa leafcutter bees. Trichlorfon is known to be highly unstable at neutral or high pH levels.

Some studies have analyzed the sub-lethal effects of pesticide exposure to solitary bees and found reproductive effects on bee larvae. For instance, Abbott et al. (2008) found that when feeding *O. lignaria* larvae food dosed with 30-300 $\mu g/kg^{-1}$ of imidacloprid, the time for them to

develop into adults was prolonged. Torchio (1983) concluded that a field treatment of trichlorfon (Dylox) did not affect survivorship of nesting alfalfa leafcutter bees (*M. rotundata*). The number of cells completed per day decreased, however, the mortality of eggs and young larvae increased in cells following trichlorfon treatment. More recently, Peach et al. (1995) found that alfalfa leafcutter bees ingesting 2 mg of carbaryl in a 25% honey bran bait solution had no significant effects on adult nesting performance, brood care, offspring size and sex ratio.

In a recent study, Sandrock et al. (2014) fed adult female *O. bicornis* sugar solution contaminated with 2.87 ppb thiamethoxam and 0.45 ppb clothianidin plus untreated pollen. Although exposure resulted in no significant effects on female longevity and body weight, treated bees constructed 22% fewer nests that contained 43.7% fewer cells, for which relative offspring mortality was significantly higher (15% versus 8.5% for treated and control bees respectively). This study concluded that adult female *O. bicornis* chronically treated with neonicotinoid laced sugar solution resulted in a significant reduction of their offspring emergence per nest, marked by a 47.7% reduction of offspring production in treated bees. Rundlöf et al. (2015) analyzed the effects of clothianidin-treated oilseed rape on *O. bicornis*. Treated fields were sowed with seeds treated with 25 ml of the Bayer product Elado® (400 g/l clothianidin + 80 g/l beta-cyfluthrin) and the fungicide thiram at a rate of 10 g clothianidin/kg of seed, while control fields were sown with seeds only treated with the fungicide thiram. They found that while none of the *O. bicornis* placed adjacent to the treated oilseed rape fields engaged in nesting behavior and brood cell construction, *O. bicornis* placed adjacent to six of the eight control fields did.

D. Toxicity of Fungicides to Bees

In addition to insecticides, bees are exposed to several other chemicals used in agricultural crop production, that may influence general bee health. For instance, in a study investigating the foliar spray toxicity of commonly used fungicides, Fisher et al. (2017) showed that exposure to tank mixes of formulated Iprodione 2SE Select® (iprodione) and either formulated Pristine® (boscalid and pyraclostrobin) or Quadris® (azoxytrobin) significantly decreased honey bee forager survival by synergism. In a separate study investigating the synergistic effects of the fungicide Pristine® (boscalid and pyraclostrobin) with the spray adjuvant Break-Thru® (polyether-polymethylsiloxane-copolymer) on queen rearing success, Johnson and Percel (2013) determined that neither chemical nor their combination had a significant effect on immature queen survival. Low levels of pyraclostrobin (50 ppb) but not boscalid, however, were found in royal jelly secreted by nurse bees feeding on treated pollen. Johnson et al. (2013) concluded that four sterol biosynthesis inhibiting fungicides increased the acute toxicity of the acaricide tau-fluvalinate to honey bee adult workers in a dose-dependent manner. They attribute this synergism to the inhibition of cytochrome P450 monooxygenase enzyme activity. This study also found that the sterol biosynthesis inhibiting fungicide prochloraz increased the acute toxicity of the acaricides coumaphos and fenpyroximate, while pre-treatment with the acaricide amitraz increased the acute toxicity of three other acarcides that are P450-detoxifyed. Migdal et al. (2018) fed Carniolan race honey bees syrup contaminated with the fungicides Miedzian 50WP (active ingredient copper oxychloride) and Thiram Granuflo 80WG (thiram) over an experimental duration of 108 and 168 hours respectively. This study found a daily fungicide intake of 4.75 mm^3/bee and 16.78 mm^3/bee respectively; resulting in daily mortality of 35.15 and 5.17 bees/cage respectively.

Exposing queen-less micro-colonies of *B. terrestris* to honey water and pollen paste containing field realistic mean or field realistic maximum concentrations of the demethylation inhibitor fungicide propiconazole for 28-days, Elston et al. (2013) showed that both propiconazole concentrations reduced honey water consumption, and the lowest dose also decreased the number of wax cells produced. In another study, Bernauer et al. (2015) exposed five *B. impatiens* to flowers treated with field-realistic levels of the fungicide chlorothalonil over the course of one month. In this study, treated colonies compared to controls, produced less than a third as many workers, contained less than half the quantity of bee biomass and were queened by mothers with half the amount of body mass.

Comparing chemical toxicity of *Osmia lignara* (Say) and *M. rotundata*, Artz and Pitts-Singer (2015) found that *O. lignaria* exposure to the fungicides Rovral 4F® (iprodione) and Pristine® (25.2% boscalid and 12.8% pyraclostrobin) and the non-ionic spray adjuvant N-90® (90% polyethoxylated nonylphenol) in cage studies negatively affected nest recognition ability. Comparing *O. lignaria* and *A. mellifera* exposure to five fungicides in cage studies, Ladurner et al. (2005) determined that oral ingestion of Orbit® (propiconazole) resulted in the acute toxicity of both species, while oral and contact exposure to Captan 50WP® (captan) was chronically toxic to *O. lignaria* by severely impacting their survival 72 hours to 7 days post exposure. Conversely, in semi-field studies using formulated product they found that *O. lignaria* foraging and nesting behavior was unaffected when sprayed in cages with one of the fungicides Rovral® (iprodione), Orbit® (propiconazole), Benlate® (benomyl), and Captan 50WP® (captan) (Ladurner et al. 2008). Foraging and nesting behavior was only affected for several hours post treatment when sprayed with a mixture of Rovral® (iprodione) + Dyne-amic (surfactant) + Bayfolan Plus®.

E. Impact of Pesticide Residues to Bees

Several studies have examined pesticide residues in crop and non-crop flowers and their impact on bees. David et al. (2016) conducted a field study quantifying the amounts of neonicotinoids present in oilseed rape, wildflowers adjacent to winter wheat and oilseed rape and the pollen collected by honey bees, and found at least six different neonicotinoid and fungicide residues in samples. In wildflower samples, they found significantly lower levels of thiamethoxam and thiacloprid compared to those found in oilseed rape. Using pollen traps to collect honey bee forager pollen from the hives used in this experiment, they found pollen contaminated with 14 pesticides collected during oilseed rape bloom, and contamination with 10 pesticide compounds after bloom. Implementing pollen traps in standard Langstroth honey bee hives placed in maize fields for 16 weeks, Long and Krupke (2016) found pollen samples contaminated with 32 different pesticides (in ppb), several of which were insecticides. In non-agricultural areas, the pyrethroid insecticides prallethrin and phenothrin were detected in 46.7% and 30% of the pollen samples respectively, while carbamates, neonicotinoids and organophosphates were detected in 3-16.7% of pollen samples. In pollen collected adjacent to untreated maize fields, the insecticide thiamethoxam was detected in 33% of samples, while carbamates, other neonicotinoids and organophosphates were present in 4-33% of samples. In pollen collected adjacent to pesticide treated maize fields, the insecticides phenothrin, acetamiprid, and carbaryl were detected in 25-28% of pollen samples. In all three experimental field settings, detection of the pyrethroid phenothrin spiked during the later collection period (August-September). However, most of the detection levels in this study (especially for the non-treated maize and non-agricultural areas) are below any observable effect by EPA standards (U.S. Environmental Protection Agency 2013).

Multiple field studies feeding honey bees sugar syrup and nectar laced with imidacloprid showed no significant increase in mortality (e.g., Schmuck et al. 2001; Faucon et al. 2005). A key point is that recently, numerous field studies have been done to analyze the actual level of pesticides that bees are exposed to. For example, Sandrock et al. (2014) found that exposing honey bees to chronic levels of in hive pollen containing clothianidin and thiamethoxam negatively impacted colony performance and queen supersedure. In agreement, Fischer et al. (2014) concluded that contact exposure with thiacloprid, imidacloprid or clothianidin impaired honey bee navigation. However, Cutler et al. (2014) assessed no overall sub-lethal effects of chronic exposure to clothianidin seed-treated canola on honey bee colonies, and even observed strong colonies during and after exposure.

After exposing honey bee colonies to thiamethoxam seed-treated maize and oilseed rape over a four-year field trial in multiple locations, Pilling et al. (2013) determined that systemic residues in pollen, nectar and hive products pose low risk to honey bees. Median levels of both thiamethoxam and its first metabolite clothianidin (CGA322704) in whole plant material for both crops were overall higher than those in bee collected pollen and nectar. Moreover, thiamethoxam and CGA322704 residues in bee bread and hive nectar were always equal to or lower than those in bee collected pollen or nectar for all matrices tested. Conversely, Hoppe et al. (2015) responded to the study of Pilling et al. (2013) by detailing several flaws. For instance, Pilling et al. (2013) only used thiamethoxam active ingredients for seed dressings, instead of testing a field-realistic formulated product. Also, no assessment was made of previous field pesticide contamination. During the experimental procedure, pollen stores were only examined for thiamethoxam and clothianidin, but not other pesticides. Colony losses throughout the experiment were incorrectly calculated.

Like honey bees, bumble bees are also exposed to pesticides in different ecosystems. In net collecting 150 specimens of five wild bumble bee species in spring, early-summer and mid-summer in the United Kingdom and agricultural and urban settings, Botías et al. (2017) found some interesting trends in neonicotinoid residue level and frequency. While overall higher pesticide quantities were detected in bumble bees foraging in agricultural settings (6.8 ± 9.5 ng/g), the highest level and frequency of imidacloprid (10 ng/g) were found on a *B. terrestris* specimen collected from an urban setting during early-summer. More generally, all five neonicotinoids registered for application in the United Kingdom were detected in at least one bumble bee, and overall, neonicotinoid residues were detected in more bumble bees net collected in urban settings (9.3%) than in agricultural settings (2.7%). They also found specimens of *B. pratorum*, the species with the smallest body mass and shortest tongue length, contained statistically lower agrochemical residue levels compared to the other species. Interestingly, pesticide quantities detected significantly decreased in midsummer, which agrees with other studies such as (David et al. 2016). Placing commercial *B. terrestris audax* nests in rural and urban areas, David et al. (2016) detected much higher insecticide levels in both pollen samples and the bees themselves in rural settings after four weeks of free foraging. Imidacloprid was the most frequently detected insecticide found in 6% of pollen samples from rural areas, while in urban areas, thiamethoxam, thiacloprid, acetamiprid were also found in addition to imidacloprid (David et al. 2016).

F. Interactions Between Multiple Pesticides

Bees and other pollinators are commonly exposed to multiple pesticides and other stressors. Therefore, it is plausible to hypothesize that exposure to multiple stressors results in additive or synergistic interactions that exacerbate the detrimental effects to afflicted bees (Sih et

al. 2004; Gill et al. 2012; Johnson and Percel 2013). Simulating foliar exposure of honey bees to imidacloprid alone or in binary mixtures combined with pesticides of different classes, Zhu et al. (2017a) determined synergistic toxicity from imidacloprid + tetraconazole, imidacloprid + sulfoxaflor and imidacloprid + oxamyl, and additive toxicity from imidacloprid + acephate and imidacloprid + lambda-cyhalothrin. Significant esterase and acetylcholinesterase enzymatic activity was suppressed following exposure to imidacloprid + acephate. Conversely, by simulating honey bee oral uptake of imidacloprid alone and in binary combinations with pesticides of different classes, Zhu et al. (2017b) demonstrated that resulting mortality was statistically similar to the control, thereby showing that pesticide residues found in hives and pollen may not adversely impact honey bees. Assessing chronic oral toxicity of pesticides commonly found in hive pollen and wax to honey bee larvae, Zhu et al. (2014) found synergistic toxicity following exposure to the binary mixture of chlorothalonil (34 mg/L) + fluvalinate (3 mg/L), and an antagonistic interaction when the mixture was diluted by 10-fold. This study also confirmed synergism between chlorothalonil (34 mg/L) + coumaphos (8 mg/L).

Testing oral toxicity of six concentrations each for a range of pesticides alone and in binary combinations to *A. mellifera*, *B. terrestris* and *O. bicornis* following prolonged exposure (240-hours), Spurgeon et al. (2016) determined a consistent level of toxicity (based on calculating LC_{50} values)for the three-bee species. These results were characterized by additive toxicity following exposure to most mixtures, slightly increased toxicity following exposure to clothianidin + propiconazole and a weak antagonistic interaction following *B. terrestris* and *O. bicornis* exposure to clothianidin + dimethoate. Analyzing the impact of *B. terrestris* oral dietary exposure to the fungicide imazalil in binary combinations with four insecticides, Raimets et al. (2018) concluded imizalil synergized mortality following uptake of mixtures with fipronil,

cypermethrin or thiamethoxam, but not imidacloprid. However, this had no synergistic impact on *B. terrestris* feeding rate. Collection of 150 wild adults representing five species of wild bumble bees revealed that 71% contained residues of multiple pesticide compounds in their tissues, thereby indicating that foraging bumble bees are routinely exposed to more than one pesticide and that their contact with these chemicals might be more widespread than previously believed (Botías et al. 2017). However, pesticide detections on bees do not necessarily indicate toxicity and resulting mortality.

G. Assessing the Toxicity of New Insecticides to Bees

The Food Quality Protection Act of 1996 (US Congress, 1996) curtailed use of broad-spectrum pesticides and particularly those within the carbamate and organophosphate classes. As a result, the suite of agricultural chemicals available for growers to use in integrated pest management programs was largely reduced. Development of insect pest resistance to existing insecticide chemistries has dramatically increased in recent years. As a result, synthesis of new premix insecticides comprised of multiple active ingredients (each with a different mode of action) has been aimed at providing growers effective pest management tools that combat insect resistance buildup.

Premixes are increasingly being used for controlling a diversity of insect pests in field crops (Ramsey et al. 2016; Jones et al. 2017) and orchard crops (Leskey et al. 2014; Alston and Murray 2017). Honey bees and solitary bees pollinate several field and orchard crops (Klein et al. 2007; Bosch et al. 2006) and thereby are potentially threatened if premixes of toxic insecticides are applied within the time frame of when bees are actively foraging. However, since premix insecticide applications are prohibited in orchards during bloom, bee exposure in orchards will be from systemic movement of pre-bloom sprays or early petal fall sprays.

Conversely, for long-blooming crops such as oilseed rape or pumpkins where insecticides are applied during bloom, it is possible for foraging bees to be exposed to premix insecticides. To our knowledge, the ecotoxicology of these insecticide mixtures has not been thoroughly tested on honey bees and not at all tested on non-*Apis* bees. In a honey bee mortality screening analysis, Zhu et al. (2015) found that contact exposure to premix insecticides by spray application resulted in high mortality within 24 and 48-hours after treatment.

To address this gap in our knowledge of insecticide toxicology to bees with premixes, we simulated contact exposure of four premix insecticides to honey bee foragers (an *Apis*-bee) and male and female blue orchard bees (a non-*Apis* bee).

H. Research Objectives

My research objectives are:

To expand upon preliminary work investigating the acute contact toxicity of premix insecticides to honey bees in the following three scenarios: (1) when bees are foraging in a crop field and are directly exposed to insecticides during an application; (2) when bees are exposed to insecticide sprays while passing through a crop field; and (3) when bees are foraging in a crop field that was sprayed with insecticides during the previous day.

To contribute to our understanding of insecticide risk to non-*Apis* bees by assessing the acute contact toxicity of premix insecticides (and the individual ingredients comprising one of these insecticides) to male and female blue orchard bees.

Obtaining these results will provide us with an improved baseline for the realistic risk that premix insecticides pose to bees. Studying these bee species should foster future work investigating whole body and oral exposure to multiple doses of these premix insecticides. My

goal is to contribute to improving our protection of bees in field settings in a manner that enables growers to safely apply insecticides for maintaining insect pests at tolerable levels.

I. References

Abbott, V. A., J. L. Nadeau, H. A. Higo, and M. L. Winston. **2008**. Lethal and Sublethal Effects of Imidacloprid on *Osmia lignaria* and Clothianidin on *Megachile rotundata* (Hymenoptera: Megachilidae). Journal of Economic Entomology. 101: 13.

Abrol, D. P. 2006. Defensive Behavior of *Apis cerana* F. Against Predatory Wasps. Journal of Apicultural Science. 50: 39–46.

Ahmad, Z., and C. Johansen. **1973**. Selective Toxicity of Carbophenothion and Trichlorfon to the Honey Bee and the Alfalfa Leafcutting Bee. Environmental Entomology. 2: 27–30.

Aizen, M. A., L. A. Garibaldi, S. A. Cunningham, and A. M. Klein. **2008**. Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. Current Biology. 18: 1572–1575.

Aliouane, Y., A. K. el Hassani, V. Gary, C. Armengaud, M. Lambin, and M. Gauthier. **2009**. Subchronic Exposure of Honeybees to Sublethal Doses of Pesticides: Effects on Behavior. Environmental Toxicology and Chemistry. 28: 113.

Alston, D., and M. Murray. **2017**. Leafrollers in Fruit Orchards (No. ENT-185-17). Utah State University.

Arena, M., and F. Sgolastra. **2014**. A meta-analysis comparing the sensitivity of bees to pesticides. Ecotoxicology. 23: 324–334.

Artz, D. R., and T. L. Pitts-Singer. **2015**. Effects of Fungicide and Adjuvant Sprays on Nesting Behavior in Two Managed Solitary Bees, *Osmia lignaria* and *Megachile rotundata*. PLOS ONE. 10: e0135688.

Bakonyi, T., R. Farkas, A. Szendröi, M. Dobos-Kovács, and R. Rusvai. **2002**. Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* field samples: Rapid screening of representative Hungarian apiaries. Apidologie. 33: 63–74.

Bartomeus, I., J. S. Ascher, D. Wagner, B. N. Danforth, S. Colla, S. Kornbluth, and R. Winfree. **2011**. Climate-associated phenological advances in bee pollinators and bee-pollinated plants. Proceedings of the National Academy of Sciences. 108: 20645–20649.

Beers, E. H., R. A. Van Steenwyk, P. W. Shearer, W. W. Coates, and J. A. Grant. **2011**. Developing *Drosophila suzukii* management programs for sweet cherry in the western United States. Pest Management Science. 67: 1386–1395.

Bernauer, O., H. Gaines-Day, and S. Steffan. **2015**. Colonies of Bumble Bees (*Bombus impatiens*) Produce Fewer Workers, Less Bee Biomass, and Have Smaller Mother Queens Following Fungicide Exposure. Insects. 6: 478–488.

Biddinger, D. J., J. L. Robertson, C. Mullin, J. Frazier, S. A. Ashcraft, E. G. Rajotte, N. K. Joshi, and M. Vaughn. **2013**. Comparative Toxicities and Synergism of Apple Orchard Pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). PLoS ONE. 8: e72587.

Biddinger, D., E. G. Rajotte, N. K. Joshi, and A. Ritz. **2011**. Wild Bees as Alternative Pollinators in Pennsylvania Apple Orchards. Fruit Times: Penn State Extension. 30.

Biesmeijer, J. C. 2006. Parallel Declines in Pollinators and Insect-Pollinated Plants in Britain and the Netherlands. Science. 313: 351–354.

Bosch, J., and W. P. Kemp. **1999**. Exceptional cherry production in an orchard pollinated with blue orchard bees. Bee World. 80: 163–173.

Bosch, J., and W. P. Kemp. **2001**. How to manage the blue orchard bee: as an orchard pollinator, Sustainable Agriculture Network handbook series. Sustainable Agriculture Network, Beltsville, MD.

Bosch, J., W. P. Kemp, and G. E. Trostle. **2006**. Bee Population Returns and Cherry Yields in an Orchard Pollinated with *Osmia lignaria* (Hymenoptera: Megachilidae). Journal of Economic Entomology. 99: 408–413.

Bosch, J., F. Sgolastra, and W. P. Kemp. **2010**. Timing of eclosion affects diapause development, fat body consumption and longevity in *Osmia lignaria*, a univoltine, adult-wintering solitary bee. Journal of Insect Physiology. 56: 1949–1957.

Botías, C., A. David, E. M. Hill, and D. Goulson. **2017**. Quantifying exposure of wild bumblebees to mixtures of agrochemicals in agricultural and urban landscapes. Environmental Pollution. 222: 73–82.

Brito, R. M., F. O. Francisco, S. Y. W. Ho, and B. P. Oldroyd. **2014**. Genetic architecture of the *Tetragonula carbonaria* species complex of Australian stingless bees (Hymenoptera: Apidae: Meliponini): Hybridization in Australian Stingless Bees. Biological Journal of the Linnean Society. 113: 149–161.

Brown, M. J. F. 2011. Conservation: the trouble with bumblebees. Nature. 469: 169–170.

Byatt, M. A., N. C. Chapman, T. Latty, and B. P. Oldroyd. **2016**. The genetic consequences of the anthropogenic movement of social bees. Insectes Sociaux. 63: 15–24.

Calderone, N. W. 2012. Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. PLoS ONE. 7: 1–32.

Cameron, S. A., H. C. Lim, J. D. Lozier, M. A. Duennes, and R. Thorp. **2016**. Test of the invasive pathogen hypothesis of bumble bee decline in North America. Proceedings of the National Academy of Sciences. 113: 4386–4391.

Cane, J. H. 1991. Soils of Ground-Nesting Bees (Hymenoptera: Apoidea): Texture, Moisture, Cell Depth and Climate. Journal of the Kansas Entomological Society. 64: 406–413.

Cane, J. H. 2001. Habitat Fragmentation and Native Bees: a Premature Verdict? Conservation Ecology. 5: 1–10.

Cane, J. H., R. R. Snelling, and L. J. Kervin. **1996**. A New Monolectic Coastal Bee, *Hesperapis oraria* Snelling and Stage (Hymenoptera: Melittidae), with a Review of Desert and Neotropical Disjunctives in the Southeastern U.S. Journal of the Kansas Entomological Society. 69: 238–247.

Cane, J. H., and V. J. Tepedino. **2017**. Gauging the Effect of Honey Bee Pollen Collection on Native Bee Communities: *Apis* pollen depletion and native bees. Conservation Letters. 10: 205–210.

Carrié, R., E. Andrieu, S. A. Cunningham, P. E. Lentini, M. Loreau, and A. Ouin. **2017**. Relationships among ecological traits of wild bee communities along gradients of habitat amount and fragmentation. Ecography. 40: 85–97.

Corbet, S. A., M. Fussell, R. Ake, A. Fraser, C. Gunson, A. Savage, and K. Smith. **1993**. Temperature and the pollinating activity of social bees. Ecological Entomology. 18: 17–30.

Costa, L. M. da, T. C. Grella, R. A. Barbosa, O. Malaspina, and R. C. F. Nocelli. **2015**. Determination of acute lethal doses (LD_{50} and LC_{50}) of imidacloprid for the native bee *Melipona scutellaris* Latreille, 1811 (Hymenoptera: Apidae). Sociobiology. 62.

Cresswell, J. E., F.-X. L. Robert, H. Florance, and N. Smirnoff. **2014**. Clearance of ingested neonicotinoid pesticide (imidacloprid) in honey bees (*Apis mellifera*) and bumblebees (*Bombus terrestris*). Pest Management Science. 70: 332–337.

Crown Bees: The Native Bee Experts. **2018**. Crown Bees the Native Bee Experts. (https://crownbees.com/shop/bees).

Cutler, G. C., C. D. Scott-Dupree, M. Sultan, A. D. McFarlane, and L. Brewer. **2014**. A large-scale field study examining effects of exposure to clothianidin seed-treated canola on honey bee colony health, development, and overwintering success. PeerJ. 2: e652.

Dafni, A., P. Kevan, C. L. Gross, and K. Goka. **2010**. *Bombus terrestris*, pollinator, invasive and pest: An assessment of problems associated with its widespread introductions for commercial purposes. Applied Entomology and Zoology. 45: 101–113.

Dara, S. K. 2018. Dara 2018.webarchive. An update on the invasive spotted lanternfly, *Lycorma delicatula*: current distribution, pest detection, efforts, and management strategies. (file:///Users/josephbelsky/Desktop/Dara%202018.webarchive).

David, A., C. Botías, A. Abdul-Sada, E. Nicholls, E. L. Rotheray, E. M. Hill, and D. Goulson. **2016**. Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. Environment International. 88: 169–178.

Decourtye, A., J. Devillers, E. Genecque, K. L. Menach, H. Budzinski, S. Cluzeau, and M. H. Pham-Delgue. **2005**. Comparative Sublethal Toxicity of Nine Pesticides on Olfactory Learning Performances of the Honeybee *Apis mellifera*. Archives of Environmental Contamination and Toxicology. 48: 242–250.

DeGrandi-Hoffman, G., Y. Chen, E. Huang, and M. H. Huang. **2010**. The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.). Journal of Insect Physiology. 56: 1184–1191.

Derato, J., K. Gump, N. Himmelsbach, S. Lank, M. Peterson, B. Simpson, M. Wartman, and T. Zhang. **2018**. Susceptibility of Montgomery Park Trees to Emerging Invasive Pests. Capstone in Environmental Science and Policy, University of Maryland-College Park.

Devillers, J., A. Decourtye, H. Budzinski, M. H. Pham-Delegue, S. Cluzeau, and G. Maurin. **2003**. Comparative toxicity and hazards of pesticides to *Apis* and non-*Apis* bees. A chemometrical study. SAR and QSRR in Environmental Research. 14: 389–403.

Devoto, M., M. Zimmermann, and D. Medan. **2007**. Robustness of plant-flower visitor webs to simulated climate change. Ecol. Aust. 17: 37–50.

Di Pasquale, G., M. Salignon, Y. Le Conte, L. P. Belzunces, A. Decourtye, A. Kretzschmar, S. Suchail, J.-L. Brunet, and C. Alaux. **2013**. Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? PLoS ONE. 8: 1–13.

Di Prisco, G., D. Annoscia, M. Margiotta, R. Ferrara, P. Varricchio, V. Zanni, E. Caprio, F. Nazzi, and F. Pennacchio. **2016**. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proceedings of the National Academy of Sciences. 113: 3203–3208.

Di Prisco, G., V. Cavaliere, D. Annoscia, P. Varricchio, E. Caprio, F. Nazzi, G. Gargiulo, and F. Pennacchio. **2013**. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proceedings of the National Academy of Sciences. 110: 18466–18471.

Drummond, F. 2012. Commercial Bumble Bee Pollination of Lowbush Blueberry. International Journal of Fruit Science. 12: 54–64.

Eckhardt, M., M. Haider, S. Dorn, and A. Müller. **2014**. Pollen mixing in pollen generalist solitary bees: a possible strategy to complement or mitigate unfavourable pollen properties? Journal of Animal Ecology. 83: 588–597.

Eischen and Graham. **2008**. Feeding overwintering honey bee colonies infected with *Nosema ceranae*. *In* American Bee Journal. Presented at the American Bee Research Conference.

Eischen et al. 2008. Overwintering nutritionally stressed honey bee colonies infested with *Varroa destructor*. American Bee Journal. 148: 555–556.

Elston, C., H. M. Thompson, and K. F. A. Walters. **2013**. Sub-lethal effects of thiamethoxam, a neonicotinoid pesticide, and propiconazole, a DMI fungicide, on colony initiation in bumblebee (*Bombus terrestris*) micro-colonies. Apidologie. 44: 563–574.

Evans, E. 2017. From Humble Bee to Greenhouse Pollination Workhorse: Can We Mitigate Risks for Bumble Bees? Bee World. 94: 34–41.

Faucon, J.-P., C. Aurières, P. Drajnudel, L. Mathieu, M. Ribière, A.-C. Martel, S. Zeggane, M.-P. Chauzat, and M. F. Aubert. **2005**. Experimental study on the toxicity of imidacloprid given in syrup to honey bee (*Apis mellifera*) colonies: Toxicity of imidacloprid given in syrup to honey bee colonies. Pest Management Science. 61: 111–125.

Fauser-Misslin, A., B. M. Sadd, P. Neumann, and C. Sandrock. **2014**. Influence of combined pesticide and parasite exposure on bumblebee colony traits in the laboratory. Journal of Applied Ecology. 51: 450–459.

Ferreira, P. A., D. Boscolo, L. G. Carvalheiro, J. C. Biesmeijer, P. L. B. Rocha, and B. F. Viana. **2015**. Responses of bees to habitat loss in fragmented landscapes of Brazilian Atlantic Rainforest. Landscape Ecology. 30: 2067–2078.

Ferrier, P. M. 2018. Economic Effects and Responses to Changes in Honey Bee Health. USDA Economic Research Service. 1–54.

Fischer, J., T. Müller, A.-K. Spatz, U. Greggers, B. Grünewald, and R. Menzel. **2014**. Neonicotinoids Interfere with Specific Components of Navigation in Honeybees. PLoS ONE. 9: e91364.

Fischman, B. J., T. L. Pitts-Singer, and G. E. Robinson. **2017**. Nutritional Regulation of Phenotypic Plasticity in a Solitary Bee (Hymenoptera: Megachilidae). Environmental Entomology. 46: 1070–1079.

Fisher, A., C. Coleman, C. Hoffmann, B. Fritz, and J. Rangel. **2017**. The Synergistic Effects of Almond Protection Fungicides on Honey Bee (Hymenoptera: Apidae) Forager Survival. Journal of Economic Entomology. 110: 802–808.

Food Quality Protection Act of 1996. Public Law 104-170. **1996**. Food Quality Protection Act of 1996. Public Law 104-170. U.S. Government Printing Office, Washington, D.C.

Forfert, N., M. E. Natsopoulou, E. Frey, P. Rosenkranz, R. J. Paxton, and R. F. A. Moritz. **2015**. Parasites and Pathogens of the Honeybee (*Apis mellifera*) and Their Influence on Inter-Colonial Transmission. PLOS ONE. 10: 1–14.

Forister, M. L., and A. M. Shapiro. **2003**. Climatic trends and advancing spring flight of butterflies in lowland California. Global Change Biology. 9: 1130–1135.

Forrest, J. R. K. 2015. Plant-pollinator interactions and phenological change: what can we learn about climate impacts from experiments and observations? Oikos. 124: 4–13.

Forrest, J. R. K., R. W. Thorp, C. Kremen, and N. M. Williams. **2015**. Contrasting patterns in species and functional-trait diversity of bees in an agricultural landscape. Journal of Applied Ecology. 52: 706–715.

Forup, M. L., and J. Memmott. **2005**. The relationship between the abundances of bumblebees and honeybees in a native habitat. Ecological Entomology. 30: 47–57.

Francisco, F. O., L. R. Santiago, R. M. Brito, B. P. Oldroyd, and M. C. Arias. **2014**. Hybridization and asymmetric introgression between *Tetragonisca angustula* and *Tetragonisca fiebrigi*. Apidologie. 45: 1–9.

Franck, P., E. Cameron, G. Good, J.-Y. Rasplus, and B. P. Oldroyd. **2004**. Nest architecture and genetic differentiation in a species complex of Australian stingless bees: Genetic Structure of Australian *Trigona*. Molecular Ecology. 13: 2317–2331.

Genersch, E., C. Yue, I. Fries, and J. R. de Miranda. **2006**. Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (*Bombus terrestris* and *Bombus pascuorum*) with wing deformities. Journal of Invertebrate Pathology. 91: 61–63.

Gill, R. J., and N. E. Raine. **2014**. Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. Functional Ecology. 28: 1459–1471.

Gill, R. J., O. Ramos-Rodriguez, and N. E. Raine. **2012**. Combined pesticide exposure severely affects individual- and colony-level traits in bees. Nature. 491: 105–108.

Goka, K. 1998. Influence of invasive species on native species: will the European bumble bee, *Bombus terrestris*, bring genetic pollution into the Japanese native species? Bulletin of the Biogeographical Society of Japan. 53: 91–101.

Goodwin, R. M., M. A. Taylor, H. M. Mcbrydie, and H. M. Cox. **2006**. Drift of *Varroa destructor*-infested worker honey bees to neighbouring colonies. Journal of Apicultural Research. 45: 155–156.

Gordo, O., and J. J. Sanz. **2005**. Phenology and climate change: a long-term study in a Mediterranean locality. Oecologia. 146: 484–495.

Gordo, O., and J. J. Sanz. **2006**. Temporal trends in phenology of the honey bee *Apis mellifera* (L.) and the small white *Pieris rapae* (L.) in the Iberian Peninsula (1952-2004). Ecological Entomology. 31: 261–268.

Gordon, R., N. Bresolin-Schott, and I. East. **2014**. Nomadic beekeeper movements create the potential for widespread disease in the honeybee industry. Australian Veterinary Journal. 92: 283–290.

Goulson, D. 2003. Effects of Introduced Bees on Native Ecosystems. Annual Review of Ecology, Evolution, and Systematics. 34: 1–26.

Goulson, D. 2010. Impacts of non-native bumblebees in Western Europe and North America. Applied Entomology and Zoology. 45: 7–12.

Goulson, D., G. C. Lye, and B. Darvill. **2008**. Decline and Conservation of Bumble Bees. Annual Review of Entomology. 53: 191–208.

Goulson, D., E. Nicholls, C. Botias, and E. L. Rotheray. **2015**. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 347: 1435–1446.

Grixti, J. C., L. T. Wong, S. A. Cameron, and C. Favret. **2009**. Decline of bumble bees (*Bombus*) in the North American Midwest. Biological Conservation. 142: 75–84.

Gross, M. 2008. Bee gloom deepens. Current Biology. 18: 1.

Guez, D. 2013. A common pesticide decreases foraging success and survival in honey bees: questioning the ecological relevance. Frontiers in Physiology. 4.

Halcroft, M. T., R. Spooner-Hart, A. M. Haigh, T. A. Heard, and A. Dollin. **2013**. The Australian stingless bee industry: a follow-up survey, one decade on. Journal of Apicultural Research. 52: 1–7.

Hannah, L., M. Steele, E. Fung, P. Imbach, L. Flint, and A. Flint. **2017**. Climate change influences on pollinator, forest, and farm interactions across a climate gradient. Climatic Change. 141: 63–75.

Harmon-Threatt, A. N., and C. Kremen. **2015**. Bumble bees selectively use native and exotic species to maintain nutritional intake across highly variable and invaded local floral resource pools: Bee nutrition in invaded landscapes. Ecological Entomology. 40: 471–478.

Hedtke, S. M., E. J. Blitzer, G. A. Montgomery, and B. N. Danforth. **2015**. Introduction of Non-Native Pollinators Can Lead to Trans-Continental Movement of Bee-Associated Fungi. PLOS ONE. 10: 1–18.

Hegland, S. J., A. Nielsen, A. Lázaro, A.-L. Bjerknes, and Ø. Totland. **2009**. How does climate warming affect plant-pollinator interactions? Ecology Letters. 12: 184–195.

Heinrich, B. 1979. "Majoring" and "Minoring" by Foraging Bumblebees, *Bombus vagans*: An Experimental Analysis. Ecology. 60: 245–255.

Henry, M., M. Beguin, F. Requier, O. Rollin, J.-F. Odoux, P. Aupinel, J. Aptel, S. Tchamitchian, and A. Decourtye. **2012**. A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. Science. 336: 348–350.

Holden, C. 2006. Ecology: Report Warns of Looming Pollination Crisis in North America. Science. 314: 397–397.

Hoppe, P. P., A. Safer, V. Amaral-Rogers, J.-M. Bonmatin, D. Goulson, R. Menzel, and B. Baer. **2015**. Effects of a neonicotinoid pesticide on honey bee colonies: a response to the field study by Pilling et al. (2013). Environmental Sciences Europe. 27.

Hopwood, J., A. Code, M. Vaughan, D. Biddinger, M. Shepherd, S. H. Black, E. Lee-Mäder, and C. Mazzacano. **2016**. How Neonicotinoids Can Kill Bees: The Science Behind the Role These Insecticides Play in Harming Bees (No. Second Edition, Revised and Expanded). Xerces Society for Invertebrate Conservation, Portland, (OR) USA.

Howard, D. C., J. W. Watkins, R. T. Clarke, C. L. Barnett, and G. J. Stark. **2003**. Estimating the extent and change in Broad Habitats in Great Britain. Journal of Environmental Management. 67: 219–227.

Huang, Z. 2010. Honey Bee Nutrition (Jointly published in the American Bee Journal and in Bee Culture). Michigan State University.

Huang, Z. 2012. Pollen nutrition affects honey bee stress resistance. Terrestrial Arthropod Reviews. 5: 175–189.

Hudewenz, A., and A.-M. Klein. **2015**. Red mason bees cannot compete with honey bees for floral resources in a cage experiment. Ecology and Evolution. 5: 5049–5056.

Huryn, V. M. B. 1997. Ecological Impacts of Introduced Honey Bees. The Quarterly Review of Biology. 72: 275–297.

Ichino, H., and I. Okada. **1994**. Japanese honey bees living in hornet nest. Honeybee Science. 15: 123–124.

Ings, T. C., N. L. Ward, and L. Chittka. **2006**. Can commercially imported bumble bees out-compete their native conspecifics?: Performance of commercial bumble bees in the wild. Journal of Applied Ecology. 43: 940–948.

Iwasa, T., N. Motoyama, J. T. Ambrose, and R. M. Roe. **2004**. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Protection. 23: 371–378.

Jacob, C. R. O., H. M. Soares, R. F. C. Nocelli, and O. Malaspina. **2015**. Impact of fipronil on the mushroom bodies of the stingless bee *Scaptotrigona postica*. Pest Management Science. 71: 114–122.

Johnson, R. M., L. Dahlgren, B. D. Siegfried, and M. D. Ellis. **2013**. Acaricide, Fungicide and Drug Interactions in Honey Bees (*Apis mellifera*). PLoS ONE. 8: e54092.

Johnson, R. M., M. D. Ellis, C. A. Mullin, and M. Frazier. **2010**. Pesticides and honey bee toxicity – USA. Apidologie. 41: 312–331.

Johnson, R. M., and E. G. Percel. **2013**. Effect of a Fungicide and Spray Adjuvant on Queen-Rearing Success in Honey Bees (Hymenoptera: Apidae). Journal of Economic Entomology. 106: 1952–1957.

Jones, M., M. Duckworth, and J. Duckworth. **2017**. Evaluation of Foliar Insecticides for Control of Rice Stink Bug, 2016. Arthropod Management Tests. 42.

Kammerer, M. A., D. J. Biddinger, E. G. Rajotte, and D. A. Mortensen. **2016**. Local Plant Diversity Across Multiple Habitats Supports a Diverse Wild Bee Community in Pennsylvania Apple Orchards. Environmental Entomology. 45: 32–38.

Kanbe, Y., I. Okada, M. Yoneda, K. Goka, and K. Tsuchida. **2008**. Interspecific mating of the introduced bumblebee *Bombus terrestris* and the native Japanese bumblebee *Bombus hypocrita* sapporoensis results in inviable hybrids. Naturwissenschaften. 95: 1003–1008.

Kerr, J. T., A. Pindar, P. Galpern, L. Packer, S. G. Potts, S. M. Roberts, P. Rasmont, O. Schweiger, S. R. Colla, L. L. Richardson, D. L. Wagner, L. F. Gall, D. S. Sikes, and A. Pantoja. **2015**. Climate change impacts on bumblebees converge across continents. Science. 349: 177–180.

King, M. J., and S. L. Buchmann. **2003**. Floral Sonication by Bees: Mesosomal Vibration by *Bombus* and *Xylocopa*, but Not *Apis* (Hymenoptera: Apidae), Ejects Pollen from Poricidal Anthers. Journal of the Kansas Entomological Society. 76: 295–305.

Kleijn, D., R. Winfree, I. Bartomeus, L. G. Carvalheiro, M. Henry, R. Isaacs, A.-M. Klein, C. Kremen, L. K. M'Gonigle, R. Rader, T. H. Ricketts, N. M. Williams, N. Lee Adamson, J. S. Ascher, A. Báldi, P. Batáry, F. Benjamin, J. C. Biesmeijer, E. J. Blitzer, R. Bommarco, M. R. Brand, V. Bretagnolle, L. Button, D. P. Cariveau, R. Chifflet, J. F. Colville, B. N. Danforth, E. Elle, M. P. D. Garratt, F. Herzog, A. Holzschuh, B. G. Howlett, F. Jauker, S. Jha, E. Knop, K. M. Krewenka, V. Le Féon, Y. Mandelik, E. A. May, M. G. Park, G. Pisanty, M. Reemer, V. Riedinger, O. Rollin, M. Rundlöf, H. S. Sardiñas, J. Scheper, A. R. Sciligo, H. G. Smith, I. Steffan-Dewenter, R. Thorp, T. Tscharntke, J. Verhulst, B. F. Viana, B. E. Vaissière, R. Veldtman, K. L. Ward, C. Westphal, and S. G. Potts. **2015**. Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. Nature Communications. 6: 1–9.

Klein, A.-M., B. E. Vaissiere, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen, and T. Tscharntke. **2007**. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences. 274: 303–313.

Kraus, F. B., S. Wolf, and R. F. A. Moritz. **2009**. Male flight distance and population substructure in the bumblebee *Bombus terrestris*. Journal of Animal Ecology. 78: 247–252.

Kudo, G., and T. Y. Ida. **2013**. Early onset of spring increases the phenological mismatch between plants and pollinators. Ecology. 94: 2311–2320.

Kudo, G., Y. Nishikawa, T. Kasagi, and S. Kosuge. **2004**. Does seed production of spring ephemerals decrease when spring comes early? Ecological Research. 19: 255–259.

Ladurner, E., J. Bosch, W. P. Kemp, and S. Maini. **2005**. Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* Say and *Apis mellifera*. Apidologie. 36: 449–460.

Ladurner, E., J. Bosch, W. P. Kemp, and S. Maini. **2008**. Foraging and Nesting Behavior of *Osmia lignaria* (Hymenoptera: Megachilidae) in the Presence of Fungicides: Cage Studies. Journal of Economic Entomology. 101: 7.

Larson, J. L., C. T. Redmond, and D. A. Potter. **2013**. Assessing Insecticide Hazard to Bumble Bees Foraging on Flowering Weeds in Treated Lawns. PLoS ONE. 8: e66375.

Laurino, D., A. Manino, A. Patetta, and M. Porporato. **2013**. Toxicity of neonicotinoid insecticides on different honey bee genotypes. Bulletin of Insectology. 66: 119–126.

Lawson, S. P., S. L. Helmreich, and S. M. Rehan. **2017**. Effects of nutritional deprivation on development and behavior in the subsocial bee *Ceratina calcarata* (Hymenoptera: Xylocopinae). The Journal of Experimental Biology. 220: 4456–4462.

Leskey, T. C., G. C. Hamilton, A. L. Nielsen, D. F. Polk, C. Rodriguez-Saona, J. C. Bergh, D. A. Herbert, T. P. Kuhar, D. Pfeiffer, G. P. Dively, C. R. R. Hooks, M. J. Raupp, P. M. Shrewsbury, G. Krawczyk, P. W. Shearer, J. Whalen, C. Koplinka-Loehr, E. Myers, D. Inkley, K. A. Hoelmer, D.-H. Lee, and S. E. Wright. **2012**. Pest Status of the Brown Marmorated Stink Bug, Halyomorpha Halys in the USA. Outlooks on Pest Management. 23: 218–226.

Leskey, T. C., B. D. Short, and D.-H. Lee. **2014**. Efficacy of insecticide residues on adult *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) mortality and injury in apple and peach orchards: Residual insecticide efficacy on Halyomorpha halys. Pest Management Science. 70: 1097–1104.

Lichtenberg, E. M., C. D. Mendenhall, and B. Brosi. **2017**. Foraging traits modulate stingless bee community disassembly under forest loss. Journal of Animal Ecology. 86: 1404–1416.

Long, E. Y., and C. H. Krupke. **2016**. Non-cultivated plants present a season-long route of pesticide exposure for honey bees. Nature Communications. 7.

Losey, J. E., and M. Vaughan. **2006**. The Economic Value of Ecological Services Provided by Insects. BioScience. 56: 311–323.

Lourenço, C. T., S. M. Carvalho, O. Malaspina, and R. C. F. Nocelli. **2012**. Oral Toxicity of Fipronil Insecticide Against the Stingless Bee *Melipona scutellaris* (Latreille, 1811). Bulletin of Environmental Contamination and Toxicology. 89: 921–924.

Mader, E., M. Shepherd, M. Vaughan, and S. Black. **2011**. Attracting Native Pollinators: The Xerces Society Guide, Protecting North America's Bees and Butterflies, 2nd ed. Storey Publishing, Portland, (OR) USA.

Manley, R., M. Boots, and L. Wilfert. **2015**. Review: Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors. Journal of Applied Ecology. 52: 331–340.

Mason Bees for Sale.com. Mason Bees for Sale.com. **2018**. Mason Bees for Sale.com. (https://masonbeesforsale.com).

McMahon, D. P., M. A. Fürst, J. Caspar, P. Theodorou, M. J. F. Brown, and R. J. Paxton. **2015**. A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees. Journal of Animal Ecology. 84: 615–624.

Memmott, J., P. G. Craze, N. M. Waser, and M. V. Price. **2007**. Global warming and the disruption of plant? Pollinator interactions. Ecology Letters. 10: 710–717.

Memmott, J., N. M. Waser, and M. V. Price. **2004**. Tolerance of pollination networks to species extinctions. Proceedings of the Royal Society B: Biological Sciences. 271: 2605–2611.

Menzel, A., T. H. Sparks, N. Estrella, and D. B. Roy. **2006**. Altered geographic and temporal variability in phenology in response to climate change. Global Ecology and Biogeography. 15: 498–504.

Michener, C. D. 2007. The bees of the world, 2nd ed. Johns Hopkins University Press, Baltimore.

Migdal, P., A. Roman, E. Popiela-Pleban, M. Kowalska-Góralska, and S. Opaliński. **2018**. The Impact of Selected Pesticides on Honey Bees. Polish Journal of Environmental Studies. 27: 787–792.

Miller-Struttmann, N. E., J. C. Geib, J. D. Franklin, P. G. Kevan, R. M. Holdo, D. Ebert-May, A. M. Lynn, J. A. Kettenbach, E. Hedrick, and C. Galen. **2015**. Functional mismatch in a bumble bee pollination mutualism under climate change. Science. 349: 1541–1544.

Moquet, L., R. Bacchetta, E. Laurent, and A. Jacquemart. **2017**. Spatial and temporal variations in floral resource availability affect bumblebee communities in heathlands. Biodiversity and Conservation. 26: 687–702.

Müller, A., S. Diener, S. Schnyder, K. Stutz, C. Sedivy, and S. Dorn. **2006**. Quantitative pollen requirements of solitary bees: Implications for bee conservation and the evolution of bee–flower relationships. Biological Conservation. 130: 604–615.

Mullin, C. A., M. Frazier, J. L. Frazier, S. Ashcraft, R. Simonds, D. vanEngelsdorp, and J. S. Pettis. **2010**. High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. PLoS ONE. 5: e9754.

Nascimento, V. A., S. H. Matusita, and W. E. Kerr. **2000**. Evidence of hybridization between two species of *Melipona* bees. Genetics and Molecular Biology. 23: 79–81.

National Agricultural Statistics Service: 2016 California Almond Acreage Report. **2017**. National Agricultural Statistics Service: 2016 California Almond Acreage Report. California Department of Food Agriculture, National Agricultural Statistics Service, Pacific Regional Office.

Nogueira, J., J. de C. Ramos, J. Benevenuto, T. M. Fernandes-Salomão, H. C. Resende, L. A. de O. Campos, and M. G. Tavares. **2014**. Conservation study of an endangered stingless bee (*Melipona capixaba*—Hymenoptera: Apidae) with restricted distribution in Brazil. Journal of Insect Conservation. 18: 317–326.

Ono, M., I. Okada, and M. Sasaki. **1987**. Heat production by balling in the Japanese honeybee, *Apis cerana japonica* as a defen-sive behavior against the hornet, *Vespa simillima xanthoptera* (Hymenoptera: Vespidae). Experientia. 43: 1031–1032.

Ovaskainen, O., S. Skorokhodova, M. Yakovleva, A. Sukhov, A. Kutenkov, N. Kutenkova, A. Shcherbakov, E. Meyke, and M. d. M. Delgado. **2013**. Community-level phenological response to climate change. Proceedings of the National Academy of Sciences. 110: 13434–13439.

Owen, R. 2017. Role of Human Action in the Spread of Honey Bee (Hymenoptera: Apidae) Pathogens. Journal of Economic Entomology. 110: 797–801.

Park, M. G., E. J. Blitzer, J. Gibbs, J. E. Losey, and B. N. Danforth. **2015**. Negative effects of pesticides on wild bee communities can be buffered by landscape context. Proceedings of the Royal Society B: Biological Sciences. 282: 20150299.

Park, M. G., N. K. Joshi, E. G. Rajotte, D. J. Biddinger, J. E. Losey, and B. N. Danforth. **2018**. Apple grower pollination practices and perceptions of alternative pollinators in New York and Pennsylvania. Renewable Agriculture and Food Systems. 1–14.

Peach, M. L., D. G. Alston, and V. J. Tepedino. **1995**. Sublethal Effects of Carbaryl Bran Bait on Nesting Performance, Parental Investment, and Offspring Size and Sex Ratio of the Alfalfa Leafcutting Bee (Hymenoptera: Megachilidae). Environmental Entomology. 24: 34–39.

Pilling, E., P. Campbell, M. Coulson, N. Ruddle, and I. Tornier. **2013**. A Four-Year Field Program Investigating Long-Term Effects of Repeated Exposure of Honey Bee Colonies to Flowering Crops Treated with Thiamethoxam. PLoS ONE. 8: e77193.

Pisa, L. W., V. Amaral-Rogers, L. P. Belzunces, J. M. Bonmatin, C. A. Downs, D. Goulson, D. P. Kreutzweiser, C. Krupke, M. Liess, M. McField, C. A. Morrissey, D. A. Noome, J. Settele, N. Simon-Delso, J. D. Stark, J. P. Van der Sluijs, H. Van Dyck, and M. Wiemers. **2015**. Effects of neonicotinoids and fipronil on non-target invertebrates. Environmental Science and Pollution Research. 22: 68–102.

Pitts-Singer, T. L., and J. H. Cane. **2011**. The Alfalfa Leafcutting Bee, *Megachile rotundata*: The World's Most Intensively Managed Solitary Bee. Annual Review of Entomology. 56: 221–237.

Ploquin, E. F., J. M. Herrera, and J. R. Obeso. **2013**. Bumblebee community homogenization after uphill shifts in montane areas of northern Spain. Oecologia. 173: 1649–1660.

Plourde, J. D., B. C. Pijanowski, and B. K. Pekin. **2013**. Evidence for increased monoculture cropping in the Central United States. Agriculture, Ecosystems & Environment. 165: 50–59.

Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, and W. E. Kunin. **2010**. Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution. 25: 345–353.

Raimets, R., R. Karise, M. Mänd, T. Kaart, S. Ponting, J. Song, and J. E. Cresswell. **2018**. Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.): Synergistic interactions between a variety of insecticides and fungicide. Pest Management Science. 74: 541–546.

Ramsey, J., A. L. Catchot, D. Bao, A. Adams, B. P. Adams, N. R. Bateman, W. D. Crow, C. Darnell, T. D. Dill, S. H. Graham, J. H. North, and A. Whalen. **2016**. Efficacy of Selected Insecticides for Control of Soybean Loopers and Southern Green Stink Bugs in Soybeans (Test 2), 2013: Table 1. Arthropod Management Tests. 41: tsw074.

Ravoet, J., L. De Smet, I. Meeus, G. Smagghe, T. Wenseleers, and D. C. de Graaf. **2014**. Widespread occurrence of honey bee pathogens in solitary bees. Journal of Invertebrate Pathology. 122: 55–58.

Remnant, E. J., A. Koetz, K. Tan, E. Hinson, M. Beekman, and B. P. Oldroyd. **2014**. Reproductive interference between honeybee species in artificial sympatry. Molecular Ecology. 23: 1096–1107.

Ricketts, T. H., J. Regetz, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen, A. Bogdanski, B. Gemmill-Herren, S. S. Greenleaf, A. M. Klein, M. M. Mayfield, L. A. Morandin, A. Ochieng', and B. F. Viana. **2008**. Landscape effects on crop pollination services: are there general patterns? Ecology Letters. 11: 499–515.

Rinderer, T., and K. Elliott. **1977**. Worker Honey Beel Response to Infection with *Nosema apis*: Influence of Diet. Journal of Economic Entomology. 70: 431–433.

Roberts, K. E., S. E. F. Evison, B. Baer, and W. O. H. Hughes. **2015**. The cost of promiscuity: sexual transmission of *Nosema* microsporidian parasites in polyandrous honey bees. Scientific Reports. 5: 1–7.

Roland, J., and S. F. Matter. **2013**. Variability in winter climate and winter extremes reduces population growth of an alpine butterfly. Ecology. 94: 190–199.

Rosa, A. de S., J. S. G. Teixeira, A. Vollet-Neto, E. P. Queiroz, B. Blochtein, C. S. S. Pires, and V. L. Imperatriz-Fonseca. **2016**. Consumption of the neonicotinoid thiamethoxam during the larval stage affects the survival and development of the stingless bee, *Scaptotrigona aff. depilis*. Apidologie. 47: 729–738.

Rosenkranz, P., P. Aumeier, and B. Ziegelmann. **2010**. Biology and control of *Varroa destructor*. Journal of Invertebrate Pathology. 103: S96–S119.

Roubik, D. W., and R. Villanueva-Gutiérrez. **2017**. Have native Hymenoptera or Africanized bees become aggressive foragers due to resource competition? Trends in Entomology. 13: 95–102.

Roy, D. B., and T. H. Sparks. **2000**. Phenology of British butterflies and climate change. Global Change Biology. 6: 407–416.

Rundlöf, M., G. K. S. Andersson, R. Bommarco, I. Fries, V. Hederström, L. Herbertsson, O. Jonsson, B. K. Klatt, T. R. Pedersen, J. Yourstone, and H. G. Smith. **2015**. Seed coating with a neonicotinoid insecticide negatively affects wild bees. Nature. 521: 77–80.

Ryabov, E. V., A. K. Childers, Y. Chen, S. Madella, A. Nessa, D. vanEngelsdorp, and J. D. Evans. **2017**. Recent spread of Varroa destructor virus-1, a honey bee pathogen, in the United States. Scientific Reports. 7: 1–10.

Sachman-Ruiz, B., V. Narváez-Padilla, and E. Reynaud. **2015**. Commercial *Bombus impatiens* as reservoirs of emerging infectious diseases in central México. Biological Invasions. 17: 2043–2053.

Samson, F., and F. Knopf. **1994**. Prairie Conservation in North America. BioScience. 44: 418–421.

Sánchez-Bayo, F., L. Belzunces, and J.-M. Bonmatin. **2017**. Lethal and sublethal effects, and incomplete clearance of ingested imidacloprid in honey bees (*Apis mellifera*). Ecotoxicology. 26: 1199–1206.

Sánchez-Bayo, F., and K. Goka. **2014**. Pesticide Residues and Bees – A Risk Assessment. PLoS ONE. 9: e94482.

Sandrock, C., M. Tanadini, L. G. Tanadini, A. Fauser-Misslin, S. G. Potts, and P. Neumann. **2014**. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. PLoS ONE. 9: e103592.

Schmidt. **1984**. Feeding Preferences of *Apis mellifera* L. (Hymenoptera: Apidae): Individual Versus Mixed Pollen Species. Journal of the Kansas Entomological Society. 57: 323–327.

Schmidt et al. 1987. Survival of Honey Bees, Apis mellifera (Hymenoptera: Apidae), Fed Various Pollen Sources. Annals of the Entomological Society of America. 80: 176–183.

Schmidt, L. S., J. O. Schmidt, H. Rao, W. Wang, and L. Xu. **1995**. Feeding Preference and Survival of Young Worker Honey Bees (Hymenoptera: Apidae) Fed Rape, Sesame, and Sunflower Pollen. Journal of Economic Entomology. 88: 1591–1595.

Schmuck, R., R. Schoning, A. Stork, and O. Schramel. **2001**. Risk posed to honey bees (*Apis mellifera* L., Hymenoptera) by an imidacloprid seed dressing of suflowers. Pest Management Science. 57: 225–238.

Schoonvaere, K., G. Smagghe, F. Francis, and D. C. de Graaf. **2018**. Study of the Metatranscriptome of Eight Social and Solitary Wild Bee Species Reveals Novel Viruses and Bee Parasites. Frontiers in Microbiology. 9: 1–12.

Sgolastra, F., P. Medrzycki, L. Bortolotti, M. T. Renzi, S. Tosi, G. Bogo, D. Teper, C. Porrini, R. Molowny-Horas, and J. Bosch. **2017**. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species: Synergistic interactions between pesticides in three bee species. Pest Management Science. 73: 1236–1243.

Sih, A., A. M. Bell, and J. L. Kerby. **2004**. Two stressors are far deadlier than one. Trends in Ecology & Evolution. 19: 274–276.

Simone-Finstrom, M., H. Li-Byarlay, M. H. Huang, M. K. Strand, O. Rueppell, and D. R. Tarpy. **2016**. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. Scientific Reports. 6: 1–10.

Smith, T. J., and M. M. Mayfield. **2018**. The effect of habitat fragmentation on the bee visitor assemblages of three Australian tropical rainforest tree species. Ecology and Evolution.

Sparks, T. H., E. P. Jeffree, and C. E. Jeffree. **2000**. An examination of the relationship between flowering times and temperature at the national scale using long-term phenological records from the UK. International Journal of Biometeorology. 44: 82–87.

Spiesman, B. J., A. Bennett, R. Isaacs, and C. Gratton. **2017**. Bumble bee colony growth and reproduction depend on local flower dominance and natural habitat area in the surrounding landscape. Biological Conservation. 206: 217–223.

Spurgeon, D., E. Lahive, C. Svendsen, J. Baas, A. Robinson, A. Horton, and M. Heard. **2016**. Chronic oral lethal and sub-lethal toxicities of different binary mixtures of pesticides and contaminants in bees (*Apis mellifera*, *Osmia bicornis* and *Bombus terrestris*). EFSA Supporting Publications. 13: 1–66.

Stanley, D. A., M. P. D. Garratt, J. B. Wickens, V. J. Wickens, S. G. Potts, and N. E. Raine. **2015**. Neonicotinoid pesticide exposure impairs crop pollination services provided by bumblebees. Nature. 528: 548–550.

Steinhauer, N., K. Rennich, D. M. Caron, K. S. Delaplane, J. Rangel, R. Rose, R. R. Sagili, J. Skinner, J. T. Wilkes, M. E. Wilson, J. Pettis, and D. van Engelsdorp. **2016**. Bee Informed Partnership: Colony Loss 2015-2016: Preliminary Results. Tenth Annual National Survey of Honey Bee Colony Losses. 10: 1–7.

Stoner, K. A. 2016. Current Pesticide Risk Assessment Protocols Do Not Adequately Address Differences between Honey Bees (*Apis mellifera*) and Bumble Bees (*Bombus* spp.). Frontiers in Environmental Science. 4.

Sumner, D. A., and H. Boriss. **2006**. Bee-conomics and the Leap in Pollination Fees. University of California.

Tan, K., W. Chen, S. Dong, X. Liu, Y. Wang, and J. C. Nieh. **2014**. Imidacloprid Alters Foraging and Decreases Bee Avoidance of Predators. PLoS ONE. 9: e102725.

Thompson, H. M., S. Wilkins, S. Harkin, S. Milner, and K. F. Walters. **2015**. Neonicotinoids and bumblebees (*Bombus terrestris*): effects on nectar consumption in individual workers: Neonicotinoids and bumblebees. Pest Management Science. 71: 946–950.

Tomé, H. V. V., G. F. Martins, M. A. P. Lima, L. A. O. Campos, and R. N. C. Guedes. **2012**. Imidacloprid-Induced Impairment of Mushroom Bodies and Behavior of the Native Stingless Bee *Melipona quadrifasciata anthidioides*. PLoS ONE. 7: e38406.

Torchio, P. F. 1973. Relative Toxicity of Insecticides to the Honey Bee, Alkali Bee, and Alfalfa Leafcutting Bee (Hymenoptera: Apidae, Halictidae, Megachilidae). Journal of the Kansas Entomological Society. 46: 446–453.

Torchio, P. F. 1983. The Effects of Field Applications of Naled and Trichlorfon on the Alfalfa Leafcutting Bee, *Megachile rotundata* (Fabricius). Journal of the Kansas Entomological Society. 56: 62–68.

Torné-Noguera, A., A. Rodrigo, S. Osorio, and J. Bosch. **2016**. Collateral effects of beekeeping: Impacts on pollen-nectar resources and wild bee communities. Basic and Applied Ecology. 17: 199–209.

Torretta, J. P., D. Medan, and A. H. Abrahamovich. **2006**. First record of the invasive bumblebee *Bombus terrestris* (L.) (Hymenoptera, Apidae) in Argentina. Transactions of the American Entomological Society. 132: 285–289.

Tripodi, A. D., X. Cibils-Stewart, B. P. McCornack, and A. L. Szalanski. **2014**. *Nosema bombi* (Microsporidia: Nosematidae) and Trypanosomatid Prevalence in Spring Bumble Bee Queens (Hymenoptera: Apidae: Bombus) in Kansas. Journal of the Kansas Entomological Society. 87: 225–233.

Ueno, T. 2014. Establishment of the Invasive Hornet *Vespa velutina* (Hymenoptera: Vespidae) in Japan. 2: 3.

U.S. Environmental Protection Agency. **2013**. A Summary of Analytical Methods Used for the Analyses of Pesticide Residues in Bees and Hive Matrices. (https://www.epa.gov/sites/production/files/2013-09/documents/bee-analytical-methods.pdf).

Valdovinos-Núñez, G. R., J. J. Quezada-Euán, P. Ancona-Xiu, H. Moo-Valle, A. Carmona, and E. R. Sánchez. **2009**. Comparative Toxicity of Pesticides to Stingless Bees (Hymenoptera: Apidae: Meliponini). Journal of Economic Entomology. 102: 1737–1742.

Van Timmeren, S., and R. Isaacs. **2013**. Control of spotted wing drosophila, *Drosophila suzukii*, by specific insecticides and by conventional and organic crop protection programs. Crop Protection. 54: 126–133.

vanEngelsdorp, D., J. D. Evans, C. Saegerman, C. Mullin, E. Haubruge, B. K. Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, R. Underwood, D. R. Tarpy, and J. S. Pettis. **2009**. Colony Collapse Disorder: A Descriptive Study. PLoS ONE. 4: 1–17.

vanEngelsdorp, D., and M. D. Meixner. **2010**. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology. 103: S80–S95.

Vaudo, A. D., L. M. Farrell, H. M. Patch, C. M. Grozinger, and J. F. Tooker. **2018**. Consistent pollen nutritional intake drives bumble bee (*Bombus impatiens*) colony growth and reproduction across different habitats. Ecology and Evolution. 8: 5765–5776.

Vaudo, A. D., H. M. Patch, D. A. Mortensen, J. F. Tooker, and C. M. Grozinger. **2016**. Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. Proceedings of the National Academy of Sciences. 113: E4035–E4042.

Vaudo, A. D., J. F. Tooker, C. M. Grozinger, and H. M. Patch. **2015**. Bee nutrition and floral resource restoration. Current Opinion in Insect Science. 10: 133–141.

Velthuis, H. H. W., and A. van Doorn. **2006**. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie. 37: 421–451.

Vidau, C., M. Diogon, J. Aufauvre, R. Fontbonne, B. Viguès, J.-L. Brunet, C. Texier, D. G. Biron, N. Blot, H. El Alaoui, L. P. Belzunces, and F. Delbac. **2011**. Exposure to Sublethal Doses of Fipronil and Thiacloprid Highly Increases Mortality of Honeybees Previously Infected by *Nosema ceranae*. PLoS ONE. 6: 1–8.

Visscher, P. K., R. S. Vetter, and R. Orth. **1994**. Benthic Bees? Emergence Phenology of *Calliopsis pugionis* (Hymenoptera: Andrenidae) at a Seasonally Flooded Site. Annals of the Entomological Society of America. 87: 941–945.

Walther-Hellwig, K., G. Fokul, R. Frankl, and R. Buchler. **2006**. Increased density of honeybee colonies affects foraging bumblebees. Apidologie. 37: 517–532.

Warzecha, D., T. Diekötter, V. Wolters, and F. Jauker. **2016**. Intraspecific body size increases with habitat fragmentation in wild bee pollinators. Landscape Ecology. 31: 1449–1455.

Wilfert, L., G. Long, H. C. Leggett, P. Schmid-Hempel, R. Butlin, S. J. M. Martin, and M. Boots. **2016**. Deformed wing virus is a recent global epidemic in honeybees driven by *Varroa* mites. Science. 351: 594–597.

Williams, N. M., E. E. Crone, T. H. Roulston, R. L. Minckley, L. Packer, and S. G. Potts. **2010**. Ecological and life-history traits predict bee species responses to environmental disturbances. Biological Conservation. 143: 2280–2291.

Williams, N. M., J. Regetz, and C. Kremen. **2012**. Landscape-scale resources promote colony growth but not reproductive performance of bumble bees. Ecology. 93: 1049–1058.

Willmer, P. G., A. A. M. Bataw, and J. P. Hughes. **1994**. The superiority of bumblebees to honeybees as pollinators: insect visits to raspberry flowers. Ecological Entomology. 19: 271–284.

Wilson, J. S., and O. Messinger Carril. **2016**. The bees in your backyard: a guide to North America's bees. Princeton University Press, Princeton.

Winfree, R., R. Aguilar, D. P. Vázquez, G. LeBuhn, and M. A. Aizen. **2009**. A meta-analysis of bees' responses to anthropogenic disturbance. Ecology. 90: 2068–2076.

Zhu, W., D. R. Schmehl, C. A. Mullin, and J. L. Frazier. **2014**. Four Common Pesticides, Their Mixtures and a Formulation Solvent in the Hive Environment Have High Oral Toxicity to Honey Bee Larvae. PLoS ONE. 9: 1–11.

Zhu, Y. C., J. Adamczyk, T. Rinderer, J. Yao, R. Danka, R. Luttrell, and J. Gore. **2015**. Spray Toxicity and Risk Potential of 42 Commonly Used Formulations of Row Crop Pesticides to Adult Honey Bees (Hymenoptera: Apidae). Journal of Economic Entomology. 108: 2640–2647.

Zhu, Y. C., J. Yao, J. Adamczyk, and R. Luttrell. **2017b**. Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (*Apis mellifera*). PLOS ONE. 12: 1–19.

Zhu, Y. C., J. Yao, J. Adamczyk, and R. Luttrell. **2017a**. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). PLOS ONE. 12: 1–16.

Chapter II: Toxicity of Formulated Insecticide Mixtures to Honey Bees, *Apis mellifera* (Linnaeus)

Toxicity of Formulated Insecticide Mixtures to Honey Bees, *Apis mellifera* (Linnaeus)

Abstract

Honey bees, *Apis mellifera* (Linnaeus) are crucial pollinators, however large overwinter losses of managed hives and declines in feral colonies have recently occurred. Increased usage of pesticides has been proposed to contribute to these declines. Previous studies have demonstrated contact toxicity of single active ingredient insecticides to honey bees. However, field realistic toxicity of formulated insecticide mixtures containing multiple active ingredients (each with a different mode of action) to honey bees has not been thoroughly assessed. Here, we simulate whole body contact to spray application of thiamethoxam + lambda-cyhalothrin, imidacloprid + beta-cyfluthrin, chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram to honey bees under three realistic scenarios in field crops: (1) continuous exposure, (2) spray-only exposure, and (3) residual exposure. A customized spray tower in a laboratory setting was used to apply insecticides at the lowest recommended dose on the EPA label of each insecticide. Acute toxicity was determined by screening resulting mortality up to 96-hours post treatment. Statistical significance was found between treatments ($p<0.05$) at all screening periods for all experiments except residual exposure at 48-hours given complete mortality. For continuous and spray-only exposure, thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin resulted in 80-90% mortality at 48-hours, while at 96-hours we observed close to complete mortality for all treatments. This difference in mortality onset between treatments highlights the importance of extended observation. Our finding of overall high mortality demonstrates the importance of comparing both combined and individual active ingredients in determining insecticide risk to honey bees.

Keywords: continuous exposure toxicity, spray-only exposure toxicity, residual toxicity, neonicotinoids, pyrethroids, honey bees, premix insecticides

Introduction

Honey bees, [*Apis mellifera* (Linnaeus) (Hymenoptera: Apidae)] provide integral pollination services for the production of food, fiber and fuel crops worldwide (Klein et al. 2007). They are ideally suited for monoculture pollination events given their foraging activity throughout an entire growing season, their biology as generalist pollinators and relative availability as a domesticated species (vanEngelsdorp and Meixner 2010). Therefore, we have become increasingly reliant on honey bees, which is evidenced by the increased trajectory of bee-dependent crop cultivation over time (Aizen et al. 2008). As a result, honey bees are commercially domesticated by beekeepers and leased to agricultural producers for their pollination services. For example, almond, (*Prunus dulcis*, Miller and Webb) production in California is almost completely reliant on the beekeeping industry, requiring annual transportation of 1.9 million beehives to California's Central Valley during bloom (Pitts-Singer et al. 2018). This month-long pollination event is spread over 433,014 bearing hectares generating 80% of global almond yield worth $5.16 billion USD in revenue (USDA NASS 2018). Economically, honey bee pollination was estimated to contribute $11.68 billion USD in direct crop pollination and $5.39 billion USD in indirect crop pollination in the United States in 2009 (Calderone 2012). Beyond their importance to commercial agriculture, honey bees are also pollinators of wild plants and are therefore critical for natural ecosystem conservation (Gallai et al. 2009). Honey bees additionally provide resources including honey and honeycomb and hive products such as wax, propolis, lip balm and hand creams (Crane 1990; Flottum 2014).

The increased reliance on honey bees for pollination services coincides with high overwinter declines of domesticated hives and reductions in feral colonies over the last decade. Several theories including a range of environmental stressors characterized by diseases, parasites, pesticide exposure, habitat destruction, insufficient nutrition and cultural practices have been postulated to explain these losses (Duay et al. 2002; vanEngelsdorp et al. 2009; Mussen 2011; Huang 2012; Goulson et al. 2015; Lundin et al. 2017). The Bee Informed Partnership has conducted annual surveys of honey bee colony loss in the United States every year beginning in 2006-2007 (Bruckner et al. 2018). According to these surveys, commercial beekeepers have experienced approximately 30% annual losses of colonies, marked by a high of 45% in 2012-2013 (Steinhauer et al. 2016). Data for 2017-2018 (the most recent year) indicate an annual loss of 40%, thereby indicating that colony decline is persistent (Bruckner et al. 2018). In 2014-2015, for the first time, summer losses were higher (26.2%) than winter losses (20.5%), which is attributed to the large losses of commercial beekeepers (Seitz et al. 2016). Therefore, the supply of available honey bee hives for pollination events is largely lagging demand, thereby dramatically increasing the average cost of hive rentals. The steepest increase in hive rental fees are for almond pollination. For example, between 2004-2006, the average price a California almond grower paid per hive increased from $54 USD to $136 USD (Sumner and Boriss 2006). Hive rental fees have similarly increased for other crops. For example, between 2005-2007, average hive rental fees increased from $35 USD to $100 USD for pollinating apples (Biddinger, pers. comm.). As a result, scientists, growers and other crop operators are increasingly motivated to isolate specific stressors affecting honey bees and identify practices that will improve honey bee health for the long term.

Assessing the effects of pesticides commonly applied to agricultural crops on honey bees is one area that has received a lot of research interest. Currently, the majority of research on pesticide toxicity to honey bees has focused on acute, sub-lethal and molecular-level toxicity or detoxification processes following oral and contact exposure to single active ingredient pesticide chemistries. However, these studies generally use non-realistic exposure of technical grade materials dissolved in acetone (OECD-library.org) rather than using formulated products dissolved in water to more accurately reflect field-realistic exposure scenarios. For example, Henry et al. (2012) determined that feeding honey bee foragers sub-lethal doses of the neonicotinoid thiamethoxam impaired their ability to successfully locate their hive. Fischer et al. (2014) concluded that oral uptake of one of the neonicotinoids imidacloprid, clothianidin or thiacloprid in sucrose solution interfered with honey bee navigation. Separately, Sandrock et al. (2014) determined that chronic exposure to pollen stores within hives containing clothianidin and thiamethoxam negatively affected colony performance and queen supersedure. Di Prisco et al. (2013) showed that topical contact exposure to the LD_{50} dose of clothianidin enhances transcription of the gene inhibiting nuclear factor-κB immune signaling in insects and correlated this to increased deformed wing virus infections in honey bees. Oral exposure to a combination of a nonlethal dose of the fungicide propiconazole and a dose in the range of an LD_{10} for clothianidin was determined to cause significant synergistic mortality of honey bees at 4 and 24-hours after treatment (Sgolastra et al. 2017). Analyzing the toxicity of imidacloprid alone and in binary mixtures with seven other pesticides to honey bees, Zhu et al. (2017) found increased mortality from synergism for imidacloprid + tetraconazole, imidacloprid + sulfoxaflor and imidacloprid + oxamyl.

Several studies on honey bee pesticide exposure have been conducted in field settings. David et al. (2016) found large mean quantities of 20 neonicotinoids and fungicides in pollen collected from both oilseed rape, (*Brassica napus*, Linnaeus) flowers (0.5-80 ppb) and wildflowers (0.6-47 ppb) growing around tested fields. This study also found a large mixture of neonicotinoid and fungicide mean residue concentrations in pollen samples collected by honey bees foraging the oilseed rape fields analyzed during bloom (0.15-12 ppb) and after bloom (0.1-2.5 ppb). Dively et al. (2015) showed that honey bee colonies chronically exposed to sub-lethal doses of imidacloprid had decreased health and overwintering success, although the more field realistic sub-lethal dose had negligible effects. Conversely, Pilling et al. (2013) concluded that long-term exposure to thiamethoxam residues poses a low risk to honey bees foraging systemically treated maize and oilseed rape, while Cutler et al. (2014) found that exposing honey bees to clothianidin seed-treated canola posed a low risk to health, development and colony overwintering success. Experimental designs testing the toxicity of field-realistic pesticide doses and formulations to bees are necessary to accurately quantify the real impacts to bees following exposure to these chemicals (Carreck and Ratnieks 2014; Biddinger and Rajotte 2015). For example, up to 100-fold differences in pesticide toxicity to bees were found in comparing formulated pesticides dissolved in water (field setting) to technical-grade pesticides dissolved in acetone (laboratory setting) (Hopwood et al. 2012; Biddinger et al. 2013).

The effects of other agricultural chemicals have also been assessed on honey bees. Spraying honey bee foragers with fungicides using a wind tunnel atomizer, Fisher et al. (2017) showed significant decreases in forager survival following exposure to simulated tank mixes of iprodione, and synergistic effects resulting from iprodione combined with pyraclostrobin and azoxystrobin. Analyzing the effects of exposure to boscalid + pyraclostrobin and the

organosilicone spray adjuvant polyether-polymethylsiloxane-copolymer on honey bee queen-rearing success, Johnson and Percel (2013) found low levels of pyraclostrobin but not boscalid in royal jelly secreted by nurse bees feeding on treated pollen. However, neither pyraclostrobin nor the organosilicone spray adjuvant reduced the survival of immature queens. Analyzing toxic synergisms between fungicides, acaricides and antimicrobial drugs applied to honey bees by thoracic contact, Johnson et al. (2013) concluded that exposure to several sterol biosynthesis inhibiting fungicides increased the toxicity of previous exposure to acaricides commonly used in beehives.

The Food Quality Protection Act of 1996 (United States Congress, 1996) curtailed use of broad-spectrum pesticides and particularly those within the carbamate and organophosphate classes. As a result, the suite of agricultural chemicals available for growers to use in integrated pest management programs was largely reduced. Neonicotinoids (a new class of insecticide characterized by their mode of action as nicotinic acetylcholine receptor agonists) were synthesized and made commercially available during this same time (Elbert et al. 2008). Given the high efficacy of neonicotinoids against insect pests, their use has surged and currently accounts for approximately 25% of the global pesticide market, with sales worth $1.9 billion USD (Jeschke et al. 2011). Neonicotinoids can be applied in a variety of methods: foliar spray applications, seed coatings, soil drenches, trunk injections (for trees), granules, bait and topical applications (Hopwood et al. 2016). However, a major question that has circulated around neonicotinoids is their toxicity to bees. Over the past decade, a myriad of studies have investigated different aspects of bee exposure to these insecticides and their resulting acute, chronic and sub-lethal and toxicity. As previously described, some studies have clearly shown that neonicotinoids pose high risk to bees, while other have found that they pose little or no risk

to bees. Also, some neonicotinoids have been found to be more toxic to bees than others. Therefore, the question of whether or not neonicotinoids are hazardous to bees is still being debated among the scientific community.

Development of insect pest resistance to existing insecticide chemistries has dramatically increased in recent years. For example, upregulation of cytochrome P450 detoxification enzyme related genes has been correlated with neonicotinoid resistance in whiteflies, [*Bemisia tabaci*, (Hemiptera: Aleyrodidae) (Gennadius, 1889)] (Ilias et al. 2015). Similarly, knock-down resistance gene evolution has resulted in cabbage stem flea beetle, [*Psylliodes chrysocephala*, (Coleoptera: Chrysomelidae) (Linnaeus, 1758)] resistance to pyrethroids (Højland et al. 2015). Synthesis of new premix insecticides comprised of multiple active ingredients (each with a different mode of action) which are commonly referred to as insecticide mixtures, has been aimed at combatting insect resistance buildup.

The ecotoxicology of premix insecticides containing multiple active ingredients (some of which are neonicotinoids) to honey bees has not been tested thoroughly. For example, Zhu et al. (2015) demonstrated high levels of contact toxicity by insecticide mixtures to honey bees for both premix products and their individual active ingredients in mortality assessments up to 48-hours after treatment. Here, we explore the toxicity of premix insecticides to honey bees over an extended period by screening forager mortality up to 96-hours following contact exposure. The main objectives of this study were to assess the risk of insecticide exposure to honey bee foragers to premix insecticides in three experiments simulating the following possible scenarios: (1) when bees are foraging in a crop field and are directly exposed to insecticides during an application; (2) when bees are exposed to insecticide sprays while passing through a crop field; and (3) when bees are foraging in a crop field that was sprayed with insecticides during the previous day.

Considering these scenarios, this study investigates field realistic acute contact toxicity of formulated premix insecticides that are commonly used for pest management in field crops to honey bees. The findings of this study will aid growers and insecticide applicators in making insecticide selection and application decisions that minimize their impact on honey bees.

Materials and Methods

Honey bees. Honey bee foragers of the Italian race (*A. mellifera mellifera*) were obtained from beehives maintained at a research farm located within the University of Arkansas Experiment Station, Fayetteville, AR. These beehives were managed according to University of Arkansas Cooperative Extension Service recommendations (University of Arkansas Cooperative Extension Service Beekeeping Publications). Bees were collected from the beehives using cage-jars (mason jars with wire mesh covering the opening). Immediately afterwards, cages containing honey bees were transferred to a cooler and were brought to a laboratory setting. Once in the laboratory, honey bees were placed into an incubator (33 ± 0.5˚C; 65% ± 2 RH) until used for experiments.

Cage design. Experimental cages were comprised of transparent polypropylene jars (500 mL, D by H: 9.3 by 10 cm). Immediately before the experiment, honey bees were immobilized by placing them in a cooler for approximately two minutes (except for experiment 3 where they were directly placed into previously treated cages). After immobilization, honey bees were quickly transferred to the experimental cages. A plastic vial containing 50% sucrose solution (6-7 mL per vial) was inserted in each cage to allow honey bees to feed *ad libitum* immediately post-treatment in all experiments.

Spray tower. We constructed a spray tower based on a previous design as described in Zhu et al. (2015). This spray tower was designed to simulate spray contact exposure experiments

within a laboratory environment. Insecticides were sprayed using a spray nozzle (Burkard Scientific, Uxbridge, Middx, United Kingdom), and were administered to honey bees in cages (0.5 mL/cage, except in experiment 3, where we used 1 mL/cage). The sprayer was set to regulated air pressure (69 kpa) and fixed spray distance (22 cm) for each treatment including control (Zhu et al. 2015). Distilled water was used as the solvent in each treatment to simulate field-realistic grower insecticide spray applications and honey bee exposure scenarios.

Post treatment observations. Cages containing treated honey bees were placed on plastic trays, and were maintained in an incubator (33 ± 0.5˚C; 65% ± 2 RH) for the duration of the experiment. Honey bee mortality was screened and recorded at 24, 48, 72, and 96-hours after treatment for all treatments, except in experiment 3, which ended after 48-hours. Honey bees that did not move after being touched by a paintbrush were considered dead. Moribund bees capable of moving their legs and antennae but incapable of flying were considered alive.

Experiment 1: Honey Bee Forager Acute Contact Toxicity in Continuous Exposure Scenario

Insecticide treatments. Contact toxicity of four formulated insecticide mixtures (Table 2.1 and Figure 2.1) to honey bee foragers was assessed by directly spraying honey bees in experimental cages. Each of these insecticide mixtures contained multiple active ingredients (each with a different mode of action). Low-range recommended application rates listed on the EPA approved insecticide labels were used (Table 2.2). We mixed each insecticide solute in 100 mL of a distilled water solvent. Insecticides were applied to honey bees at a low spray volume (0.5 mL/cage). Distilled water with the same spray volume as insecticide treatments was used as a control. A completely randomized design was used to test the five treatments in this experiment, each of which contained twelve replicates. Twelve cages containing 20 honey bees

each (n = 20/cage, total 240 bees) were utilized for each of the five treatments, with each individual cage representing an experimental unit. Each experimental unit was randomly assigned to one of the five treatments.

Experiment 2: Honey Bee Forager Acute Contact Toxicity in Spray-Only Exposure Scenario

Insecticide treatments. Honey bee foragers in treatment cages were sprayed with four formulated insecticide mixtures (Table 2.1 and Figure 2.1) and then immediately transferred to clean experimental cages for the remainder of the experiment. These insecticides are each comprised of two active ingredients (each with a different mode of action). We applied low-range application rates following EPA approved insecticide label recommendations for each respective insecticide (Table 2.2). We mixed each insecticide solute in 100 mL of a distilled water solvent. Insecticide sprays were administered at a low volume (0.5 mL/cage). Our control consisted of distilled water sprays at the same volume as insecticide treatments. We applied additional 50% sucrose solution (approximately 7 mL) to the feeders made of plastic vials inserted in each cage for *ad libitum* feeding at 24 and 72-hours after treatment. The five treatments in this experiment which each contained seven replicates, were tested using a completely randomized design. We randomly utilized seven cages containing 15 honey bees each (n = 15/cage, total 105 bees) for each of the five treatments, with each individual cage representing an experimental unit.

Experiment 3: Honey Bee Forager Acute Contact Toxicity in Residual Exposure Scenario

Insecticide treatments. We exposed honey bee foragers to cages sprayed 18-hours prior with one of four tested insecticide mixtures (Table 2.1 and Figure 2.1). Premix insecticides containing multiple active ingredients (each with a different mode of action) were tested in this

experiment. Low-range application rates were used as per the label for each respective insecticide (Table 2.2). We mixed each insecticide solute in 100 mL of a distilled water solvent. A high spray volume (1 mL/cage) of insecticides was applied to ensure complete coverage of each experimental cage. For a control, we sprayed distilled water at the same high spray volume. A completely randomized design was used to test the five treatments in this experiment, each of which contained eight replicates. Eight cages containing 12 honey bees each (n = 12/cage, total 96 bees) were randomly utilized for each of the five treatments, with each individual cage representing an experimental unit.

Statistical analysis

For each experiment, we corrected the check (control) honey bee forager mortality at each screening period by using the Schneider-Orelli's formula as in (Bibbs et al. 2015; Williams et al. 2015). Using equation 1:

$$\text{Corrected \% Mortality} = \left(\frac{\text{Mortality \% in treatment} - \text{Mortality \% in control}}{100 - \text{Mortality \% in control}}\right) * 100 \ (\text{eq. } 1)$$

where the percent mortality for treatment replication is compared to the percent mortality for each respective control replication.

As a result of correcting our data using Schneider-Orelli's formula, we removed the control from each experiment, thereby only analyzing the honey bee forager mortality in insecticide treatments. Corrected percent mortality data were normalized using an arcsine transformation as in (Glazer and Navon 1990). Normality and lack of heteroscedasticity of data were confirmed with Normal Q-Q and Residual vs. Fitted plots.

One-way ANOVA was used to determine statistical significances in the percent of honey bee forager mortality following acute contact exposure to each of the four premix insecticides tested at each screening period as in Wise et al. (2017). We used a Tukey's HSD post-hoc

multiple pairwise comparison analysis to determine statistical differences in the percent of honey bee forager mortality following acute contact exposure to each premix insecticide treatment during each screening period as in Wise et al. (2017). All statistical analyses were completed using the R Studio statistical software (R version 3.5.1 and R Studio version 1.1.463).

Results

Experiment 1: Honey Bee Forager Acute Contact Toxicity in Continuous Exposure Scenario

Continuous honey bee forager exposure to all four premix insecticides (Tables 2.1-2.2 and Figure 2.1) resulted in mortality that was statistically significant between treatments at 24-hours ($F=58.332$, $p<0.05$), 48-hours ($F=50.082$, $p<0.05$), 72-hours ($F=73.565$, $p<0.05$) and 96-hours ($F=31.131$, $p<0.05$) (Figure 2.2 and Appendix 2.1). At 24 and 48-hours, mortality of bees due to exposure to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin was significantly higher from chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram ($p<0.05$) (Figure 2.2 and Appendix 2.1). At 72 and 96-hours, thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin resulted in higher bee mortality than chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram ($p<0.05$).

Experiment 2: Honey Bee Acute Contact Toxicity in Spray-Only Exposure Scenario

In the second scenario of honey bee forager spray-only exposure to all four premix insecticides (Tables 2.1-2.2 and Figure 2.1), we observed statistically significant mortality between treatments at 24-hours ($F=50.855$, $p<0.05$), 48-hours ($F=26.533$, $p<0.05$), 72-hours ($F=86.967$, $p<0.05$) and 96-hours ($F=64.629$, $p<0.05$) (Figure 2.3 and Appendix 2.2). During all post-treatment mortality observations, bee mortality in the thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin treatments was higher than in the chlorantraniliprole +

lambda-cyhalothrin and methoxyfenozide + spinetoram treatments, however, bee mortality following exposure to chlorantraniliprole + lambda-cyhalothrin was also higher than methoxyfenozide + spinetoram ($p<0.05$) (Figure 2.3 and Appendix 2.2).

Experiment 3: Honey Bee Acute Contact Toxicity in Residual Exposure Scenario

Honey bee forager residual exposure to all four premix insecticides (Tables 2.1-2.2 and Figure 2.1) resulted in statistical significance between treatments at 24-hours ($F=11.213$, $p<0.05$), however not at 48-hours ($F=1$, $p=0.407$) given the complete mortality in all treatments during this screening period (Figure 2.4 and Appendix 2.3), thereby terminating the experiment early. At 24-hours, we observed higher mortality in the thiamethoxam + lambda-cyhalothrin treatment compared to all other treatments ($p<0.05$) (Figure 2.4 and Appendix 2.3). At 48-hours, no significantly different mortality was observed between treatments ($p=0.5013$ for thiamethoxam + lambda-cyhalothrin compared to all other treatments, and $p=1$ for imidacloprid + beta-cyfluthrin and chlorantraniliprole + lambda-cyhalothrin compared to methoxyfenozide + spinetoram). In this experiment, we applied a high spray volume (1 mL) of insecticides to ensure complete coverage of experimental cages.

Discussion

Experiment 1: Honey Bee Forager Acute Contact Toxicity in Continuous Exposure Scenario

Simulation of continuous honey bee foraging in a crop field and direct insecticide exposure while insecticides are being sprayed in that field resulted in the most rapid onset of mortality for bees treated with thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin. At 24-hours, we observed approximately 80% and 70% mortality respectively, while at 48-hours, we observed approximately 95% and 90% mortality respectively. We attribute this

fast onset of mortality leading to complete mortality at 72-hours to the combined active ingredient chemistries with particular emphasis on the pyrethroids, given their characteristic knockdown mode of action as sodium channel modulators (Table 2.1; Vijverberg et al. 1982). The systemic and translaminar properties of neonicotinoids (Elbert et al. 2008) facilitating their movement into leaf tissue results in reduced bee contact after residues dry. The low LD_{50} values of these active ingredient chemistries to honey bees (Table 2.3) further indicate their high toxicity. Our results are suggestive of an additive or synergistic toxicity arising from exposure to both a neonicotinoid and a pyrethroid, however, future analysis of multiple doses to create dose-mortality curves is necessary to prove this. These findings could also be due to continuous bee exposure to insecticide residues after treatment, given that bees were not moved to separate clean cages after insecticide application. It is evident that foraging honey bees experiencing prolonged exposure to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin in field crops will be unlikely to survive.

Differentially, honey bees continuously exposed to chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram experienced a slower onset of mortality. However, their resulting mortalities at 96-hours were approximately 95% and 90% respectively. We primarily attribute this upward trajectory in mortality for the chlorantraniliprole + lambda-cyhalothrin treatment to the presence of the pyrethroid chemistry in this insecticide mixture. The higher LD_{50} values of chlorantraniliprole, methoxyfenozide and spinetoram to honey bees (Table 2.3) further indicate their lower toxicity. Chlorantraniliprole, which is a ryanodine receptor agonist (Table 2.1; irac-online.org) has also been shown to adversely impact bees as in Smagghe et al. (2013) where *Bombus terrestris* [(Hymenoptera: Apidae) (Linnaeus, 1758)] oral consumption resulted in acute and chronic toxicity. Conversely Zhu et al. (2015) found a very

high chlorantraniliprole contact LD_{50} in honey bees. Similarly, we attribute the overall decreased mortality in the methoxyfenozide + spinetoram treatment to the lack of a pyrethroid-like active ingredient. However, these results on adult Hymenoptera are not entirely surprising given that methoxyfenozide is a diacylhydrazine ecdysone receptor agonist (Table 2.1; irac-online.org) designed to induce premature molting in immature Lepidoptera (Suiter and Scharf 2015). The findings of Mommaerts et al. (2006) demonstrating no contact or oral toxicity of ecdysone receptor agonists (including methoxyfenozide) to adult and immature *B. terrestris* and those of Besard et al. (2011) demonstrating reduced oral and contact toxicity of spinetoram (compared to spinosad) to adult *B. terrestris* further support these results. Conversely, spraying honey bee foragers with methoxyfenozide using a wind tunnel atomizer, Fisher et al. (2018) documented significantly increased mortality following treatment above the label dose at 24-hour screenings over a 10-day period. Examining the toxicity of a range of spinetoram doses to honey bees, Chen et al. (2017) determined a low LD_{50} for contact exposure at 48-hours after treatment.

Additionally, the translaminar movement of spinetoram (Sato et al. 2012) reduces bee contact after residues have dried. Interestingly, average mortality for methoxyfenozide + spinetoram was slightly higher than that for chlorantraniliprole + lambda-cyhalothrin at 24-hours, however, it was less at all other screening periods. Our results suggest that foraging honey bees continuously exposed to chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram in field crops will likely experience delayed mortality. Removing check (control) mortality from our data analysis by using the Schneider-Orelli's formula (eq. 1) enables us to demonstrate that honey bee forager mortality is increasing as a result of exposure to insecticide treatments.

Experiment 2: Honey Bee Acute Contact Toxicity in Spray-Only Exposure Scenario

In the second scenario, our analysis of spray-only honey bee forager exposure to insecticide sprays while passing through a crop field similarly demonstrated the overall highest and fastest onset of mortality for the thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin treatments. Similar to continuous exposure, we observed approximately 80% mortality at 24-hours and 90% mortality at 48-hours. Complete mortality was determined at 72-hours. Spray-only contact led rapid honey bee knockdown approximately 20-25 minutes after treatment which we primarily attribute to the pyrethroid component in each of these two insecticides. The high toxicity of these active ingredient chemistries to honey bees is further indicated by their low LD_{50} values (Table 2.3). As with continuous exposure, these findings are indicative of additive or synergistic toxicity arising from the mixture of neonicotinoid and pyrethroid active ingredients, however assessment of multiple doses to generate dose mortality curves is required to prove this. Similar to continuous exposure, our results indicate that honey bee foragers exposed to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin while passing through a crop field while it is being sprayed will likely die quickly.

At 24 and 48-hours, we observed less mortality for honey bee foragers treated with chlorantraniliprole + lambda-cyhalothrin (approximately 45% and 70% respectively) and methoxyfenozide + spinetoram (approximately 30% and 40% respectively). As in continuous exposure, mortality substantially increased at 96-hours and specifically for chlorantraniliprole + lambda-cyhalothrin (approximately 90% mortality). Similar to continuous exposure, we primarily attribute the higher observed toxicity for chlorantraniliprole + lambda-cyhalothrin to the presence of a pyrethroid chemistry. The higher LD_{50} values of these active ingredient chemistries to honey bees (Table 2.3) further indicate their lower toxicity. Interestingly, bees

exposed to spray-only contact with methoxyfenozide + spinetoram had substantially less mortality at 96-hours compared bees continuously exposed, suggesting somewhat lower toxicity for bees passing through a crop field. The findings of Mommaerts et al. (2006) and particularly Besard et al. (2011) indicate reduced toxicity for these active ingredients to *B. terrestris* under continuous exposure. Therefore, it would be interesting to examine differences in toxicity arising from continuous versus spray-only exposure as we have done here. These results indicate that honey bee foragers coming into spray-only contact with chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram will likely experience delayed mortality that will be more severe for chlorantraniliprole + lambda-cyhalothrin. Removing check (control) mortality from our data analysis by using the Schneider-Orelli's formula (eq. 1) enables us to demonstrate that honey bee forager mortality is increasing as a result of exposure to insecticide treatments.

Experiment 3: Honey Bee Acute Contact Toxicity in Residual Exposure Scenario

Honey bee forager residual contact exposure to premix insecticides simulating foraging in a crop field sprayed during the previous day resulted in high mortality at 24-hours for all treatments. Similar to continuous and spray-only exposure, the highest mortality was observed for thiamethoxam + lambda-cyhalothrin (approximately 70%) and imidacloprid + beta-cyfluthrin (approximately 55%). Specifically, we noted fast knockdown beginning approximately 20 minutes after initial exposure, which we primarily attribute to the pyrethroid in both of these insecticide mixtures. Slightly less mortality was observed for chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram (approximately 50% and 45% respectively) at 24-hours. Further demonstrating the high acute toxicity of premix insecticide residual contact exposure to honey bees, we observed complete mortality at 48-hours for all treatments, thereby

terminating the experiment. Although we removed control data from our analysis, we did notice significantly less mortality in the control treatment at both screening periods.

Several factors might in part explain this finding. First, our usage of double the insecticide spray volume (1 mL) to ensure complete coverage of experimental cages increased the proportion of insecticides that honey bees were exposed to by two-fold. Second, the high temperature (27.22-28.89°C) and humidity (71-92%) (timeanddate.com) on the day honey bees were collected from beehives might have liquified insecticide residues, thereby increasing their toxicity. We noticed bee proboscis extension inside cages, suggesting possible oral consumption of insecticides in addition to the contact exposure being measured in the experiment. Placing bees in cages in a field setting during high humidity conditions likely increased their energy expenditure and stress level. This explains our observation of increased bee fanning activity after being placed within cages. High relative humidity is known as a major factor influencing insect mortality Mishra et al. (2015), thereby potentially contributing to the high bee mortality we observed. Although we did not include the control in our analysis, it is meaningful to mention that we observed less than 10% control mortality at 24-hours. Our results indicate the potentially high risk posed to honey bee foragers coming into contact with insecticide residues. Building upon our findings, future studies should examine the toxicity of insecticide applications made at sunset (when bees are not actively foraging) to foraging bees the following morning when dew may liquify dried insecticide residues on leaf and flower tissue. Further, dried insecticide residues applied at sunset may subsequently become liquified in plant guttation fluids which have been shown to contain high levels of neonicotinoids in seed-treated maize (Girolami et al. 2009) and melons (Hoffmann and Castle 2012). Honey bees have been found to collect guttation fluid (Hopwood et al. 2016),

thereby potentially exposing them to dried insecticide residues that have liquified in guttation fluids.

Widescale insect pest resistance buildup for neonicotinoids as described in Ilias et al. (2015) and for pyrethroids in Højland et al. (2015) has in part led to development of new insecticide chemistries including premixes. Insecticide mixtures blending multiple active ingredients (each with a different mode of action) combat resistance spread as described in Soderlund (2008) for pyrethroids. Lowering the percentage of active ingredient of each chemistry in a premix insecticide (compared to that in a single active ingredient insecticide) facilitates maintenance of older insecticide chemistries. Moreover, this approach equips growers with one bottle of insecticide that provides broad-spectrum coverage. Alternating application of insecticide products between generations of insect pests within a field is an additional measure that growers can use to minimize resistance buildup. However, given that premixes provide high efficacy, both approaches should be considered when implementing an integrated pest management program.

The insecticide mixtures tested in this study are increasingly being utilized for controlling insect pests in field crops. For example, a premix insecticide containing thiamethoxam + lambda-cyhalothrin had highest efficacy for controlling rice stink bug, [*Oebalus pugnax* (Hemiptera: Pentatomidae) (Fabricius, 1775)] up to 28-days after treatment (Jones et al. 2017). Similarly, thiamethoxam + lambda-cyhalothrin had high efficacy while imidacloprid + beta-cyfluthrin had some efficacy for controlling kudzu bug, [*Megacopta cribraria* (Hemiptera: Plataspidae) (Fabricius, 1789)] in soybeans (Brown et al. 2015). Insecticides containing imidacloprid + beta-cyfluthrin, and chlorantraniliprole + lambda-cyhalothrin have been reported to effectively control southern green stink bug, [*Nezara viridula* (Hemiptera: Pentatomidae)

(Linnaeus, 1758)] while chlorantraniliprole + lambda-cyhalothrin was also effective against soybean looper [*Chrysodeixis includens* (Lepidoptera: Noctuidae) (Walker, 1858)] (Ramsey et al. 2016). Another insecticide premix (methoxyfenozide + spinetoram) evaluated in our study, had been found to significantly reduce soybean looper larvae in soybeans (Cook and Gore 2018). Several of these field crops are frequently visited by different pollinator species including honey bees (Klein et al. 2007). Therefore, it is potentially hazardous to honey bees when they are exposed to direct spray applications or dried residues of these insecticide mixtures while collecting pollen and nectar.

In recent past, some preliminary work has demonstrated the toxicity of the premix insecticides we tested on honey bees. For example, Zhu et al. (2015) demonstrated high contact toxicity based on low LD_{50} values for both premix insecticides and individual active ingredients at 48-hours after treatment. Similarly, in Zhu et al. (2017) binary insecticide mixtures of imidacloprid with seven other pesticides posed varying levels of toxicity to honey bees. While this study simulated field-realistic scenarios by using formulated as opposed to technical grade insecticides, it does not assess mortality over an extended period (96-hours) as we have done. Our finding of slow onset of acute contact toxicity for chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram demonstrates the importance of extended observation to thoroughly assess the risk posed by insecticides to honey bees.

Most research on insecticide contact toxicity to bees has focused on technical grade individual active ingredients dissolved in acetone. This approach does not assess the impact of ‘inert’ or ‘inactive’ ingredients within formulated insecticides on bees. Addressing this variable is important given preliminary results as in Mullin (2015) showing the toxicity of ‘inert’ ingredients to bees. An advantage of testing technical grade insecticides is the true analysis of

active ingredient toxicity through elimination of cofounding effects resulting from formulations. However, technical grade insecticides might pose higher toxicity to bees given their higher percentage of active ingredient compared to formulations. The increased toxicity of technical products might in part be explained by using acetone solvents, which burn through the insect cuticle and increase the quantity of insecticide absorbed by the insect.

The following studies are examples of testing both technical grade and formulated insecticides on honey bees. Using nine technical grade insecticides (including neonicotinoids and pyrethroids) dissolved in acetone to compare sensitivity among honey bee races, Rinkevich et al. (2015) showed that Italian honey bees were overall the most sensitive; this is an interesting finding given that we tested Italian honey bees in this study. Sandrock et al. (2014) reported that chronic exposure to pollen stores within hives containing technical grade thiamethoxam dissolved in water negatively affected colony performance and queen supersedure. Tavares et al. (2017) show that larval consumption of different technical grade thiamethoxam concentrations dissolved in water decreased larval and pupal survival and subsequent eclosion of adult honey bees. Aliouane et al. (2009) showed a series of behavioral impacts on honey bees exposed to technical grade thiamethoxam, acetamiprid and fipronil dissolved in acetone. Zhang et al. (2017) found that honey bee contact exposure to different rates of lambda-cyhalothrin dissolved in acetone resulted in significantly decreased lifespan, proboscis extension reflex performance and homing ability at the highest tested doses. Applying 1 µL quantities of technical grade pyrethroids dissolved in acetone to honey bees, Johnson et al. (2006) showed the importance role of cytochrome P450 monooxygenases in detoxifying these chemicals. Interestingly, assessing the impact of imidacloprid on *A. cerana*, Tan et al. (2014) found that following oral consumption of nectar mixed with formulated imidacloprid (40 µg/L) dissolved in water, treated honey bees

foraged a feeder containing a hornet predator at a rate of 1.8-fold more than controls. Similarly, Dively et al. (2015) showed that honey bee colonies chronically exposed to sub-lethal doses of formulated imidacloprid dissolved in water had decreased health and overwintering success, although a more field realistic sub-lethal dose had negligible effects. Sánchez-Bayo et al. (2017) determined that oral consumption of syrup laced with technical grade imidacloprid (125 µg/L) over a 10-day period resulted in a maximum of 45% honey bee mortality where exposed bees contained imidacloprid residues on their bodies with incomplete clearance and displayed abnormal behaviors. Liao et al. (2017) found that feeding honey bees syrup with quercetin or *p*-coumaric acid enhanced their tolerance of technical grade beta-cyfluthrin, which alone was associated with a reduced bee lifespan. Fisher et al. (2018) documented significantly increased mortality for honey bees exposed to an above label dose of formulated methoxyfenozide dissolved in water at 24-hour screenings over a 10-day period. Chen et al. (2017) determined low LD_{50} for technical grade spinetoram dissolved in acetone following honey bee contact exposure 48-hours after treatment.

Conclusion

This study embarks in a new research direction by exploring contact toxicity of premix insecticides as a result of continuous, spray-only and residual contact exposure to honey bee foragers.

Unlike orchards, insecticides are applied to field crops during flowering (and potentially when honey bees forage) as a result of continuous bloom. Continuous insecticide exposure simulates honey bees concentrating on a particular flower patch providing rewarding nectar and pollen resources (Flottum 2014). Spray-only insecticide exposure simulates honey bees searching for a suitable patch of flowers (Flottum 2014), where foragers commonly venture up to

4.82 kilometers from the hive (Ribbands 1951). Residual insecticide exposure simulates honey bees coming into contact with dried insecticide residues sprayed at sunset during the previous day (when bees are not foraging). Given that commercially managed honey bee hives are typically placed within or surrounding the fields they are pollinating, this experiment begins to address the proper timing for replacing hives to previously sprayed fields.

Our results demonstrating the high toxicity of formulated premix insecticides to honey bees are comparable to those showing high toxicity of technical grade insecticides to honey bees (Sandrock et al. 2014; Chen et al. 2017; Liao et al. 2017; Sánchez-Bayo et al. 2017). We have also found standard LD_{50} rates for the technical grade insecticides tested in this study (Table 2.3). Based on these comparisons, it is not overall surprising that the formulated premix insecticides tested (and particularly thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin) posed high risk to honey bee foragers. However, all studies do have limitations and cannot perfectly simulate reality in every circumstance. To address this and build upon our findings, future work should explore such risk in actual field settings.

While our study begins to address questions related to the acute toxicity of formulated premix insecticides to honey bees, several important aspects warrant further investigation. Future work should investigate the toxicity of these insecticides to bees via oral ingestion. Additional studies are needed to assess the toxicity of multiple doses of dried residues of the premix insecticides tested and the individual active ingredients that comprise them to honey bees. This will enable us to compare the results to our data and additionally establish possible additive or synergistic toxicities. Future studies should additionally investigate the acute toxicity of these premix insecticides to honey bee queens, drones (males) and immature brood (eggs, larvae and pupae). Generating this data will further strengthen insecticide risk assessment for honey bees.

A further step is to assess sub-lethal toxicity by means of behavioral changes to honey bees coming into direct contact with these insecticide chemistries. In particular, it would be helpful to assess whether contact exposure to these insecticide mixtures impairs the ability of forager bees to collect pollen and nectar, queens to oviposit, nurse bees to feed developing brood, workers to properly progress through their various hive tasks and for immatures to properly develop into adult bees.

Acknowledgements

Authors are thankful to Dr. Y. C. Zhu (USDA-ARS Lab) for valuable advice on the customized sprayer and experimental cage design. Authors are also thankful to Drs. N. Seiter and J. Smith for statistical advice, Dr. N. Seiter for providing insecticides, V. Piazza for customizing and constructing certain materials used to conduct experiments in this study, and M-K. Williams for assistance in collecting honey bee foragers from beehives.

Tables

Table 2.1: Active ingredients and modes of action of formulated premix insecticides assessed for acute contact toxicity to honey bee foragers.

Treatment	Formulation	Active Ingredient(s)	Mode of Action 1st Insecticide*	Mode of Action 2nd Insecticide*	Manufacturer
A	Control				
B	Endigo ZC®	Thiamethoxam (12.6%) + Lambda-cyhalothrin (9.48%)	Thiamethoxam -Nicotinic acetylcholine receptor (nAChR) competitive modulator	Lambda-cyhalothrin -Sodium channel modulator	Syngenta Crop Protection, LLC Greensboro, NC
C	Leverage 360®	Imidacloprid (21.0%) + Beta cyfluthrin (10.5%)	Imidacloprid -Nicotinic acetylcholine receptor (nAChR) competitive modulator	Beta-cyfluthrin -Sodium channel modulator	BayerCropScience LP Research Triangle Park, NC
D	Besiege®	Chlorantraniliprole (9.26%) + Lambda-cyhalothrin (4.63%)	Chlorantraniliprole -Ryanodine receptor modulator	Lambda-cyhalothrin -Sodium channel modulator	Syngenta Crop Protection, LLC Greensboro, NC
E	Intrepid Edge®	Methoxyfenozide (28.3%) + Spinetoram (5.66%)	Methoxyfenozide -Ecdysone receptor agonist (molt accelerating compound)	Spinetoram -Nicotinic acetylcholine receptor (nAChR) allosteric modulator	Dow AgroSciences LLC Indianapolis, IN

*Active ingredient modes of action were confirmed with the IRAC Mode of Action Classification Scheme version 8.4 (www.irac-online.org) and the Compendium of Pesticide Common Names: Insecticides (http://www.alanwood.net/pesticides/class_insecticides.html).

Table 2.2: Application rates administered, highest recommended application rates, and active ingredient concentrations of formulated premix insecticides assessed for acute contact toxicity to honey bee foragers.

Treatment	Active Ingredient(s)	Application Rate of Formulated Product (mL/Ha)	Highest Application Rate on Label (mL/Ha)*	AI Concentration (ppm) in Formulated Product (per L)
A	Control			
B	Thiamethoxam (12.6%) + Lambda-cyhalothrin (9.48%)	328.71	438.28	Thiamethoxam = 495 Lambda-cyhalothrin = 369
C	Imidacloprid (21.0%) + Beta cyfluthrin (10.5%)	204.53	233.75	Imidacloprid = 105 Beta-cyfluthrin = 53
D	Chlorantraniliprole (9.26%) + Lambda-cyhalothrin (4.63%)	438.28	913.08	Chlorantraniliprole = 470 Lambda-cyhalothrin = 230
E	Methoxyfenozide (28.3%) + Spinetoram (5.66%)	803.51	876.56	Methoxyfenozide = 2600 Spinetoram = 520

*Highest recommended application rate for field crops listed on the EPA approved insecticide label for each respective insecticide mixture.

Table 2.3: Toxicity classification (LD_{50}) of insecticide technical grade active ingredients to honey bees.

Insecticide	**Toxicity Classification**	**Contact LD_{50}**	**Oral LD_{50}**
Carbaryl*	H	1.3 µg[1]	0.14 µg[1]
Thiamethoxam	H	0.024 µg[2] - 0.029 µg[3]	0.005 µg[2]
Imidacloprid	H	0.0179 µg[4] - 0.243 µg[5]	0.0037 µg[5] - 0.081 µg[6]
Lambda-cyhalothrin	H	0.483 µg[7]	
Beta-cyfluthrin	H	0.025 µg[8] - 0.037 µg[9]	
Chlorantraniliprole	N	>100 µg[10]	>119 µg[10]
Methoxyfenozide	N	>100 µg[11]	
Spinetoram	H	0.024 µg[12]	0.14 µg[12]

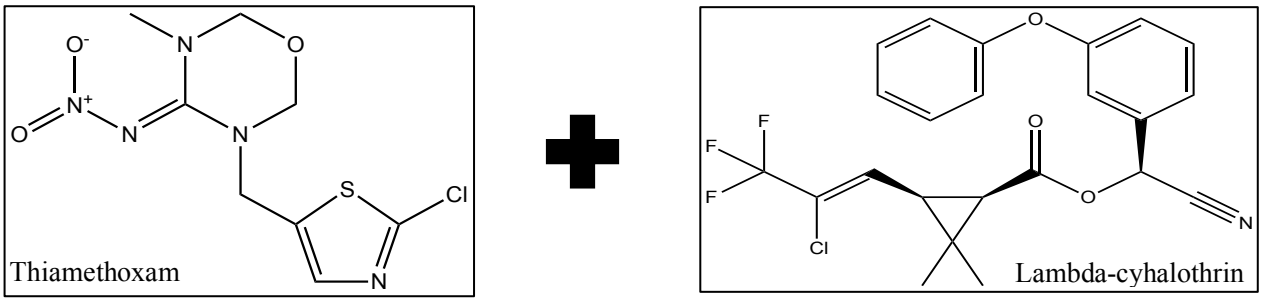
*Standard comparison reference for insecticide contact and oral LD_{50} to honey bees.

Toxicity Classification[13, 14, 15]
Highly toxic (H) = LD_{50} <2 µg
Moderately toxic (M) = LD_{50} 2-10.99 µg
Slightly toxic (S) = LD_{50} 11-100 µg
Practically nontoxic (N) = LD_{50} >100 µg

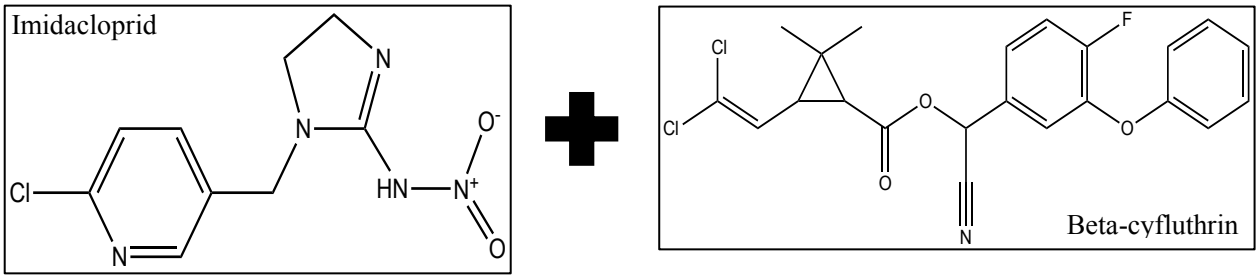
Sources
1. Stevenson 1978
2. Syngenta Group 2005
3. Iwasa et al. 2004
4. EPA 2003
5. Schmuck et al. 2001
6. Nauen et al. 2001
7. Gough et al. 1984
8. FAO 1999
9. EPA 1987
10. EPA 2008
11. Methoxyfenozide: CA Dept. Pesticide Regulation 2003
12. Spinetoram: CA Dept. Pesticide Regulation 2007-2010
13. Hopwood et al. 2016
14. EPA 2012 (Sappington and Ruhman)
15. EPA 2015

Figures

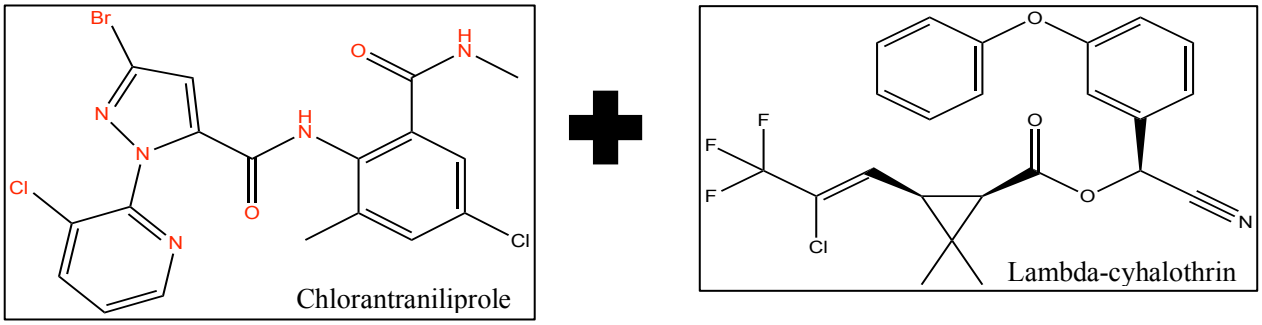
Endigo ZC®



Leverage 360®



Besiege®



Intrepid Edge®

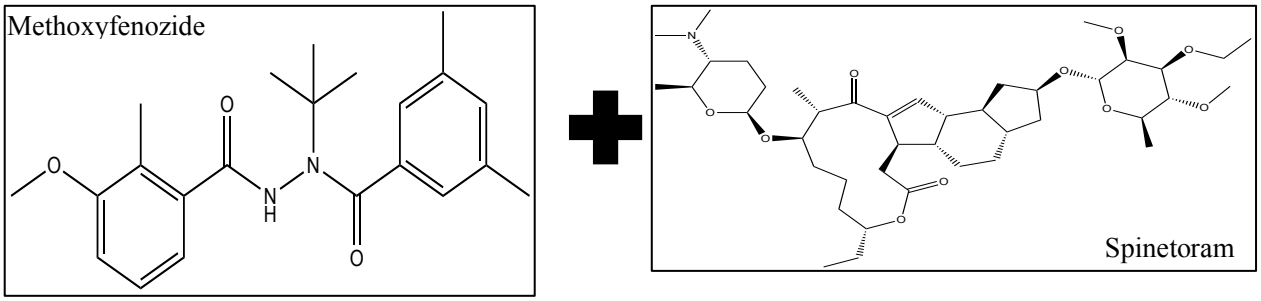


Figure 2.1: Chemical Structure of Premix Insecticides* Tested for Acute Contact Toxicity to Honey Bee Foragers. *Source of chemical structures/diagrams: Chem Draw Pro® and verified in Reaxys®.

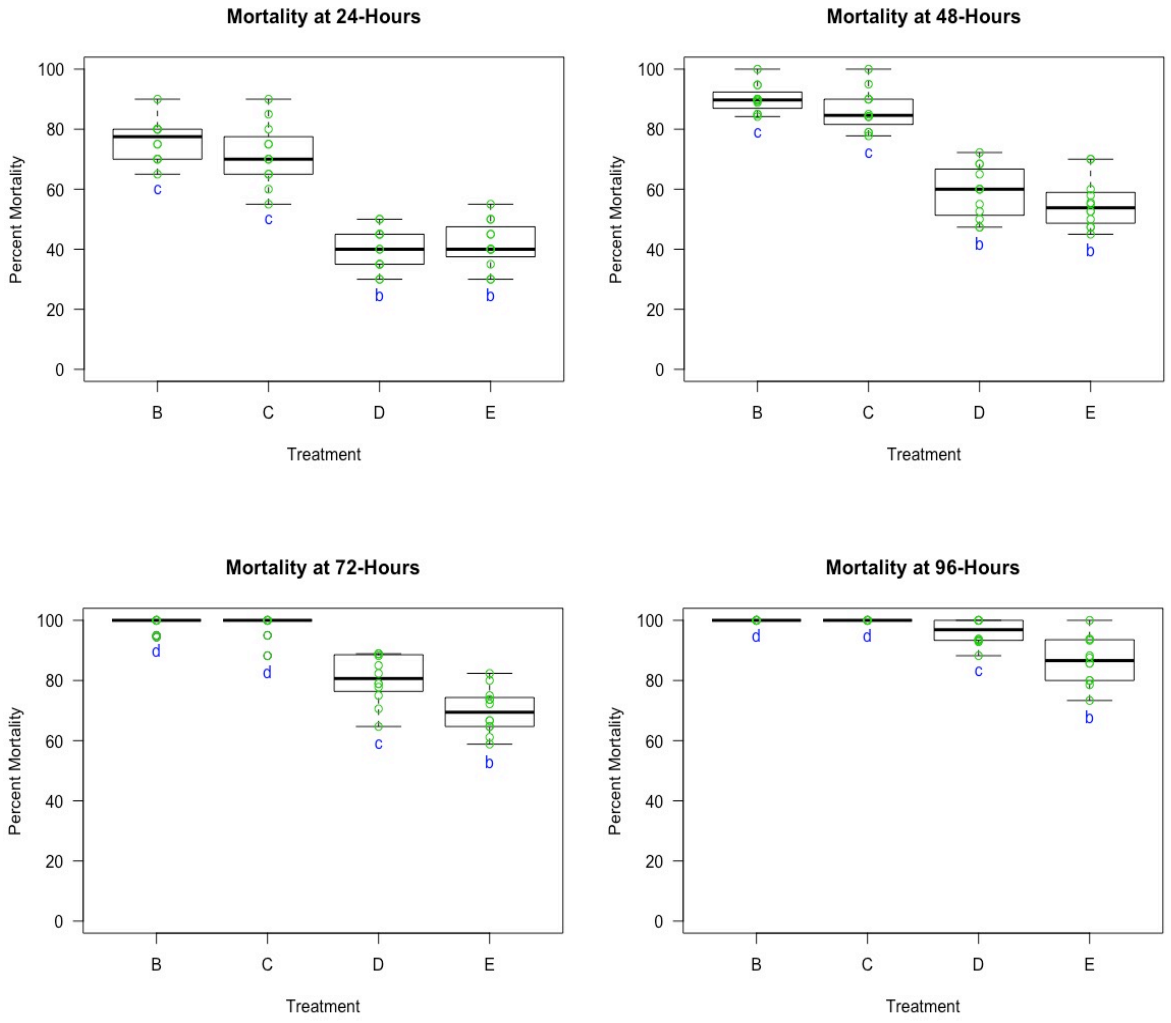


Figure 2.2: Honey Bee Forager Acute Contact Toxicity in Continuous Exposure Scenario: Mean, 1st and 3rd quartile honey bee forager percent mortality following continuous contact exposure to each insecticide mixture (experiment 1) at 24, 48, 72 and 96-hours. Data on the x-axis indicate each insecticide treatment, and data the y-axis indicate the percent mortality. Insecticide treatments: B = thiamethoxam + lambda-cyhalothrin, C = imidacloprid + beta-cyfluthrin, D = chlorantraniliprole + lambda-cyhalothrin, E = methoxyfenozide + spinetoram. Each green point represents all cages in a treatment containing the respective percent mortality. Letters (b, c, d) indicate significant difference at *$p<0.05$ (pairwise comparison).

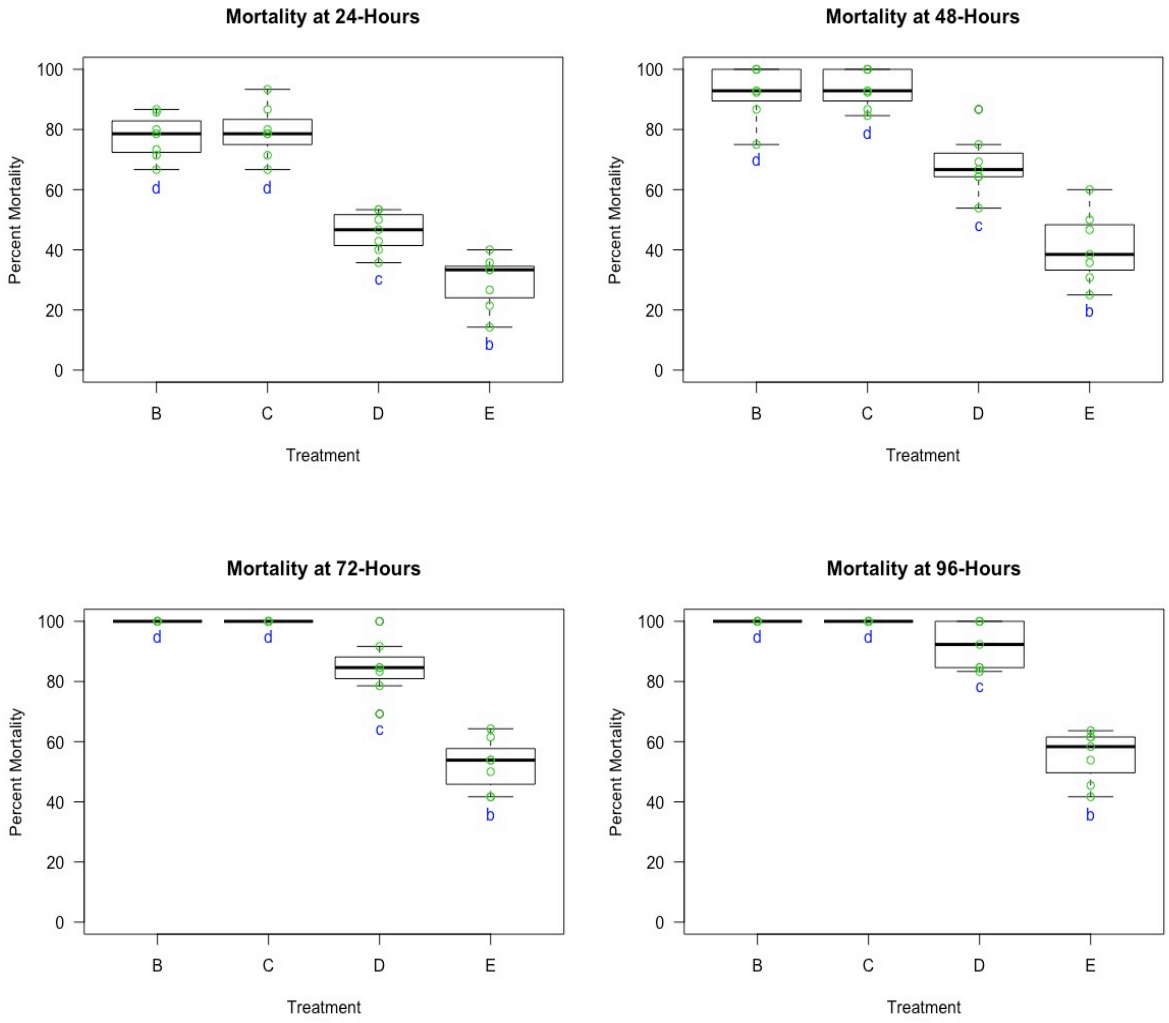


Figure 2.3: Honey Bee Forager Acute Contact Toxicity in Spray-Only Exposure Scenario: Mean, 1st and 3rd quartile honey bee forager percent mortality following spray-only contact exposure to each insecticide mixture (experiment 2) at 24, 48, 72 and 96-hours. Data on the x-axis indicate each insecticide treatment, and data the y-axis indicate the percent mortality. Insecticide treatments: B = thiamethoxam + lambda-cyhalothrin, C = imidacloprid + beta-cyfluthrin, D = chlorantraniliprole + lambda-cyhalothrin, E = methoxyfenozide + spinetoram. Each green point represents all cages in a treatment containing the respective percent mortality. Letters (b, c, d) indicate significant difference at *$p<0.05$ (pairwise comparison).

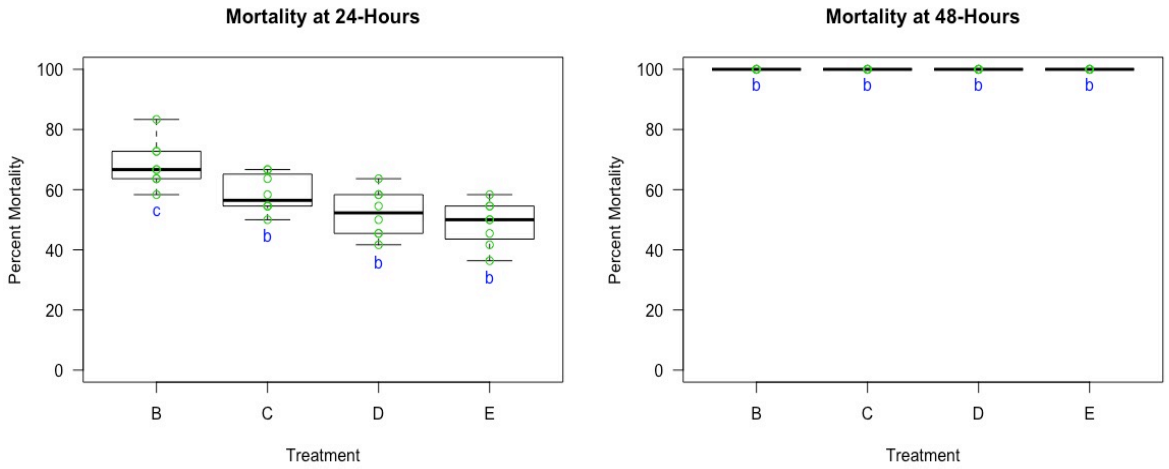


Figure 2.4: Honey Bee Forager Acute Contact Toxicity in Residual Exposure Scenario: Mean, 1st and 3rd quartile honey bee forager percent mortality following residual contact exposure to each insecticide mixture (experiment 3) at 24 and 48-hours. Data on the x-axis indicate each insecticide treatment, and data the y-axis indicate the percent mortality. Insecticide treatments: B = thiamethoxam + lambda-cyhalothrin, C = imidacloprid + beta-cyfluthrin, D = chlorantraniliprole + lambda-cyhalothrin, E = methoxyfenozide + spinetoram. Each green point represents all cages in a treatment containing the respective percent mortality. Letters (b, c) indicate significant difference at *$p<0.05$ (pairwise comparison).

References

Aizen, M. A., L. A. Garibaldi, S. A. Cunningham, and A. M. Klein. **2008**. Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. Current Biology. 18: 1572–1575.

Aliouane, Y., A. K. El Hassani, V. Gary, C. Armengaud, M. Lambin, and M. Gauthier. **2009**. Subchronic Exposure of Honeybees to Sublethal Doses of Pesticides: Effects on Behavior. Environmental Toxicology and Chemistry. 28: 113.

Besard, L., V. Mommaerts, G. Abdu-Alla, and G. Smagghe. **2011**. Lethal and sublethal side-effect assessment supports a more benign profile of spinetoram compared with spinosad in the bumblebee *Bombus terrestris*. Pest Management Science. 67: 541–547.

Bibbs, C. S., A. Fulcher, and R.-D. Xue. **2015**. Allethrin-Based Mosquito Control Device Causing Knockdown, Morbidity, and Mortality in Four Species of Field-Caught Mosquitoes (Diptera: Culicidae). Journal of Medical Entomology. 52: 739–742.

Biddinger, D. J., and E. G. Rajotte. **2015**. Integrated pest and pollinator management — adding a new dimension to an accepted paradigm. Current Opinion in Insect Science. 10: 204–209.

Biddinger, D. J., J. L. Robertson, C. Mullin, J. Frazier, S. A. Ashcraft, E. G. Rajotte, N. K. Joshi, and M. Vaughn. **2013**. Comparative Toxicities and Synergism of Apple Orchard Pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). PLoS ONE. 8: e72587.

Brown, S. A., D. L. Kerns, T. S. Williams, K. Emfinger, and N. Jones. **2015**. Evaluation of Foliar Insecticides for Kudzu Bug Control in Soybeans, 2014: Table 1. Arthropod Management Tests. 40: F14.

Bruckner, S., N. Steinhauer, K. Rennich, S. Dan Aurell, D. M. Caron, J. D. Ellis, and A. M. Fauvel. **2018**. Honey Bee Colony Losses 2017-2018: Preliminary Results. (beeinformed.org).

Calderone, N. W. 2012. Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. PLoS ONE. 7: e37235.

California Department of Pesticide Regulation Public Report: Active Ingredient: Spinetoram. **2007**. California Department of Pesticide Regulation Public Report: Active Ingredient: Spinetoram (No. Tracking ID Numbers 218411, 218412, 218413, 218414, 218415).

California Department of Pesticide Regulation Public Report: Methoxyfenozide. 2003. California Department of Pesticide Regulation Public Report: Methoxyfenozide (No. Tracking ID Numbers 195668 and 196546).

Carreck, N. L., and F. L. W. Ratnieks. **2014**. The dose makes the poison: have "field realistic" rates of exposure of bees to neonicotinoid insecticides been overestimated in laboratory studies? Journal of Apicultural Research. 53: 607–614.

ChemDraw® Prime. PerkinElmer Informatics, Inc.

Chen, X. D., T. A. Gill, K. S. Pelz-Stelinski, and L. L. Stelinski. **2017**. Risk assessment of various insecticides used for management of Asian citrus psyllid, *Diaphorina citri* in Florida citrus, against honey bee, *Apis mellifera*. Ecotoxicology. 26: 351–359.

Cook, D. R., and J. Gore. **2018**. Impact of Selected Insecticides Against Soybean Looper Infesting Soybean, 2017. Arthropod Management Tests. 43.

Crane, E. 1990. Bees and beekeeping: science, practice, and world resources. Heinemann Newnes, Gerrards Cross, Bucks, U.K.

Cutler, G. C., C. D. Scott-Dupree, M. Sultan, A. D. McFarlane, and L. Brewer. **2014**. A large-scale field study examining effects of exposure to clothianidin seed-treated canola on honey bee colony health, development, and overwintering success. PeerJ. 2: e652.

David, A., C. Botías, A. Abdul-Sada, E. Nicholls, E. L. Rotheray, E. M. Hill, and D. Goulson. **2016**. Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. Environment International. 88: 169–178.

Di Prisco, G., V. Cavaliere, D. Annoscia, P. Varricchio, E. Caprio, F. Nazzi, G. Gargiulo, and F. Pennacchio. **2013**. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proceedings of the National Academy of Sciences. 110: 18466–18471.

Dively, G. P., M. S. Embrey, A. Kamel, D. J. Hawthorne, and J. S. Pettis. **2015**. Assessment of Chronic Sublethal Effects of Imidacloprid on Honey Bee Colony Health. PLOS ONE. 10: e0118748.

Duay, P., D. D. Jong, and W. Engels. **2002**. Decreased flight performance and sperm production in drones of the honey bee (*Apis mellifera*) slightly infested by *Varroa destructor* mites during pupal development. Genetics and Molecular Research. 6.

Ecological Risk Assessment for Section 3 Registration for Fruit, Vegetable, Selected Field Crop, Turf and Ornamental Uses of Chlorantraniliprole. (PC Code 090100). **2008**. Ecological Risk Assessment for Section 3 Registration for Fruit, Vegetable, Selected Field Crop, Turf and Ornamental Uses of Chlorantraniliprole (PC Code 090100) (No. EPA-HQ-OPP-2007-0275-0005). USEPA/Office of Pesticide Programs.

Elbert, A., M. Haas, B. Springer, W. Thielert, and R. Nauen. **2008**. Applied aspects of neonicotinoid uses in crop protection. Pest Management Science. 64: 1099–1105.

FAO Specifications and Evaluations for Plant Protection Products: Beta-cyfluthrin. **1999**. FAO Specifications and Evaluations for Plant Protection Products: Beta-cyfluthrin. Food and Agriculture Organization of the United Nations.

Fischer, J., T. Müller, A.-K. Spatz, U. Greggers, B. Grünewald, and R. Menzel. **2014**. Neonicotinoids Interfere with Specific Components of Navigation in Honeybees. PLoS ONE. 9: e91364.

Fisher, A., C. Coleman, C. Hoffmann, B. Fritz, and J. Rangel. **2017**. The Synergistic Effects of Almond Protection Fungicides on Honey Bee (Hymenoptera: Apidae) Forager Survival. Journal of Economic Entomology. 110: 802–808.

Fisher, A., C. Colman, C. Hoffmann, B. Fritz, and J. Rangel. **2018**. The Effects of the Insect Growth Regulators Methoxyfenozide and Pyriproxyfen and the Acaricide Bifenazate on Honey Bee (Hymenoptera: Apidae) Forager Survival. Journal of Economic Entomology. 111: 510–516.

Flottum, K. 2014. The Backyard Beekeeper: An absolute beginner's guide to keeping bees in your yard and garden third edition., 3rd ed. Quarry Books Inc., Beverly, MA.

Food Quality Protection Act of 1996. Public Law 104-170. **1996**. Food Quality Protection Act of 1996. Public Law 104-170. U.S. Government Printing Office, Washington, D.C.

Gallai, N., J.-M. Salles, J. Settele, and B. E. Vaissière. **2009**. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics. 68: 810–821.

Girolami, V., L. Mazzon, A. Squatini, N. Mori, M. Marzaro, A. Dibernardo, M. Greatti, C. Giorio, and A. Tapparo. **2009**. Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: a novel way of intoxication for bees. Journal of Economic Entomology. 102: 1808–1815.

Glazer, I., and A. Navon. **1990**. Activity and Persistence of Entomoparasitic Nematodes Tested Against *Heliothis armigera* (Lepidoptera: Noctuidae). Journal of Economic Entomology. 83: 1795–1800.

google earth. **2018**. google earth. (https://www.google.com/earth/).

Gough, H. J., L. G. Collins, C. J. Everett, and W. Wilkinson. **1984**. Acute contact and oral toxicity to honey bees (*Apis mellifera*). (No. Reg. No. 10182-0A. Ace. No. 400524-09). ICI Americas Inc., Wilmington, DE.

Goulson, D., E. Nicholls, C. Botias, and E. L. Rotheray. **2015**. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 347: 1255957–1255957.

Guidance on Exposure and Effects Testing for Assessing Risks to Bees. **2016**. Guidance on Exposure and Effects Testing for Assessing Risks to Bees. Office of Pesticide Programs U.S. Environmental Protection Agency.

Henry, M., M. Beguin, F. Requier, O. Rollin, J.-F. Odoux, P. Aupinel, J. Aptel, S. Tchamitchian, and A. Decourtye. **2012**. A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. Science. 336: 348–350.

Hoffmann, E. J., and S. J. Castle. **2012**. Imidacloprid in melon guttation fluid: a potential mode of exposure for pest and beneficial organisms. Journal of Economic Entomology. 105: 67–71.

Højland, D. H., R. Nauen, S. P. Foster, M. S. Williamson, and M. Kristensen. **2015**. Incidence, Spread and Mechanisms of Pyrethroid Resistance in European Populations of the Cabbage Stem Flea Beetle, *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae). PLOS ONE. 10: e0146045.

Hopwood, J., M. Vaughan, M. Shepherd, D. Biddinger, E. Mader, S. H. Black, and C. Mazzacano. **2012**. Are Neonicotinoids Killing Bees? A Review of Research into the Effects of Neonicotinoid Insecticides on Bees, with Recommendations for Action. The Xerces Society for Invertebrate Conservation, Portland, (OR) USA.

Hopwood, J., A. Code, M. Vaughan, D. Biddinger, M. Shepherd, S. H. Black, E. Lee-Mäder, and C. Mazzacano. **2016**. How Neonicotinoids Can Kill Bees: The Science Behind the Role These Insecticides Play in Harming Bees (No. Second Edition, Revised and Expanded). The Xerces Society for Invertebrate Conservation, Portland, (OR) USA.

Huang, Z. 2012. Pollen nutrition affects honey bee stress resistance. Terrestrial Arthropod Reviews. 5: 175–189.

Ilias, A., J. Lagnel, D. E. Kapantaidaki, E. Roditakis, C. S. Tsigenopoulos, J. Vontas, and A. Tsagkarakou. **2015**. Transcription analysis of neonicotinoid resistance in Mediterranean (MED) populations of *B. tabaci* reveal novel cytochrome P450s, but no nAChR mutations associated with the phenotype. BMC Genomics. 16.

IRAC International MoA Working Group: Approved by IRAC Executive. **2018**. IRAC Mode of Action Classification Scheme (Classification Scheme No. Version 8.4).

Iwasa, T., N. Motoyama, J. T. Ambrose, and R. M. Roe. **2004**. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Protection. 23: 371–378.

Jeschke, P., R. Nauen, M. Schindler, and A. Elbert. **2011**. Overview of the status and global strategy for neonicotinoids. Journal of Agricultural and Food Chemistry. 59: 2897–2908.

Johnson, R. M., L. Dahlgren, B. D. Siegfried, and M. D. Ellis. **2013**. Acaricide, Fungicide and Drug Interactions in Honey Bees (*Apis mellifera*). PLoS ONE. 8: e54092.

Johnson, R. M., and E. G. Percel. **2013**. Effect of a Fungicide and Spray Adjuvant on Queen-Rearing Success in Honey Bees (Hymenoptera: Apidae). Journal of Economic Entomology. 106: 1952–1957.

Johnson, R. M., Z. Wen, M. A. Schuler, and M. R. Berenbaum. **2006**. Mediation of Pyrethroid Insecticide Toxicity to Honey Bees (Hymenoptera: Apidae) by Cytochrome P450 Monooxygenases. Journal of Economic Entomology. 99: 5.

Jones, M., M. Duckworth, and J. Duckworth. **2017**. Evaluation of Foliar Insecticides for Control of Rice Stink Bug, 2016. Arthropod Management Tests. 42.

Klein, A.-M., B. E. Vaissière, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen, and T. Tscharntke. **2007**. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences. 274: 303–313.

Leverage 360® EPA Regulated Insecticide Label. **2015**. Leverage 360® EPA Regulated Insecticide Label.

Liao, L.-H., W.-Y. Wu, and M. Berenbaum. **2017**. Impacts of Dietary Phytochemicals in the Presence and Absence of Pesticides on Longevity of Honey Bees (*Apis mellifera*). Insects. 8: 22.

Lundin, O., K. L. Ward, D. R. Artz, N. K. Boyle, T. L. Pitts-Singer, and N. M. Williams. **2017**. Wildflower Plantings Do Not Compete with Neighboring Almond Orchards for Pollinator Visits. Environmental Entomology. 46: 559–564.

Mishra, S., P. Kumar, and A. Malik. **2015**. Effect of temperature and humidity on pathogenicity of native *Beauveria bassiana* isolate against *Musca domestica* L. Journal of Parasitic Diseases. 39: 697–704.

Mommaerts, V., G. Sterk, and G. Smagghe. **2006**. Bumblebees can be used in combination with juvenile hormone analogues and ecdysone agonists. Ecotoxicology. 15: 513–521.

Mullin, C. A. 2015. Effects of 'inactive' ingredients on bees. Current Opinion in Insect Science. 10: 194–200.

Mussen, E. 2011. Diagnosing and Treating Nosema Disease. U.C. Davis, Davis, CA.

Nauen, R., U. Ebbinghaus-Kintscher, and R. Schmuck. **2001**. Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: Apidae). Pest Management Science. 57: 577–586.

OECD-214 Test Guidelines: OECD Guidelines for the Testing of Chemicals: Honey Bees, Acute Contact Toxicity Test. **1998**. (OECD-214 Test Guidelines): OECD Guidelines for the Testing of Chemicals: Honey Bees, Acute Contact Toxicity Test. OECD/OCDE.

Pesticide Fact Sheet Number 164: Cyfluthrin. **1987**. Pesticide Fact Sheet Number 164: Cyfluthrin. US Environmental Protection Agency, U.S. EPA, Office of Pesticide Programs, Washington, D.C.

Pilling, E., P. Campbell, M. Coulson, N. Ruddle, and I. Tornier. **2013**. A Four-Year Field Program Investigating Long-Term Effects of Repeated Exposure of Honey Bee Colonies to Flowering Crops Treated with Thiamethoxam. PLoS ONE. 8: e77193.

Pitts-Singer, T. L., D. R. Artz, S. S. Peterson, N. K. Boyle, and G. I. Wardell. **2018**. Examination of a Managed Pollinator Strategy for Almond Production Using *Apis mellifera* (Hymenoptera: Apidae) and *Osmia lignaria* (Hymenoptera: Megachilidae). Environmental Entomology. 47: 364–377.

Ramsey, J., A. L. Catchot, D. Bao, A. Adams, B. P. Adams, N. R. Bateman, W. D. Crow, C. Darnell, T. D. Dill, S. H. Graham, J. H. North, and A. Whalen. **2016**. Efficacy of Selected Insecticides for Control of Soybean Loopers and Southern Green Stink Bugs in Soybeans (Test 2), 2013: Table 1. Arthropod Management Tests. 41: tsw074.

Reaxys®. **2018**. Reaxys®. (https://0-www-reaxys-com.library.uark.edu/#/search/quick).

Ribbands, C. R. 1951. The Flight Range of the Honey-Bee. The Journal of Animal Ecology. 20: 220.

Rinkevich, F. D., J. W. Margotta, J. M. Pittman, R. G. Danka, M. R. Tarver, J. A. Ottea, and K. B. Healy. **2015**. Genetics, Synergists, and Age Affect Insecticide Sensitivity of the Honey Bee, *Apis mellifera*. PLOS ONE. 10: e0139841.

Sánchez-Bayo, F., L. Belzunces, and J.-M. Bonmatin. **2017**. Lethal and sublethal effects, and incomplete clearance of ingested imidacloprid in honey bees (*Apis mellifera*). Ecotoxicology. 26: 1199–1206.

Sandrock, C., M. Tanadini, L. G. Tanadini, A. Fauser-Misslin, S. G. Potts, and P. Neumann. **2014**. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. PLoS ONE. 9: e103592.

Sappington, K. G., and M. A. Ruhman. **2012**. (U.S. Environmental Protection Agency). Environmental Fate and Ecological Risk Assessment for Sulfoxaflor Registration. (No. EPA-HQ-OPP-2010-0889). US Environmental Protection Agency, Washington, D.C.: EPA.

Sato, N., Y. Shimokawatoko, T. Yamaguchi, and H. Tanaka. **2012**. Development of the Novel Insecticide Spinetoram (DIANA®). Sumitomo Kagaku. 14.

Schmuck, R., R. Schoning, A. Stork, and O. Schramel. **2001**. Risk posed to honey bees (*Apis mellifera* L., Hymenoptera) by an imidacloprid seed dressing of sun flowers. Pest Management Science. 57: 225–238.

Seitz, N., K. S. Traynor, N. Steinhauer, K. Rennich, M. E. Wilson, J. D. Ellis, R. Rose, D. R. Tarpy, R. R. Sagili, D. M. Caron, K. S. Delaplane, J. Rangel, K. Lee, K. Baylis, J. T. Wilkes, J. A. Skinner, J. S. Pettis, and D. vanEngelsdorp. **2016**. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. Journal of Apicultural Research. 54: 292–304.

Sgolastra, F., P. Medrzycki, L. Bortolotti, M. T. Renzi, S. Tosi, G. Bogo, D. Teper, C. Porrini, R. Molowny-Horas, and J. Bosch. **2017**. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species: Synergistic interactions between pesticides in three bee species. Pest Management Science. 73: 1236–1243.

Smagghe, G., J. Deknopper, I. Meeus, and V. Mommaerts. **2013**. Dietary chlorantraniliprole suppresses reproduction in worker bumblebees: Dietary chlorantraniliprole suppresses reproduction in worker bumblebees. Pest Management Science. 69: 787–791.

Soderlund, D. M. 2008. Pyrethroids, knockdown resistance and sodium channels. Pest Management Science. 64: 610–616.

Steinhauer, N., K. Rennich, D. M. Caron, K. S. Delaplane, J. Rangel, R. Rose, R. R. Sagili, J. Skinner, J. T. Wilkes, M. E. Wilson, J. Pettis, and D. vanEngelsdorp. **2016**. Bee Informed Partnership: Colony Loss 2015-2016: Preliminary Results. Tenth Annual National Survey of Honey Bee Colony Losses. 10.

Stevenson, J. H. 1978. The Acute Toxicity of Un-formulated Pesticides to Worker Honey Bees (*Apis mellifera* L.). Plant Pathology. 27: 38–40.

Suiter, D. R., and M. E. Scharf. **2015**. Insecticide basics for the pest management professional. (UGA Bulletin No. 1352). University of Georgia.

Sumner, D. A., and H. Boriss. **2006**. Bee-conomics and the Leap in Pollination Fees. University of California.

Syngenta Group. 2005. Thiamethoxam: Used to formulate Patinum®, Actara®, Centric®, Cruiser®, Flagship®, and Helix®. (Envirofacts: Syngenta Crop Protection Fact Sheet). Syngenta Crop Protection, Inc., Greensboro, NC.

Tan, K., W. Chen, S. Dong, X. Liu, Y. Wang, and J. C. Nieh. **2014**. Imidacloprid Alters Foraging and Decreases Bee Avoidance of Predators. PLoS ONE. 9: e102725.

Tavares, D. A., C. Dussaubat, A. Kretzschmar, S. M. Carvalho, E. C. M. Silva-Zacarin, O. Malaspina, G. Bérail, J.-L. Brunet, and L. P. Belzunces. **2017**. Exposure of larvae to thiamethoxam affects the survival and physiology of the honey bee at post-embryonic stages. Environmental Pollution. 229: 386–393.

timeanddate. **2018**. timeanddate.(https://www.timeanddate.com/weather/usa/fayetteville-ar/historic?month=9&year=2018).

University of Arkansas Cooperative Extension Beekeeping Publications. **2018**. University of Arkansas Cooperative Extension Beekeeping Publications. (https://www.uaex.edu/farm-ranch/special-programs/beekeeping/beepubs.aspx).

U.S. Environmental Protection Agency. **2003**. Pesticide Fact Sheet: Clothianidin. EPA-OPPTS., Washington, D.C.

U.S. Environmental Protection Agency. Registration Decision for the New Active Ingredients Flupyradifurone). **2015**. (U.S. Environmental Protection Agency). Registration Decision for the New Active Ingredients Flupyradifurone (No. EPA-HQ-OPP-2013-0226). Washington, D.C.: EPA.

USDA NASS 2018. **2018**. 2018 California Almond Forecast. USDA National Agricultural Statistics Service, Pacific Regional Office, Sacramento, CA.

vanEngelsdorp, D., J. D. Evans, C. Saegerman, C. Mullin, E. Haubruge, B. K. Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, R. Underwood, D. R. Tarpy, and J. S. Pettis. **2009**. Colony Collapse Disorder: A Descriptive Study. PLoS ONE. 4: e6481.

vanEngelsdorp, D., and M. D. Meixner. **2010**. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology. 103: S80–S95.

Vijverberg, H. P. M., J. M. van der Zalm, and J. van den Bercken. **1982**. Similar mode of action of pyrethroids and DDT on sodium channel gating in myelinated nerves. Nature. 295: 601–603.

Williams, H., H. Zoller, R. Roepke, E. Zschiesche, and A. R. Heckeroth. **2015**. Fluralaner activity against life stages of ticks using *Rhipicephalus sanguineus* and *Ornithodoros moubata* IN in vitro contact and feeding assays. Parasites & Vectors. 8: 90.

Wise, J. C., D. Hulbert, and C. Vandervoort. **2017**. Rainfall influences performance of insecticides on the codling moth (Lepidoptera: Tortricidae) in apples. The Canadian Entomologist. 149: 118–128.

Wood, A. 2018. Insecticides. Compendium of Pesticide Common Names. (http://www.alanwood.net/pesticides/class_insecticides.html).

Zhang, B., C. H. Liao, J. H. Hu, and X. B. Wu. **2017**. Effects of lambda-cyhalothrin on the viability and memory-related traits of the western honey bee, *Apis mellifera* (Hymenoptera: Apidae). Acta Entomologica Sinica. 60: 189–196.

Zhu, Y. C., J. Adamczyk, T. Rinderer, J. Yao, R. Danka, R. Luttrell, and J. Gore. **2015**. Spray Toxicity and Risk Potential of 42 Commonly Used Formulations of Row Crop Pesticides to Adult Honey Bees (Hymenoptera: Apidae). Journal of Economic Entomology. 108: 2640–2647.

Zhu, Y. C., J. Yao, J. Adamczyk, and R. Luttrell. **2017**. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). PLOS ONE. 12: e0176837.

Appendices

Appendix 2.1: Honey Bee Forager Acute Contact Toxicity in Continuous Exposure
Scenario: One-way ANOVA test of statistical significance between honey bee forager mortality following exposure to each insecticide treatment and Tukey HSD post-hoc test of statistical difference between treatments on honey bee forager mortality. Statistical significance at *p<0.05.

One-way ANOVA test: 24-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	2.14453	0.71484	58.332	2.24E-15
Residuals	44	0.53921	0.01225		

Tukey HSD post-hoc test: 24-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	0.06331215	0.04519372	44	1.401	0.5055
B - D	0.46112673	0.04519372	44	10.203	<.0001
B - E	0.44250254	0.04519372	44	9.791	<.0001
C - D	0.39781458	0.04519372	44	8.802	<.0001
C - E	0.37919039	0.04519372	44	8.39	<.0001
D - E	-0.0186242	0.04519372	44	-0.412	0.9761

One-way ANOVA test: 48-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	3.04646	1.01549	50.082	3.09E-14
Residuals	44	0.89217	0.02028		

Tukey HSD post-hoc test: 48-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	0.07081657	0.05813286	44	1.218	0.6188
B - D	0.51369767	0.05813286	44	8.837	<.0001
B - E	0.55789329	0.05813286	44	9.597	<.0001
C - D	0.4428811	0.05813286	44	7.618	<.0001
C - E	0.48707672	0.05813286	44	8.379	<.0001
D - E	0.04419562	0.05813286	44	0.76	0.8718

One-way ANOVA test: 72-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	7337.3	2445.77	73.565	< 2.2e-16
Residuals	44	1462.8	33.25		

Tukey HSD post-hoc test: 72-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	0.5174292	2.353949	44	0.22	0.9962
B - D	18.4352425	2.353949	44	7.832	<.0001
B - E	29.1517601	2.353949	44	12.384	<.0001
C - D	17.9178133	2.353949	44	7.612	<.0001
C - E	28.6343309	2.353949	44	12.164	<.0001
D - E	10.7165176	2.353949	44	4.553	0.0002

One-way ANOVA test: 96-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	1.9141	0.63804	31.131	5.92E-11
Residuals	44	0.9018	0.0205		

Tukey HSD post-hoc test: 96-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	4.16E-16	0.05844575	44	0	1
B - D	1.93E-01	0.05844575	44	3.301	0.01
B - E	4.88E-01	0.05844575	44	8.351	<.0001
C - D	1.93E-01	0.05844575	44	3.301	0.01
C - E	4.88E-01	0.05844575	44	8.351	<.0001
D - E	2.95E-01	0.05844575	44	5.05	<.0001

Appendix 2.2: Honey Bee Forager Acute Contact Toxicity in Spray-Only Exposure Scenario: One-way ANOVA test of statistical significance between honey bee forager mortality following exposure to each insecticide treatment and Tukey HSD post-hoc test of statistical difference between treatments on honey bee forager mortality. Statistical significance at *p<0.05.

One-way ANOVA test: 24-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	2.03881	0.6796	50.855	1.50E-10
Residuals	24	0.32073	0.01336		

Tukey HSD post-hoc test: 24-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.0363614	0.06179157	24	-0.588	0.9346
B - D	0.41486308	0.06179157	24	6.714	<.0001
B - E	0.59603248	0.06179157	24	9.646	<.0001
C - D	0.45122444	0.06179157	24	7.302	<.0001
C - E	0.63239384	0.06179157	24	10.234	<.0001
D - E	0.18116939	0.06179157	24	2.932	0.0343

One-way ANOVA test: 48-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	3.8201	1.27335	26.533	8.54E-08
Residuals	24	1.1518	0.04799		

Tukey HSD post-hoc test: 48-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.0229521	0.1170978	24	-0.196	0.9973
B - D	0.51706775	0.1170978	24	4.416	0.001
B - E	0.85677422	0.1170978	24	7.317	<.0001
C - D	0.54001981	0.1170978	24	4.612	0.0006
C - E	0.87972629	0.1170978	24	7.513	<.0001
D - E	0.33970648	0.1170978	24	2.901	0.0367

One-way ANOVA test: 72-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	4.9802	1.66007	86.967	4.94E-13
Residuals	24	0.4581	0.01909		

Tukey HSD post-hoc test: 72-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-6.30E-16	0.07385027	24	0	1
B - D	5.13E-01	0.07385027	24	6.952	<.0001
B - E	1.02E+00	0.07385027	24	13.762	<.0001
C - D	5.13E-01	0.07385027	24	6.952	<.0001
C - E	1.02E+00	0.07385027	24	13.762	<.0001
D - E	5.03E-01	0.07385027	24	6.809	<.0001

One-way ANOVA test: 96-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	4.5211	1.50703	64.629	1.22E-11
Residuals	24	0.5596	0.02332		

Tukey HSD post-hoc test: 96-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-2.66E-16	0.08162298	24	0	1
B - D	3.01E-01	0.08162298	24	3.684	0.006
B - E	9.84E-01	0.08162298	24	12.054	<.0001
C - D	3.01E-01	0.08162298	24	3.684	0.006
C - E	9.84E-01	0.08162298	24	12.054	<.0001
D - E	6.83E-01	0.08162298	24	8.37	<.0001

Appendix 2.3: Honey Bee Forager Acute Contact Toxicity in Residual Exposure Scenario: One-way ANOVA test of statistical significance between honey bee forager mortality following exposure to each insecticide treatment and Tukey HSD post-hoc test of statistical difference between treatments on honey bee forager mortality. Statistical significance at *p<0.05.

One-way ANOVA test: 24-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	0.2853	0.095099	11.213	5.24E-05
Residuals	28	0.23748	0.008482		

Tukey HSD post-hoc test: 24-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	0.13114661	0.04604756	28	2.848	0.0385
B - D	0.20827065	0.04604756	28	4.523	0.0006
B - E	0.24724124	0.04604756	28	5.369	0.0001
C - D	0.07712404	0.04604756	28	1.675	0.3554
C - E	0.11609464	0.04604756	28	2.521	0.0783
D - E	0.0389706	0.04604756	28	0.846	0.8319

One-way ANOVA test: 48-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	3.70E-30	1.23E-30	1	4.07E-01
Residuals	28	3.45E-29	1.23E-30		

Tukey HSD post-hoc test: 48-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-7.85E-16	5.55E-16	28	-1.414	0.5013
B - D	-7.85E-16	5.55E-16	28	-1.414	0.5013
B - E	-7.85E-16	5.55E-16	28	-1.414	0.5013
C - D	-6.90E-31	5.55E-16	28	0	1
C - E	-2.96E-31	5.55E-16	28	0	1
D - E	3.94E-31	5.55E-16	28	0	1

Chapter III: Toxicity of Formulated Insecticide Mixtures to Blue Orchard Bees, *Osmia lignaria* (Say)

Toxicity of Formulated Insecticide Mixtures to Blue Orchard Bees, *Osmia lignaria* (Say)

Abstract

Blue orchard bees, *Osmia lignaria* (Say) have been developed as an important pollinator for orchard crops in North America over the last 40 years. The toxicity of several pesticides to blue orchard bees and other *Osmia* species has been previously reported. However, the field-realistic toxicity of formulated premix insecticides comprised of multiple active ingredients (each with a different mode of action) to blue orchard bees has not been assessed. Here, we used a customized spray tower in a laboratory setting to simulate male and female whole-body contact exposure to thiamethoxam + lambda-cyhalothrin, imidacloprid + beta-cyfluthrin, chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram. We separately simulated male and female contact exposure to imidacloprid, beta-cyfluthrin and their 1:1 binary combination. We consulted the recommended dose regimen on the EPA approved label of each insecticide for selecting application rates. Resulting mortality was screened up to 96-hours post treatment to determine acute contact toxicity. Thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin resulted in statistically higher mortality at a fast onset at 24 and 48-hours, which often resulted in complete mortality by 72-hours. Chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram resulted in high mortality at extended observation (96-hours). We did observe statistical differences between chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram. Exposure to the 1:1 binary combination of imidacloprid + beta-cyfluthrin overall caused statistically higher mortality than exposure to imidacloprid or beta-cyfluthrin. However, at extended observation, we observed high mortality in both single active ingredient treatments.

Keywords: direct contact exposure, contact toxicity, neonicotinoids, pyrethroids, blue orchard bees, insecticide mixtures

Introduction

Blue orchard bees, [*Osmia lignaria* (Say) (Hymenoptera: Megachilidae)] are solitary nesting bees native to North America that are increasingly being utilized for their pollination services. Unlike honey bees [*Apis mellifera* (Linnaeus) (Hymenoptera: Apidae)], blue orchard bees are univoltine spring bees that provision their nests in pre-established cavities (usually old beetle burrows in timber, although they will nest in artificial reed tubes) with moistened nectar and pollen balls for their offspring (Torchio 1982; Bosch and Kemp 2001). Females oviposit approximately six eggs per nest at a ratio of two females placed behind four male offspring; these nests are plugged with mud. Blue orchard bee development occurs over several months with fifth instar larvae spinning a cocoon in late summer to early fall where they will subsequently molt into adult bees and remain dormant until the following spring (Bosch et al. 2000; Bosch and Kemp 2001). Pupal emergence begins in early spring but can be artificially initiated by incubation (at higher than ambient temperature to better time emergence for early blooming crops and at colder than ambient temperature to delay adult eclosion for later blooming crops), thereby rendering blue orchard bees an excellent pollinator for early blooming crops when few other bees are present (Bosch et al. 2000; Horth and Campbell 2018). Males emerge first and initiate foraging while simultaneously aggregating around nests containing female cocoons; mating begins immediately following female emergence and wing drying (Bosch et al. 2006).

Initially viewed as a supplement to commercially managed honey bees, blue orchard bees have in some cases been found to provide superior pollination services leading to improved fruit

set and yield. For example, in comparing commercial sweet cherry (*Prunus avium*, Linnaeus) yield from honey bee versus blue orchard bee pollination over five respective growing seasons, Bosch et al. (2006) determined that blue orchard bee pollination increased production by an average of 2.2 times. This study also found increased female progeny in the orchard each subsequent year (with the exception of 2003 due to heavy bird predation) compared to the quantity of released blue orchard bees the previous spring. Brittain et al. (2013) found that diverse bee communities as opposed to a single species (honey bees) resulted in bee species interactions that improved pollination in almond, (*P. dulcis*, Miller and Webb). In a separate experiment in this study, higher fruit set was demonstrated when honey bees and blue orchard bees were combined in cages, compared to cages containing only one bee species. Similarly, by augmenting commercial almond orchards throughout the Central Valley in California with both honey bees and blue orchard bees, Pitts-Singer et al. (2018) demonstrated significantly increased nut set in orchards with both bee species, compared to those only containing honey bees. Moreover, this study found that blue orchard bees placed in almond orchards were overall reproductively successful. Horth and Campbell (2018) concluded that blue orchard bees can also be used for pollinating strawberries (*Fragaria* species, Duchesne) after demonstrating significantly higher growth rates and larger fruit size for berries grown adjacent to blue orchard bee nests, thereby suggesting that blue orchard bees can be utilized for pollinating herbaceous berry crops. Conducting an economic analysis of blue orchard bee almond pollination, Koh et al. (2018) demonstrated increased profit when blue orchard bees were present; the highest profit increase of $6,916 USD per hectare was correlated with the highest nest density of 25 nest boxes per hectare. Therefore, blue orchard bees are commercially reared and managed to provide pollination services to orchard crops. However, blue orchard bee populations are not stable in

almond systems as a result of crop bloom time frames being too short to complete the lifecycle and insufficient forage surrounding orchards (Biddinger; pers. comm.). As a result, blue orchard bees utilized in almonds are still largely collected from feral populations in mountainous regions. Analyzing the effects of environmental stressors on blue orchard bee health and their impact on reproductive success, overall population decline and ultimately the ability to provide effective pollination services is a developing area of research interest.

One potential environmental stressor of interest is assessing the effects of exposure to pesticides commonly used in orchards before and after bloom on pollinating blue orchard bee and closely related *Osmia* species. Although insecticide applications in orchards are restricted during bloom (epa.gov; Leverage 360® insecticide label), pollinators can still be exposed to systemic insecticide residues present in flowers. Secondarily, insecticide application in cases of premature petal fall while other flowers are simultaneously in bloom can also result in pollinator exposure.

Dosing blue orchard bee larval provisions with the neonicotinoid imidacloprid, Abbott et al. (2008) observed prolonged development and cocoon spinning times for larvae exposed to the higher doses, however no lethal effects were detected. Similarly, no effects on development were recorded when dosing the larval provisions of red mason bees, [*Osmia bicornis* (Hymenoptera: Megachilidae) (Linnaeus, 1758)], with the neonicotinoid clothianidin (Nicholls et al. 2017). Conversely, feeding adult *O. bicornis* sugar water laced with field-realistic amounts of the neonicotinoid thiamethoxam and clothianidin in flight cages resulted in decreased reproduction characterized by increased mortality and male-biased sex ratios (Sandrock et al. 2014). Assessing the toxicity of insecticides applied in canola pest management on blue orchard bees, Scott-Dupree et al. (2009) found that imidacloprid and clothianidin was more toxic than the spinosyn

spinosad or the pyrethroid deltamethrin. Comparing the topical toxicities of five insecticides commonly sprayed in apple orchards to honey bees and Japanese orchard bees, [*Osmia cornifrons* (Hymenoptera: Megachilidae) (Radoszkowski, 1887)] Biddinger et al. (2013) determined large differences in the order of decreasing toxicity for bee species, that in some cases differed between species for resulting LD_{50} and LD_{90} values. In this same study, topically exposing both species of bees to mixtures of the minimally toxic fungicide fenbuconazole with either acetamiprid or imidacloprid resulted in synergistic toxicity for only imidacloprid that was significant. Orally exposing *O. bicornis* to non-lethal doses of the fungicide propiconazole, clothianidin, or a mixture of both, Sgolastra et al. (2017) found synergistic toxicity of the combined mixture demonstrated by significant differences in survival curves for each treatment over the 96-hour experimental duration. A field study assessing the effects of seed coating oilseed rape (*Brassica napus*, Linnaeus) with clothianidin + the pyrethroid beta-cyfluthrin found reduced *O. bicornis* nest construction and provisioning adjacent to fields sowed with insecticide-coated seeds (Rundlöf et al. 2015). Conversely, Ruddle et al. (2018) found no significant effects on *O. bicornis* nest establishment, cell production and adult eclosion following exposure to fields containing oilseed rape grown from thiamethoxam-treated seeds. Evaluating contact and oral toxicity of five fungicides to blue orchard bees, Ladurner et al. (2005) found acute and delayed oral toxicity from single high doses of propiconazole and high acute and delayed toxicity from captan irrespective of mode of application. Analyzing the effects of sprayed pesticide exposure on almond trees within a cage enclosure in a field setting, Artz and Pitts-Singer (2015) demonstrated that the fungicides iprodione, pyraclostrobin + boscalid and the non-ionic wetting agent N-9® (90% polyethoxylated nonylphenol) inhibited blue orchard bee female nest recognition.

The ecotoxicology of formulated premix insecticides comprised of multiple active ingredients (each with a different mode of action; some of which are neonicotinoids) to blue orchard bees has not been tested. Preliminary studies investigating spray contact exposure of premix insecticides to honey bees have demonstrated high toxicity by measure of low LD_{50} values in mortality screenings up to 48-hours after treatment (Zhu et al. 2015). We expand assessment of the toxicity of these insecticide mixtures to bees by simulating adult male and female blue orchard bee contact exposure to middle range EPA regulated insecticide label recommended doses for orchard crops to four premix insecticides (Tables 3.1-3.2 and Figure 2.1). This study separately examines male and female blue orchard bee resulting mortality under two insecticide application scenarios in orchard settings (during pre-bloom or early petal fall with continued residual bloom) or in field crops where insecticides are applied during bloom. We tested (1) spray-only exposure to premix insecticide sprays while bees are foraging in an orchard or a field; and (2) spray-only exposure to the individual active ingredients (and their 1:1 binary combination) comprising one of the most toxic premix insecticides tested while bees are foraging in an orchard or a field. We evaluated each scenario in two separate experiments (analyzing male and female blue orchard bees separately), therefore a total of four experiments were conducted in this study.

Simulating these scenarios for orchards or field crops, we analyzed the acute contact toxicity of formulated insecticide premixes (under field-realistic conditions) to male and female blue orchard bees that are increasingly being used in orchard and field crop integrated pest management. Our findings will aid orchardists and field crop operators in their selection and use of insecticides that have a minimized impact on blue orchard bees.

Materials and Methods

Blue orchard bees. Cocoons containing pupae with adult male and female blue orchard bees in diapause stage were purchased from Watts Solitary Bees (Bothell, WA United States) during spring 2018. Upon receival, cocoons were placed in an incubator at (5 ± 0.5˚C; 65% ± 5 RH) within a laboratory setting until they were used for experiments. Four days prior to beginning each experiment, blue orchard bees were placed in an incubator with higher temperature (14 ± 0.5˚C; 65% ± 5 RH) to stimulate adult pupal eclosion. Blue orchard bees used for all experiments eclosed from their pupae 24-hours prior to being treated.

Cage design. Transparent polypropylene jars (500 mL, D by H: 9.3 by 10 cm) were used as experimental cages. Fine netting cloth was securely placed with rubber bands over cages used for all treatments on male blue orchard bees to prevent their ability to escape. Blue orchard bees were immobilized in separate storage cages for approximately two minutes by being placed in a cooler prior to being used in an experiment. Immobilized blue orchard bees were quickly transferred to petri dishes for treatment, and then placed in clean cages for the duration of each experiment. We fed treated blue orchard bees by inserting a plastic vial containing 50% sucrose solution (6-7 mL per vial) into the side of each cage for *ad libitum* feeding. A cotton swab soaked in 50% sucrose solution was placed on top of each cage providing a second food resource.

Spray tower. Insecticides were administered using a customized spray tower design as described in (Zhu et al. 2015). Using this spray tower, we simulated direct foliar spray exposure in a laboratory setting while meeting laboratory safety and efficiency. A spray nozzle (Burkard Scientific, Uxbridge, Middx, United Kingdom), was used for applying insecticides to blue orchard bees in cages at a low spray volume (0.5 mL/cage). Regulated air pressure (69 kpa) and

fixed spray distance (22 cm) were utilized for each treatment including the control (Zhu et al. 2015). We used a distilled water solvent for all treatments to simulate realistic grower insecticide spray applications in orchards and resulting blue orchard bee exposure.

Post treatment observations. Treated blue orchard bees were transferred to cages placed on plastic trays, where they were maintained in an incubator (21 ± 0.5°C; 65% ± 5 RH) for the duration of the experiment. We screened and recorded resulting mortality at 24, 48, 72, and 96-hours after treatment for all treatments, except in experiment 3, which was terminated after 48-hours. Blue orchard bees were considered dead if they did not move after being touched by a paintbrush. Moribund bees capable of moving their legs and antennae but incapable of flying were considered alive.

Experiment 1: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides

Insecticide treatments. Four formulated insecticide mixtures (Table 3.1 and Figure 2.1) were assessed for their contact toxicity to adult male blue orchard bees directly sprayed in experimental cages. Each of these insecticides is comprised of two active ingredients (each with a different mode of action). A single rate for each insecticide was chosen that was in the middle of the range of rates listed on the EPA regulated insecticide label for orchard crops (Table 3.2). We mixed each insecticide solute in 100 mL of a distilled water solvent. A low spray volume (0.5 mL/cage) was used for insecticide administration. The control treatment was sprayed with distilled water at the same spray volume. Treated male blue orchard bees were fed by inserting a feeder made of a plastic vial containing 50% sucrose solution (6-7 mL per vial) into the side of each cage for *ad libitum* feeding. A cotton swab soaked in 50% sucrose solution was placed on top of each cage providing a second food resource. For all treatments we filled the plastic vial

feeder inserted in each cage with an additional 2 mL of 50% sucrose solution at both 48 and 72-hours for continued male blue orchard bee feeding. A completely randomized design was used to test the five treatments in this experiment, each of which contained seven replicates. Seven cages containing 10 male blue orchard bees each (n = 10/cage, total 70 bees) were utilized for each of the five treatments, with each individual cage representing an experimental unit. Each experimental unit was randomly assigned to one of the five treatments.

Experiment 2: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides

Insecticide treatments. Spray contact toxicity of four formulated premix insecticides (Table 3.1 and Figure 2.1) to adult female blue orchard bees was assessed by applying insecticides to bees placed in experimental cages. Each of these insecticide mixtures contains multiple active ingredients (each with a different mode of action). We consulted the insecticide label for each respective insecticide in selecting a rate within the middle-range of recommended rates for orchard crops (Table 3.2). Each insecticide solute was diluted in 100 mL of a distilled water solvent. Insecticides were applied to blue orchard bees at a low spray volume (0.5 mL/cage). Distilled water sprayed at the same volume was used as a control. Treated female blue orchard bees were fed by inserting a plastic vial containing 50% sucrose solution (6-7 mL per vial) into the side of each cage for *ad libitum* feeding. A cotton swab soaked in 50% sucrose solution was placed on top of each cage providing a second food resource. At both 48 and 72-hours, we administered 2 mL of 50% sucrose solution to the cotton swab placed on top of each cage for continued female blue orchard bee feeding in all treatments. A completely randomized design was used to test the five treatments in this experiment, each of which contained six replicates. Six cages containing 9 female blue orchard bees each (n = 9/cage, total 54 bees) were

utilized for each of the five treatments, with each individual cage representing an experimental unit. Each experimental unit was randomly assigned to one of the five treatments.

Experiment 3: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides

Insecticide treatments. Contact toxicity of the formulated individual active ingredient insecticides and their 1:1 binary combination comprising one premix insecticide tested (Table 3.3 and Figure 2.1) to adult male blue orchard bees was assessed by direct spray application to bees in experimental cages. We selected a rate within the low-range of rates on the insecticide label recommendations for orchard crops (Table 3.4). Insecticide solutes were mixed with 100 mL of distilled water, which served as a solvent. Spray application was made using a low spray volume (0.5 mL/cage). Administration of distilled water at the same spray volume was used as a control. Treated male blue orchard bees were fed by inserting a plastic vial containing 50% sucrose solution (6-7 mL per vial) into the side of each cage for *ad libitum* feeding. A cotton swab soaked in 50% sucrose solution was placed on top of each cage providing a second food resource. We supplied an additional 3-4 mL of 50% sucrose solution to the cotton swab placed on top of each cage at 24-hours for continued male blue orchard bee feeding in all treatments. A completely randomized design was used to test the four treatments in this experiment, each of which contained six replicates. Six cages containing 8 male blue orchard bees each (n = 8/cage, total 48 bees) were utilized for each of the five treatments, with each individual cage representing an experimental unit. Each experimental unit was randomly assigned to one of the five treatments.

Experiment 4: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides

Insecticide treatments. Adult female blue orchard bees placed in experimental cages were sprayed with the formulated individual active ingredients and their 1:1 binary combination comprising one insecticide mixture tested (Table 3.3 and Figure 2.1) to determine resulting contact toxicity. A rate within the low-range recommended application rates for orchard crops were used as per the EPA regulated insecticide label for each respective insecticide (Table 3.4). We diluted each insecticide solute in a distilled water (100 mL) solvent. Using a spray volume (0.5 mL/cage), we applied insecticide treatments to blue orchard bees. The same volume of distilled water was used as a control. Treated female blue orchard bees were fed by inserting a plastic vial containing 50% sucrose solution (6-7 mL per vial) into the side of each cage for *ad libitum* feeding. A cotton swab soaked in 50% sucrose solution was placed on top of each cage providing a second food resource. We did not apply additional 50% sucrose solution for continued female blue orchard bees feeding at any post treatment screening period for all treatments. A completely randomized design was used to test the five treatments in this experiment, each of which contained six replicates. Six cages containing 8 female blue orchard bees each (n = 8/cage, total 48 bees) were randomly utilized for each of the four treatments, with each individual cage representing an experimental unit.

Statistical analysis

We corrected the check (control) blue orchard bee mortality for each experiment at each screening period by using the Schneider-Orelli's formula (Bibbs et al. 2015; Williams et al. 2015) as below:

$$\text{Corrected \% Mortality} = \left(\frac{\text{Mortality \% in treatment} - \text{Mortality \% in control}}{100 - \text{Mortality \% in control}}\right) * 100 \quad (\text{eq. } 1)$$

where the percent mortality for treatment replication is compared to the percent mortality for each respective control replication.

We removed the control from each experiment as a result of correcting our data using the Schneider-Orelli's formula. Therefore, we only analyzed the blue orchard bee mortality for insecticide treatments. An arcsine transformation was used to normalize corrected percent mortality data as in (Glazer and Navon 1990). Normal Q-Q and Residual vs. Fitted plots were used to confirm normality and lack of heteroscedasticity.

Statistical significances in the percent of blue orchard bee mortality following acute contact exposure to each of the four premix insecticides (experiments 1-2) and to each individual active ingredient insecticide and their 1:1 binary combination (experiments 3-4) tested at each screening period were determined using one-way ANOVA as in Wise et al. (2017).

A Tukey's HSD post-hoc multiple pairwise comparison analysis was used to determine statistical differences in the percent of blue orchard bee mortality following acute contact exposure to each premix insecticide (experiments 1-2) and to each individual active ingredient insecticide and their binary combination (experiments 3-4) treatment during each screening period as in Wise et al. (2017). The R Studio statistical software (R version 3.5.1 and R Studio version 1.1.463) was used for completing all statistical analyses.

Results

Experiment 1: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides

Male blue orchard bee spray-only exposure to all four premix insecticides (Tables 3.1-3.2 and Figure 2.1) resulted in mortality that was statistically significant between treatments at 24-hours ($F=18.059$, $p<0.05$), 48-hours ($F=48.053$, $p<0.05$), 72-hours ($F=35.37$, $p<0.05$) and 96-

hours ($F=12.824$, $p<0.05$) (Figure 3.1 and Appendix 3.1). At 24-hours, bees exposed to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin had higher mortality than chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram ($p<0.05$) (Figure 3.1 and Appendix 3.1). At 48-hours, we observed higher mortality for thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin than for chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram, however, mortality in chlorantraniliprole + lambda-cyhalothrin was also higher than methoxyfenozide + spinetoram ($p<0.05$). At 72 and 96-hours, bee mortality was higher for thiamethoxam + lambda-cyhalothrin, imidacloprid + beta-cyfluthrin and chlorantraniliprole + lambda-cyhalothrin compared to methoxyfenozide + spinetoram ($p<0.05$).

Experiment 2: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides

Spray-only exposure to the four premix insecticides (Tables 3.1-3.2 and Figure 2.1) resulted in female blue orchard bee mortality that was statistically significant between treatments at 24-hours ($F=14.859$, $p<0.05$), 48-hours ($F=22.135$, $p<0.05$) and 72-hours ($F=14.869$, $p<0.05$) (Figure 3.2 and Appendix 3.2). At 96-hours, we did not observe significant mortality for all treatments ($F=1$, $p=0.413$) given the complete mortality at this screening period. However, one replication in methoxyfenozide + spinetoram was an outlier at 96-hours with 60% mortality. At 24 and 72-hours, mortality in the thiamethoxam + lambda-cyhalothrin, imidacloprid + beta-cyfluthrin, and chlorantraniliprole + lambda-cyhalothrin treatments was higher than in the methoxyfenozide + spinetoram treatment ($p<0.05$) (Figure 3.2 and Appendix 3.2). At 48-hours, we observed higher bee mortality following exposure to thiamethoxam + lambda-cyhalothrin compared to methoxyfenozide + spinetoram, while bee mortality in the imidacloprid + beta-

cyfluthrin treatment was higher than that in the chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram treatment, and bee mortality in the chlorantraniliprole + lambda-cyhalothrin treatment was higher than that in the methoxyfenozide + spinetoram treatment ($p<0.05$). At 96-hours, no significant differences were observed between insecticide treatments given the observation of complete mortality ($p=1$ for thiamethoxam + lambda-cyhalothrin compared to imidacloprid + beta-cyfluthrin and chlorantraniliprole + lambda-cyhalothrin, and for imidacloprid + beta-cyfluthrin compared to chlorantraniliprole + lambda-cyhalothrin; $p=0.5054$ for thiamethoxam + lambda-cyhalothrin, imidacloprid + beta-cyfluthrin and chlorantraniliprole + lambda-cyhalothrin compared to methoxyfenozide + spinetoram).

Experiment 3: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides

Mortality for male blue orchard bees following spray-only exposure to individual active ingredient insecticides and their 1:1 binary combination (Tables 3.3-3.4 and Figure 2.1) was statistically significant between treatments at 24-hours ($F=13.098$, $p<0.05$), however not at 48-hours ($F=1$, $p=0.391$) given the complete mortality at this screening period which terminated the experiment early (Figure 3.3 and Appendix 3.3). Lower mortality was observed in imidacloprid and beta-cyfluthrin compared to the 1:1 binary combination (imidacloprid + beta-cyfluthrin) at 24-hours ($p<0.05$) (Figure 3.3 and Appendix 3.3). At 48-hours, no significant difference in mortality was observed between treatments given the complete mortality at this screening period ($p=0.4574$ for imidacloprid compared to beta-cyfluthrin and the 1:1 binary combination, while $p=1$ for beta-cyfluthrin compared to the 1:1 binary combination).

Experiment 4: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides

Female blue orchard bee spray-only exposure to the individual active ingredient insecticides and their 1:1 binary combination (Tables 3.3-3.4 and Figure 2.1) resulted in statistically significant mortality between treatments at 24-hours ($F=6.3879$, $p<0.05$), 48-hours ($F=11.266$, $p<0.05$), 72-hours ($F=10.972$, $p<0.05$) and 96-hours ($F=8.2398$, $p<0.05$) (Figure 3.4 and Appendix 3.4). At 24-hours, we observed lower mortality for bees exposed to imidacloprid compared to the 1:1 binary combination (imidacloprid + beta-cyfluthrin) ($p<0.05$) (Figure 3.4 and Appendix 3.4). At 48, 72 and 96-hours, imidacloprid demonstrated lower bee mortality than beta-cyfluthrin and the 1:1 binary combination ($p<0.05$).

Discussion

Experiment 1: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides

Examining the scenario of spray-only insecticide exposure, we found that male blue orchard bees sprayed with thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin experienced the most rapid onset of mortality. At 24-hours approximately 40% mortality resulted for both treatments. Interestingly, we observed less mortality in male blue orchard bees than for females only at 24-hours, which is surprising given the larger body size and increased bodily hair of females. At 48-hours, we observed approximately 80% male mortality for both treatments, while complete mortality resulted for both treatments at 72-hours. We observed quick knockdown characterized by trembling and an incapacity to fly within 25 minutes of application of these two treatments. Male blue orchard bees displayed the same behavior at 24 and 48-hours. Given the characteristic knockdown mode of action of pyrethroids

as sodium channel modulators (Table 3.1; Vijverberg et al. 1982), we primarily attribute this rapid knockdown to the presence of pyrethroid active ingredients. Neonicotinoids have systemic and translaminar properties (Elbert et al. 2008) which facilitate their movement into leaf tissue, thereby reducing bee contact to these chemicals after residues dry. Standard LD_{50} values of these active ingredient chemistries have been quantified for honey bees (Table 2.3). Although honey bees cannot be considered as a surrogate for non-*Apis* bees including blue orchard bees (Biddinger et al. 2013), these standard LD_{50} values provide a preliminary indication of the high toxicity of these insecticides to bees. It would be helpful to generate standard LD_{50} values for insecticide toxicity to *Osmia* species bees for accurate comparison in future studies. Our results are suggestive of additive or synergistic toxicity arising from male blue orchard bee exposure to both a neonicotinoid and a pyrethroid, however, future analysis of multiple doses to create dose-mortality curves is necessary to prove this. Nonetheless, it is evident that male blue orchard bees will likely experience rapid mortality following spray-only exposure to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin.

Male blue orchard bee treatment with chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram resulted in reduced mortality. At 24-hours, we observed approximately 20% mortality for both treatments, while at 48-hours, approximately 45% mortality was observed for bees treated with chlorantraniliprole + lambda-cyhalothrin, and approximately 30% mortality was observed for bees treated with methoxyfenozide + spinetoram. By 96-hours, we recorded complete mortality for the chlorantraniliprole +lambda-cyhalothrin treatment, and approximately 65% mortality for the methoxyfenozide + spinetoram treatment. Here we observed some knockdown effect (characterized by inability to fly) for male blue orchard bees treated with chlorantraniliprole + lambda-cyhalothrin at 24-hours. This could be

due to the presence of a pyrethroid active ingredient. However, this knockdown was less pronounced than that for thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin, which we attribute to the lack of a neonicotinoid active ingredient. Moreover, the delayed toxicity of chlorantraniliprole + lambda-cyhalothrin to male blue orchard bees is evident by our only recording complete mortality at 96-hours. Interestingly, we recorded complete mortality (with one outlier) for females at 72-hours, which is surprising given their physiological differences (discussed in experiment 2). As previously mentioned, the standard LD_{50} values of these active ingredient chemistries to honey bees (Table 2.3) are not a direct comparison because honey bees can differ in response to toxicants compared to non-*Apis* bees (Biddinger et al. 2013). However, these standard LD_{50} values are a starting point and do indicate lower toxicity of these insecticides to bees. Interestingly, Smagghe et al. (2013) found acute and chronic toxicity of chlorantraniliprole to *Bombus terrestris* [(Hymenoptera: Apidae) (Linnaeus, 1758)] (a non-*Apis* bee) following oral consumption. This finding combined with ours supports the need for more research regarding the risk posed to non-*Apis* bees by chlorantraniliprole, which is a ryanodine receptor agonist (Table 3.1; irac-online.org). Conversely, we observed the least amount of mortality for male blue orchard bees treated with methoxyfenozide + spinetoram (and no knockdown activity), which we primarily attribute to the lack of a pyrethroid chemistry. As with chlorantraniliprole + lambda-cyhalothrin, our recording of approximately 65% mortality at 96-hours indicates the slow onset of toxicity for methoxyfenozide + spinetoram. However, it is surprising that we observed complete mortality for females exposed to methoxyfenozide + spinetoram at 96-hours. For males, these results are not entirely surprising given the classification of methoxyfenozide as a diacylhydrazine ecdysone receptor agonist (Table 3.1; irac-online.org) designed to induce premature molting in immature Lepidoptera (Suiter and

Scharf 2015). Studies demonstrating the benign toxicity of methoxyfenozide (Mommaerts et al. 2006) and reduced toxicity of spinetoram (Besard et al. 2011) to *B. terrestris* (a non-*Apis* bee) further support our results. Additionally, the translaminar movement of spinetoram (Sato et al. 2012) reduces bee contact after residues have dried. These results demonstrate that male blue orchard bees will likely experience delayed mortality following spray-only exposure to chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram. Removing check (control) mortality from our data analysis by using the Schneider-Orelli's formula (eq. 1) enables us to demonstrate that male blue orchard bee mortality is increasing as a result of exposure to insecticide treatments. Even though we removed control data from our analysis, our observation that male blue orchard bees in the control were alive six days (144-hours) post treatment bolsters our conclusion of the toxicity of premix insecticides to male blue orchard bees.

Experiment 2: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides

For female blue orchard bees, we similarly observed the most rapid onset of mortality for the thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin treatments. Approximately 50% mortality resulted at 24-hours for both treatments. It is surprising that the females experienced higher mortality than males at 24-hours, given their larger body size and increased bodily hair. Therefore, future studies analyzing physiological and metabolic differences between male and female adult blue orchard bees are warranted. At 48-hours we observed approximately 85% female mortality, and by 72-hours complete mortality for both treatments (except for one outlier at 83.3% mortality at 72-hours for imidacloprid + beta-cyfluthrin). Similar to males, we observed quick female knockdown within 25 minutes after application of these two insecticides, as well as for alive bees during the 24 and 48-hour

mortality screenings. We primarily attribute this rapid knockdown to the presence of pyrethroids. As mentioned before, the low standard LD_{50} values for these insecticides to honey bees (Table 2.3) are not a direct comparison to blue orchard bees, however they do indicate the overall high toxicity posed to bees. While our findings in this experiment suggest an interaction between neonicotinoid and pyrethroid chemistries, further analysis of multiple doses to create dose mortality curves is necessary to prove this. However, similar to males, these findings indicate that female blue orchard bees will likely experience high mortality at a rapid pace following spray-only exposure to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin.

Female blue orchard bees sprayed with chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram experienced a slower onset of initial mortality, and specifically for methoxyfenozide + spinetoram. At 24-hours, we observed approximately 45% and 10% mortality, and by 48-hours we observed approximately 60% and 25% mortality for both treatments respectively. At 72-hours, we observed complete mortality for chlorantraniliprole + lambda-cyhalothrin (except for one outlier at 83.3% mortality), and at 96-hours we observed complete mortality for methoxyfenozide + spinetoram (except for one outlier at 60% mortality). Interestingly, for females, chlorantraniliprole + lambda-cyhalothrin resulting mortality was close to that of thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin, which is a trend we did not observe for males. Moreover, compared to males, we observed higher mortality for females exposed to chlorantraniliprole + lambda-cyhalothrin at 24 and 48-hours, and for females exposed to methoxyfenozide + spinetoram at 72 and 96-hours. As with thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin at 24-hours, this is surprising given the physiological differences between males and females. Therefore, as previously mentioned, future studies should investigate these differences. Female blue orchard bees treated with

chlorantraniliprole + lambda-cyhalothrin also displayed some knockdown behavioral effects (characterized by inability to fly) at 24-hours. As before, we attribute this finding to the pyrethroid active ingredient. However, this knockdown was less pronounced than that for the thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin treatments which we attribute to the lack of a neonicotinoid. Conversely, we observed no knockdown activity for females treated with methoxyfenozide + spinetoram, which we primarily attribute to the lack of a pyrethroid. Our results for females agree with the findings of Smagghe et al. (2013) demonstrating the risk of chlorantraniliprole to a non-*Apis* bee, however, are contrary to the findings of Mommaerts et al. (2006) showing the benign toxicity of methoxyfenozide and Besard et al. (2011) depicting the reduced toxicity of spinetoram to a non-*Apis* bee. Given the rapid versus slow onset of mortality in our study, it would be helpful to explore blue orchard bee detoxification at 24-hours for highly toxic insecticides (thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin) and at 72 and 96-hours for less toxic insecticides (chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram). Generating this data might contribute to creating standard LD_{50} values for non-*Apis* bees, similar to those for honey bees (Table 2.3). Nonetheless, our observation of alive bees in the control treatment at six days (144-hours) after insecticide application further supports our conclusion of high risk posed by premix insecticides to female blue orchard bees.

Experiment 3: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides

Under the scenario simulating spray-only exposure to individual active ingredients and their 1:1 binary combination, we found high mortality for male blue orchard bees in all treatments. At 24-hours, we observed approximately 57.5%, 45% and 80% mortality for

imidacloprid, beta-cyfluthrin and imidacloprid + beta-cyfluthrin, respectively. We observed complete mortality at 48-hours which terminated the experiment. At 24-hours for all treatments, we noticed that all alive male bees tremored, characterized by their wings, legs and antennae slightly moving. These behaviors are consistent with symptoms from exposure to pyrethroid active ingredients. The statistically higher mortality in the 1:1 binary combination of imidacloprid + beta-cyfluthrin at 24-hours suggests an additive or synergistic toxicity posed to male blue orchard bees exposed to this insecticide mixture. However, future work testing multiple doses to generate a dose mortality curve would be necessary to prove this. Moreover, our laboratory synthesized 1:1 binary combination is not an identical simulation of Leverage 360® (the premix insecticide comprised of these two active ingredients) which contains 0.24 g/mL imidacloprid and 0.12 g/mL beta-cyfluthrin (Leverage 360® label). Additionally, the higher toxicity observed in this experiment may in part be explained by the higher percentage of active ingredients in the single active ingredient products (Tables 3.3-3.4) compared to those in the premix insecticide products (Tables 3.1-3.2). Nonetheless, our results demonstrate that male blue orchard bees coming into spray-only contact with imidacloprid, beta-cyfluthrin and particularly imidacloprid + beta-cyfluthrin will be unlikely to survive. However, future assessment of single active ingredient insecticides containing equal quantities of active ingredients as the premix insecticides we tested would be helpful in specifically determining the risk posed by exposure to insecticide mixtures to blue orchard bees. Although we adjusted for the control mortality using Schneider-Orelli's formula (eq. 1), we observed 16% male mortality in the control at 24-hours. We primarily attribute this to bee age at treatment (24-hours post pupal eclosion) and living in an artificial cage environment with a single food source (50% sucrose solution). Interestingly,

though, we did notice some male survival in the control at six days (144-hours) after treatment, which further supports the notion of mortality induced by insecticide toxicity.

Experiment 4: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides

Although we ran this experiment for female blue orchard bees for the entire 96-hour duration, we observed similar results to the males marked by overall high mortality. At 24-hours, we recorded approximately 40%, 50% and 70% mortality for imidacloprid, beta-cyfluthrin and imidacloprid + beta-cyfluthrin respectively. At 48-hours, average imidacloprid mortality remained unchanged, while beta-cyfluthrin increased to 90% and imidacloprid + beta-cyfluthrin increased to complete mortality. At 72-hours imidacloprid increased to approximately 60% mortality, while beta-cyfluthrin increased to complete mortality (except for a couple outliers at 86% mortality). By 96-hours, imidacloprid mortality was approximately 77.5% while beta-cyfluthrin increased to complete mortality. As with males, we noticed that all females at 24-hours tremored in all treatments, characterized by their wings, legs and antennae slightly moving. Interestingly, we observed high knockdown for females sprayed with imidacloprid at 24-hours, suggesting a similarity in acute contact toxicity to that of pyrethroids. This finding warrants further exploration of dynamics governing female blue orchard bee neonicotinoid bodily uptake given their systemic and translaminar properties (Elbert et al. 2008). Additionally, future studies should investigate female blue orchard bee metabolic detoxification of imidacloprid in efforts to explain the physiological basis for the onset of tremors we observed in females 24-hours after spray exposure to imidacloprid. Similar to males, the numerically higher mortality for imidacloprid + beta-cyfluthrin at all screening periods is indicative of additive or synergistic toxicity. Since we are unable to prove this in our study, future work testing multiple doses to

generate dose mortality curves is required to validate this claim. Additionally, correcting for the unequal proportions of insecticides in our 1:1 binary combination compared to those in the premix (Leverage 360® label) and the higher percentage of active ingredients in the single ingredient products (Tables 3.3-3.4) compared to the premix products (Tables 3.1-3.2) should be factored into future studies. Incorporating these corrections will create a more direct comparison between premix and single ingredient products, thereby enriching assessment of whether exposure to premix chemistries poses increased risk to female blue orchard bees. However, our results serve as a preliminary base point indicating that female blue orchard bees coming into spray-contact with imidacloprid, beta-cyfluthrin and particularly imidacloprid + beta-cyfluthrin likely experience high mortality. Removing control mortality from our data ensured demonstration of mortality resulting from insecticide exposure. Although, it is worth mentioning that similar to males, we did observe some female survival at six days (144-hours) after treatment, thereby further supporting that female mortality was induced by insecticide treatment.

The insecticide mixtures we tested in this study are increasingly being utilized for controlling insect pests in orchard and berry crops. For example, a premix insecticide containing thiamethoxam + lambda-cyhalothrin was found to provide effective control of brown marmorated stink bugs [*Halyomorpha halys* (Hemiptera: Pentatomidae) (Stål, 1855)] in a USDA apple (*Malus* species, Mill) and peach (*Prunus persica*, Linnaeus) orchard field trial (Leskey et al. 2013). At zero days after treatment in this study, mid-season application resulted in 66.7% mortality in 2011, while in 2012 early-season application resulted in 73.3% mortality and mid-season application resulted in 80.0% mortality. Similarly, applying thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin to orchard locations baited with *H. halys* aggregation pheromones, Morrison et al. (2016) found 63% increased mortality in locations

where high pheromone doses were applied. Tanigoshi et al. (2011) found high efficacy for thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin for spotted wing drosophila [*Drosophila suzukii* (Diptera: Drosophilidae) (Matsumura, 1931)] control in red raspberry (*Rubus idaeus*, Linnaeus). Similarly, Tanigoshi et al. (2013) found imidacloprid + beta-cyfluthrin exceeded the threshold of 90% spotted wing drosophila mortality, while thiamethoxam + lambda-cyhalothrin provided 89.7% efficacy at seven days after treatment in duke or northern highbrush blueberry (*Vaccinium corymbosum*, Linnaeus). Insecticide control of oblique banded leaf-roller [*Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae)] in sweet cherry orchards includes chlorantraniliprole, methoxyfenozide and spinetoram (Alston and Murray 2017). Isaacs (2010) showed that growers applying methoxyfenozide + spinetoram following a degree-day model reported low fruit-worm infestation in blueberry. Several of these orchard and berry crops are pollinated by bee species including blue orchard bees (Bosch et al. 2006; Pitts-Singer et al. 2018). An analysis of apple pollination in New York State and Pennsylvania demonstrated grower interest in managing orchards for native pollinators given their high effectiveness in instigating fruit-set (Park et al. 2018). This study also found that grower interest in native bee pollination increased when provided appropriate guidelines and resources. Therefore, usage of non-*Apis* alternatives to honey bees is increasing. As a result, non-*Apis* bees including blue orchard bees may potentially be exposed to these insecticides while foraging for pollen and nectar to provision their nests. However, this would only occur either before or after bloom in an orchard, although, this may occur during bloom for a berry crop given the continuous blossom of berries during a growing season.

It is important to note that application of these premix insecticides in orchards is prohibited during bloom (Leverage 360® label; epa.gov) when pollinators including blue orchard

bees are most likely to be foraging. Therefore, insecticide labels specifically instruct applicators to only administer insecticides in orchards before bloom (prepink of flowers) or after flower petal fall. However, spraying before bloom does not guarantee pollinator safety as systemic and translaminar insecticides such as neonicotinoids (Elbert et al. 2008) and spinetoram (Sato et al. 2012) may remain in plant tissues (Doering et al. 2004; Oliver et al. 2010) and subsequently move from leaf and stem surfaces into the pollen and nectar of blossoming flowers (Mogren and Lundgren 2016). Also, dried insecticide residues applied before bloom may become liquified in plant guttation fluids which have been shown to contain high levels of neonicotinoids in seed-treated maize (Girolami et al. 2009) and melons (Hoffmann and Castle 2012). Bees have been found to collect guttation fluid (Hopwood et al. 2016), thereby potentially exposing them to insecticides. Pollinating insects foraging these flowers would be subjected to direct contact with insecticide-contaminated pollen and nectar. Our experimental design of spray-only insecticide exposure to blue orchard bees directly simulates this scenario. Although blue orchard bees are not currently used for pollinating maize and melons, they potentially could come into contact with insecticide contaminated guttation fluid in crops they do pollinate. Future work should explore this scenario in orchard and particularly berry crops. Second, the practice of applying insecticides in orchards after flower petal fall is a variable definition since flowering can occur in different stages and separate times as a result of ambient temperature as shown in (Rodrigo and Herrero 2002). Therefore, it is possible that subsequent flowers may be in full bloom (and hence attractive to pollinators) after the majority of flower petal fall. Under this scenario, pollinators (including blue orchard bees) could forage later blooming flowers in an orchard while insecticides are being applied. We designed our spray-only exposure bioassay in a laboratory setting to also simulate this scenario where blue orchard bees are exposed to insecticide sprays

while foraging through an orchard. Additionally, applying insecticides after petal fall might be hazardous to other native bees such as *Andrena* species [(Hymenoptera: Andrenidae) (Fabricius)], *Bombus* and *Osmia* species naturally found within orchard settings (Cane 1997; Park et al. 2010). Field studies exploring routes of insecticide exposure to these native bees within orchards before and after bloom that particularly emphasize how differences in nesting biology (i.e. *Bombus* and *Osmia* species excavate nests in cavities above ground while *Andrena* species excavate nests below ground) impact exposure to insecticides would greatly contribute to our understanding of insecticide flow through an orchard agro-ecosystem post application.

Blue orchard bees have also been shown to be effective pollinators of berry crops as in Horth and Campbell (2018) where their use increased strawberry size and growth rate on small farms. Similarly, Sampson et al. (2013) demonstrated reliance on the closely related *Osmia ribifloris* [(Hymenoptera: Megachilidae) (Cockerell, 1900)] for 80-100% of fruit yield in seven wild and cultivated blueberry species. Given that insecticide applications are not prohibited during bloom of berry crops (Leverage 360® label; epa.gov) blue orchard bees that are used to pollinate berries are at risk for insecticide exposure. Moreover, since bloom in berries can occur multiple times throughout a growing season in a fluctuating pattern (Melito et al. 2015), it is possible for blue orchard bees foraging berry agro-ecosystems to come into contact with insecticides as we have simulated in our laboratory bioassays. Testing the acute contact toxicity of the insecticides we used in this study at field realistic rates for berry crops to blue orchard bees should be investigated in future studies.

Some preliminary work has demonstrated the toxicity of the premix insecticides we tested on honey bees. Examining impact of spray exposure to honey bees, Zhu et al. (2015) demonstrated high toxicity by means of low LD_{50} values at 48-hours after administration.

However, to our knowledge, no data exists regarding the acute contact toxicity of these premix insecticides to blue orchard bees, and particularly for an extended period (up to 96-hours after application). The slower onset of male and female mortality for bees exposed to chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram in our study highlights the importance of extended mortality observation.

Additionally, most research on insecticide contact toxicity to bees has focused on technical grade individual active ingredients dissolved in acetone. This approach does not assess the impact of 'inert' or 'inactive' ingredients within formulated insecticides on bees. Addressing this variable is important given preliminary results as in Mullin (2015) showing the toxicity of 'inert' ingredients to bees. Although honey bees have been the subject of most bee toxicity work (including that of Mullin 2015), accounting for technical versus formulated products is an important variable that should be incorporated into studies of insecticide toxicity to blue orchard bees and other non-*Apis* bees. Moreover, honey bees cannot serve as a surrogate species for other bee species given differences in response between *Apis* and non-*Apis* bees following insecticide exposure (Biddinger et al. 2013; Rundlöf et al. 2015). While using technical grade insecticides allows for a true analysis of active ingredient toxicity, they use may pose an increased risk to bees due to the higher percentages of active ingredients they contain compared to formulated products. Acetone solvents may also increase toxicity by burning through the bee cuticle and thereby increasing the quantity of insecticide that the bee absorbs. Therefore, side-by-side analysis of both technical grade and formulated products should be conducted in comparisons between honey bees and non-*Apis* bees to accurately gauge insecticide risk to non-*Apis* bees.

Previous studies have demonstrated the toxicity of individual active ingredient insecticides to commercially managed *Osmia* species bees. Given that less studies have been

conducted on *Osmia* species bees (compared to honey bees) variations in study design for current data exist. For example, the most realistic studies have been conducted in field settings, while others in laboratory settings have used either formulated products or technical grade products dissolved in water. Assessing the effects of neonicotinoids in different countries on *O. bicornis*, Woodcock et al. (2017) found a negative correlation between egg cell production and combined nest residues of imidacloprid + thiamethoxam + clothianidin. Oral consumption of sugar water laced with field-realistic amounts of thiamethoxam (2.87 µg/kg) resulted in decreased *O. bicornis* reproduction characterized by increased mortality and male-biased sex ratios (Sandrock et al. 2014). *O. cornifrons* contact exposure to formulated lambda-cyhalothrin dissolved in water demonstrated significantly higher toxicity compared to imidacloprid, acetamiprid and phosmet at LD_{50}, and significantly increased susceptibility at LD_{90} (Biddinger et al. 2013). Since LD_{90} represents the lethal dose that kills 90% of the bees, this finding of increased *O. cornifrons* susceptibility at LD_{90} is meaningful. Dosing blue orchard bee larval provisions with technical grade imidacloprid dissolved in water, Abbott et al. (2008) observed prolonged development and cocoon spinning times for larvae exposed to higher doses (30-300 ppm), however no lethal effects were detected. Placing *O. bicornis* adjacent to clothianidin + beta-cyfluthrin seed-coated fields decreased nest construction and provisioning (Rundlöf et al. 2015). Conversely, in conducting a similar study exposing *O. bicornis* to clothianidin + beta-cyfluthrin Peters et al. (2016) concluded no negative impacts as a result of high reproduction and low parasitism levels. Similarly, Ruddle et al. (2018) found no effects of exposure to oilseed rape grown from thiamethoxam-treated seeds on *O. bicornis* nest production and cell provisioning through adult emergence. Analyzing different routes of solitary bee pesticide exposure, Kopit and Pitts-Singer

(2018) mention that anthranilic diamides such as chlorantraniliprole are long-lasting in soil and therefore might be present in mud obtained by *Osmia* species bees for nest construction.

Conclusion

The results of this study demonstrate the high toxicity of formulated premix insecticides and their individual active ingredients to blue orchard bees. Our finding of differences in male versus female mortality should also be further explored. Work investigating the physiological and metabolic basis for blue orchard bee insecticide detoxification over an extended period is warranted. A future study might expose blue orchard bees to both insecticides and detoxification enzyme inhibitors to assess the impact of these enzymes on blue orchard bee tolerance or susceptibility of different agricultural chemicals. A study of this fashion would be similar to the work of Johnson et al. (2006) showing the importance of cytochrome P450 monooxygenases in honey bee detoxification of pyrethroids.

While our study begins to address questions related to the acute toxicity of formulated premix insecticides to blue orchard bees, several important aspects warrant further investigation. An analysis of oral acute toxicity of the premix insecticides we tested (and if possible their individual active ingredients) would be the next logical step. Assessment of residual blue orchard bee exposure to these chemicals is warranted, especially in cases where insecticide residues in orchards sprayed before bloom linger and subsequently pose toxicity to bees foraging flower blossoms. Testing insecticide toxicity to immatures (eggs, larvae and pupae) including contact and oral exposure from their food provisions will further strengthen risk assessment protocols. As previously mentioned, generating this data for blue orchard bees will aid our ability to correctly assess the effects of insecticide exposure on other non-*Apis* bee species.

Finally, future work should analyze sub-lethal and chronic insecticide toxicity to blue orchards bees. Specifically, it would be interesting to observe if exposure to smaller doses of the premix insecticides we tested negatively impairs normal behaviors of this bee species. It is critical to determine if contact exposure to these insecticides impairs male and female blue orchard bee's ability to collect pollen and nectar, female egg oviposition, nest provisioning and proper development of immature brood into adult bees.

Acknowledgements

Authors are thankful to Dr. Y. C. Zhu (USDA-ARS Lab) for valuable advice on the customized sprayer and experimental cage design. Authors are also thankful to Drs. N. Seiter and J. Smith for statistical advice, Dr. N. Seiter for providing insecticides, V. Piazza for customizing and constructing certain materials used to conduct experiments in this study, and B. Gibson for assistance in preparing blue orchard bees for treatment.

Tables

Table 3.1: Active ingredients and modes of action of formulated premix insecticides assessed for acute contact toxicity to adult male (experiment 1) and female blue orchard bees (experiment 2).

Treatment	Formulation	Active Ingredient(s)	Mode of Action 1st Insecticide*	Mode of Action 2nd Insecticide*	Manufacturer
A	Control				
B	Endigo ZC®	Thiamethoxam (12.6%) + Lambda-cyhalothrin (9.48%)	Thiamethoxam -Nicotinic acetylcholine receptor (nAChR) competitive modulator	Lambda-cyhalothrin -Sodium channel modulator	Syngenta Crop Protection, LLC Greensboro, NC
C	Leverage 360®	Imidacloprid (21.0%) + Beta cyfluthrin (10.5%)	Imidacloprid -Nicotinic acetylcholine receptor (nAChR) competitive modulator	Beta-cyfluthrin -Sodium channel modulator	BayerCropScience LP Research Triangle Park, NC
D	Besiege®	Chlorantraniliprole (9.26%) + Lambda-cyhalothrin (4.63%)	Chlorantraniliprole -Ryanodine receptor modulator	Lambda-cyhalothrin -Sodium channel modulator	Syngenta Crop Protection, LLC Greensboro, NC
E	Intrepid Edge®	Methoxyfenozide (28.3%) + Spinetoram (5.66%)	Methoxyfenozide -Ecdysone receptor agonist (molt accelerating compound)	Spinetoram -Nicotinic acetylcholine receptor (nAChR) allosteric modulator	Dow AgroSciences LLC Indianapolis, IN

*Active ingredient modes of action were confirmed with the IRAC Mode of Action Classification Scheme version 8.4 (www.irac-online.org) and the Compendium of Pesticide Common Names: Insecticides (http://www.alanwood.net/pesticides/class_insecticides.html).

Table 3.2: Application rates administered, highest recommended application rates, and active ingredient concentrations of formulated premix insecticides assessed for acute contact toxicity to adult male (experiment 1) and female (experiment 2) blue orchard bees.

Treatment	Active Ingredient(s)	Application Rate Administered (mL/Ha)	Highest Application Rate on Label (mL/Ha)*	AI Concentration (ppm) in Formulated Product (per L)
A	Control			
B	Thiamethoxam (12.6%) + Lambda-cyhalothrin (9.48%)	401.76	438.28	Thiamethoxam = 120 Lambda-cyhalothrin = 91
C	Imidacloprid (21.0%) + Beta cyfluthrin (10.5%)	189.92	204.53	Imidacloprid = 98 Beta-cyfluthrin = 49
D	Chlorantraniliprole (9.26%) + Lambda-cyhalothrin (4.63%)	657.42	876.56	Chlorantraniliprole = 56 Lambda-cyhalothrin = 28
E	Methoxyfenozide (28.3%) + Spinetoram (5.66%)	657.42	876.56	Methoxyfenozide = 140 Spinetoram = 28

*Highest recommended application rate for orchard crops listed on the EPA regulated insecticide label for each respective insecticide.

Table 3.3: Active ingredients and modes of action of individual active ingredient insecticides assessed for acute contact toxicity to adult male (experiment 3) and female blue orchard bees (experiment 4).

Treatment	Formulation	Active Ingredient	Insecticide Mode of Action*	Manufacturer
A	Control			
B	Admire Pro®	Imidacloprid (42.8%)	Imidacloprid -Nicotinic acetylcholine receptor (nAChR) competitive modulator	BayerCropScience LP Research Triangle Park, NC
C	Baythroid XL®	Beta-cyfluthrin (12.7%)	Beta-cyfluthrin -Sodium channel modulator	BayerCropScience LP Research Triangle Park, NC
D	Admire Pro® + Baythroid XL®	Imidacloprid + Beta-cyfluthrin		

*Active ingredient modes of action were confirmed with the IRAC Mode of Action Classification Scheme version 8.4 (www.irac-online.org) and the Compendium of Pesticide Common Names: Insecticides (http://www.alanwood.net/pesticides/class_insecticides.html).

Table 3.4: Application rates administered, highest recommended application rates, and active ingredient concentrations of individual active ingredient insecticides assessed for acute contact toxicity to adult male (experiment 3) and female (experiment 4) blue orchard bees.

Treatment	Active Ingredient	Application Rate Administered (mL/Ha)*	Highest Application Rate on Label (mL/Ha)*	AI Concentration (ppm) in Formulated Product (per L)
A	Control			
B	Imidacloprid (42.8%)	102.27	204.53	Imidacloprid = 120
C	Beta-cyfluthrin (12.7%)	102.27	204.53	Beta-cyfluthrin = 26
D	Imidacloprid + Beta-cyfluthrin			

*Highest recommended application rate for orchard crops listed on the EPA regulated insecticide label for each respective insecticide.

Figures

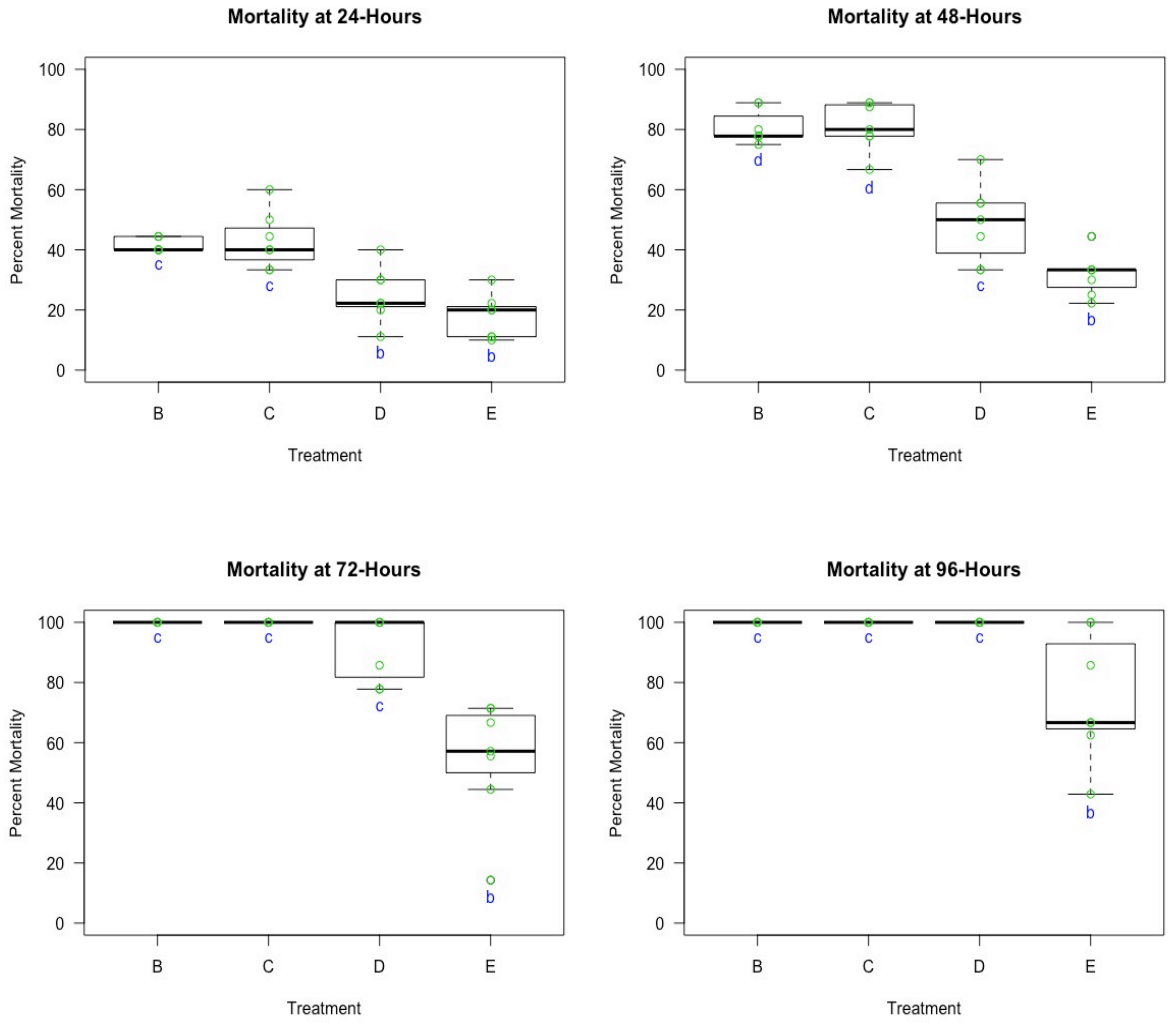


Figure 3.1: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides: Male blue orchard bee mean, 1st and 3rd quartile percent mortality following spray-only contact exposure to each insecticide mixture (experiment 1) at 24, 48, 72 and 96-hours. Data on the x-axis indicate each insecticide treatment, and data the y-axis indicate percent mortality. Insecticide treatments: B = thiamethoxam + lambda-cyhalothrin, C = imidacloprid + beta-cyfluthrin, D = chlorantraniliprole + lambda-cyhalothrin, E = methoxyfenozide + spinetoram. All cages in a treatment containing the respective percent mortality are represented by a single green point. Letters (b, c, d) indicate significant difference at *p<0.05 (pairwise comparison).

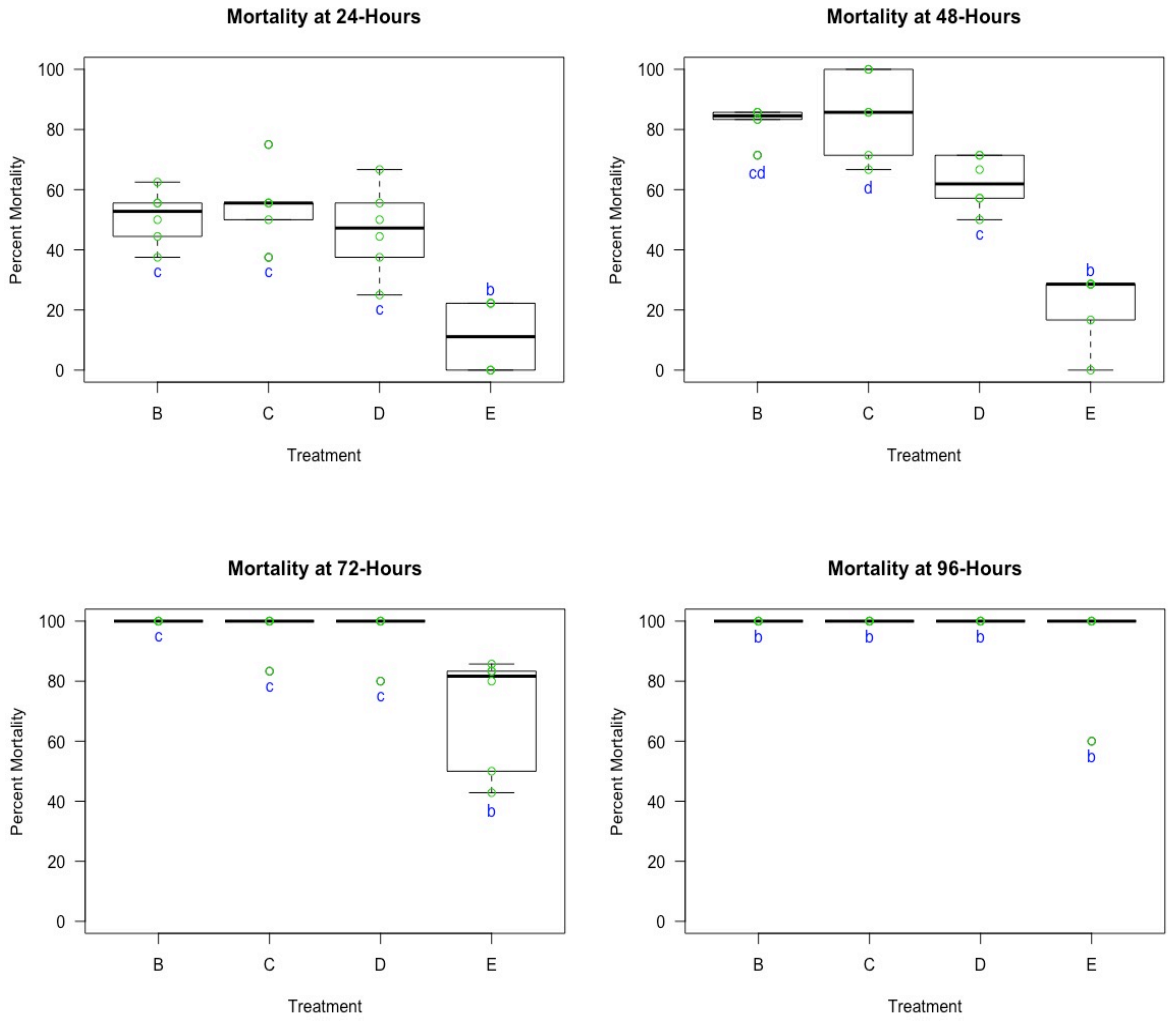


Figure 3.2: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides: Female blue orchard bee mean, 1st and 3rd quartile percent mortality following spray-only contact exposure to each insecticide mixture (experiment 2) at 24, 48, 72 and 96-hours. Data on the x-axis indicate each insecticide treatment, and data the y-axis indicate percent mortality. Insecticide treatments: B = thiamethoxam + lambda-cyhalothrin, C = imidacloprid + beta-cyfluthrin, D = chlorantraniliprole + lambda-cyhalothrin, E = methoxyfenozide + spinetoram. All cages in a treatment containing the respective percent mortality are represented by a single green point. Letters (b, c, d) indicate significant difference at *p<0.05 (pairwise comparison).

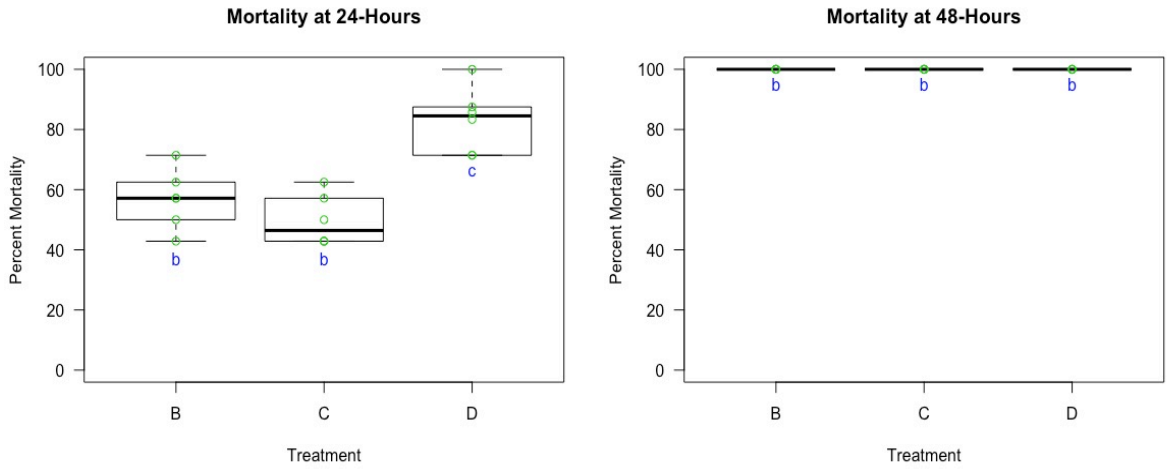


Figure 3.3: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides and their 1:1 Binary Combination: Male blue orchard bee mean, 1st and 3rd quartile percent mortality following spray-only contact exposure to each individual active ingredient insecticide (experiment 3) at 24 and 48-hours. Data on the x-axis indicate each insecticide treatment, and data the y-axis indicate percent mortality. Insecticide treatments: B = imidacloprid, C = beta-cyfluthrin, D = imidacloprid + beta-cyfluthrin. All cages in a treatment containing the respective percent mortality are represented by a single green point. Significant difference at *$p<0.05$ (pairwise comparison) is indicated by different letters (b, c).

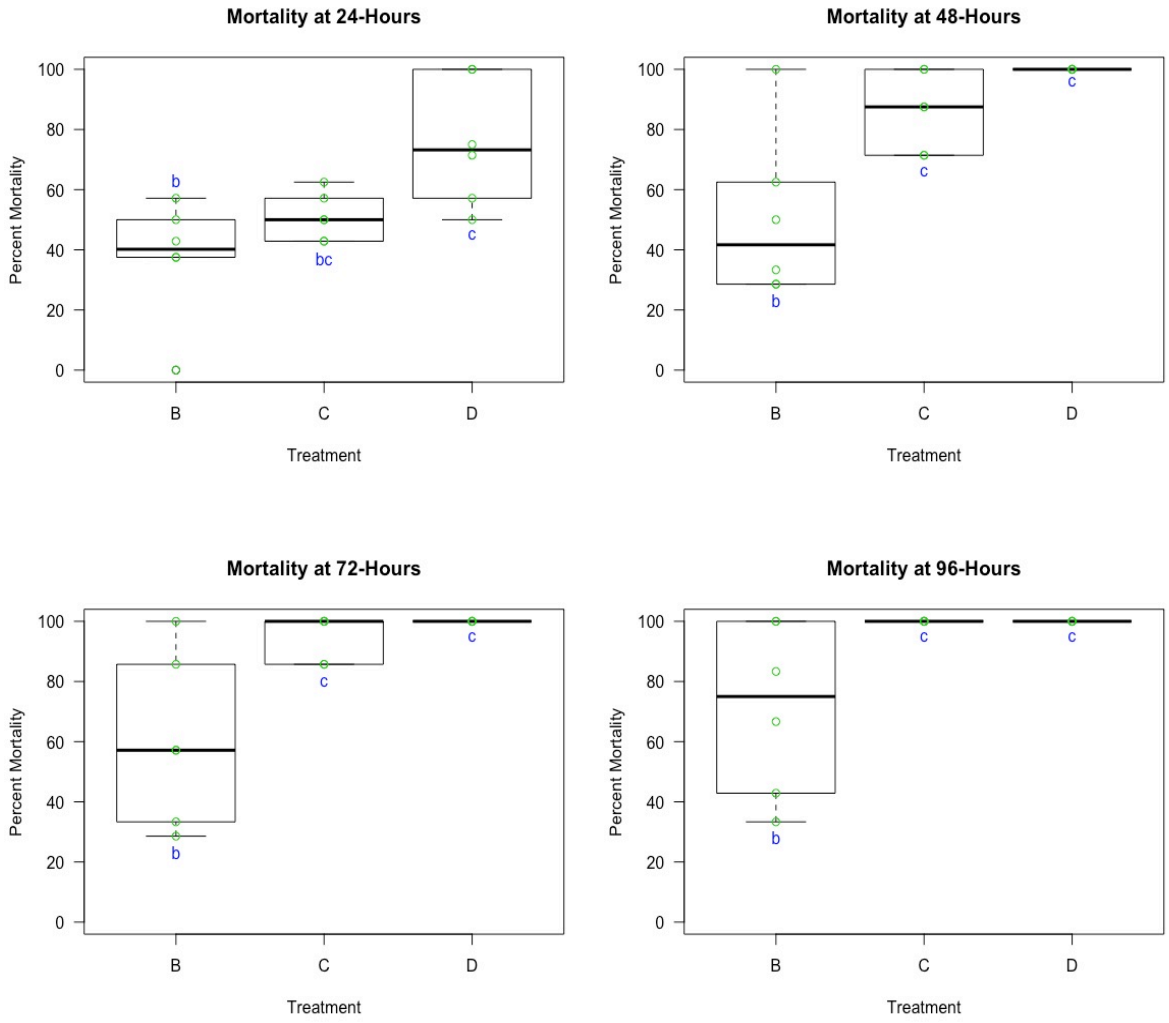


Figure 3.4: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides and their 1:1 Binary Combination: Female blue orchard bee mean, 1st and 3rd quartile percent mortality following spray-only contact exposure to each individual active ingredient insecticide (experiment 3) at 24 and 48-hours. Data on the x-axis indicate each insecticide treatment, and data the y-axis indicate percent mortality. Insecticide treatments: B = imidacloprid, C = beta-cyfluthrin, D = imidacloprid + beta-cyfluthrin. All cages in a treatment containing the respective percent mortality are represented by a single green point. Significant difference at *$p<0.05$ (pairwise comparison) is indicated by different letters (b, c).

References

Abbott, V. A., J. L. Nadeau, H. A. Higo, and M. L. Winston. **2008**. Lethal and Sublethal Effects of Imidacloprid on *Osmia lignaria* and Clothianidin on *Megachile rotundata* (Hymenoptera: Megachilidae). Journal of Economic Entomology. 101: 13.

Alston, D., and M. Murray. **2017**. Leafrollers in Fruit Orchards (No. ENT-185-17). Utah State University.

Artz, D. R., and T. L. Pitts-Singer. **2015**. Effects of Fungicide and Adjuvant Sprays on Nesting Behavior in Two Managed Solitary Bees, *Osmia lignaria* and *Megachile rotundata*. PLOS ONE. 10: e0135688.

Besard, L., V. Mommaerts, G. Abdu-Alla, and G. Smagghe. **2011**. Lethal and sublethal side-effect assessment supports a more benign profile of spinetoram compared with spinosad in the bumblebee *Bombus terrestris*. Pest Management Science. 67: 541–547.

Bibbs, C. S., A. Fulcher, and R.-D. Xue. **2015**. Allethrin-Based Mosquito Control Device Causing Knockdown, Morbidity, and Mortality in Four Species of Field-Caught Mosquitoes (Diptera: Culicidae). Journal of Medical Entomology. 52: 739–742.

Biddinger, D. J., J. L. Robertson, C. Mullin, J. Frazier, S. A. Ashcraft, E. G. Rajotte, N. K. Joshi, and M. Vaughn. **2013**. Comparative Toxicities and Synergism of Apple Orchard Pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). PLoS ONE. 8: e72587.

Bosch, J., and W. P. Kemp. **2001**. How to manage the blue orchard bee: as an orchard pollinator, Sustainable Agriculture Network handbook series. Sustainable Agriculture Network, Beltsville, MD.

Bosch, J., W. P. Kemp, and S. S. Peterson. **2000**. Management of *Osmia lignaria* (Hymenoptera: Megachilidae) Populations for Almond Pollination: Methods to Advance Bee Emergence. Environmental Entomology. 29: 874–883.

Bosch, J., W. P. Kemp, and G. E. Trostle. **2006**. Bee Population Returns and Cherry Yields in an Orchard Pollinated with *Osmia lignaria* (Hymenoptera: Megachilidae). Journal of Economic Entomology. 99: 408–413.

Brittain, C., N. Williams, C. Kremen, and A.-M. Klein. **2013**. Synergistic effects of non-*Apis* bees and honey bees for pollination services. Proceedings of the Royal Society B: Biological Sciences. 280: 20122767–20122767.

California Department of Pesticide Regulation Public Report: Active Ingredient: Spinetoram. **2007**. California Department of Pesticide Regulation Public Report: Active Ingredient: Spinetoram (No. Tracking ID Numbers 218411, 218412, 218413, 218414, 218415).

California Department of Pesticide Regulation Public Report: Methoxyfenozide. **2003**. California Department of Pesticide Regulation Public Report: Methoxyfenozide. (No. Tracking ID Numbers 195668 and 196546).

Cane, J. H. 1997. Ground-nesting bees: the neglected pollinator resource for agriculture. Proc. Int'l Symp. on Pollination: Acta Hort. 437.

ChemDraw® Prime. **2018**. ChemDraw® Prime. PerkinElmer Informatics, Inc.

Doering, J., C. Maus, and R. Schoening. **2004**. Residues of Imidacloprid WG 5 in Blossom and Leaf Samples of Apple Trees After Soil Treatment in the Field. Application: 2003, Sampling: 2004 (No. Bayer CropScience AG Report No. G201819). Bayer: AG, Monheim, Germany.

Ecological Risk Assessment for Section 3 Registration for Fruit, Vegetable, Selected Field Crop, Turf and Ornamental Uses of Chlorantraniliprole. PC Code 090100. **2008**. Ecological Risk Assessment for Section 3 Registration for Fruit, Vegetable, Selected Field Crop, Turf and Ornamental Uses of Chlorantraniliprole (PC Code 090100) (No. EPA-HQ-OPP-2007-0275-0005). USEPA/Office of Pesticide Programs.

Elbert, A., M. Haas, B. Springer, W. Thielert, and R. Nauen. **2008**. Applied aspects of neonicotinoid uses in crop protection. Pest Management Science. 64: 1099–1105.

FAO Specifications and Evaluations for Plant Protection Products: Beta-cyfluthrin. **1999**. FAO Specifications and Evaluations for Plant Protection Products: Beta-cyfluthrin. Food and Agriculture Organization of the United Nations.

Girolami, V., L. Mazzon, A. Squatini, N. Mori, M. Marzaro, A. Dibernardo, M. Greatti, C. Giorio, and A. Tapparo. **2009**. Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: a novel way of intoxication for bees. Journal of Economic Entomology. 102: 1808–1815.

Glazer, I., and A. Navon. **1990**. Activity and Persistence of Entomoparasitic Nematodes Tested Against *Heliothis armigera* (Lepidoptera: Noctuidae). Journal of Economic Entomology. 83: 1795–1800.

Gough, H. J., L. G. Collins, C. J. Everett, and W. Wilkinson. **1984**. Acute contact and oral toxicity to honey bees (*Apis mellifera*). (No. Reg. No. 10182-0A. Ace. No. 400524-09). ICI Americas Inc., Wilmington, DE.

Guidance on Exposure and Effects Testing for Assessing Risks to Bees. **2016**. Guidance on Exposure and Effects Testing for Assessing Risks to Bees. Office of Pesticide Programs U.S. Environmental Protection Agency.

Hoffmann, E. J., and S. J. Castle. **2012**. Imidacloprid in melon guttation fluid: a potential mode of exposure for pest and beneficial organisms. Journal of Economic Entomology. 105: 67–71.

Hopwood, J., A. Code, M. Vaughan, D. Biddinger, M. Shepherd, S. H. Black, E. Lee-Mäder, and C. Mazzacano. **2016**. How Neonicotinoids Can Kill Bees: The Science Behind the Role These Insecticides Play in Harming Bees (No. Second Edition, Revised and Expanded). The Xerces Society for Invertebrate Conservation, Portland, (OR) USA.

Horth, L., and L. A. Campbell. **2018**. Supplementing small farms with native mason bees increases strawberry size and growth rate. Journal of Applied Ecology. 55: 591–599.

IRAC International MoA Working Group: Approved by IRAC Executive. **2018**. IRAC Mode of Action Classification Scheme. Classification Scheme No. Version 8.4.

Isaacs, R. 2010. Blueberry insect management overview for 2010. No. 22, Berry Notes. University of Massachusetts Amherst Extension, Amherst, Massachusetts.

Iwasa, T., N. Motoyama, J. T. Ambrose, and R. M. Roe. **2004**. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Protection. 23: 371–378.

Johnson, R. M., Z. Wen, M. A. Schuler, and M. R. Berenbaum. **2006**. Mediation of Pyrethroid Insecticide Toxicity to Honey Bees (Hymenoptera: Apidae) by Cytochrome P450 Monooxygenases. Journal of Economic Entomology. 99: 5.

Koh, I., E. V. Lonsdorf, D. R. Artz, T. L. Pitts-Singer, and T. H. Ricketts. **2018**. Ecology and Economics of Using Native Managed Bees for Almond Pollination. Journal of Economic Entomology. 111: 16–25.

Kopit, A. M., and T. L. Pitts-Singer. **2018**. Routes of Pesticide Exposure in Solitary, Cavity-Nesting Bees. Environmental Entomology. 47: 499–510.

Ladurner, E., J. Bosch, W. P. Kemp, and S. Maini. **2005**. Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* (Say) and *Apis mellifera*. Apidologie. 36: 449–460.

Leskey, T. C., B. D. Short, and D.-H. Lee. **2013**. Efficacy of insecticide residues on adult *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) mortality and injury in apple and peach orchards: Residual insecticide efficacy on *Halyomorpha halys*. Pest Management Science. 70: 1097–1104.

Leverage 360® EPA Regulated Insecticide Label. **2015**. Leverage 360® EPA Regulated Insecticide Label.

Melito, S., S. La Bella, F. Martinelli, I. Cammalleri, T. Tuttolomondo, C. Leto, and M. Mulas. **2015**. Phenology study of *Myrtus communis* accessions selected from wild populations of Sicily: preliminary results. ISHA Acta Horticulture, XVI EUCARPIA Symposium on Fruit Breeding and Genetics. 1172.

Mogren, C. L., and J. G. Lundgren. **2016**. Neonicotinoid-contaminated pollinator strips adjacent to cropland reduce honey bee nutritional status. Scientific Reports. 6.

Mommaerts, V., G. Sterk, and G. Smagghe. **2006**. Bumblebees can be used in combination with juvenile hormone analogues and ecdysone agonists. Ecotoxicology. 15: 513–521.

Morrison, W. R., D.-H. Lee, B. D. Short, A. Khrimian, and T. C. Leskey. **2016**. Establishing the behavioral basis for an attract-and-kill strategy to manage the invasive *Halyomorpha halys* in apple orchards. Journal of Pest Science. 89: 81–96.

Mullin, C. A. 2015. Effects of 'inactive' ingredients on bees. Current Opinion in Insect Science. 10: 194–200.

Nauen, R., U. Ebbinghaus-Kintscher, and R. Schmuck. **2001**. Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: Apidae). Pest Management Science. 57: 577–586.

Nicholls, E., R. Fowler, J. E. Niven, J. D. Gilbert, and D. Goulson. **2017**. Larval exposure to field-realistic concentrations of clothianidin has no effect on development rate, over-winter survival or adult metabolic rate in a solitary bee, *Osmia bicornis*. PeerJ. 5: e3417.

OECD-214 Test Guidelines: OECD Guidelines for the Testing of Chemicals: Honey Bee Acute Contact Toxicity. **1998**. (OECD-214 Test Guidelines): OECD Guidelines for the Testing of Chemicals: Honey Bee Acute Contact Toxicity. OECD/OCDE.

Oliver, J. B., D. C. Fare, N. Yousef, S. S. Scholl, M. E. Reding, C. M. Ranger, J. J. Moyseenko, and M. A. Halcomb. **2010**. Evaluation of a Single Application of Neonicotinoid and Multi-Application Contact Insecticides for Flatheaded Borer Management in Field Grown Red Maple Cultivars. Journal of Environmental Horticulture. 28: 135–149.

Park, M. G., N. K. Joshi, E. G. Rajotte, D. J. Biddinger, J. E. Losey, and B. N. Danforth. **2018**. Apple grower pollination practices and perceptions of alternative pollinators in New York and Pennsylvania. Renewable Agriculture and Food Systems. 1–14.

Park, M. G., M. C. Orr, and B. N. Danforth. **2010**. The Role of Native Bees in Apple Pollination. New York Fruit Quarterly. 18: 5.

Pesticide Fact Sheet Number 164: Cyfluthrin. **1987**. Pesticide Fact Sheet Number 164: Cyfluthrin. U.S. Environmental Protection Agency, U.S. EPA, Office of Pesticide Programs. Washington, DC.

Peters, B., Z. Gao, and U. Zumkier. **2016**. Large-scale monitoring of effects of clothianidin-dressed oilseed rape seeds on pollinating insects in Northern Germany: effects on red mason bees (*Osmia bicornis*). Ecotoxicology. 25: 1679–1690.

Pitts-Singer, T. L., D. R. Artz, S. S. Peterson, N. K. Boyle, and G. I. Wardell. **2018**. Examination of a Managed Pollinator Strategy for Almond Production using *Apis mellifera* (Hymenoptera: Apidae) and *Osmia lignaria* (Hymenoptera: Megachilidae). Environmental Entomology. 47: 364–377.

Reaxys®. **2018**. Reaxys®. (https://0-www-reaxys-com.library.uark.edu/#/search/quick.

Rodrigo, J., and M. Herrero. **2002**. Effects of pre-blossom temperatures on flower development and fruit set in apricot. Scientia Horticulturae. 92: 125–135.

Ruddle, N., C. Elston, O. Klein, A. Hamberger, and H. Thompson. **2018**. Effects of exposure to winter oilseed rape grown from thiamethoxam-treated seed on the red mason bee *Osmia bicornis*: Thiamethoxam-treated oilseed rape and *Osmia bicornis* reproduction. Environmental Toxicology and Chemistry. 37: 1071–1083.

Rundlöf, M., G. K. S. Andersson, R. Bommarco, I. Fries, V. Hederström, L. Herbertsson, O. Jonsson, B. K. Klatt, T. R. Pedersen, J. Yourstone, and H. G. Smith. **2015**. Seed coating with a neonicotinoid insecticide negatively affects wild bees. Nature. 521: 77–80.

Sampson, B. J., S. J. Stringer, and D. A. Marshall. **2013**. Blueberry Floral Attributes and Their Effect on the Pollination Efficiency of an Oligolectic Bee, *Osmia ribifloris* Cockerell (Megachilidae: Apoidea). 48: 7.

Sandrock, C., L. G. Tanadini, J. S. Pettis, J. C. Biesmeijer, S. G. Potts, and P. Neumann. **2014**. Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success: Loss of pollinator fitness. Agricultural and Forest Entomology. 16: 119–128.

Sappington, K. G., and M. A. Ruhman. **2012**. (U.S. Environmental Protection Agency). Environmental Fate and Ecological Risk Assessment for Sulfoxaflor Registration. (No. EPA-HQ-OPP-2010-0889). US Environmental Protection Agency, Washington, D.C.: EPA.

Sato, N., Y. Shimokawatoko, T. Yamaguchi, and H. Tanaka. **2012**. Development of the Novel Insecticide Spinetoram (DIANA®). Sumitomo Kagaku. 14.

Schmuck, R., R. Schoning, A. Stork, and O. Schramel. **2001**. Risk posed to honey bees (*Apis mellifera* L., Hymenoptera) by an imidacloprid seed dressing of sun flowers. Pest Management Science. 57: 225–238.

Scott-Dupree, C. D., L. Conroy, and C. R. Harris. **2009**. Impact of Currently Used or Potentially Useful Insecticides for Canola Agroecosystems on *Bombus impatiens* (Hymenoptera: Apidae), *Megachile rotundata* (Hymentoptera: Megachilidae), and *Osmia lignaria* (Hymenoptera: Megachilidae). Journal of Economic Entomology. 102: 177–182.

Sgolastra, F., P. Medrzycki, L. Bortolotti, M. T. Renzi, S. Tosi, G. Bogo, D. Teper, C. Porrini, R. Molowny-Horas, and J. Bosch. **2017**. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species: Synergistic interactions between pesticides in three bee species. Pest Management Science. 73: 1236–1243.

Smagghe, G., J. Deknopper, I. Meeus, and V. Mommaerts. **2013**. Dietary chlorantraniliprole suppresses reproduction in worker bumblebees: Dietary chlorantraniliprole suppresses reproduction in worker bumblebees. Pest Management Science. 69: 787–791.

Stevenson, J. H. 1978. The Acute Toxicity of Un-formulated Pesticides to Worker Honey Bees (*Apis mellifera* L.). Plant Pathology. 27: 38–40.

Suiter, D. R., and M. E. Scharf. **2015**. Insecticide basics for the pest management professional. (UGA Bulletin No. 1352). University of Georgia.

Syngenta Group. 2005. Thiamethoxam: Used to formulate Patinum®, Actara®, Centric®, Cruiser®, Flagship®, and Helix®. (Envirofacts: Syngenta Crop Protection Fact Sheet.). Syngenta Crop Protection, Inc., Greensboro, NC.

Tanigoshi et al., L. K., G. Spitler Hollis, and B. S. Gerdeman. **2013**. Field Efficacy of Several Labled and Experimental Insecticides for Spotted Wing Drosophila Control in Blueberry, Section V: Pests of Wine Grapes & Small Fruits. Washington State University, Mount Vernon, WA.

Tanigoshi, L. K., B. S. Gerdeman, and G. Hollis Spitler. **2011**. Overview of Our First Season's Experiences to Chemically Manage the Spotted Wing Drosophila Through Lab and Field Research on Red Raspberry in Western Washington, Section I: Invasive & Emerging Pests. Washington State University, Mount Vernon, WA.

Torchio, P. F. 1982. Field experiments with *Osmia lignaria propinqua* Cresson as a pollinator in almond orchards: III, 1977 studies (Hymenoptera: Megachilidae). J. Kans. Entomol. Soc. 55: 101–116.

U.S. Environmental Protection Agency. **2003**. Pesticide Fact Sheet: Clothianidin. EPA-OPPTS., Washington, D.C.

U.S. Environmental Protection Agency. Registration Decision for the New Active Ingredients Flupyradifurone). **2015**. (U.S. Environmental Protection Agency). Registration Decision for the New Active Ingredients Flupyradifurone. No. EPA-HQ-OPP-2013-0226. US Environmental Protection Agency, Washington, D.C.: EPA.

US EPA Pesticide Labels. **2018**. US EPA Pesticide Labels. (https://www.epa.gov/pesticide-labels).

Vijverberg, H. P. M., J. M. van der Zalm, and J. van der Bercken. **1982**. Similar mode of action of pyrethroids and DDT on sodium channel gating in myelinated nerves. Nature. 295: 601–603.

Williams, H., H. Zoller, R. Roepke, E. Zschiesche, and A. R. Heckeroth. **2015**. Fluralaner activity against life stages of ticks using *Rhipicephalus sanguineus* and *Ornithodoros moubata* IN in vitro contact and feeding assays. Parasites & Vectors. 8: 90.

Wise, J. C., D. Hulbert, and C. Vandervoort. **2017**. Rainfall influences performance of insecticides on the codling moth (Lepidoptera: Tortricidae) in apples. The Canadian Entomologist. 149: 118–128.

Wood, A. 2018. Insecticides. Compendium of Pesticide Common Names. (http://www.alanwood.net/pesticides/class_insecticides.html).

Woodcock, B. A., J. M. Bullock, R. F. Shore, M. S. Heard, M. G. Pereira, J. Redhead, L. Ridding, H. Dean, D. Sleep, P. Henrys, J. Peyton, S. Hulmes, L. Hulmes, M. Sárospataki, C. Saure, M. Edwards, E. Genersch, S. Knäbe, and R. F. Pywell. **2017**. Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science. 356: 1393–1395.

Zhu, Y. C., J. Adamczyk, T. Rinderer, J. Yao, R. Danka, R. Luttrell, and J. Gore. **2015**. Spray Toxicity and Risk Potential of 42 Commonly Used Formulations of Row Crop Pesticides to Adult Honey Bees (Hymenoptera: Apidae). Journal of Economic Entomology. 108: 2640–2647.

Appendices

Appendix 3.1: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides: One-way ANOVA test of statistical significance between male blue orchard bee mortality following exposure to each insecticide treatment and Tukey HSD post-hoc test of statistical difference between treatments on male blue orchard bee mortality. Statistical significance at *p<0.05.

One-way ANOVA test: 24-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	0.36872	0.122905	18.059	2.39E-06
Residuals	24	0.16333	0.006806		

Tukey HSD post-hoc test: 24-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.0146617	0.04409593	24	-0.332	0.987
B - D	0.17799173	0.04409593	24	4.036	0.0025
B - E	0.25333976	0.04409593	24	5.745	<.0001
C - D	0.19265344	0.04409593	24	4.369	0.0011
C - E	0.26800148	0.04409593	24	6.078	<.0001
D - E	0.07534803	0.04409593	24	1.709	0.3412

One-way ANOVA test: 48-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	2.11602	0.70534	48.053	2.68E-10
Residuals	24	0.35228	0.01468		

Tukey HSD post-hoc test: 48-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.007997	0.0647598	24	-0.123	0.9993
B - D	0.43160957	0.0647598	24	6.665	<.0001
B - E	0.62528889	0.0647598	24	9.656	<.0001
C - D	0.43960655	0.0647598	24	6.788	<.0001
C - E	0.63328588	0.0647598	24	9.779	<.0001
D - E	0.19367933	0.0647598	24	2.991	0.0301

One-way ANOVA test: 72-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	4.5158	1.50528	35.37	5.70E-09
Residuals	24	1.0214	0.04256		

Tukey HSD post-hoc test: 72-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-2.18E-16	0.1102699	24	0	1
B - D	2.71E-01	0.1102699	24	2.462	0.0921
B - E	9.82E-01	0.1102699	24	8.905	<.0001
C - D	2.71E-01	0.1102699	24	2.462	0.0921
C - E	9.82E-01	0.1102699	24	8.905	<.0001
D - E	7.10E-01	0.1102699	24	6.443	<.0001

One-way ANOVA test: 96-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	1.9323	0.64411	12.824	3.35E-05
Residuals	24	1.2055	0.05023		

Tukey HSD post-hoc test: 96-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-5.21E-16	0.1197947	24	0	1
B - D	-6.17E-16	0.1197947	24	0	1
B - E	6.07E-01	0.1197947	24	5.064	0.0002
C - D	-9.62E-17	0.1197947	24	0	1
C - E	6.07E-01	0.1197947	24	5.064	0.0002
D - E	6.07E-01	0.1197947	24	5.064	0.0002

Appendix 3.2: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Premix Insecticides: One-way ANOVA test of statistical significance between female blue orchard bee mortality following exposure to each insecticide treatment and Tukey HSD post-hoc test of statistical difference between treatments on female blue orchard bee mortality. Statistical significance at *p<0.05.

One-way ANOVA test: 24-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	0.845	0.281667	14.859	2.55E-05
Residuals	20	0.37911	0.018956		

Tukey HSD post-hoc test: 24-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.0502346	0.07948911	20	-0.632	0.9205
B - D	0.0469591	0.07948911	20	0.591	0.9337
B - E	0.42491062	0.07948911	20	5.346	0.0002
C - D	0.09719369	0.07948911	20	1.223	0.6202
C - E	0.47514521	0.07948911	20	5.977	<.0001
D - E	0.37795152	0.07948911	20	4.755	0.0006

One-way ANOVA test: 48-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	2.84269	0.94756	22.135	1.45E-06
Residuals	20	0.85616	0.04281		

Tukey HSD post-hoc test: 48-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.1452337	0.1194542	20	-1.216	0.6244
B - D	0.2989819	0.1194542	20	2.503	0.09
B - E	0.7547433	0.1194542	20	6.318	<.0001
C - D	0.4442156	0.1194542	20	3.719	0.0068
C - E	0.899977	0.1194542	20	7.534	<.0001
D - E	0.4557614	0.1194542	20	3.815	0.0055

One-way ANOVA test: 72-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	2.16541	0.7218	14.869	2.53E-05
Residuals	20	0.97088	0.04854		

Tukey HSD post-hoc test: 72-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	0.09761426	0.1272057	20	0.767	0.8681
B - D	0.10725019	0.1272057	20	0.843	0.8333
B - E	0.75517576	0.1272057	20	5.937	<.0001
C - D	0.00963593	0.1272057	20	0.076	0.9998
C - E	0.6575615	0.1272057	20	5.169	0.0003
D - E	0.64792557	0.1272057	20	5.094	0.0003

One-way ANOVA test: 96-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	3	0.10748	0.035828	1	4.13E-01
Residuals	20	0.71656	0.035828		

Tukey HSD post-hoc test: 96-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	6.22E-16	0.1092828	20	0	1
B - D	6.11E-16	0.1092828	20	0	1
B - E	1.55E-01	0.1092828	20	1.414	0.5054
C - D	-1.16E-17	0.1092828	20	0	1
C - E	1.55E-01	0.1092828	20	1.414	0.5054
D - E	1.55E-01	0.1092828	20	1.414	0.5054

Appendix 3.3: Male Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides and their 1:1 Binary Combination: One-way ANOVA test of statistical significance between male blue orchard bee mortality following exposure to each insecticide treatment and Tukey HSD post-hoc test of statistical difference between treatments on male blue orchard bee mortality. Statistical significance at *p<0.05.

One-way ANOVA test: 24-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	2	0.92329	0.46165	13.098	5.12E-04
Residuals	15	0.5287	0.03525		

Tukey HSD post-hoc test: 24-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	0.08633774	0.108392	15	0.797	0.7108
B - D	-0.4314184	0.108392	15	-3.98	0.0033
C - D	-0.5177561	0.108392	15	-4.777	0.0007

One-way ANOVA test: 48-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	2	9.86E-32	4.93E-32	1	3.91E-01
Residuals	15	7.40E-31	4.93E-32		

Tukey HSD post-hoc test: 48-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	1.57E-16	1.28E-16	15	1.225	0.4574
B - D	1.57E-16	1.28E-16	15	1.225	0.4574
C - D	-7.40E-32	1.28E-16	15	0	1

Appendix 3.4: Female Blue Orchard Bee Acute Contact Toxicity Following Spray-Only Exposure to Individual Active Ingredient Insecticides and their 1:1 Binary Combination: One-way ANOVA test of statistical significance between female blue orchard bee mortality following exposure to each insecticide treatment and Tukey HSD post-hoc test of statistical difference between treatments on female blue orchard bee mortality. Statistical significance at *p<0.05.

One-way ANOVA test: 24-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	2	1.157	0.5785	6.3879	9.84E-03
Residuals	15	1.3584	0.09056		

Tukey HSD post-hoc test: 24-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.1454746	0.1737444	15	-0.837	0.6864
B - D	-0.5955922	0.1737444	15	-3.428	0.0098
C - D	-0.4501175	0.1737444	15	-2.591	0.0506

One-way ANOVA test: 48-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	2	2.7522	1.37609	11.266	1.03E-03
Residuals	15	1.8322	0.12215		

Tukey HSD post-hoc test: 48-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.5291339	0.2017821	15	-2.622	0.0477
B - D	-0.9559852	0.2017821	15	-4.738	0.0007
C - D	-0.4268513	0.2017821	15	-2.115	0.1202

One-way ANOVA test: 72-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	2	2.2851	1.14257	10.972	1.16E-03
Residuals	15	1.5621	0.10414		

Tukey HSD post-hoc test: 72-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-0.6493343	0.186315	15	-3.485	0.0088
B - D	-0.8297008	0.186315	15	-4.453	0.0013
C - D	-0.1803665	0.186315	15	-0.968	0.6073

One-way ANOVA test: 96-hour mortality screening

	DF	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Treatment	2	1.5923	0.79615	8.2398	3.85E-03
Residuals	15	1.4493	0.09662		

Tukey HSD post-hoc test: 96-hour mortality screening

Contrast	Estimate	SE	DF	t-ratio	p-value
B - C	-6.31E-01	0.1794653	15	-3.516	0.0083
B - D	-6.31E-01	0.1794653	15	-3.516	0.0083
C - D	2.22E-16	0.1794653	15	0	1

Chapter IV: Conclusions

The goal of our research was to expand upon previous work investigating the ecotoxicology of insecticides commonly used in agro-ecosystems to *Apis* and non-*Apis* bees. Implementation of the Food Quality Protection Act of 1996 combined with increased arthropod pest pressures, invasive species, and subsequent resistance buildup have spurred the development of a new generation of insecticide chemistries. These insecticides are specifically designed to aid growers in maintaining agricultural insect pests at tolerable levels. Neonicotinoids (which function as nicotinic acetylcholine receptor agonists) are among this new generation of insecticides. Premix insecticides containing multiple active ingredients (each with a different mode of action; some of which are neonicotinoids) are newly registered insecticides that are currently used in a variety of agro-ecosystems. Bees commonly forage agricultural settings in which these premix insecticides are applied, therefore a thorough assessment of the hazards they pose to bees and other pollinators is warranted. Although contact toxicity following exposure to premix insecticides has been preliminarily explored in adult honey bee workers, to our knowledge, no detail analysis comparing honey bees to non-*Apis* bees that tests various realistic exposure scenarios has been published.

To fill this gap in bee toxicology, we assessed the acute contact toxicity following whole body contact exposure to four premix insecticides for honey bee foragers and male and female blue orchard bees. Honey bees were exposed to insecticides in a laboratory setting simulating three possible field-realistic scenarios: (1) when bees continuously forage in a crop field, and are directly exposed to insecticides while they are applied in that crop field; (2) when bees are exposed to insecticide applications while passing through a crop field; and (3) when bees are foraging in a crop field where insecticides were applied during the previous day. Using a similar experimental design, we assessed the relative toxicity of different insecticides to blue orchard

bees in a laboratory setting simulating two possible field-realistic scenarios: (1) the four premix insecticides and (2) individual active ingredients (and their 1:1 binary combination) comprising one of the premix insecticides tested. Male and female blue orchard bees were tested separately in each scenario therefore we conducted a total of four experiments on this bee.

For all three honey bee experiments, we observed statistical significance between treatments at all screening periods, except for residual toxicity at the 48-hour observation (due to the complete mortality observed in this experiment at this time frame). We found that exposure to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin posed the highest toxicity and induced a rapid onset of mortality. We attribute this result to the combined neonicotinoid and pyrethroid active ingredients in both of these insecticide mixtures. However, the pyrethroid chemistries, which are sodium channel modulators probably accentuated this response given their characteristic fast knockdown. These results are suggestive of an additive or synergistic toxicity arising from exposure to the combination of both active ingredients. Conversely, contact exposure to chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram induced a more gradual onset of mortality. However, by extended observation (96-hours) honey bee mortality was close to complete. Considering insecticide mode of action, we attribute the higher mortality in chlorantraniliprole + lambda-cyhalothrin to the presence of a pyrethroid. Oral consumption of chlorantraniliprole has also been found to interfere with bumble bee feeding, so it is possible that a similar effect might have inhibited honey bee feeding in our bioassays. Overall, we found that methoxyfenozide + spinetoram posed the lowest toxicity to honey bees, which we attribute to the lack of a pyrethroid. This result on adult Hymenopterans is not entirely surprising given the presence of methoxyfenozide, which is a narrow-spectrum ecdysone receptor agonist intended for immature Lepidopterans. Secondarily, the relatively

benign toxicity of methoxyfenozide and spinetoram to bumble bees has been demonstrated in recent literature, so it is plausible that these insecticides also pose low risk to honey bees. However, more research is needed to confirm the low toxicity of these insecticides to honey bees.

Our finding of complete honey bee mortality at 48-hours for residual exposure might in part have been influenced by several variables. First, we sprayed cages with double the volume of insecticide to ensure complete coverage. Second, we collected honey bees from hives in high temperature and humidity conditions. Therefore, the high humidity in cage environments while still in the field might have increased the energetic stress placed on honey bees. Also, we believe that the humid conditions liquified the dried insecticide residues within cages. Third, we observed honey bee proboscis extension after being placed in cages, so it is possible that bees obtained both oral and contact exposure to insecticides.

For all four blue orchard bee experiments, resulting mortality was statistically significant between treatments at all screening periods except for female premix exposure at 96-hours and male individual active ingredient exposure at 48-hours given complete mortality in all treatments. For the premix insecticide exposure scenario, we found that thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin were the most toxic and resulted in a rapid onset of mortality. We observed quick knockdown characterized by trembling and an incapacity to fly for both male and female blue orchard bees within 25 minutes of treatment. As with honey bees, we primarily attribute these results to the combination of neonicotinoid and pyrethroid chemistries, but with an emphasis on the pyrethroids given their characteristic knockdown.

Similar to honey bees, male and female blue orchard bees exposed to chlorantraniliprole + lambda-cyhalothrin and methoxyfenozide + spinetoram had a slower onset of mortality. As

with honey bees, high mortality was observed for these treatments at extended observation (96-hours) in blue orchard bees. Interestingly, we observed that blue orchard bees exposed to chlorantraniliprole + lambda-cyhalothrin had similar mortality levels to those exposed to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin treatments at extended observation. We noticed some knockdown activity at 24-hours for this treatment, which we attribute to the presence of a pyrethroid active ingredient. However, knockdown in this treatment was less pronounced than that observed for bees exposed to thiamethoxam + lambda-cyhalothrin and imidacloprid + beta-cyfluthrin. Conversely, we did not observe knockdown activity for blue orchard bees treated with methoxyfenozide + spinetoram, which we attribute to the lack of a pyrethroid active ingredient.

Male and female blue orchard bee whole body contact exposure exposure to imidacloprid, beta-cylfuthrin and their 1:1 binary combination resulted in overall high mortality. We observed complete mortality for males at 48-hours post treatment, and for females at 96-hours. At 24-hours, male and females displayed trembling behaviors in their wings, legs and antennae, thereby suggesting knockdown activity. As before, we primarily attribute this behavior to the presence of a pyrethroid. Interestingly, females exposed to imidacloprid also displayed trembling behaviors at 24-hours, indicating that exposure to this neonicotinoid might cause similar sub-lethal toxicity. Blue orchard bees treated with the 1:1 binary combination had statistically higher mortality, thereby futher supporting that an interaction occurs between these two insecticides that increases the toxicity of their combination. However, the higher proportion of active ingredient concentration in the individual active ingredients, compared to that in the premix insecticides might in part explain this result.

Our results support the evaluation of several current regulatory guidelines for assessing

the toxicity of insecticides to bees. First, requiring extended mortality observation will account for insecticides that result in high bee mortality, but at a slower rate, as we have demonstrated. Second, mandating simultaneous testing of both technical grade insecticides dissolved in acetone and formulated insecticides dissolved in water allows for an independent evaluation of the impact of active ingredient and 'inert' products on bees. Third, requiring separate testing of *Apis* and non-*Apis* bees enables a more realistic assessment of toxicity assessment that accounts for differences in biology between bee species. Fourth, placing more emphasis on examining the impacts of dried insecticide residues on *Apis* and non-*Apis* bees will aid in determining appropriate times to apply insecticides to different crops that bees forage. This information will also help us determine the correct time to remove or otherwise protect beehives or nests from agro-ecosystems in advance of insecticide application and the appropriate time to replace them.

Future studies should build upon our work on honey bees and blue orchard bees by specifically testing multiple insecticide doses to generate dose mortality curves. This analysis will enable determination of additive or synergistic toxicity arising from the combination of insecticide chemistries in premixes by testing the hypothesis of parallel or equal slopes. A more robust analysis of more insecticides and the individual active ingredients and their 1:1 binary combination (that tests single active ingredients containing the same percentage of active ingredient as the premixes) is warranted. Finally, an analysis of acute oral toxicity and sub-lethal toxicity of these insecticides to *Apis* and non-*Apis* bees would enhance our understanding of the effect of these insecticides for risk assessment.