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Examining Risks to Honey Bee Pollinators Foraging in Agricultural Landscapes

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Examining Risks to Honey Bee Pollinators Foraging in Agricultural Landscapes

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by

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Abstract

Bee pollinators provide essential ecological services to wild plant communities, and add tremendous economic value to agriculture by improving both the quality and quantity of crop yield. Beekeepers are often contracted by growers to provide colonies of honey bees for pollination of high-value produce (fruits, vegetables and nuts). Many of the major commodity crops produced in the central and mid-southern United States are wind-pollinated (rice, corn, grain sorghum, wheat), or are sufficiently self-fertile (soybeans, cotton), and so do not require bee pollination in order to produce yield. Beekeepers still rely on these agricultural landscapes to support honey bee colonies when not actively pollinating farms or orchards because these landscapes remain irrigated and productive while other areas may endure a long seasonal nectar dearth. However, intensely managed agricultural landscapes can also expose bees to a variety of detrimental risks, including reduced plant diversity and nutrition, and increased pesticide exposure. Neonicotinoid insecticides have been blamed for recent widespread losses of honey bee colonies in the U.S. and abroad. The planting of insecticide-coated seeds to protect plant growth from early season insect damage has come under particular scrutiny as a potentially significant factor in honey bee declines. Previous investigations have concluded with inconsistent results, based on varying methods employed, seasons and environments, and the scale of the experiments. This study characterized the landscape where seed treatments were common, in terms of floral resources available to bees, sources of contamination. A radius of 2 miles (3.2 km) around an apiary was surveyed for 2 seasons to determine the land use by crop, and to quantify the proportion planted with treated seeds, and what other products were applied during the cropping season, and which of these compounds were found in bee hives. Our survey found that approximately 81% of the landscape was under cultivation, of which 70% was planted

with neonicotinoid treated seeds. However, no neonicotinoids were detected in samples of bee hive products. Because pollen could be sampled directly from foraging bees at discrete intervals, and traced back to plant origin, it was used as a bioindicator to determine when neonicotinoids might be present in crops or wild plants. Bees collected relatively little pollen from crops except for a brief period of hot, dry weather. Neonicotinoids were detected infrequently and at low levels, and not at all when bees were visiting crop plants. To test the effects of neonicotinoid ingestion on individual bees *in situ*, a method was devised to continuously monitor the activities of individual honey bees fed with a sublethal concentration of imidacloprid. Bees that consumed 20 ppb imidacloprid did not suffer acute mortality, but actually appeared to survive 1.7 times as long as untreated bees. This work suggests that neonicotinoids, when properly utilized, may not necessarily pose a greater risk to honey bees than other agricultural chemicals, provided colonies have access to sufficient alternative nutritional sources in the surrounding landscape.

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Dedication

This dissertation is dedicated to the memory of my mother. She was the first person to engender in me a sense of wonder and curiosity about the natural world. She also filled me with a foundation of faith, and encouraged my creativity. She helped to shape the person I became.

Thanks, Mom!

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Zawislak, J., Adamczyk, J., Johnson, D.R., Lorenz, G., Black, J., Hornsby, Q., Stewart, S.D. and Joshi, N. (2019). Comprehensive survey of area-wide agricultural pesticide use in Southern United States row crops and potential impact on honey bee colonies. *Insects*, 10(9): 280. doi.org/10.3390/insects10090280

Chapter III:

Zawislak, J., Lorenz, G., Adamczyk, J., Wiedenmann, R. and Joshi, N.K. (2021). Proportion of commodity crop pollens and pesticide contamination in honey bee diets in two different landscapes. *Environmental Advances*, 5:100116. doi.org/10.1016/j.envadv.2021.100116

Chapter I

Introduction and Literature Review

The importance of bee pollinators

Pollinators are essential components of agriculture for many crops, either as a necessary input for fruit, seed or nut production, or as an input to increase and optimize the quality and quantity of that yield (Reilly et al. 2020). Calderone (2012) estimated that insect pollination, directly and indirectly, contributed $29 billion to the U.S. agricultural economy in 2010, and that the contribution of just one species, the honey bee (*Apis mellifera*), accounted for $19.2 billion of that total. Pollination by other insects, including bumble bees, alfalfa leafcutter bees, and mason bees, accounted for another $9.9 billion. A review by Jordan et al. (2021) more recently estimated the economic value of insect pollination services in the U.S. to be $34 billion. While they did not establish the value of honey bees specifically, using the same proportion as Calderone (2012), the contribution of honey bees could be estimated to add $22.5 billion to U.S. agriculture annually.

The economic value of beeswax and honey production is minor compared to the value of the fruits, vegetables, seeds, nuts and fiber that are optimized by the actions of sufficient pollinators (Delaplane et al. 2000). Around 60% of global food crops produced do not require animal pollination (including self-fertile legumes and anemophilous cereal grains such as wheat, corn and rice), but 35% of world crops do need pollinator assistance, and 5% were unevaluated (Klein et al. 2006). The agricultural products that are most dependent on insect pollination tend to be fruits, vegetables and nuts that are produced on smaller acreages, but command higher

market value, and are worth an average of five times as much per ton than commodities that are self- or wind-pollinated (Gallai et al. 2009). For crops that are dependent on insect pollination, maximizing pollinator visits are the most effective way to maximize production. Most other agricultural inputs, such as fertilizer, pest control and weed management, are primarily used to minimize losses subsequent to the pollination of the flowers, as seeds and fruits develop.

Honey Bees and Commercial Agriculture

The honey bee, *Apis mellifera*, remains the most commonly utilized managed pollinator for commercial agricultural production for numerous reasons. Honey bees maintain large social colonies, each of which represents a significant workforce unit. Because their colonies are perennial, unlike most solitary bees or bumble bees, colonies of honey bees can be made available for pollination services at practically any point during a growing season.

This is particularly important for the California almond industry, where trees begin blooming in mid-February when few other pollinators are available (Traynor 2017). Almonds are completely dependent on bee pollination to produce yield, which was valued at $5.6 billion in 2020 (USDA-NASS 2021). The demand for honey bee pollination for almonds in 2020 was 2.4 million colonies, with 1.9 million of those being shipped in from other states (Goodrich and Durant 2021). Once almond bloom has finished, these colonies are transported to other crops in need of pollination, or placed in areas for spring honey production.

Because honey bees are naturally cavity dwellers, suitable manmade hives can easily and conveniently be transported where and when they are needed. Honey bees are also generalist feeders, and can therefore be utilized for pollination in a wide variety of crop situations, unlike

some other bee species which may be more highly specialized pollinators for a particular flower shape or structure. The honey bee keeping industry is well developed and widespread, and the pollination requirements of many important crops have been well-established and documented (Delaplane et al. 2000). Bee colonies are also widely maintained for honey production as well as for pollination. As a result, honey bee colonies can usually be obtained for pollination on demand.

A well-developed industry of migratory beekeepers can provide pollination services when and where necessary. These mobile operations, as well as stationary commercial beekeepers, all require suitable foraging territory for their honey bee stocks that are not actively pollinating contracted crop land. Honey bee colonies are often placed on uncultivated land adjacent to agricultural production areas. Area crops may or may not benefit from the presence of bee pollinators, but the crops and flowering plants on surrounding uncultivated land can provide suitable forage to sustain large honey bee populations (Alburaki et al. 2018, Durant 2019, Sponsler and Johnson 2015, Zawislak et al. 2021).

Over the past half century, the demand for pollinator-dependent fruits and other crops has increased dramatically (Aizen et al. 2009), while populations of pollinators and other insects have simultaneously been declining (VanEngelsdorp et al. 2009, Sánchez-Bayo and Wyckhuys 2019). Due to the scale and geographical distribution of modern intensive agricultural production, some of the areas that require the largest numbers of honey bee colonies for brief periods of commercial pollination are often areas that cannot support sufficient honey bee populations throughout the rest of the year (Koh *et al* 2016). Approximately 20% of U.S. counties require 80% of the total agricultural pollination, but these same intensively cultivated areas also have the lowest density of wild pollinators (Jordon et al. 2021).

Some native bee species are better suited than honey bees for certain specialty crop systems. Commercially reared bumble bees, mason bees, or alfalfa leafcutter bees are cultured by growers some situations (Bosch and Kemp 2002, Garibaldi et al. 2013, Richards 1990, Ryder et al. 2020), but these pollinators may not be dependable in all circumstances or may not be suitable for large scale agricultural needs. The potential for pathogen spillover from commercially-reared bumble bee colonies into native populations is a current concern to ecologists (Goulson 2010, Murray et al. 2013, Seabra et al. 2019), as well as transmission of pathogens through the introduction of novel non-native bee species (Hedtke et al. 2015).

The abundance and diversity of many native bee species are also being negatively impacted by agricultural practices (Belsky and Joshi 2019, Huang et al. 2021, Klein et al. 2006, Kremen et al. 2002, Kline and Joshi 2020, Main et al. 2020). Globally, the percentage of land devoted to agriculture has been increasing (St. Clair et al. 2020), along with the production of pollinator-dependent cropland (Aizen et al. 2009), while at the same time agricultural practices such as large-scale monoculture plantings, increased herbicide use, and pest control techniques threaten to reduce pollinator population abundance and diversity, and thus the frequency of pollinator visitation (Eeraerts et al. 2017, Forister et al. 2019, Kevan and Phillips, 2001, Kluser and Peduzzi 2007, Kovács-Hostyánszki et al. 2017, Nicholson et al. 2017).

Agricultural landscapes and Exposure of Bees to Various Pesticides

Agricultural landscapes, especially monocultures, can pose hazards to honey bee health due to limited floral nutrition (Ament et al. 2010, Earls et al. 2018, Huang 2012) and the potential for pesticide exposure (Škerl et al. 2009, Johnson et al. 2010, Otto et al. 2016, Mullin et

al. 2010, Thompson 2010, Krupke et al. 2012, Pettis et al. 2012, Johnson 2015). Changes in land management have caused a great deal of natural wildlife habitat to be converted to urban, recreational or agricultural uses. Areas of intensively cultivated and irrigated farmland, with smaller patches of semi-natural landscapes, may be the only landscapes available with sufficient floral resources to support the large numbers of honey bee colonies necessary to sustain other segments of intensive agriculture. Westphal et al. (2003) determined that wild bee density in Europe was positive correlated with the mass-flowering crops, but that these same large monocultures could also be detrimental at a large-scale densely cultivated landscape level. Improved plant diversity and the conservation of natural and semi-natural habitats within agroecosystems can provide not only nutrition (Cole et al. 2022, Kline and Joshi 2020, St. Clair et al. 2020, Vaudo et al. 2015) but also suitable nesting habitat for solitary bees and bumble bees (Kells and Goulson 2003, Kline and Joshi 2020, Svensson et al. 2000), which helps to conserve and improve pollinator species richness and abundance (Amy et al. 2018) and improve biological control (Snyder 2019).

Historically honey bees have persisted in a very clean environment, flying directly from a relatively sterile hive to visit floral food resources and then back again, contacting few contaminates or pathogens. Worker honey bees can come into contact with numerous potential toxins while foraging, but reproductive members of the colony (queens and drones) often do not. Contaminated food is usually diluted, processed and stored with food from other sources. While this behavior reduces the risks of acute toxicity to the reproductive castes, it simultaneously reduces selection pressure for honey bees to develop tolerance to a particular compound. Because of their division of labor, foraging bees which do survive exposure to toxins will not reproduce, and therefore cannot directly contribute heritable traits that may have assisted their

survival. Likewise, the deaths of these non-reproductive foragers exposed to acute toxins outside the hive only indirectly affect the reproductive fitness of the queen bee.

Insects living in large eusocial colonies tend to rely on behavioral mechanisms, rather than strong biological responses, to cope with disease as well (Cremer et al. 2007, Fefferman et al. 2007, López-Uribe et al. 2016, Naug and Camazine 2002, Simone-Finstrom et al. 2017, Wilson-Rich et al. 2009) and may not be able to respond quickly when exposed to novel or exotic pathogens or parasites. This same behavior in honey bees likely limits their ability to rapidly adapt to novel toxins in the environment. Honey bees do have an immune system to protect against pathogens, but it is relatively less developed than that of other insect species (Larsen et al. 2019). These social bees also rely on the antimicrobial properties of the propolis envelope enclosing their nest (Borba and Spivak 2017, Dalenberg et al. 2020, Simone-Finstrom et al. 2017) and on the hygienic behavior of nest mates (Gilliam et al. 1983, Khan and Ghramh 2021, Spivak and Reuter 2001) to reduce biotic contamination.

Individual honey bees also possess relatively few genes associated with detoxification, making them particularly susceptible to chemical contaminants in their environment (Berenbaum and Johnson 2015, Gong and Diao 2017). Unlike phloem- or foliage-feeding insects, honey bees have not been subjected to strong evolutionary pressures to develop complex physiological methods to detoxify dietary compounds as herbivorous arthropods have had to do (Dowd et al. 1983, Wu *et al* 2015).

Honey Bees and Environmental Stressors

Populations of honey bees and other pollinators have declined significantly in recent years, both in the U.S. and abroad (Dainat et al. 2012, Engelsdorp et al. 2010, Kluser et al. 2010, Neumann and Carreck 2010, Tylianakis 2013, Watanabe 2008, Williams et al. 2010). The reasons for their declines are numerous, but are generally thought to be caused by multiple interacting stress factors (Cox-Foster et al. 2007, VanEngelsdorp et al. 2009, Goulson et al. 2015, Johnson 2015, Speybroeck et al. 2010).

Many in the scientific community agree that the primary threat to honey bee health is a complex of the ectoparasitic mite *Varroa destructor* and numerous associated viruses which these mites are known to vector (Benaets et al. 2017, Bowen-Walker and Gunn 2001, Downey et al. 2000, Francis et al. 2013, Genersch 2010, Dietemann et al. 2012, Manley et al. 2015, Solignac et al. 2005). Other pathogens (Higes et al. 2013) and interactions between pathogens and pesticides (Harwood and Dolezal 2020, Paris et al. 2020, Tadei et al.) or the effects of nutritional stress and pathogens (Dolezal and Toth 2018, Tritschler et al. 2017) or nutritional stress and pesticide exposure (Tosi et al. 2017), or all of these together can also synergistically play a role in declining honey bee colony health. The transportation of honey bee stocks, both nationally and globally over many years, has assisted the movement of exotic pathogens and parasites from one honey bee species to another (Anderson and Trueman 2000, Hubert et al. 2017, Ke et al. 2021, Shutler et al. 2014), between continents, and between bee geographically distant population (Martin et al. 2012, Wilfert et al. 2016). In recent years, many pathogens and parasites of honey bees have become cosmopolitan in distribution within the United States.

Loss or fragmentation of honey bee habitat and forage contributes to nutritional stress (Branchiccela et al. 2019, Dozal and Toth 2018, Spiesman and Inouye 2013). At the same time

the presence of environmental chemical toxins and pesticides are additional threats to colony health (Cresswell et al. 2012, Decourtye and Devillers 2010, Fisher and Rangel 2018, Gill et al. 2012, Godfray et al. 2014, Gregorc and Ellis 2011, Kluser et al. 2010, Zhu et al. 2014). While any of these factors can impact bee health, they rarely occur in isolation, and may often synergize to increase the stress on bee health and immunocompromization (Alaux et al. 2010b, Belsky and Joshi 2019, Di Prisco et al. 2013, Pettis et al. 2012, Vidau et al. 2011).

The appearance of novel chemical compounds in the landscape has increased dramatically over the past half century (Carvalho 2017, Douglas and Tooker 2015, Fernandez-Cornejo et al. 2014, Meehan et al. 2011), and the relative toxicity of individual compounds has also increased (DiBartolomeis et al. 2019, Schulz et al. 2021). The use of pesticides has at times placed beekeepers and farmers at odds with each other when pest control products cause inadvertent harm to managed bees (Dos Santos et al. 2018, Douglas et al. 2020, Durant 2019). Uncertainties regarding the effects on bee health of the use of recently developed insecticide seed treatments, herbicides and other pesticide products has further strained the relationship between beekeepers and farmers.

Exposure to environmental chemicals is known to suppress or compromise honey bee immune response (Desneux et al. 2007, Vidau et al. 2011, DeGrandi-Hoffman et al. 2013, Di Prisco et al. 2013, Doublet et al. 2015). Some pesticides have been shown to reduce the success of queen rearing or fecundity (Chaimanee et al. 2016, Dussaubat et al. 2016, Forfert et al. 2017, Johnson and Percel 2013, Williams et al. 2015, Wu-Smart and Spivak 2016) as well as drone reproductive fitness (Abderkader et al. 2018, Fisher and Rangel 2018). A review by Thompson (2003) summarized many complex behaviors that can be negatively affected by sublethal exposure to insecticides, including foraging, conditioned responses, colony development, nest

mate recognition, and even larval behavior. Exposure to fungicides alters commensal gut microbiota and increases bees' susceptibility to microsporidian gut pathogens (Fisher et al. 2017, Gilliam 1997, Pettis et al. 2012, Tadei et al. 2020). Herbicide use reduces flowering plant abundance and can thus contribute to nutritional stress. Some herbicides are known to disrupt beneficial gut microbial communities in honey bees (Belsky and Joshi 2020, Castelli et al. 2021, Dai et al. 2018, Motta et al. 2018, Vázquez et al. 2020). Herbicides can also have lethal or sublethal effects on bees (Hoopman et al. 2018, Migdał et al. 2018, Morton et al. 1972), or can synergize with other insecticides and fungicides, resulting in higher toxicity to bees (Almasri et al. 2020, Glavan and Bozic 2013, Niedobová et al. 2019).

Neonicotinoid Pesticides and Different Routes of Toxicity Exposure to Honey Bees

While many factors have been shown to be responsible for bee population declines, public opinion has tended to narrowly follow reports in popular media that specifically highlight the effects of agricultural pesticides as the main threat to pollinator health. Much attention has been focused specifically on the class of chemicals known as neonicotinoids, and particularly those used as agricultural seed treatments (Philpot 2013, Walsh 2013, Morris 2015, Benjamin 2015).

Neonicotinoid compounds have rapidly become the most widely used class of pesticides in the world (Douglass and Tooker 2015, Seltenrich 2017). These insecticides bind readily to the postsynaptic nicotinic acetylcholine receptor of insects, resulting in prolonged excitation and death (Grünewald and Siefert 2019). Neonicotinoids also have a relatively low toxicity to mammals (compared to other insecticides), and require low application rates compared to many

other pest control compounds (Jeschke and Nauen 2008, Miranda et al. 2011, Sánchez-Bayo 2014, Sheets et al. 2016, Tomizawa and Casida 2003).

The efficacy of neonicotinoids as water-soluble seed treatments to protect crops at early growth stages has led to widespread use of these compounds in agriculture (Douglas and Tooker 2015, Radolinski et al. 2019). By applying a pesticide coating directly to the seed, which is taken up systemically by the plant, the compounds are effectively delivered specifically to pests feeding upon plant tissue during early germination and growth phases. Some investigators have demonstrated that the materials in seed treatments or soil are translocated throughout plant tissues, where they are readily available in flowers for collection by bees in acutely toxic or sub-lethal levels (Girolami et al. 2009, Hladik et al. 2018, Main et al. 2020, Rortais et al. 2005, Schmuck et al. 2001). Other studies have reported little or no measurable material detected in nectar or pollen of row crop plants by the time flowering occurs (Cutler and Scott-Dupree 2007, Stewart et al. 2014, Sur and Stork 2003, Whalen et al. 2021, Zawislak et al. 2021). Cowles and Eitzer (2017) found that ornamental horticultural plants treated with imidacloprid did contain acutely toxic concentrations when applied as a foliar spray or soil drench at nursery rates.

Another potential means of exposure to agricultural chemicals includes dust and particulate matter released from farm equipment when planting treated seeds, which can drift toxic materials to non-target areas outside of cultivated farm fields and onto wildflowers attractive to bees and other beneficial arthropods (Krupke et al. 2012, Krupke et al. 2017, Lin et al. 2021, Pisa et al. 2015, Sgolastra et al. 2012, Tapparo et al. 2011).

Gutation droplets exuded by plant foliage have also been reported to contain systemic insecticides (Girolami et al. 2009, Tapparo et al. 2011), although the detectable residues found in these droplets may have a negligible impact for free-foraging bee colonies with adequate clean

water sources available (Shawki et al. 2006). Water sources, themselves, may become contaminated with systemic neonicotinoids, which are highly soluble in water (Mörtl et al. 2020, Qiu et al. 2019, Samson-Robert et al. 2014).

After a three-year ban on neonicotinoid seed treatments on bee-attractive crops in Europe, Blacquière and van der Steen (2017) determined that declines in honey bees and other wild pollinators were not likely driven primarily by the use of neonicotinoids, but were more likely associated with pests, parasites and beekeeping practices. Separate reviews by Sánchez-Bayo and Wyckhuy (2019) and Wagner (2020) also concluded that habitat loss, and subsequent conversion of wild landscapes to intensive agriculture and urban use, was likely the primary driver of worldwide losses of insect diversity and abundance, particularly among pollinators.

While neonicotinoids and other pesticides can be acutely toxic to bees as to other pest insects, many studies that implicate their association to pollinator losses have been criticized for methods that rely on laboratory situation where unrealistic concentrations are fed to caged bees with no choice in feeding (Carreck and Ratnieks 2014, Wood and Goulson 2017). Investigators have directly exposed honey bees to pesticide compounds, either by contact (Christen et al. 2017, Iwasa et al. 2004, El Hassani et al. 2008, Gill et al. 2012, Negi et al. 2021, Škerl et al. 2010) or by ingestion in sucrose syrup (Baines et al. 2017, Bortolotti et al. 2003, Chensheng et al. 2012, El Hassani et al. 2008, Henry et al. 2012, Jacob et al. 2019, Oliveira et al. 2014, Yang et al. 2008, Zhu et al. 2014) to determine the effects of neonicotinoids on honey bee behavior or health. However, results of these tests have been variable, and exposure of small numbers of bees to isolated compounds in a laboratory does not necessarily produce results comparable to what bees would encounter when whole colonies forage in a complex environment where neonicotinoids and other pesticides have been applied in the landscape.

Attempts have been made to estimate the levels of pesticides that may be present in the honey bees' environment, and determine the effects of exposure to these field-realistic doses on honey individual honey bees and whole colonies (Cresswell 2011, Henry et al. 2012, Boily et al. 2013, Al Naggar et al. 2015, Stewart et al. 2014, Traynor et al. 2021, Zawislak et al. 2019) as well as on other types of bees (Whitehorn et al. 2012, Feltham et al. 2014). However, because conditions are highly variable across the landscape, and laboratory experiments often have different outcomes when conducted in the field, many scientists disagree about the concentrations that should be considered realistic under field conditions (Carreck and Ratnieks 2014).

A review by Wood and Goulson (2017) reported that neonicotinoids in nectar or pollen are typically found below or close to the lower limits of detection even when honey bee hives are located adjacent to seed treated crop fields. Stewart et al. (2014) found average thiamethoxam and clothianidin levels between the limit of detection (LOD) of 1 to 5.9 ng/g across a range of seed treatments. Meikle et al. (2016) found no difference in brood production in hives fed varying concentrations of imidacloprid that also had access to outside forage.

Exposure to Agricultural Pesticide Residues and Its Impact on Honey Bee Health

Exposure to sub-lethal levels of pesticide residues are believed to affect physiology and development of honey bee workers (Baines et al. 2017, Hatjina et al. 2013, Shi et al. 2017, Wessler et al. 2016, Wu et al. 2011, Wu-Smart and Spivak 2016) and queens (Dai et al. 2010, DeGrandi-Hoffman et al. 2013, Sandrock et al. 2014, Wu-Smart and Spivak 2016).

While neonicotinoids alone have not been conclusively implicated in bee colony collapse (Cresswell 2011), chronic sub-lethal pesticide exposure has been linked to deficiencies in the ability of bees to learn or process stored memories, as well as aversive learning to avoid predators and other dangers. Any factors which negatively impact navigation and reduce foraging efficiency will thus impacts overall colony efficiency, health and success (Decourtye et al. 2003, Decourtye et al. 2004a, Decourtye et al. 2004b, El Hassani et al. 2008, Eiri and Nieh 2012, Fischer et al. 2014, Guez et al. 2001, Iqbal et al. 2019, Ludicke and Nieh 2020, Meled et al. 1995, Morfin et al. 2020, Siviter et al. 2018, Tasman et al. 2021, Teeters et al. 2012, Thany et al. 2005, Tison et al. 2019, Urlacher et al. 2016, Yang et al. 2008, Zhang and Nieh 2015). In a meta-analysis of multiple studies, Cresswell (2011) concluded that field-realistic trace levels of dietary imidacloprid in nectar did not cause significant mortality, but did reduce colony performance by between 6 and 20%.

Learning and memory processing are imperative for successful long-range flight and foraging activities. Honey bees must efficiently find and exploit ephemeral floral food resources, return to the hive, and communicate the location of these resources in a complex environment to hive mates with precision (Menzel 2012, Tautz et al. 2008). Trace concentrations have also been shown to induce premature foraging in honey bees, with overall reduced lifetime foraging efficiency (Colin et al. 2019). Any condition that alters a bee's sense of direction, causing it to become disoriented outside of the hive, can prevent its return. Significant failure of foragers to return home will contribute to rapid colony depopulation, which was an early identified symptom of Colony Collapse Disorder (VanEngelsdorp et al. 2009).

While researchers may be able to determine the levels of compounds contained in specific plant tissues, or may be available in bee collected resources, the true exposure of an

individual bee or a whole colony is more difficult to measure. Honey bees typically forage over a large area, with many diverse food resources available to them in the landscape, across which they constantly assess and adapt their foraging habits for efficiency and to maximize the quality of food gathered (Nürnberger et al. 2019, Visscher and Seeley, 1982, Zawislak et al. 2021). Honey bees appear able to recognize dietary deficiencies in essential amino acids, and will modify their pollen foraging behaviors to include increased floral diversity, and thereby compensate for nutritional deficits (Hendriksma and Shafir 2016). The diversity of available plant species, however, may be significantly limited near intensive monoculture farming, and especially so where herbicides are widely used (Schütte et al. 2017). This reduction of plant diversity forces bees to gather more of their pollen protein from fewer plant species, which can disrupt the nutritional balance of their diets, thus causing cascading effects on overall colony health (Alaux et al. 2010a, Brodschneider and Crailsheim 2010, Huang 2012, Di Pasquale et al. 2013). If the food available to bees in these remaining plants contains pesticide residues, then the potential exposure of these bees in the field, as well as those feeding on the pollen or nectar in the hive, will be increased. Large honey bees colonies may be able to absorb and compensate deleterious effects of periodic neonicotinoid exposure (Wu-Smart and Spivak 2016) but bumble bees and solitary bees may be less able to compensate

Research Gaps and Potential Solutions

There are many gaps in our knowledge regarding precise lethal concentrations of pesticides for honey bees. These limitations are due to the complexities of the bees' own biology and behaviors as well as the complexity of the environments they must navigate. Studies vary in their methods of contact or ingestion, the specific chemicals or their formulations, the age or

health of individual bees, and how different chemicals interact with other factors, including synergistic chemicals and bee pathogens (Johnson 2015, Vidau et al. 2011). The effects of sub-lethal, and chronic sub-lethal doses of chemicals, or combinations of chemicals, on honey bee health and their behaviors, particularly learning and memory processing, is also uncertain, and sometimes contradictory, or data may be inadequate to establish clear causality (Almeida et al. 2021, Decourtye and Devillers 2010, Mullin et al. 2010, Noi et al. 2021, Pohorecka et al. 2012, Williamson and Wright 2013).

The system of integrated pest management (IPM) was developed to be a solution for plant protection that did not rely solely and indiscriminately on the application of chemical pesticides. Meticulous scientific research into pest biology, natural enemies, ecology, technology and other innovations have helped to develop IPM strategies for different crops and pests (Hokkanen 2015). Long-term sustainable pest management solutions encourage an integrated approach to maintaining pest populations below economic thresholds, and not reliance on a single "silver bullet" product that will provide only short term, and often inadequate control (Lewis et al. 1997).

An IPM approach to pest management should implement insecticide use only when and where the pest population is predicted to reach economic threshold, and no other effective management tools are available (Mourtzinis et al. 2019). It could also be refined further to integrate pollinator health in an integrated pest and pollinator management (IPPM) framework, to balance minimizing plant damage from pests with the ecosystem services of pollinators and other beneficial arthropods (Biddinger et al. 2018 Belien et al. 2021, Egan et al. 2020, Lundin et al. 2021, Penn and Penn 2021). Repeated application of a few products with similar modes of action will only increase selection pressure on pest species to develop heritable tolerance to these

treatments, and ultimately render them ineffective. Farmers already face herbicide-resistant weeds developing within their herbicide-resistant crops (Brabham et al. 2019, Perotti et al. 2020), as well as arthropod pests developing resistance to both chemical controls and *Bt*-engineered crops (Huseth et al. 2018, Pavlidi et al. 2018, Sosa-Gómez et al. 2020, Tabashnik and Carrière 2017, Umina et al. 2019).

Neonicotinoid seed treatments have become widely used to systemically protect plants from pest damage early in the season when other applications may not be effective (Gore et al. 2014). During 2012-2014, approximately 90% of corn, 76% of soybeans, 62% of cotton, and 56% of winter wheat planted in the United States received one or more seed treatment product (Hitaj et al. 2020). If farmers have limited choices available for purchasing seeds, they may pay a premium for a product or technology they neither want nor need (Tooker et al. 2017). Growers may deem seed coatings as inexpensive insurance against a potential pest problem, for which there is no evidence at the time of use (Krupke and Tooker 2020). The acceptance of widespread prophylactic use of neonicotinoid seed treatments on commodity crops is a violation of fundamental IPM principles, by abandoning decision making on monitoring and thresholds (Barzman et al. 2015, Goulson 2013, Tooker et al. 2017).

Neonicotinoids have been linked to significant risks to pollinators and other non-target species at multiple trophic levels (Berheim et al. 2019, Blacquière et al. 2012, Byholm et al. 2018, Eng et al. 2019, Bredeson and Lundgren 2019, Reisig et al. 2012, Seagraves and Lundgren 2012, Singh and Leppanen 2020, Yamamuro et al. 2019). Some investigators have suggested that neonicotinoid seed treatments provide negligible benefit in terms of improved crop yield (Mourtzinis et al. 2019, Krupke et al. 2017, Smith et al. 2020, Stevens and Jenkins 2014). Others, however, have calculated clear economic benefit from seed treatment practices (Gore et

al. 2014, Hurley and Mitchell 2017, North et al. 2016, North et al. 2018,). These differing conclusions seem to be largely dependent on planting dates and on a geographic basis. Where northern and northeastern farms may not see a benefit, growers in the south and southeast, where early season pest pressure can be a variable but significant factor in yield reduction, may be able to realize a measurable benefit by using seed treatments (Allen et al. 2018). The use of insecticidal seed treatments should be reserved for locations where early season pests are regular and predictable, and this technique can be expected to provide economic benefit to yield. In places where these pests occur sporadically, farmers should be encouraged to refrain from unnecessary seed treatments as part of responsible land stewardship and as a tenant of IPM practices.

An outright ban on neonicotinoids will require growers to rely on other products for pest control strategies, such as organophosphates, pyrethroids and carbamates, which dominated the agrochemical market prior to the adoption of neonicotinoids (Bass and Field 2018, Blake 2018, Jeschke et al. 2011). This could have implications for resistance management strategies, which relies on the ability to alternate treatments with different modes of actions. Reliance on fewer product will increase selection pressure on pest populations to develop resistance to products in rotational use.

Off-target effects of neonicotinoids and other pesticide classes will continue to be a concern for the health of pollinators and other beneficial arthropods. There remains a need to continue exploring other alternative pest management strategies such as biopesticides and other low-risk compounds, biological control tactics and breeding resistant crop varieties in conjunction with judicious use of pesticide products. All of these all belong to a comprehensive integrated pest management plan that can reduce dependence on pesticides, reduce negative

effects on non-target species, and still mitigate crop loss successfully. Research is also needed on the interactions of multiple and interacting contributing factors of pollinator declines.

Dedication to IPM principles combined with deliberate efforts to conserve and enhance areas of pollinator forage and nesting habitats within agricultural landscapes, will both promote pollinator health and protect plant health, together enhancing sustainable agriculture.

This work examines the risks to honey bee pollinators in agricultural environments. A survey of the area around an apiary was conducted to determine quantify the land use and to determine what compounds were applied to this landscape which could pose a risk to honey bees. Bee-collected pollen was sampled throughout an entire season to establish a chronological record of what plants honey bees visited, and when pesticide residues were detected while bees were actively foraging in an agricultural landscape. A method was also developed to monitor individual honey bees over their lifespans to elucidate the effects of a particular compound on honey bee behavior and longevity.

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Chapter II:

Survey of Area-Wide Agricultural Pesticide Use in Southern United States Row Crops and Potential Impact on Honey Bee Colonies

Abstract

Honey bees forage across a large area, continually scouting the local landscape for ephemeral food resources. Beekeepers often rely on flowering plants in and around irrigated farmland to maintain their colonies during dry seasons, despite the potential risk of pesticide exposure. Recent declines in pollinator abundance and diversity have focused attention on the role of pesticides and their effects on honey bee health. This investigation examined two types of landscapes within a two-mile (3.2 km) radius of honey bee colonies: an intensive agricultural setting and a rural setting without intensive agriculture. More than 10,000 acres of agricultural land was surveyed to quantify the area of cultivated crops and the area treated with pesticides, including seed treatments and foliar applications of insecticides. Samples of honey, bee bread (stored pollen), beeswax, and adult bees were collected from hives in both landscape types and screened for pesticide residues to determine if foraging bees were transporting pesticides to hives. Some samples of bee bread and honey did contain pesticide residues, but these were below known lethal dose (LD50) levels for honey bees. Beeswax samples contained the highest levels of contamination, but most were still relatively low. Samples were screened for 174 common agricultural pesticides and metabolites, but only 26 compounds were detected during the two-year study. These included one defoliant, one insect growth regulator, five herbicides, six fungicides, six insecticides never used in beekeeping, and five insecticides/miticides and their metabolites, which are used in beekeeping and for various other agricultural purposes, as well as

two miticides exclusively used by beekeepers to control *Varroa destructor*. Bee colonies foraging in agricultural landscapes are potentially exposed to numerous pesticide applications. While the residues detected in this study did not pose an acute lethal risk to adult honey bees, this study did not measure sublethal effects on bee colony health or performance, which merit further investigation.

Introduction

Honey bees (*Apis mellifera* L.) are known to forage for food across an extensive landscape, up to three miles (5 km) or more from their hives (Visscher and Seeley 1982). While foraging distances are highly variable in different landscapes and in different seasons, as long as adequate resources are available, foragers tend to remain closer to their hives in order to conserve energy, within an average distance of about one mile (1.6 km) or less, and sometimes only a few hundred yards in agricultural settings with abundant food (Hagler et al. 2011, Steffan-Dewenter et al. 2003, Couvillon et al. 2015). However, bees can range much farther for highly desirable food (Beekman and Ratnieks 2000). Honey bees exhibit preference for visiting flowers with high sugar content in the nectar, and will fly farther for higher quality forage, while bypassing lower quality forage nearby if the net caloric gain is greater (Waddington 1982). Honey bees appear to be able to differentiate, and actively diversify their foraging, to compensate for protein deficiencies in dietary pollen (Cook et al. 2003, Hendriksma et al. 2016). Also, the floral resources available to bees are often ephemeral, with some species blooming for only a short time each season. For these reasons, bees continuously scout their territory to readily and efficiently exploit new sources of food before competitors (Visscher and Seeley 1982).

The foraging activities of bee pollinators affect the continued survival of plant species as well as the genetic structure of distinct plant populations. Pollinator preferences have likely been a long-term driver of angiosperm speciation and evolution (Schiestl et al. 2013). Both the long-range foraging habits of honey bees, and the relatively limited foraging range of solitary bee species, may be essential to the survival of plants in disturbed or fragmented habitats (Greenleaf et al. 2007, Levin 1981), such as those surrounding agricultural production areas. Small uncultivated areas within crop production landscapes can also serve as important refuge habitats for pollinators and other beneficial insects, as well as other wildlife species (Gillespie et al. 2016, Heidel-Baker et al. 2014, Ramsden et al. 2015). Many agricultural crops rely on insect pollination, either partially or completely, to ensure fruit and/or seed production (Calderone 2012). Cereal grains such as corn, wheat, and rice are primarily wind-pollinated and do not require insect visits (McGregor 1976), although bees may sometimes collect their pollen for food (Severson et al. 1981). Some large-scale commodity crops such as cotton and soybeans can be self-fertile and do not require insect pollination to produce yield, but there is some evidence that pollinator visits can increase yield production (Erickson et al. 1978, Chiari et al. 2005, Abrol et al. 2012, Milfont 2013).

Commercial beekeepers often rely on irrigated farmland to sustain large numbers of honey bee colonies, and to produce surplus honey during dry periods, which would otherwise be a nectar dearth outside of an agricultural setting (Coy 2016). The amount of honey that these colonies can produce is affected by multiple factors that can determine nectar production, including cultivar variety, soil conditions, and weather (Smith et al. 2017, Shuel et al. 1952). While large-scale plantings of flowering crops can be significant nectar sources, bees in agricultural areas also greatly benefit from the presence of diverse wild flowers (i.e., weeds),

which are also sustained on and around farms through dry conditions by crop irrigation. These plants can provide bees with additional pollen and nectar resources when crops may not be in bloom or when monocultures may not provide sufficient nutrition on their own (Requier et al. 2015, Di Pasquale et al. 2013). Sponsler and Reed (2015) reported that wax production and food accumulation were both positively correlated with proximity to crop land, as opposed to urban area, forest, or grassland. While intensive agricultural landscapes can greatly benefit honey bee colonies, beekeepers who maintain colonies in these areas must also be constantly wary of pesticides that can negatively affect their bees.

When foraging in an agricultural landscape, honey bees are potentially exposed to numerous insecticides, fungicides, herbicides, and other agricultural chemicals. Recent widespread declines in bee populations across the country have focused public scrutiny on the negative effects that agricultural chemicals may have on pollinator health (Desneux et al. 2007, Johnson et al. 2010). Due to their widespread use in agriculture, especially as a pre-planting seed treatment, the neonicotinoid group has received particular attention because of suspected associations with declines in honey bee populations and health. These systemic insecticides can be translocated through the plant and into pollen and nectar, which becomes available to pollinating insects in sublethal quantities, which can negatively affect the behavior, reproduction, and survival of honey bees (Blacquière et al. 2012, Lundin et al. 2015, Rortais et al. 2005, Dively et al. 2015) and bumble bees (Laycock et al. 2012, Whitehorn et al. 2012).

The mid-South region of the United States has abundant agriculture as well as an abundance of diverse agricultural pests. Intensive crop production involves the diligent and routine scouting of fields for insects, weeds, and diseases, which are conventionally managed with a variety of insecticides, herbicides, and fungicides. Pesticide application decisions are routinely based on

monitoring by crop consultants who determine appropriate pest control strategies. Honey bees from colonies in agricultural areas that are exposed to pesticides may transfer these compounds into the hive, potentially affecting the entire colony. When principles of integrated pest management (IPM) are followed, and pesticides are applied only on an as-needed basis, pests can be controlled while reducing off-target exposure to pollinators and other beneficial arthropods (Cumming et al. 2006). However, even with careful use, some level of exposure will likely be inevitable.

Pollen and/or bee bread collected from hives in numerous locations in France revealed contamination from multiple pesticides (Chauzat et al. 2006). Bernal et al. (2010) evaluated the pesticide residues in stored pollen from honey bee colonies in Spain, and found varying concentrations of numerous residues in both spring-collected and fall-collected samples. Mullin et al. (2010) analyzed samples of beeswax, pollen, and honey bees from across North America, and detected 121 pesticides and their metabolites, with most samples containing multiple residues. In all of these studies, among the most prevalent residues detected were products routinely applied to hives by beekeepers for the control of *Varroa* mites, although some of these products have other pest-control applications as well. In Canada, Codling et al. (2016) reported the detection of neonicotinoid insecticides and their breakdown metabolites in honey, pollen, and honey bees, although concentrations in most samples did not approach oral LD_{50} values for honey bees. That investigation did not screen for other classes of pesticides.

The current study describes the potential chemical exposure within the foraging territory of bee colonies located in an agricultural setting in the southern United States. The study sites were selected to represent the diversity of mid-South agriculture as well as areas with little or no agriculture. The crops in the intensive agriculture area were primarily soybeans, rice, corn, and

cotton, with a few other minor crops, which included grain sorghum and green beans. Growers utilize a diverse selection of pesticide products for conventional production in Arkansas and the mid-South region, including herbicides, fungicides, and insecticides (including neonicotinoids as both as seed-treatments and foliar applications). A detailed survey was conducted to determine which crops were grown, and which pesticides were applied, across the entire landscape within a two-mile radius around an apiary. Sample of bees, beeswax, honey, and pollen were also collected from hives and screened for the presence of pesticide residues to which worker bees may have been exposed during foraging activity, and may have been brought back to the hive in collected food.

Materials and Methods

The survey was conducted in Lonoke County, Arkansas, during the 2014 and 2015 growing seasons. An apiary ("High-Ag" site) was established in April 2014, in an area where more than 80% of the landscape was under cultivation using conventional agricultural crop production methods and pesticide use. This site was representative of conditions around honey bee colonies in agricultural areas in the region. Four bee colonies were established in new 10-frame Langstroth beehives (two deep hive bodies each), using wired-beeswax foundation. All the beehive equipment was purchased from The Walter T. Kelley Company (Clarkson, KY, USA). Hives were protected from drift on all sides by a tree line, but bees had easy flight access to extensive cultivated row crop landscape in all directions (Figure 1).

A second apiary ("Low-Ag" site) was established at the same time, with four colonies, using identical equipment from the same sources. The Low-Ag site was also in Lonoke County, approximately 20 miles (32 km) from High-Ag site. The Low-Ag landscape was composed

primarily of native grasses and forbs, pasture land, woodland, and some commercial fish farms, but was not surrounded by intensive row crop production (Figure 2).

The two sites were chosen for comparison because they were close together, with similar climate conditions, but surrounded by very different land use. Commercial beekeepers in the region favor apiary locations adjacent to agricultural land for higher honey production over non-agricultural land, despite the risk of pesticide exposure (Coy 2016).

In 2014, all the colonies in both locations were started from three-pound packages purchased from the same source. In April 2015, eight additional colonies were established at the High-Ag site from locally-sourced nucleus colonies, and transferred into new, identical hives from the same source, as in 2014.

All the colonies, both years, were initially provided with 1:1 (sugar:water) syrup ad libitum for 1 month to help them establish and produce fresh comb. After this initial period, colonies foraged within the surrounding landscape for all their nutritional needs. All the colonies were managed with standard practices, for normal honey production, with additional hive bodies added as necessary. Queen excluders were not used, so that brood nest expansion was unlimited. No *Varroa* control products were applied in 2014 prior to hive product sampling. Thymol (Apiguard®, Vita (Europe) Ltd., Basingstoke, UK) was applied, following label instructions, after hive products were sampled in 2014. In 2015, all the new nucleus colonies had been treated with amitraz (Apivar®, Véto-pharma, Palaiseau, France) for early season *Varroa* mite control prior to our purchase of them. Thymol (Apiguard®) was applied to all the colonies on 20 August, according to label instructions, approximately 5 weeks prior to taking hive product samples.

A map was created of the area surrounding the High-Ag apiary, and all the agricultural fields within a 2-mile (3.2 km) radius of the apiary were defined and measured using ArcGIS software

(Esri, Redlands, CA, USA). If fields extended beyond this radius, the acreage of the entire field was included. While the actual honey bee foraging territory is potentially much larger than the acreage surveyed, land-use and farming practices are fairly consistent throughout the area surrounding the study site; therefore, the surveyed area is representative of the conditions that foraging honey bees would encounter in the local landscape outside of the survey radius.

Each crop field within the High-Ag study site was visually inspected to determine which crops were planted for two growing seasons. Growers were personally contacted and surveyed regarding their application of insecticides on each field. The survey determined only the presence of compounds (active ingredients) and/or specific product names that were applied. Information on the application rates, number or timing of applications made to all fields, and methods of application were not collected. The information gathered was limited to that which was voluntarily supplied by growers. While this data is likely incomplete, it does represent a minimum indication of the presence of these compounds applied to this landscape. The use of insecticide seeds treatments at planting was noted, and included as an application. Herbicide applications were not included in the survey, but were likely applied to most fields as a standard practice. Particularly, glyphosate (Roundup®, Bayer Ag, Leverkusen, Germany) was assumed to have been applied to most crops with engineered tolerance (soybean, corn and cotton), except for rice, green beans, and grain sorghum.

A map of the Low-Ag landscape was also made, and land use was calculated. An extensive survey of landowners in this area was not conducted, because this area did not contain significant large-scale row crop acreage. The majority of the landscape was pasture and woodland, but also contained a small fruit and pecan operation, some home gardens, a small dairy farm, and some commercial fish farming within the bees' foraging range. While the fish farm could have been

utilized as a water source by the bees, it is unlikely, as there were numerous fresh water sources (creeks and ponds) much closer to the apiary. Some soybean production was located approximately 2.5 miles (4 km) from the apiary, and an area of wheat was located approximately 1.5 miles (2.4 km) away, which was likely ignored by bees for lack of nectar. No other row-crop agriculture was located in the vicinity.

Samples were collected from bee hive products to determine if field-applied agricultural pesticides could be detected in beehives. Prior to colony installation in 2014, two samples of beeswax foundation were collected. Pieces of beeswax were sampled from 10 randomly selected sheets of wax foundation, which were part of a bulk purchase from which all the foundation used in the study originated. Additionally, two samples of adult bees were pooled from random packages at the time of colony installation. Later in the season, additional samples were taken from hives in both study apiaries (High-Ag and Low-Ag) in 2014. These samples included newly drawn beeswax comb (not yet used for brood-rearing or food storage, removed avoiding the foundation wax), bee bread (stored pollen), and adult honey bees randomly collected from inside the hive. Each sample consisted of 3–4 g of material or bees. All the samples were collected with sterile instruments, immediately placed on ice in the field, and later stored at −12 °C. Samples were shipped frozen, with dry ice, to the USDA's National Science Laboratory in Gastonia, North Carolina, for their comprehensive apicultural pesticide screening. Sampling of live bees and hive products was repeated in 2015 only at the High-Ag site.

During 2014, samples for residue testing were collected on 6 August, and again on 24 September. On 6 August, samples of new beeswax, bee bread, and adult bees were collected from each of two hives at the High-Ag site and from each of two hives at the Low-Ag site. On

24 September, the sampling procedure was repeated from each of the same hives at both sites, with capped honey also collected from each of the same hives.

In 2015, samples of adult honey bees and beeswax from combs in nucleus colonies were collected when the colonies were initially established. However, these samples were accidently destroyed in shipment, and could not be analyzed for residue contaminants. Additional samples of hive products were collected on 29 September from 4 hives in the High-Ag area. The samples included new beeswax, bee bread, honey, and adult bees. Colonies in the Low-Ag area were not sampled in 2015, because none of the Low-Ag samples from 2014 contained detectable residues except for the new beeswax, which contained only very low levels. Resources were instead devoted to samples taken in the High-Ag apiary.

Results

The survey of the High-Ag landscape included all the area within a two-mile radius of the apiary (8038 acres). If cultivated fields extended beyond this radius, the entire field was included. The total surveyed area under cultivation varied between 2014 (12,160 acres) and 2015 (10,063 acres). The total area of the survey was slightly different between years because of changes in land management, and an inability to contact some growers for interviews. The aerial map in Figure 1 shows the High-Ag area surveyed, in the context of its surrounding landscape. Crops in the High-Ag area included a predominant commercial production of soybeans, corn, rice, cotton, and grain sorghum, as well as small areas of green beans, some commercial fish farming, woodland, wetlands, pasture, and fallow fields, which are typical of this area. The maps in Figure 2 indicate the distribution of land use by crop around the High-Ag site for both years. Slight changes in land use between growing seasons did occur, but did not significantly

modify the overall composition of the landscape. Figure 3 shows an aerial view of the landscape around the Low-Ag apiary site, which was dominated by a mixture of pasture and woodlands, with some small home gardens, commercial fish farming, and a few small fruit operations, but very little row crop agriculture. Figure 4 outlines the dominant land use within a two-mile radius of the beehives.

An average of 81% of the landscape was under cultivation in the High-Ag area during the 2014 and 2015 growing seasons (Table 1). The largest proportion (57%) was planted with soybeans, while 10% was used for rice, 8% was used for corn, and 6% was planted with minority crops (cotton, grain sorghum, green beans). The remaining landscape was comprised of 15% uncultivated land (fallow fields, pasture, woodland, wetland), with 4% devoted to commercial fish ponds. This extensive agricultural area supplied bee colonies with ample forage to build up population numbers and produce surplus honey, but also had potential for significant exposure to numerous pesticides applied throughout the season. Grower-reported applications of insecticides and fungicides in 2014 and 2015 are summarized by crop in Table 2.

The Low-Ag site, within two miles (3.2 km) of the apiary, had very little of the landscape devoted to row crop agriculture (Table 3). Less than 6% of the landscape was devoted to wheat—which is not attractive to honey bees—and fish farming. The rest of the land around the site was either woodland (54%) or grass/pasture (43%). Pastures may contain bee-attractive flowers, and are sometimes treated for fall armyworms to protect grazing and hay crops, but no products recommended for armyworm control (Studebaker, 2018) were detected in any of our samples.

Surveyed farmers reported planting 87.2% of this area (8,839 acres) with neonicotinoid-treated seeds in 2014, and 52.7% (4,181 acres) in 2015. Figure 5 illustrates the reported distribution of crops planted with neonicotinoid seed treatments. These treatments have come under particular scrutiny for their potential to translocate toxins and make them available to foraging bees in pollen and nectar, however Stewart et al. reported generally low concentrations of these products when sampling seed-treated crops growing in the mid-South (Stewart et al. 2014). The survey also determined that 73.0% of the cropland (7,400 acres) was treated with at least one foliar application of insecticide later in the 2014 season (including additional neonicotinoids). During 2015, at least 15.6% of the cropland was treated with at least one foliar application. Figure 6 illustrates the distribution of foliar pesticide applications reported around the apiary site.

Samples of package bees and beeswax foundation were taken when colonies were established and screened for pesticide residues along with hive products sampled later in the season. Both the package bees and foundation wax contained compounds that we had not applied to the hives, and were not reported as used by area farmers, but were detected (Table 4). Coumaphos and fluvalinate were both detected in package bees, which could be a result of the package bee supplier treating bees for mites prior to shipping spring packages. The presence of the herbicide atrazine in package bees is curious, and may have resulted from bees encountering the compound prior to being packaged for sale.

The highest levels of residues found in wax foundation were coumaphos and fluvalinate, which agrees with Mullin et al. (2010) and Medici et al. (2012). These products are commonly applied by beekeepers to control *Varroa* mites. These lipophilic compounds are known to be readily soluble in beeswax (Medici et al. 2012, Korta et al. 2001), and remain stable when wax is

melted and formed into new foundation sheets (Bajuk et al. 2017). Chlorpyrifos was also detected, but at a much lower level than that found by Mullin et al. (2010).

Samples of adult bees and drawn comb were also initially collected from nucleus colonies established in the High-Ag apiary in 2015; however, these samples were accidently destroyed in shipping, and could not be analyzed for residues.

Given that agricultural pesticides were routinely applied to much of the landscape around the apiary, we expected that bees would be exposed to these while foraging, and had potential to transport contaminated nectar or pollen back to the hive. Samples of beeswax, bee bread, honey, and bees were screened for 174 common agricultural pesticides and their metabolites. Of these, only 26 compounds were detected during the two-year study, including one defoliant, one insect growth regulator, five herbicides, six fungicides, six insecticides never used in beekeeping, and five insecticides/miticides and their metabolites which are used in beekeeping and for various other agricultural purposes, as well as two miticides exclusively used by beekeepers to control *Varroa destructor*. Overall, considering the widespread use of pesticides in the landscape around the apiary at the High-Ag site, bee hive samples contained fairly little contamination. The residues detected in hive samples are summarized in Table 5. A list of the compounds screened, but not detected, is reported in Table 6.

In honey sampled at the High-Ag site, the only contaminants detected were flubendiamide (in 2014) and DMPF (2,4-dimethylphenyl formamide) (in 2015). This agrees with Rissato et al. (2007) and Alburaki et al. (2018), who also found pesticide concentrations in honey to be very low or undetectable. This is likely because many synthetic pesticides are lipophilic, and readily accumulate in beeswax (Korta et al. 2001), but are not especially soluble in honey (Bajuk et al.

2017). Also, many foliar-applied insecticides work by contact, and are unlikely to be present in nectar collected by bees. Honey samples from the Low-Ag site contained no detectable residues.

Bee bread collected from hives in the High-Ag apiary contained four compounds in 2014 and three compounds in 2015, but all at low levels. A review by Bogdanov (2006) also suggests that pollen (bee bread) is more likely to be contaminated with residues than honey. Bee bread samples from the Low-Ag site contained no detectable residues.

No pesticide residues were detected in adult bee samples in 2014, from either the High-Ag or Low-Ag sites. However, because adult bees are short-lived in the summer, our limited sampling at the end of the season may not have detected applications made earlier. Similarly, in 2015, only beekeeper-applied products were detected in adult bee samples.

New beeswax contained the highest number of detected compounds at both sites, and in both years. New beeswax sampled from the Low-Ag site in 2014 contained the highest number of compounds detected (16). The sources of these contaminants in the Low-Ag landscape are unknown, but were generally well below LD_{50} values for bees. In new beeswax sampled at the High-Ag site, nine compounds were detected in 2014, and seven compounds were detected in 2015.

In 2015, a high level of the herbicide metolachlor was detected in samples of new beeswax, but not in bee bread or honey. This contamination could have been the result of foraging honey bees in contact with freshly applied material, and spreading it to wax while walking across the comb. Several fungicides were detected, again mostly in beeswax. These are commonly used to control blight and plant diseases in agriculture, and are not presumed to be acutely toxic to honey bees. However, when synergized with other compounds, the combined toxicity may increase (Mullin et al. 2010, Thompson et al. 2014, Johnson et al. 2013). Also, exposure to fungicides

appears to make honey bees more susceptible to the gut pathogen *Nosema cerana* (Pettis 2013). Also, acute toxicity is not the only concern of pollinator health. Numerous sublethal effects from exposure to single and multiple pesticides have been noted in recent literature (Desneux et al. 2007, Dively et al. 2015, Yang et al. 2008, Bryden et al. 2013, Tosi et al. 2017, Wu et al. 2011).

The highest levels of residues detected in wax were from products that are primarily applied by beekeepers for *Varroa* mite control. In 2014, coumaphos and fluvalinate were detected in new beeswax at both sites. Both of these compounds had been detected in foundation wax and package bees at the beginning of the season, but were not applied early to hives during the experiment, and were not likely to be used for any nearby field application. Both of these are known to migrate from contaminated wax (Yang et al. 2008). Their presence in newly secreted beeswax suggests that these lipophilic chemicals may have diffused from contaminated foundation or been spread by contact with the bodies of bees. In 2015, wax samples contained residues of products that were applied to colonies. Amitraz had been applied for *Varroa* control in nucleus colonies prior to purchase, according to the nucleus colony provider. No amitraz was detected in the subsequent sampling of any hive products, but DMA (2,4-dimethyl aniline) and DMPF, which are both breakdown products of amitraz (Corta 1999), were detected more than six months later in samples of adult bees, capped honey, bee bread, and new beeswax. Also, high levels of thymol were present in adult bees that were sampled after *Varroa* control application of thymol was made in the late summer. However, thymol was not detected in other hive products. Thymol is a naturally derived essential oil that is obtained from the thyme plant (*Thymus vulgaris*), and not considered toxic to bees (Vita Bee Health, 2018), but can affect the flavor of honey if applied before honey is harvested (Véto-pharma, Inc. 2018).

Absent from the list of detected compounds are any of the neonicotinoid group of insecticides, which have recently received much critical attention for their suspected role in honey bee population declines. Krupke et al. (2012) suggested that dust exhausted during planting treated seeds could potentially contaminate nearby wildflowers where bees forage, which was confirmed by Stewart et al. (2014). Dively and Kamel (2012) found that neonicotinoid treatments applied as foliar applications or through chemigation resulted in the highest residues in nectar and pollen in cucurbits, while the lowest residues were detected from seed dressings. Furthermore, Meikel et al. (2016) found that imidacloprid remained stable in hive products for at least seven months. A worldwide survey of honey as a human food product found very low levels of neonicotinoid contamination, with a mean for positive detections of 1.8 ± 0.56 (SE) ppb (Mitchell et al. 2017). In the current survey, neonicotinoid products were applied as pre-plant seed coatings (i.e., seed treatments) as well as via foliar applications on multiple crops throughout the foraging landscape around the High-Ag apiary site. Despite their widespread use in this landscape, we did not detect any neonicotinoids in our samples. However, our sampling was limited to the end of the growing season, when residues from early season treatments or other sporadic applications may not have been detectable.

Discussion

Honey bees forage over an extensive area for the nectar and pollen they utilize as food. In agricultural landscapes, there is great potential for pesticide exposure of honey bees in the field, and for contamination of the hive and hive products. The Arkansas survey of area growers, although most certainly incomplete in documenting all pesticide applications, confirms that

multiple products, in multiple chemical classes, are applied to the agricultural landscape routinely throughout the season as part of conventional agricultural production.

Despite the widespread use of these chemicals, both hobbyist and commercial beekeepers continue to maintain productive honey bee colonies in intensive agricultural areas (Coy 2016). Furthermore, colony productivity has been shown to increase with proximity to crop land (Sponsler and Reed, 2015), and research has also shown that mass flowering crops can benefit wild and managed bees, despite other risks posed by agricultural practices and land management (Westphal et al. 2003, Le Féon et al. 2010, Holzschuh et al. 2013).

The results of our limited investigation are consistent with other studies. Similar to Mullin et al. (2010), who conducted one of the broadest and most geographically diverse studies, we found that the highest concentrations of detectable compounds were a result of beekeeper-applied products. These products, by design, have low toxicity relative to the dose required for adverse effects. To a lesser degree, fungicides and herbicides also have low general toxicity to honey bees, but are known to have synergistic effects with other pesticides, which increase the toxicity of one or more of the compounds (Johnson et al. 2013, Pilling et al. 1993, Thompson and Wilkins 2003). The increasing buildup of pesticide contamination in combs over time can adversely affect honey bee health and survivorship (Haarmann et al. 2002, Di Prisco et al. 2013, Zhu et al. 2014). Chronic exposure to sublethal levels of pesticides can impact honey bee health and immune response (Pettis et al. 2013, Johnson 2015). Pesticides are rarely, if ever, encountered individually, but more often simultaneously with others (Mullin et al. 2010). Efforts have been made to explore the toxicity of combinations of pesticides that are often found together (Thompson et al. 2014, Johnson et al. 2013, Zhu et al. 2014, Johnson 2015).

Recent declines in honey bee populations cannot be attributed to any one single cause, but are likely the result of accumulated stresses from multiple causes (Bryden et al. 2013). The complex of the mite *Varroa destructor* (Anderson and Trueman) and the viruses they vector continues to be the greatest threat to honey bee health (Rosenkranz et al. 2010, Guzman-Novoa et al. 2010). Other pathogens such as *Nosema ceranae* also affect honey bee health, productivity, and survivorship (Chen et al. 2008). Additionally, bees must have access to adequate nutrition from floral resources in order to maintain health (Alaux et al. 2010). Most likely, a combination of multiple factors, including these and others, are responsible for recent declines in honey bee health and populations (Bryden et al. 2013, Vanengelsdorp et al. 2009). Optimal management of honey bee colonies must include a reduction of multiple stress factors, including sublethal exposure to pesticides, and discussions of honey bee health should not be limited to a narrow focus on pesticide exposure.

To expand upon this work, a similar survey could be conducted that includes records on the timing, formulations, and rates of pesticide applications for specific crop fields, and more frequent sampling through the season to more precisely determine when contaminants may be entering beehives, and how long particular applications may pose specific risks to bee colonies.

Acknowledgments:

I wish to thank Ples Spradley and Elmer Wilman, Jr., for hosting experimental hives on their property, as well as Tyler Fields for his assistance with apiary work. Joe Black contributed to the GIS mapping. We are also grateful to the USDA-ARS for help in funding this study.

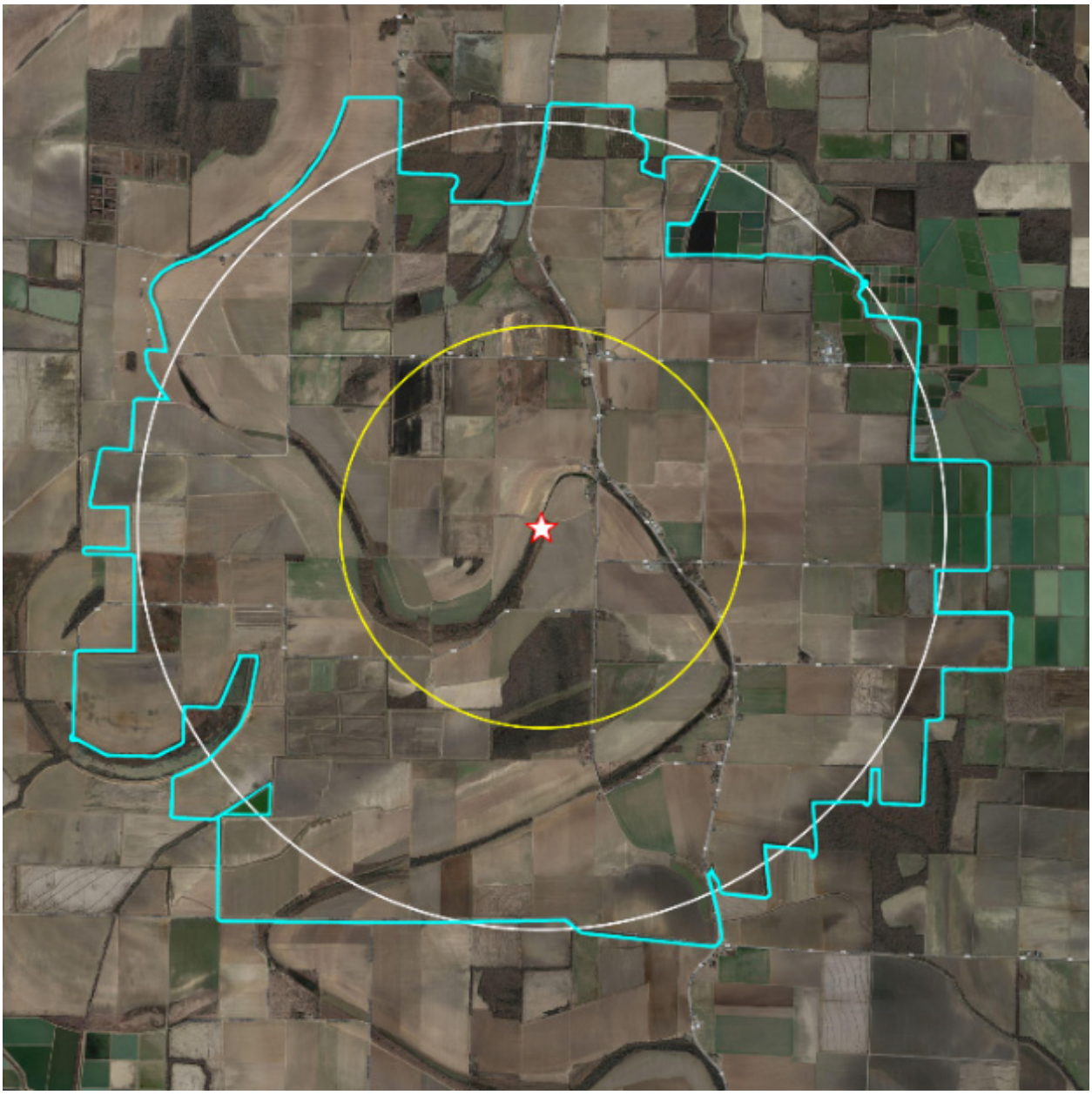

Figure 1. Aerial view of the High-Ag study site in Lonoke County, Arkansas. The star indicates the apiary location. The yellow circle indicates a one-mile radius from the beehives; the white circle indicates a two-mile radius from the hives; the blue line indicates the approximate area included in the survey. Landscape included the commercial production of soybeans, corn, rice, cotton, grain sorghum, and green beans, as well as commercial fish ponds, woodlands, grasslands, wetlands, and fallow fields. This site is representative of agricultural production land in this region (data: Google, Landsat/Copernicus, Maxar Technologies, US Geological Survey).

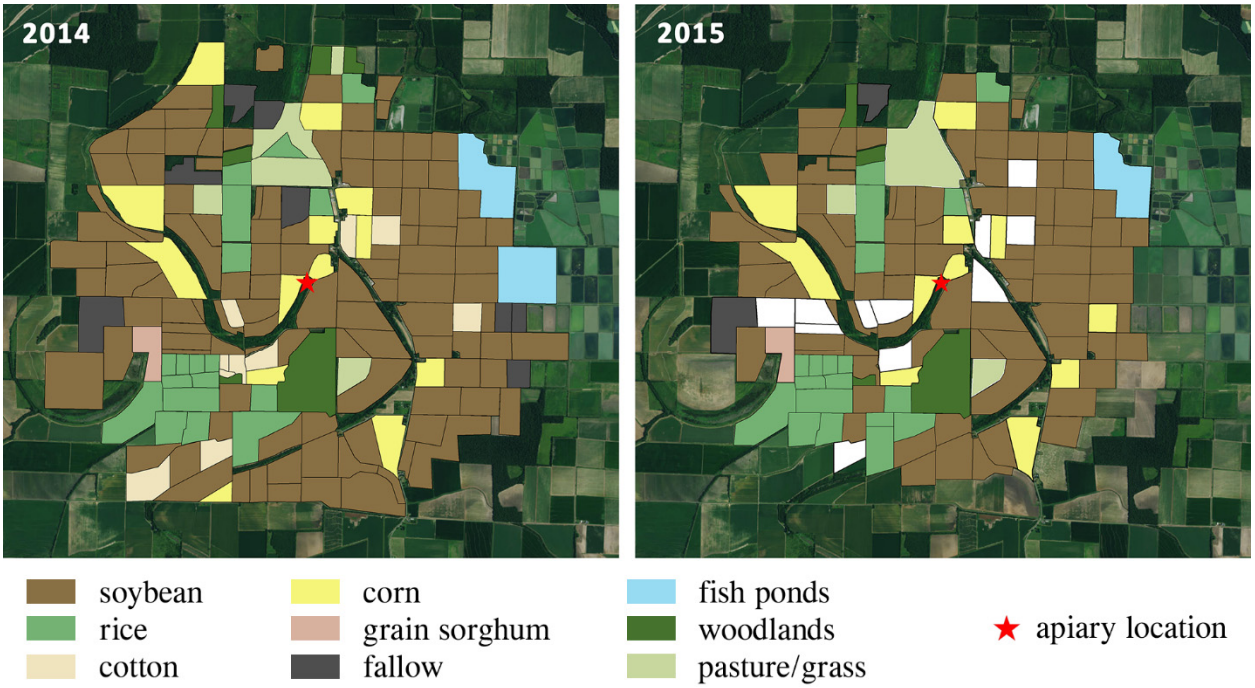

Figure 2. Land use by crop within the surveyed area around the High-Ag site during the 2014 and 2015 growing seasons. The survey area was slightly different between years due to changes in land use and an inability to contact farmers for interviews regarding all fields. However, general patterns of land use and crop production remained similar in the landscape around the apiary during both years.

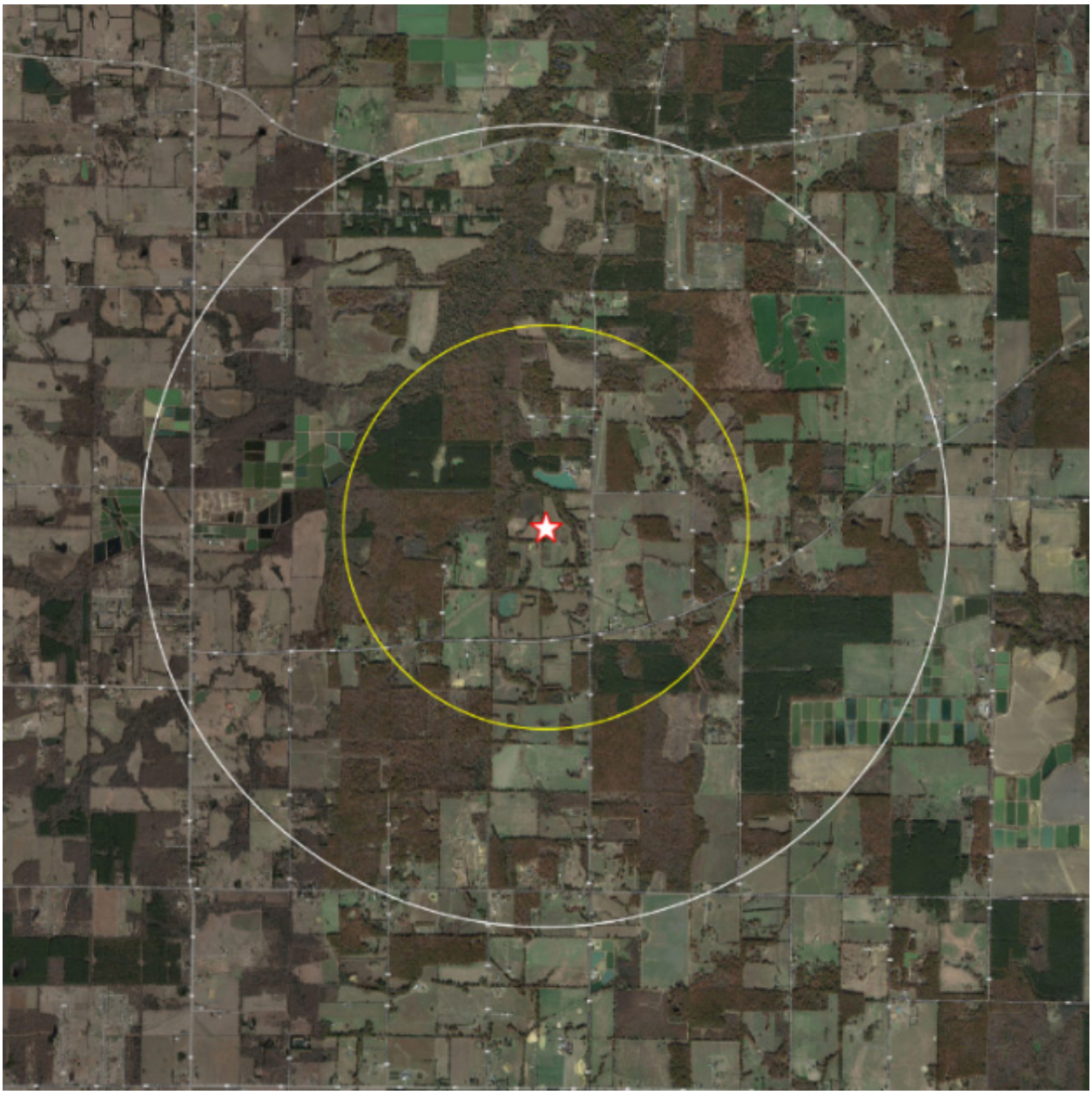

Figure 3. Aerial view of the Low-Ag study site, Lonoke County, Arkansas. The star indicates the apiary location. The yellow circle indicates a one-mile radius from the beehives; the white circle indicates a two-mile radius from the hives. The landscape included a diverse mixture of pasture, woodlands, commercial fish farming, residential gardens, and a few small fruit or orchard operations, but no significant row crop agriculture near the apiary site (data: Google, Maxar Technologies, State of Arkansas, USDA Farm Services Agencyimage source: maps.google.com).

Table 1. Summary of land use within the High-Ag survey site in 2014–2015. This site included all the agricultural fields within approximately two miles of the apiary location. Areas of crop fields that extended outside of a two-mile radius were included in the survey.

Land Use	Total Acreage		% Acreage		
	2014	2015	2014	2015	2-Year Average
Soybean	7489	5285	61.6	52.5	57.1
Rice	1110	1088	9.1	10.8	10
Corn	1005	849	8.3	8.4	8.4
Cotton	443	317	3.6	3.2	3.4
Grain Sorghum	92	91	0.8	0.9	0.9
Green Beans	0	306	0	3	1.5
Total Crop Acreage	10,139	7936	83.4	78.9	81.2
Fish Ponds	396	396	3.9	3.9	3.9
Uncultivated Land	1625	1731	12.7	17.2	15
Total Acreage	12,160	10,063	100	100	100

Table 2. Reported acreage receiving pesticide application, by crop, within High-Ag survey area during 2014 and 2015 growing seasons.

	Pesticide	class *	Soybean	Corn	Rice	Grain Sorghum	Cotton	Green Beans	Total acres treated	Percentage surveyed landscape treated
2014	Thiamethoxam	i - neo	3677	789	669	92	264	0	5491	45.2
	Imidacloprid	i - neo	884	81	0	0	203	0	1168	9.6
	Clothianidin	i - neo	1054	81	0	0	11	0	1146	9.4
	Dimethoate	i - op	54	0	0	0	0	0	54	0.4
	Cypermethrin	i - pyr	33	0	0	0	61	0	94	0.8
	Lambda-Cyhalothrin	i - pyr	685	0	347	0	192	0	1224	10.1
	Bifenthrin	i - pyr	319	81	0	0	11	0	411	3.4
	Chlorantraniliprole	i - ry	319	50	0	0	72	0	441	3.6
	Flonicamid	i - u	175	0	0	0	10	0	185	1.5
	Novaluron	igr	285	81	0	0	11	0	377	3.1
	Fludioxonil	f	3637	868	669	92	192	0	5458	44.9
	Mefenoxam	f	3637	868	669	92	192	0	5458	44.9
	Azoxystrobin	f	1608	0	347	0	323	0	2278	18.7
	Prothioconizole	f	1567	509	62	0	0	0	2138	17.6
	Trifloxystrobin	f	1567	509	62	0	0	0	2138	17.6
	Metalaxyl	f	564	0	0	0	131	0	695	5.7
	Tebuconazole	f	564	0	0	0	131	0	695	5.7
	Tiabendazole	f	519	0	0	0	0	0	519	4.3
	Pyraclostrobin	f	479	0	0	0	0	0	479	3.9
	Propiconazole	f	0	0	292	0	0	0	292	2.4
2015	Thiamethoxam	i - neo	2965	0	344	0	317	225	3851	38.3
	Clothianidin	i - neo	0	849	0	0	317	0	1166	11.6
	Acephate	i - op	0	0	0	0	317	0	317	3.2
	Chlorpyrifos	i - op	0	0	0	91	0	0	91	0.9
	Bifenthrin	i - pyr	0	0	0	0	317	0	317	3.2
	Lambda-Cyhalothrin	i - pyr	199	0	0	0	0	0	199	2
	Chlorantraniliprole	i - ry	768	0	0	0	317	93	1178	11.7
	Flubendiamide	i - ry	256	0	0	0	0	0	256	2.5
	Novaluron	igr	0	0	0	0	317	0	317	3.2
	Fludioxonil	f	2197	0	0	0	0	132	2329	23.1
	Mefenoxam	f	2197	0	0	0	0	132	2329	23.1
	Azoxystrobin	f	877	312	745	0	0	306	2240	22.3
	Propiconazole	f	0	312	344	0	0	0	656	6.5

* f = fungicide, i = insecticide, igr = insect growth regulator; neo = neonicotinoid, op = organophosphate, pyr = pyrethroid, ry = ryanoid, u = unclassified

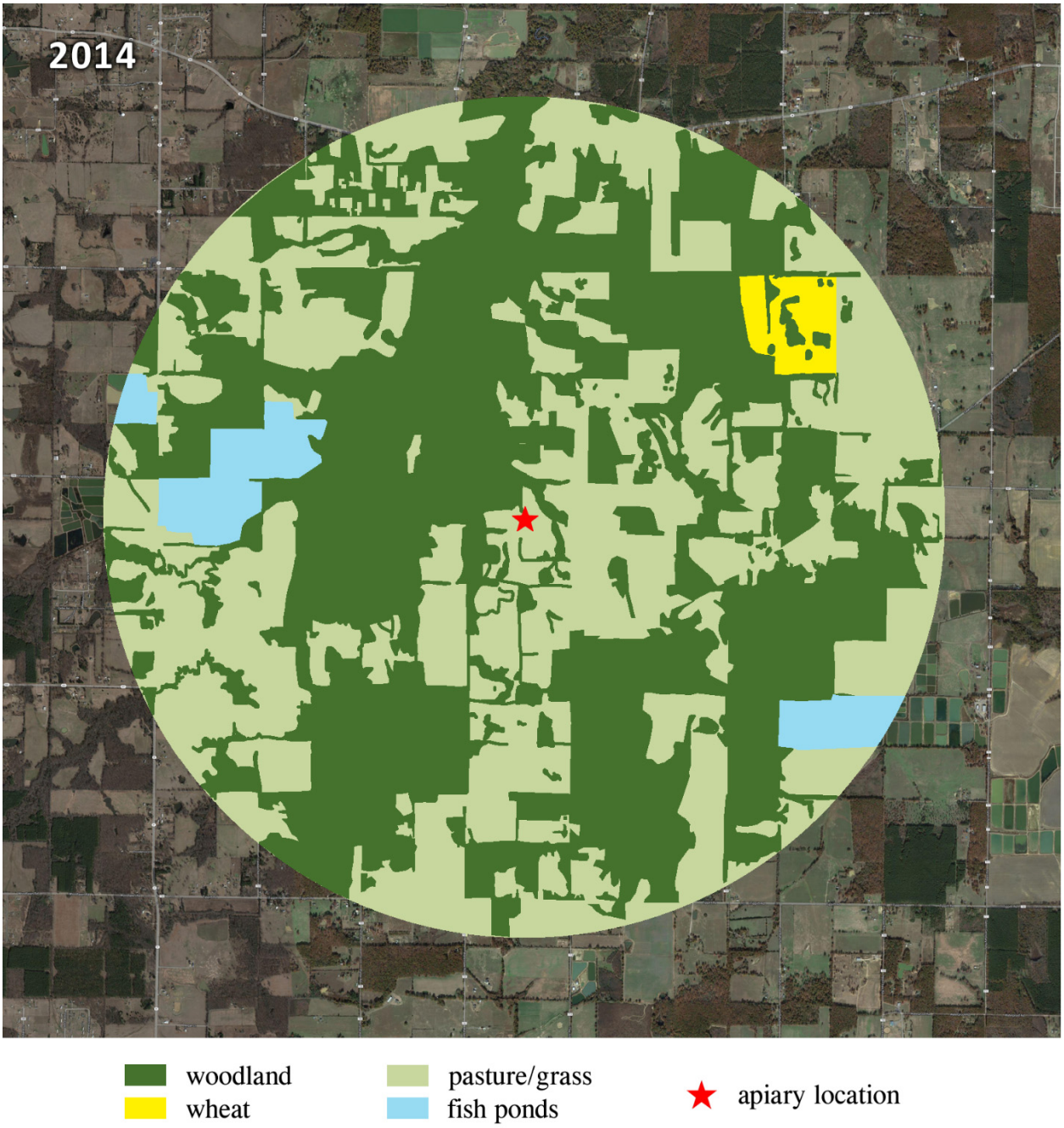

Figure 4. Dominant land use within a two-mile radius around the Low-Ag site in 2014. This landscape was primarily composed of woodland and grassland/pasture, with a small area of wheat, and some commercial fish farming.

Table 3. Summary of land use within a two-mile radius around the Low-Ag site in 2014.

Land Use	Total Acreage	% Acreage
Woodland	7,489	54.0
Grass/Pasture	1,110	42.5
Fish Ponds	1,005	3.5
Wheat	443	1.2
Total Acreage	8,043	100

Table 4. Compounds detected in initial samples of package bees and foundation wax used to establish colonies in 2014. Results reported as parts per billion (PPB), and are a mean of two separate samples randomly taken on the day of installation.

Compound	Class *	Level of Detection (ppb)	Beeswax Foundation	Package Bees
coumaphos	a	5	323.5	59
fluvalinate	a	1	273	136.5
chlorpyriphos	i	1	2.6	0
hexythiazox	igr	30	trace	0
vinclozolin	f	1	trace	0
atrazine	h	6	0	96.9

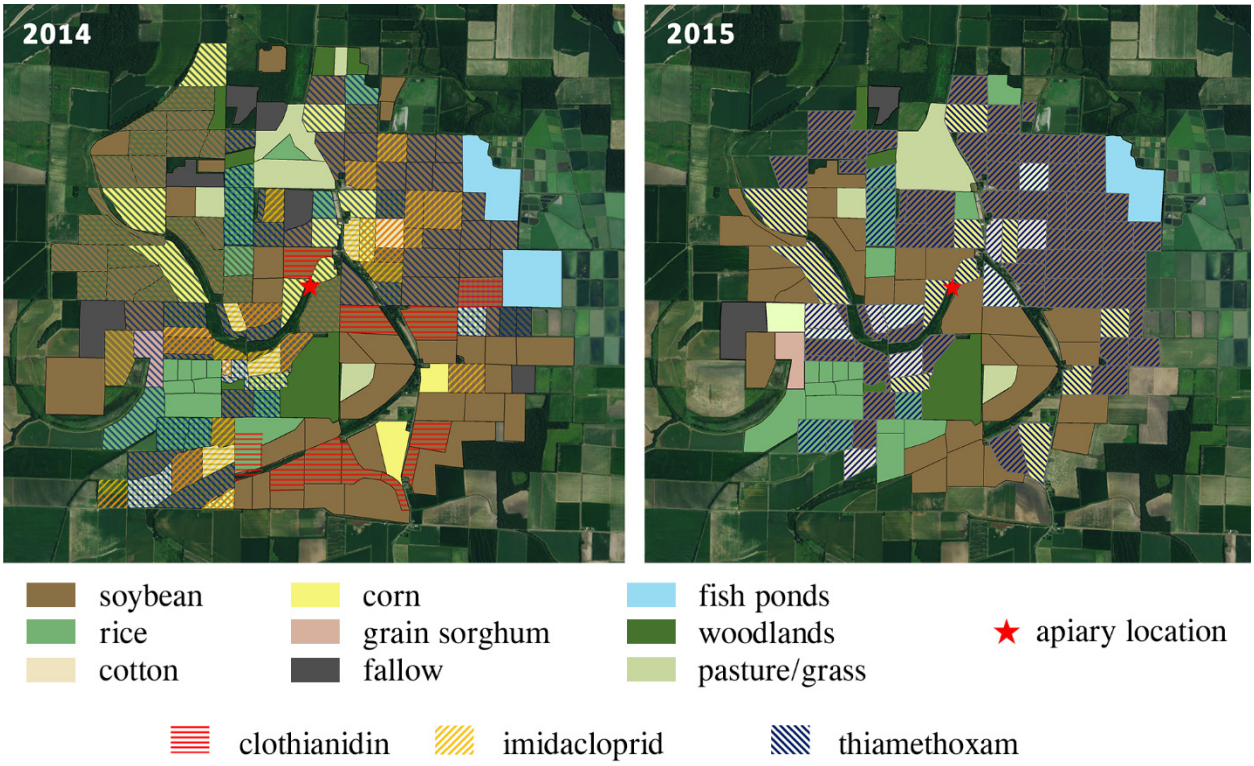

Figure 5. Reported distribution of neonicotinoid insecticides applied as seed treatments within the High-Ag survey area during the 2014 and 2015 growing seasons.

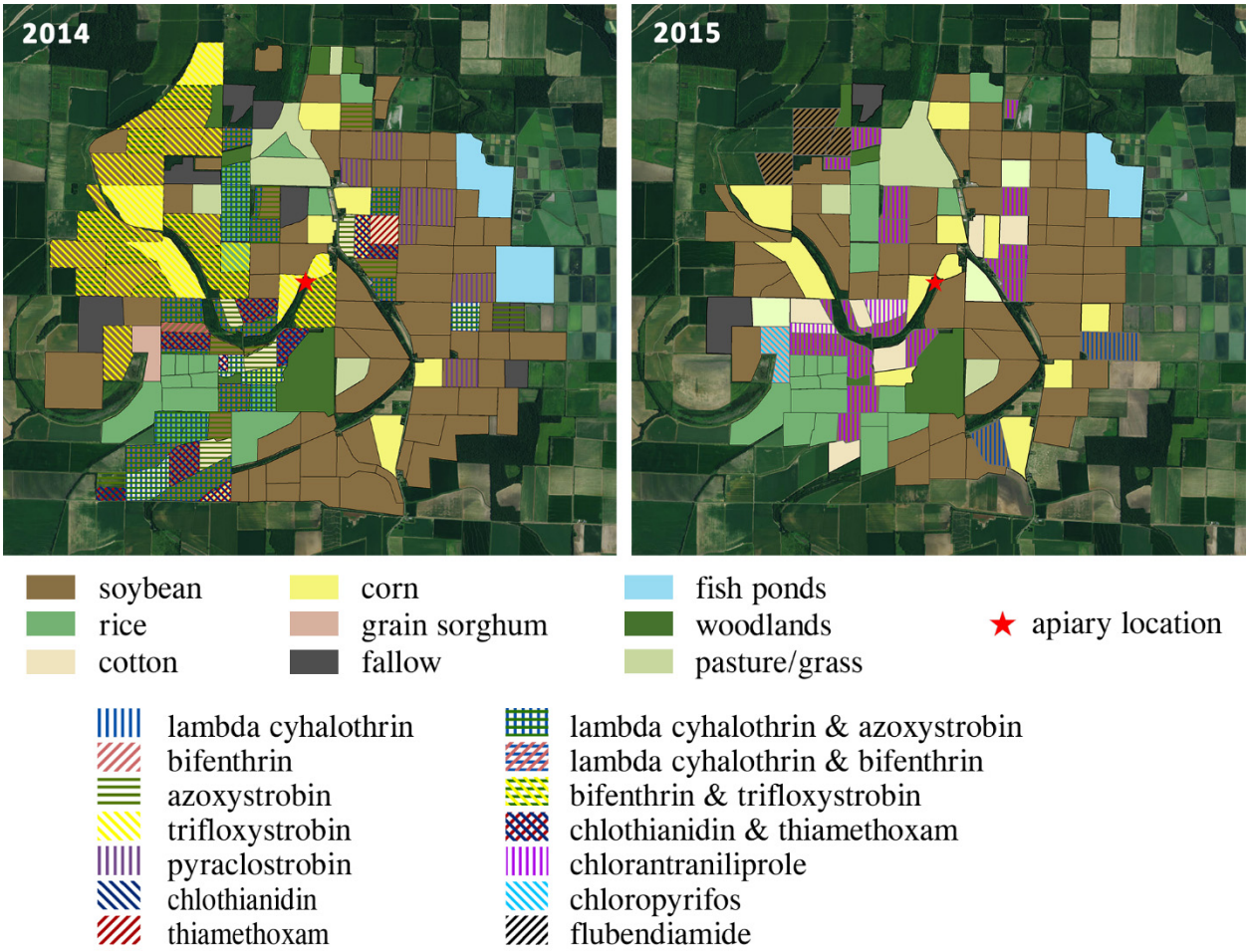

Figure 6. Reported distribution of foliar applied pesticides in the surveyed area within the High-Ag survey area during the 2014 and 2015 growing seasons.

Table 4. Pesticide residues detected in hive products. Results are given in PPB (±SE). Where results are reported as 0, compound was not detected; where are reported as "trace" the compound was detected, but at a level too low to be quantifiable.

pesticide	class*	level of detection (PPB)	2014				2015			
			Low-Ag	High-Ag			High-Ag			
			new wax	honey	pollen	new wax	honey	pollen	new wax	bees
Coumaphos	a	5	158.85 (95.38)	0	0	103.75 (73.08)	0	0	0	0
Coumaphos Oxon **	a	5	1.28 (2.55)	0	0	trace	0	0	0	0
Fluvalinate	a	1	128.53 (61.1)	0	0	63 (73.52)	0	0	0	0
Amitraz	a	4	0	0	0	0	0	0	0	0
DMA ***	a	50	0	0	0	0	0	0	0	297.5 (595)
DMPF ***	a	10	0	0	0	0	13.05 (15.66)	0.38 (0.25)	769.75 (373.05)	trace
Thymol	a	50	trace	0	0	0	0	0	0	747.5 (1495)
Bifenthrin	i	2	37 (30.2)	0	4.98 (9.95)	3.75 (4.37)	0	2.05 (4.1)	14.3 (3.03)	0
Chlorpyrifos	i	1	0.68 (1.35)	0	0	0.55 (1.1)	0	0	0	0
Cyhalothrin	i	1	0.55 (1.1)	0	3.78 (0.79)	0	0	2.48 (2.94)	0	0
Dimethoate	i	50	0.25 (0.5)	0	0	0	0	0	0	0
Flubendiamide	i	25	0	48.7 (68.87)	0	0	0	0	0	0
Methyl Parathion	i	2	0.25 (0.5)	0	0	0	0	0	0	0
Hexythiazox	igr	30	0.25 (0.5)	0	0	0.5 (0.58)	0	0	0	0
Azoxystrobin	f	2	1.13 (2.25)	0	30.25 (36.07)	2.13 (4.25)	0	0	0	0
Carbendazim	f	5	0	0	0	0	0	0	0.25 (0.29)	0
Chlorothalonil	f	30	0	0	0	0.5 (0.58)	0	0	0	0
Metalaxyl	f	2	1.55 (3.1)	0	0	0	0	0	0	0
Trifloxystrobin	f	1	0.5 (0.58)	0	0	0	0	0	0	0
Vinclozolin	f	1	0	0	0	0.25 (0.5)	0	0	0	0
Atrazine	h	6	2.35 (4.7)	0	0	0	0	0	0.25 (0.29)	0
Metolachlor	h	6	0	0	0	0	0	0	241.25 (311.42)	0
Metribuzin	h	1	0	0	0	0	0	0	10.9 (5.01)	0
Pendimethalin	h	6	8.8 (16.94)	0	0	0	0	0	0	0
Trifluralin	h	1	0.5 (0.58)	0	0	0	0	0	0	0
Tribufos	d	2	0	0	3.9 (7.8)	0	0	0	8.48 (16.95)	0

* a = acaricide, d = defoliant, f = fungicide, h = herbicide, i = insecticide, igr = insect growth regulator

** coumaphos oxon is a breakdown metabolites of coumaphos

*** DMA = 2, 4 dimethylanaline, DMPF = 2, 4 dimethylphenyl formamide; both are breakdown metabolites of amitraz

Table 6. All beehive samples were screened for 174 common agricultural chemicals and metabolites. Of these, 148 compounds that were not detected in any samples are listed, with their levels of detection (LOD) in PPB.

Compound	LOD	Compound	LOD	Compound	LOD
1-Naphthol	10	Dinotefuran	2	Parathion methyl	2
3-Hydroxycarbofuran	10	Diphenamid	20	Permethrin total	10
4,4 dibromobenzophenone	4	Endosulfan I	2	Phenothrin	10
4-Hydroxychlorothalonil	50	Endosulfan II	2	Phorate	50
Acephate	50	Endosulfan sulfate	2	Phosalone	10
Acetamiprid	2	Endrin	10	Phosmet	10
Acetochlor	50	Epoxiconazole	1	Piperonyl butoxide	50
Alachlor	10	Esfenvalerate	2	Pirimiphos methyl	20
Aldicarb	4	Ethion	10	Prallethrin	4
Aldicarb sulfone	2	Ethofumesate	10	Profenofos	10
Aldicarb sulfoxide	20	Etoxazole	1	Pronamide	1
Aldrin	10	Etridiazole	50	Propachlor	10
Allethrin	10	Famoxadone	20	Propanil	10
Amicarbazone	30	Fenamidone	10	Propargite	10
Azinphos methyl	6	Fenbuconazole	10	Propazine	20
Bendiocarb	10	Fenhexamid	6	Propetamphos	4
Benoxacor	20	Fenoxaprop-ethyl	20	Propham	20
BHC alpha	4	Fenpropathrin	10	Propiconazole	20
Bifenazate	20	Fenpyroximate	5	Pymetrozine	20
Boscalid	4	Fenthion	10	Pyraclostrobin	15
Bromuconazole	20	Fipronil	10	Pyrethrins	50
Buprofezin	20	Flonicamid	8	Pyridaben	10
Captan	10	Fludioxonil	20	Pyrimethanil	20
Carbaryl	30	Fluoxastrobin	4	Pyriproxyfen	10
Carbofuran	10	Fluridone	10	Quinoxyfen	10
Carboxin	4	Flutolanil	4	Quintozene (PCNB)	1
Carfentrazone ethyl	1	Heptachlor epoxide	10	Resmethrin total	5
Chlorfenopyr	1	Heptachlor	4	Sethoxydim	2
Chlorfenvinphos	6	Hexachlorobenzene (HCB)	1	Simazine	50
Chlorferone	50	Hydroprene	20	Spinosad	50
Chlorpropham (CIPC)	40	Imazalil	20	Spirodiclofen	2
Clofentezine	100	Imidacloprid 5-hydroxy	25	Spiromesifen	10
Clothianidin	1	Imidacloprid	1	Tebuconazole	8
Cyfluthrin	4	Imidacloprid olefin	10	Tebufenozide	10
Cypermethrin	4	Indoxacarb	3	Tebuthiuron	2
Cyphenothrin	20	Iprodione	50	Tefluthrin	1
Cyprodinil	1	Lindane	4	Tetrachlorvinphos	4
DDD p,p'	4	Linuron	20	Tetraconazole	6
DDE p,p’	2	Malathion	4	Tetradifon	1
DDT p,p'	4	Methamidophos	4	Tetramethrin	10
Deltamethrin	50	Methidathion	10	Thiabendazole	1
Diazinon	5	Methomyl	10	Thiacloprid	1
Dichlorvos (DDVP)	50	Methoxyfenozide	10	Thiamethoxam	1
Dicloran	1	MGK-264	50	THPI	50
Dicofol	1	MGK-326	10	Triadimefon	2
Dieldrin	10	Myclobutanil	15	Triadimenol	45
Difenoconazole	10	Norflurazon	6	Triflumizole	50
Diflubenzuron	10	Oxamyl	5	Triticonazole	10
Dimethenamid	10	Oxyfluorfen	1		
Dimethomorph	20	Paradichlorobenzene	10		

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Chapter III:

Proportion of commodity crop pollens and pesticide contamination in honey bee diets in two different landscapes

Abstract:

Honey bees are the most important managed pollinators in commercial agriculture. Large irrigated mixed agricultural landscapes in the mid-south United States can be vital to maintaining commercial honey bee operations during times of prolonged nectar dearth, even if those crops are not dependent on bee pollinators. Severe declines in bee populations have generated concerns about the relationship of pesticide-treated seeds and crops and the health of honey bees. To investigate the role of pollen from seed-treated crops as a component of honey bee diet and pesticide contamination in pollen diet of honey bees from agricultural and urban landscapes, we monitored honey bee colonies in both agricultural and urban areas. Pollen collection began in mid-March, before seed-treated crops were planted, and continued through the end of August, after crops had ceased blooming. Pollen samples from returning bees were identified to determine the botanical origin of the bees' pollen diet, and were also analyzed for pesticide contamination. Honey bees in the agricultural landscape visited crop sources only during the seasonal period of natural nectar dearth, when other wildflower sources were limited, and they encountered acutely toxic pesticide residues (above LD_{50}) during this period. These bees also encountered toxic levels of herbicide on multiple occasions throughout the season, in pollen from non-crop plants. Bees in the urban area were also exposed to toxic levels of insecticides on several occasions. Urban bees were exposed to herbicides throughout the season,

but not at concentrations approaching acute toxicity. Simultaneous exposure to multiple pesticides occurred at both sites on multiple occasions. The results underscore the need to conserve areas of habitat and forage for both wild and managed pollinators within both agricultural and urban landscapes.

Introduction

Pollinators, and bees in particular, serve a vital ecological role in any terrestrial ecosystem. The economic value of insect pollination in the United States alone was estimated to be $34 billion in 2012 (Jordan et al. 2021). As the primary managed pollinator, honey bees (*Apis mellifera*) have a significant role in food production (Reilly et al. 2020). Bees depend on forage (nectar and pollen) available in their surrounding landscapes. During periods of seasonal nectar dearth, non-cultivated landscapes may be unable to support the large numbers of bee colonies commercial beekeepers maintain for honey production and pollination of bee-dependent crops. Beekeepers in the mid-southern United States often choose to place bee colonies adjacent to farmland that does not require insect pollination, but can provide nutrition to bee colonies and may support the production of surplus honey (Zawislak et al. 2019). The root causes behind declines in honey bee populations are complex and controversial, but these losses are generally agreed to be caused by multiple combined stressors that include biotic and abiotic factors (Goulson et al. 2015, Klein et al. 2017, Nazzi et al. 2012, Neov et al. 2019, Potts et al. 2010). Large-scale losses of honey bee colonies over the last decade have raised concerns about the health and safety of pollinators in agricultural landscapes.

Exposure to even sublethal levels of pesticides can affect honey bee health and behavior. These effects may include reduced brood production (Dively et al. 2015, Traynor et al. 2021a), abnormal foraging behavior (Morfin et al. 2019a, Shi et al. 2020, Yang et al. 2008), impaired learning ability (Aliouane et al. 2009, Li et al. 2019), increased susceptibility to pathogens (Alaux et al. 2010, Grassl et al. 2018, López et al. 2017, Pettis et al. 2013), and even changes in gene expression (Morfin et al. 2019b, Wu et al. 2017). Insecticides in the neonicotinoid class have particularly received much recent scrutiny for their perceived role in poor bee health due to their widespread use and systemic activity (Blacquiére et al. 2012, Cresswell, 2011, Dively et al. 2015, Heller et al. 2020, Schneider et al. 2012).

While many herbicides have long been considered to pose low toxicity risks to pollinators, they have recently been directly implicated in causing sublethal effects on honey bee health (Belsky and Joshi, 2020). Herbicide exposure can disrupt the microbiota in honey bee digestive tract (Dai et al. 2018, Motta et al. 2018, Motta and Moran, 2020, may affect reproduction (Hoopman et al. 2018), can cause delayed molting and development (Vázquez et al. 2018), can impair bee cognition and navigation (Balbuena et al. 2015, Farina et al. 2019), and impair gland development (Faita et al. 2018). Herbicides also affect bees and other pollinators indirectly when they significantly reduce the floral nutrient resources available to pollinators in the landscape (Bohnenblust et al. 2015, Decourtye et al. 2010, Egan et al. 2014, Sharma et al. 2018).

Fungicides were also once considered to have low risks for pollinators, but more recently have been shown to affect pollinator health in multiple ways. Exposure to fungicides can inhibit honey bees' production of detoxification enzymes (Mao et al. 2017), can negatively impact their immune response to pathogens (Glavinic et al. 2019), and may accelerate premature foraging

behavior in adult bees, leading to lower longevity and decreased colony population (Fisher et al. 2020). Fungicides can also synergize with other pesticide compounds, increasing the toxicity of other pesticides (Almasri et al. 2017) and greatly increasing the toxicity of beekeeper-applied miticides (Johnson et al. 2013).

Bees must collect all nutritional resources from plants in their surrounding environment. Although honey contains numerous enzymes and components that regulate metabolic processes in bees (Mao et al. 2013), it is composed mainly of carbohydrates with a low proportion of other nutrients (Ball, 2007) and serves mostly as fuel for adult bee activities rather than nutrition. By contrast, pollen contains proteins, amino acids, sterols, vitamins and other dietary nutrients vital to honey bee health and development (Di Pasquale et al. 2013, 2016). Both the quality and quantity of pollen diversity are important, as no single pollen variety contains all the essential amino acids to sustain bee health (Annoscia et al. 2017, Wright et al. 2018). Honey bee productivity and health have been shown to be positively linked to the proximity of their hives to suitable habitat (Ricigliano et al. 2019). Nutritional stress in bees caused by poor diet has also been linked to greater susceptibility to pathogens (DeGrandi-Hoffman and Chen, 2015, Dolezal and Toth, 2018). Because of the close link between nutritional stress and pollinator declines, conserving and enhancing pollinator habitats may be one of the best ways to directly improve health and boost the populations of both wild and managed pollinators (Naug, 2009).

Modern agricultural landscapes are often composed of large monoculture fields, with reduced plant and floral diversity (Nicholls and Altieri, 2013, Rands and Whitney, 2010). These landscapes are often heavily managed with herbicides, which eliminate flowering weeds within the fields, and reduce the abundance wild flowering plants around the fields due to herbicide drift (Grundy et al. 2011, Roy et al. 2003). Even in landscapes dominated by abundant flowering

crops, bees will also actively forage for pollen and nectar among the non-crop plants they can find (Long and Krupke, 2016). Agricultural landscapes are also routinely exposed to multiple other pesticide applications that potentially affect pollinator health (Calatayud-Vernich et al. 2019, Desneux et al. 2007, Johnson et al. 2010, Pettis et al. 2013, Sánchez-Bayo et al. 2016, Tsvetkov et al. 2017, Yoder et al. 2013). Changes in landscape may also affect quality of bee habitat. For instance, Otto et al. (2016) found that converting land use in North and South Dakota (the top honey producing states in the U.S.) from prairie/grassland to commercial soybeans and corn production made the areas less suitable for beekeeping, due to both pesticide exposure and the loss of forage habitat. Dolezal et al. (2019) also found that intensive agriculture reduced floral resources in a landscape, which contributed to colony decline. Bee nutrition is inarguably linked to bee health (McNeil et al. 2020, Naug, 2009), reduced floral diversity in large monocultures is considered poor forage for bees and conventional crop pest management practices are often blamed for declines in pollinator health. However, in areas with a brief honey flow, beekeepers may rely on farmland and surrounding uncultivated land to help their bees survive a nectar dearth and even produce a substantial honey crop (Zawislak et al. 2019). Even though some crops, such as corn and soybeans, are not dependent on bee pollination, these can provide pollen and/or nectar for bees, and the irrigation runoff from these farm fields enhances flowering vegetation on field margins during otherwise dry conditions, which also benefits bees.

Nectar is collected by bees and returned to the hive, where it is passed to other bees, processed and concentrated into honey, and stored in honeycomb cells throughout the hive. Honey can also be analyzed for both plant origin and chemical contamination. However, because it is not stored in a sequential manner, it is not possible to determine when honey was

collected. Pollen, by contrast, can be sampled directly from returning foragers as they enter the hive, using standard beekeeping equipment (pollen traps) to collect discrete samples at specific intervals. Bee-collected pollen can be identified to plant taxa (Lau et al. 2019) to determine which plants bees were visiting on specific dates. Pollen is also known to readily absorb and contain pesticide residues, which contaminate bee colony diet (Kasiotis et al. 2014, Mullin et al. 2010, de Oliveira et al. 2016). In a recent survey conducted in the United States, only 18% of pollen samples were found to be free of pesticide residues (Traynor et al. 2021b). Calatayud et al. (2018) reported that pollen samples contained the highest number of pesticides of any sampled hive products, and those concentrations were significantly higher in colonies near intensive agriculture. In this context, the current study examines the diversity of pollen sources available in a typical agricultural landscape in the mid-southern United States, as well as the pollen sources available in a nearby mixed urban setting, and evaluates the pesticides residue contamination in bee pollen loads throughout an entire cropping season.

Materials and methods

Study sites

Two study sites were selected with established apiaries. One site ("Agri") was located in Lonoke County, Arkansas, and was surrounded by conventional agricultural production typical for the area (Fig. 1a). Within a 3 km radius of this apiary, 81.2% of the landscape was cultivated with a small number of crops (57.1% soybeans, 10.0% rice, 8.4% corn, 2.0% grain sorghum, 3.4% cotton, 1.5% green beans). Commercial fish pond operations covered an additional 3.8%. The remaining 15% of the landscape was uncultivated, with a mixture of fallow

fields, livestock pastures, woodlands, wetlands and a few residential yards (Zawislak et al. 2019). Although honey bees can forage much farther than 3 km, they typically remain within 2 km of their hive when desirable forage is adequate (Beekman and Ratnieks 2000, Couvillon et al. 2015, Hagler et al. 2011, Steffan-Dewenter and Kuhn, 2003, Traynor et al. 2021b).

The second study site ("Urban") was located approximately 48 km (30 miles) away, in Pulaski County, Arkansas. The apiary was located adjacent to a large urban community garden, surrounded by some open fields, woodlands, and residential lawns (Fig 1b). The community garden contained approximately 400 individual plots, each managed independently without restrictions on what could be planted or how weeds and pests might be controlled. This site was considered to be representative of environmental conditions to which area urban honey bee colonies are routinely exposed. Surrounding land type for the Urban site was not quantified, as was done for the Agri site.

Pollen collection

Pollen traps (Brushy Mountain Bee Supply #509, Moravian Falls, NC) were installed on three bee hives in each of the two apiaries. Traps featured a sliding panel that, when engaged, forced honey bees to enter and exit the hive through constricted openings, which mechanically remove pollen loads from their corbiculae (tibial pollen baskets). Pollen pellets collect in a separate tray below. For each sampling period, traps were engaged to collect pollen for 48 h at a time, but trap panels were removed to allow bees free access to the hive when not collecting pollen. Samples were collected in both apiaries on 13 dates, biweekly from March 16 to August 31, 2016. This included the period before, during, and after planting of pesticide-coated seeds, and continued throughout the major bloom periods of the crops around the Agri apiary location. Pollen was removed from each trap immediately after each 48 h sampling period and stored at -

12 C (10 ◦F) to preserve pesticide residues and to kill pests, such as ants and small hive beetles (*Aethina tumida*) and their eggs, which are commonly found in pollen traps in this area.

Plant identification

All pollen collected from the three hives at an apiary site was pooled and thoroughly mixed on each of 13 collection dates, and at each of the 2 sites. The resulting 26 samples provide a chronological record of plants that produced pollen attractive to bees at each site throughout the cropping season. Pooling pollen was done to provide a broader survey of the surrounding landscape vegetation than from a single hive, and because resources were limited to analyze all samples separately.

For each sample date and each apiary site, a random 50 mL subsample of pooled pollen was taken for plant taxa identification. Each of these samples was sorted by color, and then examined microscopically to determine the relative proportion of pollen from crop plants (corn, cotton, soybean, grain sorghum) in each sample, and to identify non-crop taxa where possible. Pollen sorting, quantification and identification was performed at Deschambault Animal Science Research Center, Quebec, CA, as described by Girard et al. (2012). Further identifications of some unknown pollen specimens were also conducted at the Texas A & M Palynology Laboratory, College Station, Texas.

Pesticide residue analysis

Additional 50 mL subsamples of pooled pollen from each date and location were screened for pesticide residues by the USDA-ARS National Science Laboratory, Gastonia, NC. The laboratory performed an analysis procedure (method AOAC OMA 2007.01) referred to as QuEChERS, which stands for Quick-Easy-Cheap-Effective-Rugged-Safe (Perestrelo et al. 2019).

The analytes were extracted from the samples by high-speed grinding in an acidified acetonitrile and water mixture followed by a clean-up step to remove some matrix components, and filtration to remove particulates. Separate aliquots of extract were analyzed for pesticide residue by gas chromatography (GC) and liquid chromatography (LC) techniques utilizing mass selective detection systems. This procedure screened for 214 common agricultural chemicals and metabolites as part of their standardized apicultural product analysis.

Results

Pollen sources by plant taxa

Collections made throughout the entire growing season from the honey bee colonies at the Agri hives yielded pollen from 58 distinct plant taxa, whereas only 46 unique plant taxa were identified from the pollen collected in the urban apiary. Some pollen taxa were recognized as morphologically distinct from others, but could not be precisely identified, and were labeled as unknowns, designated with a number. Other pollen types could be identified to plant family or genus, but not to species, and were designated as types within that taxon.

Honey bees collected pollen from a succession of many different plants throughout the season (Figs. 2 and 3), although a small number of plant taxa contributed the majority of pollen during each sampling period. Pollen from some plant taxa was collected at both sites simultaneously, while others were found at only one or the other site. The plant families Salicaceae, Rosaceae, Caryophyllaceae and Brassicaceae were dominant early pollen sources at both sites. Oleaceae was a significant spring pollen source at the Agri site, but absent from the Urban samples. Conversely, Sapindaceae was important at the Urban site, but absent at the Agri

site. Later in the spring (April-May), plants in Fabaceae, Rosaceae and Vitaceae were significant sources of pollen at both sites, while Magnoliaceae was a significant source in the Agri area, and Cornaceae was significant at the Urban site. Figs. 2 and 3 summarize the relative abundance of each pollen taxa collected by the bees on each date, and at each site, during the sampling period.

Pollen collected at the Agri site was categorized as being from three crop sources (soybean, corn and sorghum) and non-crop sources (Fig. 4). No pollen from cotton or rice was identified in any of the samples. For the first seven collection dates (March 16-June 8) and the last collection date (August 31), 100% of all pollen was from non-crop plant taxa. On two dates (June 22 and July 6) non-crop sources accounted for 97.1% and 99.96% of all pollen collected. Bees visited one or more crops to collect pollen on only 5 of the 13 sample dates, from June 22-August 17. Soybean pollen was collected on four dates, representing 2.9%, 0.4%, 17.1% and 41.4% of all pollen collected on June 22, July 6, July 20 and August 3, respectively. Corn pollen was collected only on July 20, when it accounted for 66.3% of all pollen collected. Sorghum pollen was collected on two dates, representing 3.8% and 8.4% of all pollen collected on August 3 and August 17, respectively. No crop-related pollens were identified from samples collected on August 31.

The Urban hives were not in proximity to commodity crops, but some sweet corn was grown in some nearby garden plots, and small amounts of corn pollen were identified on June 22 (2%) and August 17 (10%) in samples from the Urban site. On two consecutive dates (July 20 and Aug 3) the urban bees collected 100% and 86.1%, respectively, of total pollen from a single unidentified plant source (Fig. 3).

Pesticide residues

A total of 26 samples of bee-collected pollen, from each date and each apiary site, were screened for 214 common agricultural pesticides. Of these, only 16 compounds were detected in pollen from the Agri apiary, which included five insecticides, four fungicides and seven herbicides (Table 2). Only eight pesticides were detected in pollen from the Urban apiary, which included three insecticides, three fungicides and two herbicides (Table 3). Agricultural chemicals and metabolites that were not detected in any samples are presented with their limits of detection in Table S1.

At least one pesticide product was detected in pollen from the Agri site on every date sampled. Multiple products were identified on 11 of the 13 dates, with an average of 3.2 ± 1.5 (sd) compounds detected in each sample. In 14 of 55 cases, compounds were detected in only trace amounts, which could be identified, but remained below the lower limit of detection (LOD) to be accurately quantified.

Traces of fenpyroximate, imidacloprid and thiamethoxam were detected in the Agri pollen samples on only two early season dates, March 30 and April 13. Chlorantraniliprole was detected on four consecutive sampling dates (July 6-August 17) and cyhalothrin was detected three consecutive sampling dates (July 20-August 17). Residues of four fungicides (azoxystrobin, carbendazim, difenoconazole, trifloxystrobin) were detected in Agri pollen samples, but were found at only trace levels on six of the nine sample dates before July 20. All four fungicides were found at greater than trace levels on at least one of the last four sample dates (July 20-Aug 31). Seven herbicide products were detected, and at least one or more herbicides were found on every sample date throughout the season. Atrazine was present in all but one sample. In contrast, the herbicides propazine and propachlor were each detected only

once; on April 27 and on August 8, respectively. Fluometron was found at only trace levels on May 11 and May 25. Metolachlor was detected on six consecutive samples between April 13 and June 22, twice exceeding its LD_{50} level for honey bees. Propanil was detected at varying concentrations in 5 consecutive samples between April 27 and June 22, and again on July 20 and August 17, but never above LD_{50}. Complete results of pesticide residue screening for all sampling dates at Agri site are reported in Table 2.

At least one pesticide product was detected in pollen from the Urban site on 7 of 13 dates sampled, with an average of 1.4 ± 0.5 (sd) compounds detected on each of those 7 days. Of the eight compounds detected at the Urban apiary site during the season, one insecticide (imidacloprid) and two fungicides (azoxystrobin and pyraclostrobin) were detected only at trace levels. The fungicide carbendazim was detected on five consecutive sample dates (Mar 30-May 25) although only above trace level on Mar 30. The herbicide atrazine was detected in six samples, but only at or near trace levels each time. Detections of the remaining compounds were infrequent, and no residues were detected in pollens on six of the thirteen sample dates. Complete results of pesticide residue screening for all sampling dates at the Urban site are reported in Table 3.

Each sample was screened for 214 common agricultural pesticides and metabolites. Of these, 198 compounds were not detected in any samples. They are listed in Table 4, along with their lower limits of detection in ppb.

Discussion

A honey bee colony with an average of 50,000 members requires 20 kg (44 pounds) of pollen annually (Seeley, 1995). Early in the growing season, while farm fields are being prepared and before crops bloom, honey bees and other pollinators in agricultural landscapes must seek flowering plants in the relatively small proportion of the landscape outside of these fields. Pollen is particularly important to honey bees during the early spring when colony populations build up rapidly, and the bees need substantial amounts of pollen protein to rear large numbers of larvae. Without this rapid spring brood expansion, honey bee colonies may not have a sufficient population to create a surplus honey crop later in the season when nectar is abundant.

Our results are similar to Wood et al. (2018), who found that both honey bees and native bees utilized mostly native plants, but did visit non-native (crop) plants later in the summer, when these plant resources were readily available. The current study suggests that, despite the majority of this agricultural landscape being devoted to a small number of commodity crops, and being managed with extensive herbicide applications, honey bees were able to locate floral resources around the study site when crop plants were not blooming, and that crop plants that are not bee-dependent for pollination still provided significant resources for honey bees, particularly when wild forage sources were less abundant during dry conditions. But even when these abundant crop plants were in bloom, bees also continued to seek out and utilize non-crop pollen sources as well.

Pernal and Curie (2001) suggested that honey bees collect pollen from diverse plant taxa without regard to protein content. Liolios et al. (2015) also determined that bees' preference for pollen sources was not correlated to nutrient content, but was associated with its relative abundance in

the landscape. Other research, however, has concluded that bees can detect nutritional deficiencies in pollen sources, and actively adjust foraging to include other plant sources to provide missing nutrients, including proteins, amino acids and fatty acids (Cook et al. 2003, Hendriksma and Shafir 2016). Honey bees likely employ both of these strategies, taking advantage of abundant resources when available, but also purposely seeking diverse pollen sources over a wide area to compensate for nutrient deficiencies in any one plant taxon. If bees simply gathered pollen in proportion to the relative availability of plants, we would expect a much higher proportion of pollen to be collected from soybeans during their bloom period. Taxonomic identification and pesticide screening of bee-collected pollen samples provided a chronological record of both seasonal plant bloom as well as potential chemical contamination of pollinator diet.

Our data indicate that the bees in this agricultural system utilized a broad range of plant taxa from a small proportion of the land within their foraging territory throughout the season. On 8 of our 13 sample dates, bees collected no pollen from cultivated crops in the Agri area, and for most of this period, crop plants were not yet flowering. On only 2 of the other 5 dates was the proportion of crop pollen more than 8.4% of the total pollen collected (83.4% on July 20, and 45.2% on August 3). These dates correspond with a hot, dry period around both study sites, when few wildflowers were in bloom, which beekeepers refer to as a nectar dearth. These dates also correspond to the blooming period of the major commodity crops produced in this region.

Although soybeans began blooming near the end of June, they were not immediately utilized by the bees as a pollen source, despite covering more than 57% of the landscape. The percentage of soybean pollen was 2.9%, 0.4%, 17.1% and 41.4% on June 22, July 6, July 20 and August 3, respectively. Soybean pollen was not detected from the last two sample dates (August

17, August 31), likely because their flowering period had mostly ended. Although covering only 8% of the landscape, corn accounted for 66.3% of the pollen collected by bees on one sampling date (July 20). This suggests that bees responded to a sudden abundance of a novel pollen source, but they also continued to seek other pollen sources as well. Although corn is anemophilous, and its pollen contains a crude protein content of only 15% (Malerbo-Souza 2011), bees will utilize it as a pollen source. An individual corn plant can produce between 2 and 25 million grains of pollen, which it will shed in 3-7 days. With some variability in plant maturity across a field, most plants within a field will complete pollen shed within 14 days (Nielsen 2010). It is therefore not surprising that corn was detected on only one sample date. Grain sorghum is another wind-pollinated plant that produces no nectar, but was utilized by the bees for pollen. It accounted for 3.8% and 8.4% of collected pollen on August 3 and August 17 respectively. Because its bloom period is limited to 4–9 days per plant (Gerik et al. 2003) and it was cultivated on only 2% of the Agri landscape, it is not surprising that sorghum made a relatively small contribution to the bees' overall diet.

On August 3, 29.9% of sampled pollen was from Amaranthaceae pollen. While not identified to species, this pollen may have been from Palmer amaranth (*Amanarthus palmeri*), which is a fast-growing and highly competitive weed native to the area, and is commonly found in and around fields of soybeans and other crops. The ubiquitous presence of this agricultural weed and its documented resistance to glyphosate (Norsworthy et al. 2008) may account for some of the high levels of herbicides detected in pollen samples. *A. palmeri* is dioecious and anemophilous (Sosnoskie et al. 2012), but is also clearly attractive to honey bees for its pollen. Its presence in only one pollen sample suggests it also has a brief flowering period. Focusing efforts to control this weed earlier in the season, before it produces pollen, could potentially help

to reduce the reappearance of this weed in subsequent seasons and help to limit the transfer of herbicide resistance genes by wind- or insect-assisted pollination.

The brief period when bees did collect a high proportion of pollen from crop sources coincided with the seasonal nectar dearth expected in Arkansas, due to lack of rain in July and August. During this annual period, irrigated agricultural land likely hosts the most abundant food supply for pollinators, including crop plants as well as wild plants and weeds within the fields and near field margins, while other wild vegetation in the area may be limited at this time. Significant agricultural crop pollens were not expected in samples from the Urban apiary site, and only small amounts of corn were detected (2.0% on June 22 and 10.3% on August 17), likely from small garden plots.

It was surprising to find greater plant diversity in the Agri samples than in the urban samples, since more than 80% of the landscape was devoted to the production of a few plants. However, this was a limited study with only two sites, and both Agri and Urban landscapes are highly disturbed and contained managed vegetation and non-native. Plant diversity and abundance is likely to vary across even small changes in geography as well as land use, and additional sampling in other locations between both sites would likely render somewhat different results.

A single plant type contributed 100% of pollen to the Urban pollen sample on July 20, and 86.1% on Aug 3. Although it was among those pollen types not identified, it would be useful to discover the species of this plant. It is clearly attractive to honey bees, and blooms during the seasonal dearth. Its presence in the Agri landscape as well (1.9% on Aug 3) suggests it may be a native plant with wide distribution. If identified, it could be valuable for beekeepers to incorporate this plant into pollinator conservation enhancement seed mixes.

Among different classes of pesticides detected, herbicides were found throughout the season at the Agri site, reflecting their extensive use in this landscape. Their presence in every Agri apiary sample suggests that bees consistently visited wild plants (weeds) that farmers were attempting to control in the field and along field margins, or that herbicides were consistently drifting onto surrounding non-target vegetation. Most likely, both of these occurred. While herbicides are generally considered less toxic to bees than other classes of pesticides, the levels detected in our sampling exceeded published LD_{50} values for atrazine on 4 occasions, metolachlor twice, and metribuzin once. On 11 of the 13 dates, more than one herbicide was detected simultaneously. While LD_{50} values for individual products were not exceeded, the combined effect on bee health of exposure to multiple products is largely unknown.

Herbicides were detected in fewer than half the pollen samples from the Urban area, and never at levels over 6% of the known LD_{50}. These residues were likely related to consumer products for weed control applied to nearby residential lawns. The herbicide glyphosate was not reported in our results, despite its high probability of use in this landscape. Due to the polar nature of glyphosate, the extraction procedure is different and must be screened with a separate test, which was not performed due to resource limitations. An estimated 95% of corn, soybean and cotton seeds planted in this region are "Roundup-Ready" crops, engineered to be tolerant to glyphosate. Most fields are prepared with herbicides prior to planting, and many of the resistance-engineered crops do receive multiple applications during the growing season.

Fungicides were detected at Agri site throughout the season (in 9 of 13 samples), but mostly in trace amounts. Carbendazim was detected in trace levels throughout in the season, but at a quantifiable concentration only on the final sample date, and then only at 14% of LD_{50}. Only on two dates, when bees were collecting substantial amounts of corn and soybean pollens, were

the levels of fungicides detected at a quantifiable level, and then still well below LD_{50}. Although fungicides have low acute toxicity to bees on their own, they can synergize with other compounds, particularly beekeeper-applied miticides (Johnson et al. 2013), and can negatively impact immune function and digestion in honey bees (DeGrandi-Hoffman et al. 2015).

Fewer fungicide products were detected at the Urban site, although not completely absent. Carbendazim is used in residential turf and lawn formulations, and was detected in five consecutive samples from the Urban apiary, likely related to products applied to nearby residential lawns. It was detected above a trace level once, but still well below LD_{50}.

Only five insecticides were detected in pollen samples from the Agri site. Two of these, chlorantraniliprole and cyhalothrin, were only detected when bees foraging for pollen in field crops. Chlorantraniliprole is considered relatively non-toxic to honey bees, and was detected above trace levels on only three occasions, and still well below LD_{50}. Cyhalothrins, can be highly toxic (Dolezal et al. 2016), but was not detected here at a level greater than 11% of LD_{50}.

Our previous survey (Zawislak et al. 2019) indicated that the majority of all crop seeds planted near this apiary were coated with neonicotinoid insecticides prior to planting. However, imidacloprid and thiamethoxam (both neonicotinoids) were each detected only once each, and only in trace quantities, during the planting period of the growing season. No detections of these products were made later in the season from any sources, in contrast to other reports (Botías et al. 2015, 2016, Krupke et al. 2012, Krupke and Long, 2015, Pilling et al. 2013). Our results are consistent with Stewart et al. (2014), who found only low levels of neonicotinoids in bee-collected pollen from multiple apiaries in agricultural locations in the mid-south.

Traces of imidacloprid and thiamethoxam were each detected once (March 30 and April 13, respectively), which coincided with spring planting, and may have been related to contaminated dusts exhausted by equipment used to plant treated seeds (Greatti et al. 2006, Krupke and Long, 2015). For both of these compounds, the laboratory's lower limit of detection (LOD) was 1 ppb. Below this level, the compound could be confirmed to be present (reported as "trace") but could not be reliably quantified. Even though the quantity remained below LOD, the LD_{50} values for these compounds (0.004 ppb for imidacloprid and 0.05 ppb for thiamethoxam) are also well below the LOD. Therefore it could not be determined using our methods if the residue levels detected in these samples was acutely toxic, could cause sub-lethal effects, or was low enough to have no observable effect on honey bees. Fenpyroximate was also detected at a trace level, well below LD_{50}, on April 13. However, this compound is not commonly used in row crop agriculture in our area, and was more likely related to a homeowner-applied product on ornamental plants at a nearby residential home.

Insecticides were detected in Urban pollen samples. Imidacloprid was detected twice, but both were at levels below LOD. As before, these trace levels could potentially have been toxic to honey bees. Dicofol, an insecticide and miticide that is commonly used on different cultivated and on ornamental plants, was detected twice at this apiary (July 6 and July 20), at 5–7 times the published LD_{50} level. Carbaryl was detected twice (April 27 and July 6), both times at 20 times the LD_{50}. One sample (July 6) contained all three of these compounds. Simultaneous exposure to multiple pesticide compounds has been shown to increase synergistic toxicity and negatively impact honey bee health and physiology (Johnson et al. 2010, Tomé et al. 2017, Zhu et al. 2017). The specific effects of multiple or repeated exposures to mixtures of compounds is largely

unknown because there are practically unlimited combinations of product formulations and different concentrations to which pollinators could be exposed in the field.

Despite the highly disturbed and managed landscapes surrounding both sites, honey bees in our study appeared able to find ample nutrition within their foraging habitat throughout the season. The majority of the Agri landscape was dedicated to just a few crops, which are not bee-dependent and bloom only in late summer. Given the risks that some agricultural chemicals can pose to pollinator health (Mullin et al. 2010, Milone et al. 2021), the consistent applications of agricultural pesticides in this landscape and the potential for pesticide drift to occur onto non-target vegetation, the levels of fungicides and insecticides detected in our pollen samples were surprisingly low, and in most cases were well below what is acutely toxic to bees. However, because we were unable to accurately quantify small amounts of the most toxic insecticides, their specific risks to bees could not be determined for some samples, although the instances where this did occur were infrequent. One or more herbicide compounds were detected in each of the Agri samples, and on some occasions did greatly exceed LD_{50} for one or more of these.

For honey bees, pollen foraging is a specialized task different from nectar foraging. Bee colonies collect far more nectar, which is concentrated into honey, and consumed during times when fresh floral resources are not available. Contamination of nectar may vary, as it is produced continually by different plants, and usually collected from multiple flower species by different colony members simultaneously (Visscher and Seeley, 1982). Our study only considered residues found in pollen, and did not investigate nectar foraging by bees in crop sources. Total exposure to pesticides must take into account both pollen and nectar contamination, as well as other avenues of environmental exposure, to fully determine the risk of pest control practices on pollinator health.

These honey bees obtained the majority of their pollen diet from non-crop plants, found on a relatively small proportion of the landscape for most of the growing season. This behavior may potentially limit their exposure to pesticide products being applied to crops earlier in the season because bees were foraging in vegetation outside of cultivated fields, and away from heavily contaminated wildflowers. The fact that most of the Agri pollen samples contained few insecticides or fungicides suggests that, given adequate alternative forage area, honey bee colonies can be kept in predominantly agricultural landscapes without necessarily high risk from acute toxicity, although sublethal effects may exist. Herbicides, however, were detected in every pollen sample. Not only do herbicides reduce the available nutrition for pollinators in a landscape, but they have been shown to negatively impact pollinator health by disrupting beneficial gut microbes in bees (Dai et al. 2018, Motta et al. 2018, Motta and Moran, 2020). The identification of bee-collected pollen sources as non-crop for most of the season emphasizes the importance of these wild plants for honey bees, and by extension to native bees and other pollinators. The presence of herbicides in every sample underscores the extensive use of herbicides in modern agriculture, and further emphasizes the importance of maintaining and conserving areas of diverse wild vegetation within agricultural areas for the health of both native and managed pollinators.

Pollinators are keystone species, which perform valuable ecological services in terrestrial landscapes. In addition to improving fruit production and seed set in human food crops, the efforts of bees and other pollinators ensures continued wild plant reproduction and food availability for many species of wildlife, directly or indirectly. However, the most intensively cultivated agricultural areas in the U.S. have also been documented to have the lowest bee abundance (Jordan et al. 2021). A review by Sánchez-Bayo and Wyckhuys (2019) concluded

that habitat loss and conversion to intensive agriculture or urban land use was the primary driver in insect losses, followed by pollution and contamination by agricultural chemicals.

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There are numerous benefits to conserving, enhancing or expanding diverse floral resources and pollinator-friendly habitat within otherwise disturbed or highly managed landscapes. Improving forage availability around agricultural land supports bees with nutrition during times when crops are not in bloom, maintaining increased pollinator populations that can visit crops when they are in bloom. Enhanced pollinator habitat outside of crop fields may limit the exposure of bees to early season pesticide applications because bees are foraging elsewhere. Diverse floral resources promote bee health and reproduction, and can offset effects from exposure to pesticides (Klaus et al. 2021). Increased floral diversity around cropping systems can also improve the longevity and fecundity of predatory arthropods, which can help control agricultural pests (He et al. 2020).

Our study suggests that even within an intensively managed agricultural landscape such our study site, honey bees were able to locate numerous types of plants that serve as pollen resources outside of cultivated areas. While a minority of crop plants made up the majority of

the landscape, they did not serve as major pollen resources except for a brief period. These crop plants can benefit honey bees by providing nutrition during the nectar dearth, but will not support honey bee colonies nutritionally for the majority of the year. Conserving even a relatively small proportion of a landscape with enhanced pollinator habitat could greatly support bee populations and mediate some of the negative impacts that large scale agricultural production can have on pollinator health and populations.

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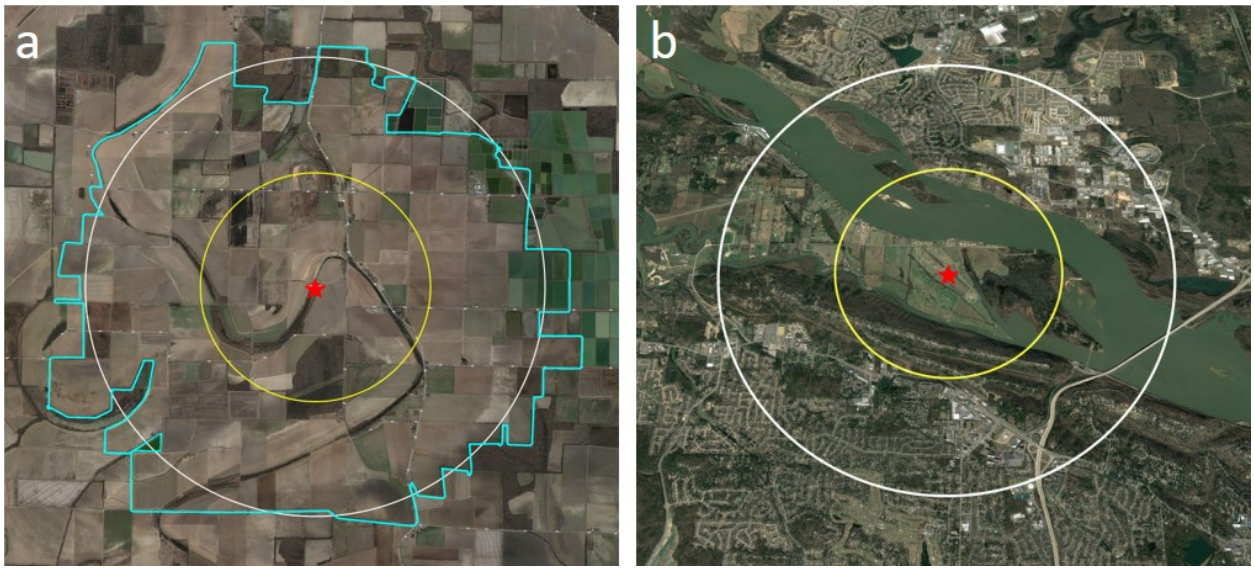

Figure 1. Aerial maps show land use surrounding apiary study sites, (a) Agri site and (b) Urban site. Red stars indicate bee hive locations. Yellow circles indicate a 1.6 km (1 mile) radius from hives; white circles indicate a 3.3 km (2 mile) radius. Blue outline on (a) indicates area surveyed for land use (see Table 1). (Imagery ©2020 Google, Landsat/Copernicus, Maxar Technologies, Pulaski Area GIS, State of Arkansas, U.S. Geological Survey, USDA Farm Service Agency.)

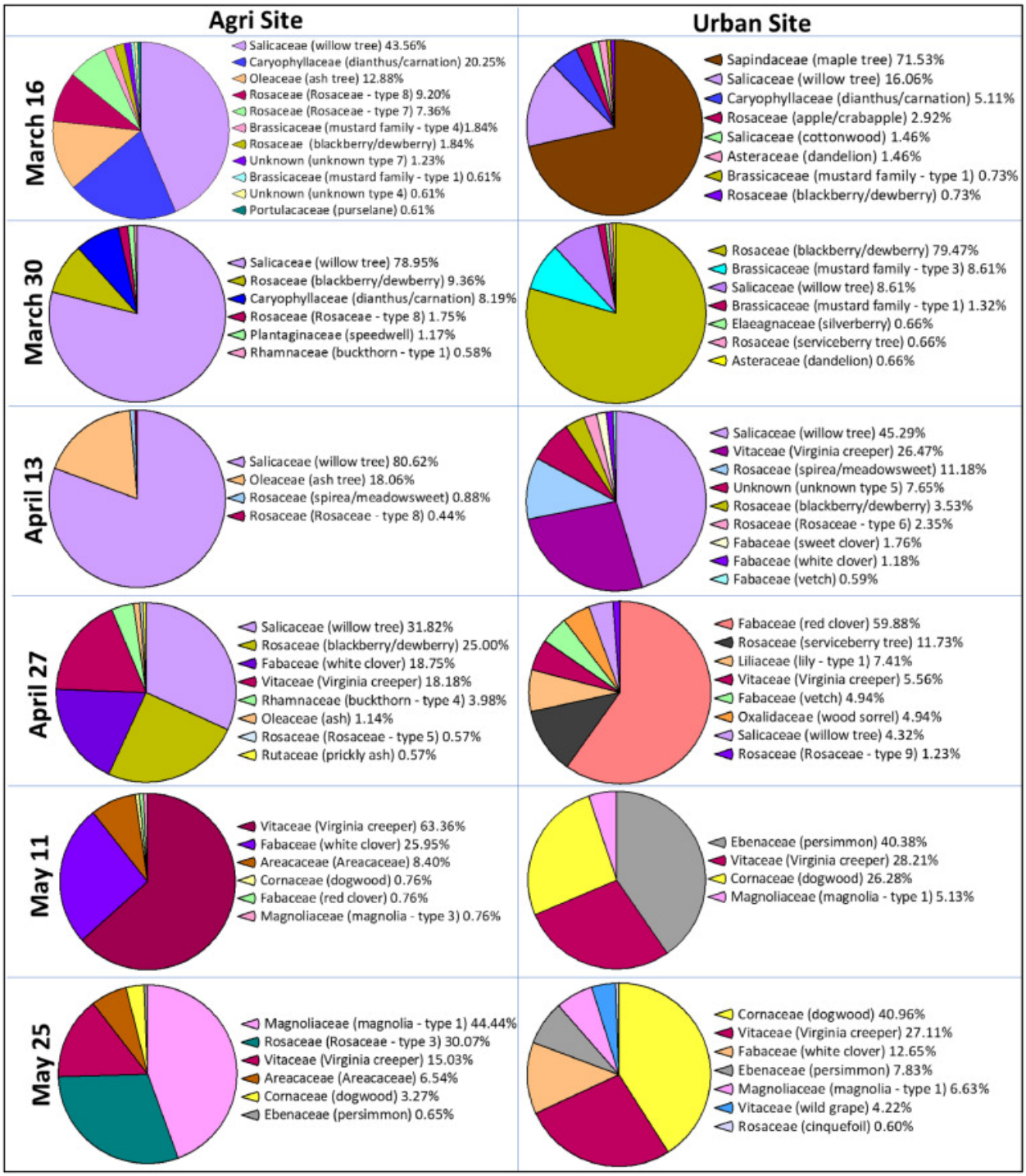

Figure 2. Relative proportion of plant taxa in pollen samples from both apiaries for each sample date during March, April and May sampling period.

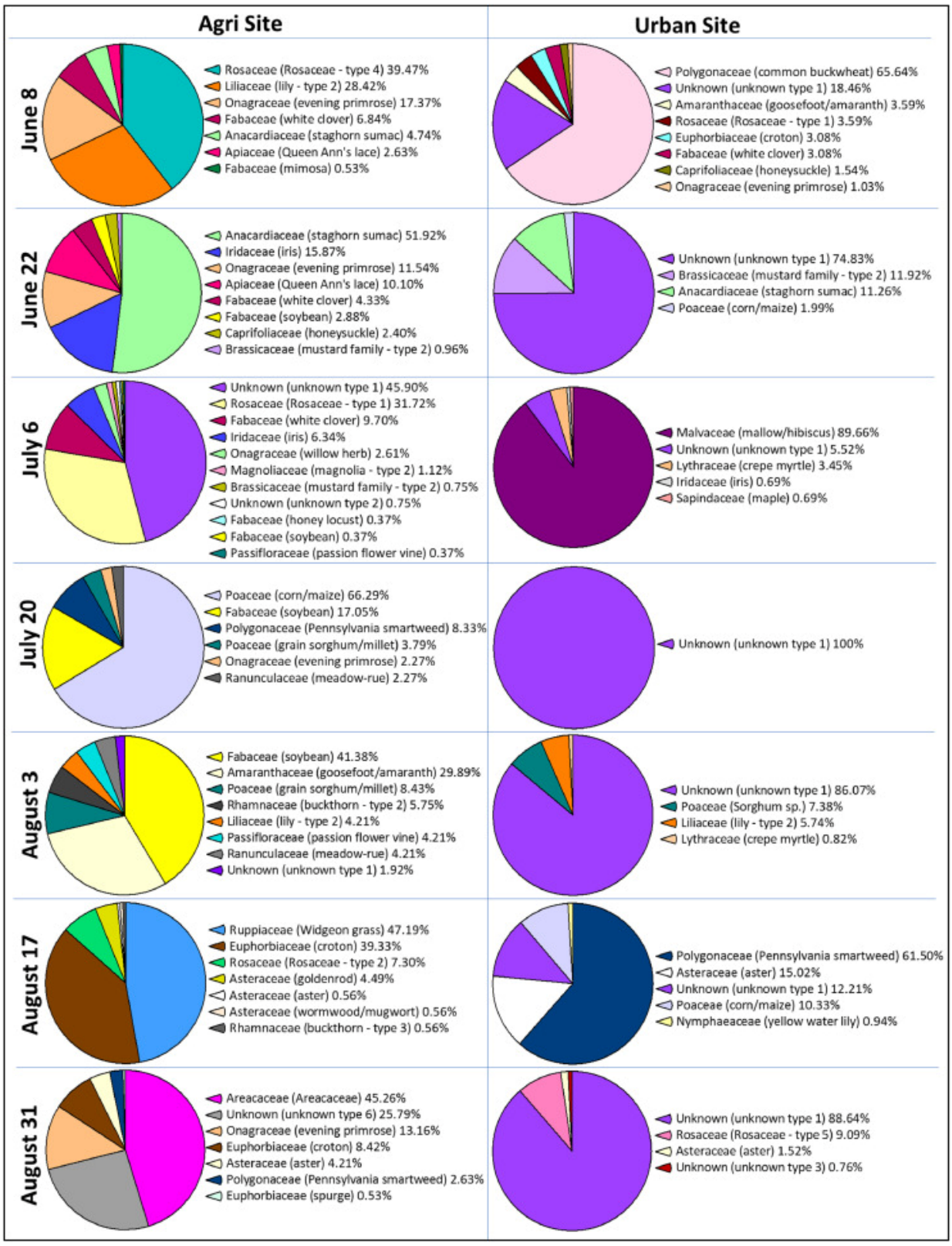

Figure 3. Relative proportion of plant taxa in pollen samples from both apiaries for each sample date during June, July and August sampling period.

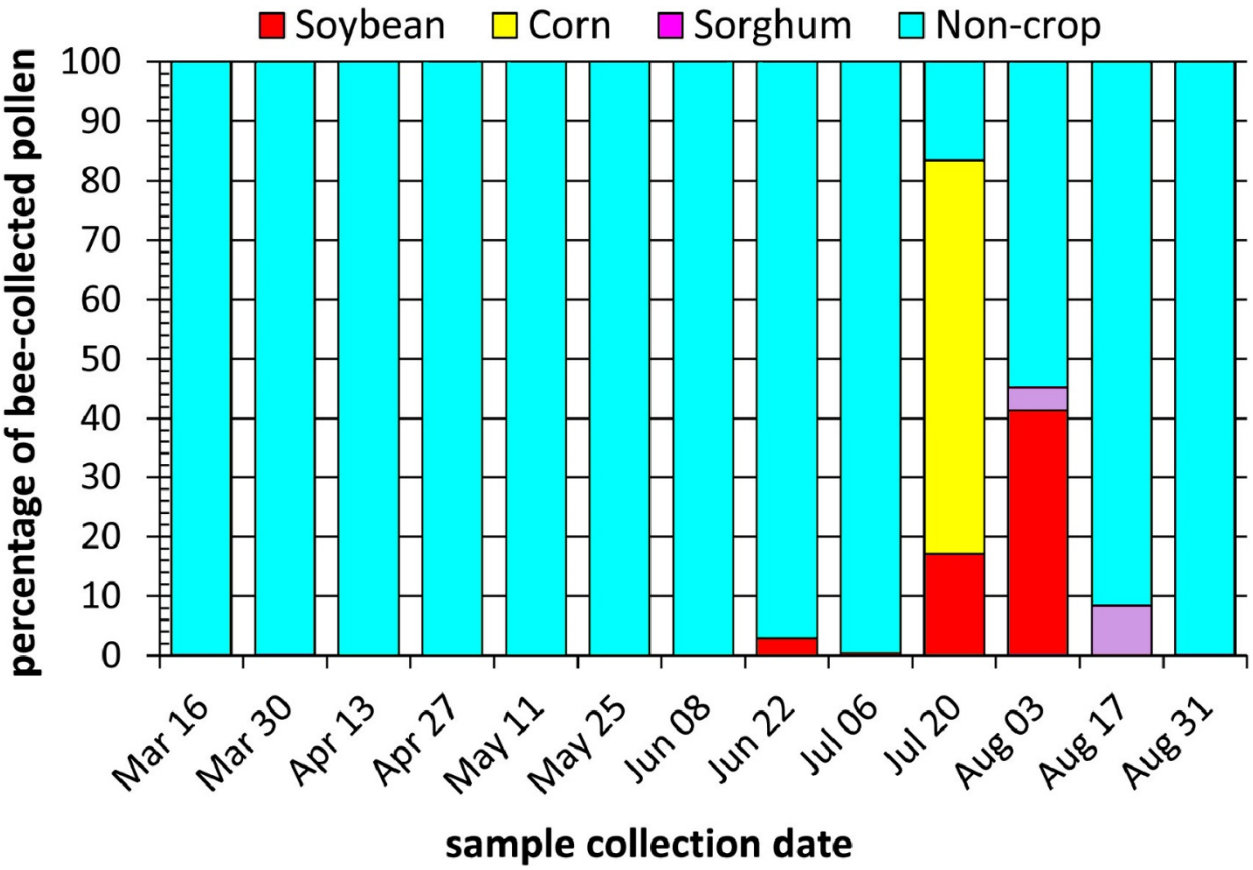

Figure 4. Percentage of bee-collected pollen from crop and non-crop sources at Agri apiary site for each sample date.

Table 1. Land use around Agri apiary site. Acreage per crop and uncultivated land within approximately a 3.3 km (2 mile) radius around the apiary during two years prior to the current study. Non-crop land included uncultivated fields, woodlands, and natural wetlands.

Crop/Land Use	Number of acres	Percentage of total acreage surveyed
Soybean	6,387	57.1
Rice	1,099	10.0
Corn	927	8.4
Cotton	380	3.4
Green beans	153	1.5
Grain sorghum	92	2.0
Total crop area	9,038	81.2
Fish ponds	396	3.8
Non-crop area	1,678	15.0
Total acreage surveyed	11,112	100

Table 2. Residues detected at Agricultural site, reported as parts per billion (ppb). Five insecticides (i), four fungicides (f) and seven herbicides (h) detected in pollen sampled between March 16 and August 31, in Lonoke County, Arkansas. Also shown are the numbers of detections, including trace levels, percentages of samples with detections, maximum detection levels and published LD_{50} values for honey bees.

compound	use	LOD (ppb)	Mar 16	Mar 30	Apr 13	Apr 27	May 11	May 25	Jun 8	Jun 22	July 6	Jul 20	Aug 8	Aug 17	Aug 31	number of detections	percentage detections	max detected (ppb)	oral (or dermal*) LD_{50} (ng/bee)
Chlorantraniliprole	i	15	0	0	0	0	0	0	0	0	trace	**36**	**19**	**30**	0	4	31%	36	104 [b]
Cyhalothrin (total)	i	1	0	0	0	0	0	0	0	0	0	**11**	trace	**10**	0	3	23%	11	0.97 [c]
Fenpyroximate	i	5	0	0	trace	0	0	0	0	0	0	0	0	0	0	1	8%	< LOD	119 [a]
Imidacloprid	i	1	0	trace	0	0	0	0	0	0	0	0	0	0	0	1	8%	< LOD	0.004 [a]
Thiamethoxam	i	1	0	0	trace	0	0	0	0	0	0	0	0	0	0	1	8%	< LOD	0.05 [a]
Azoxystrobin	f	2	0	0	0	0	0	0	0	trace	0	**11**	**17**	0	0	3	23%	17	>25 [b]
Carbendazim (MBC)	f	5	0	trace	trace	0	trace	trace	trace	0	0	trace	0	0	**7**	7	54%	7	>50* [a]
Difenoconazole	f	10	0	0	0	0	0	0	0	0	0	**10**	trace	0	0	2	15%	10	532 [b]
Trifloxystrobin	f	1	0	0	0	0	0	0	0	0	0	**11**	0	0	0	1	8%	11	>200* [a]
Atrazine	h	6	**16**	**16**	**1100**	**400**	0	**130**	**15**	**26**	**11**	**22**	**21**	**1100**	**8**	12	92%	1100	>97* [a]
Fluometuron	h	40	0	0	0	0	trace	trace	0	0	0	0	0	0	0	2	15%	< LOD	582 [a]
Metolachlor	h	6	0	0	**1710**	**238**	**31**	**56**	**43**	**41**	0	0	0	0	**13**	7	54%	1710	>110 [a]
Metribuzin	h	1	0	0	**192**	**25**	0	0	0	0	0	0	0	0	0	2	15%	192	60* [a]
Propachlor	h	10	0	0	0	0	0	0	0	0	0	0	**428**	0	0	1	8%	428	>1000* [a]
Propanil	h	10	0	0	0	**26**	**74**	**74**	**300**	**17**	0	**7**	0	**17**	0	7	54%	300	504 [a]
Propazine	h	20	0	0	0	**19**	0	0	0	0	0	0	0	0	0	1	8%	19	97* [a]
compounds detected per date > LOD			1	1	3	5	2	3	3	3	1	7	4	4	3				

use: f = fungicide, h = herbicide, i = insecticide/acaricide
LOD = level of detection (PPB)
trace = compound detected but below LOD, unable to quantify
* dermal LD50 used when oral LD50 was not found

toxicity data sources:
a https://ecotox.ipmcenters.org
b https://doi.org/10.1371/journal.pone.0094482
c http://npic.orst.edu/factsheets/archive/l_cyhalotech.pdf

Table 3. Residues detected at urban site, reported as parts per billion (ppb). Three insecticides (i), three fungicides (f) and two herbicides (h) were detected in pollen sampled between March 16 and August 31, in Pulaski County, Arkansas. Also shown are the numbers of detections, including trace levels, percentages of samples with detections, maximum detection levels and published LD_{50} values for honey bees.

compound	use	LOD (ppb)	Mar 16	Mar 30	Apr 13	Apr 27	May 11	May 25	Jun 8	Jun 22	July 6	Jul 20	Aug 8	Aug 17	Aug 31	number of detections	percentage detections	max detected (ppb)	oral (or dermal*) LD_{50} (ng/bee)
Carbaryl	i	2	0	0	0	**3**	0	0	0	0	**3**	0	0	0	0	2	15%	3	0.15 [b]
Dicofol	i	5	0	0	0	0	0	0	0	0	**71**	**58**	0	0	0	2	15%	71	10 [b]
Imidacloprid	i	1	0	0	0	0	0	0	0	0	trace	trace	0	0	0	2	15%	< LOD	0.004 [a]
Azoxystrobin	f	2	0	0	0	0	0	0	0	trace	0	0	0	0	0	1	8%	< LOD	>25 [b]
Carbendazim (MBC)	f	5	0	**22**	trace	trace	trace	trace	0	0	0	0	0	0	0	5	38%	22	>50* [a]
Pyraclostrobin	f	15	trace	0	0	0	0	0	0	0	0	0	0	0	0	1	1%	< LOD	73 [b]
Atrazine	h	4	**4**	**5**	trace	**6**	0	0	trace	0	0	0	0	**4**	0	6	46%	6	>97* [a]
Propanil	h	10	0	0	0	0	0	0	**11**	0	0	0	0	0	0	1	8%	11	504 [a]
compounds detected per date > LOD			1	2	0	1	0	0	1	0	2	1	0	2	0				

use: f = fungicide, h = herbicide, i = insecticide/acaricide
LOD = level of detection (PPB)
trace = compound detected but below LOD, unable to quantify
* dermal LD50 used when oral LD50 was not found

toxicity data sources:
a https://ecotox.ipmcenters.org
b https://doi.org/10.1371/journal.pone.0094482

Table 4. All beehive samples were screened for 214 common agricultural chemicals and metabolites. Of these, 198 compounds that were not detected in any samples are presented with their lower limit of detection (LOD) in ppb.

Compound	LOD	Compound	LOD	Compound	LOD
1-Naphthol	50	Dimethoate	15	Myclobutanil	15
2,4 Dimethylaniline	250	Dimethomorph	25	Naled	50
2,4 Dimethylphenyl formamide (DMPF)	5	Dinotefuran	10	Norflurazon	15
3-Hydroxycarbofuran	10	Diphenamid	3	Norflurazon desmethyl	25
4-Aminopyridine	4	Diuron	6	Novaluron	30
4-Hydroxychlorothalonil	10	Emamectin Benzoate	5	Omethoate	50
Abamectin0	100	Endosulfan I	10	Oxamyl	15
Acephate0	100	Endosulfan II	10	Oxyfluorfen	5
Acetamiprid	4	Endosulfan sulfate	10	Paradichlorobenzene	250
Acetochlor	15	Endrin	25	Parathion ethyl	10
Alachlor	15	Epoxiconazole	5	Parathion methyl	10
Aldicarb	25	Esfenvalerate	5	Pendimethalin	15
Aldicarb sulfone	15	Ethalfluralin	5	Permethrin total	25
Aldicarb sulfoxide	25	Ethion	15	Phenothrin	30
Aldrin	30	Ethofumesate	20	Phorate	25
Allethrin	10	Etofenprox	5	Phosalone	15
Amicarbazone	15	Etoxazole	5	Phosmet	50
Atrazine CEAT	30	Etridiazole	5	Phosmet OA	10
Atrazine CIAT	30	Famoxadone	25	Piperonyl butoxide	15
Azinphos methyl	15	Fenamidone	30	Pirimiphos methyl	15
Bendiocarb	10	Fenarimol	100	Prallethrin	20
Benfluralin	5	Fenazaquin	5	Prodiamine	100
Benoxacor	15	Fenbuconazole	15	Profenofos	30
BHC alpha	15	Fenhexamid	30	Pronamide	5
Bifenazate	15	Fenoxaprop-ethyl	15	Propargite	15
Bifenthrin	10	Fenpropathrin	10	Propetamphos	20
Boscalid	10	Fenthion	15	Propham	15
Bromacil	20	Fipronil	50	Propiconazole	15
Bromuconazole	50	Fipronil sulfide	5	Prothioconazole	125
Buprofezin	60	Fipronil sulfone	5	Pymetrozine	30
Captan	50	Flonicamid	15	Pyraclostrobin	5
Carbaryl	2	Flubendiamide	10	Pyrethrins	250
Carbofuran	10	Fludioxonil	60	Pyridaben	5
Carboxin	15	Fluopyram	5	Pyrimethanil	15
Carfentrazone ethyl	5	Fluoxastrobin	5	Pyriproxyfen	5
Chlorfenopyr	5	Flupyradifurone	25	Quinoxyfen	15
Chlorfenvinphos	10	Fluridone	5	Quintozene (PCNB)	5
Chlorothalonil0	100	Flutolanil	15	Resmethrin total	30
Chlorpropham (CIPC)	10	Fluvalinate	5	Sethoxydim	10
Chlorpyrifos	5	Heptachlor	15	Simazine	50
Chlorpyrifos methyl	5	Heptachlor epoxide	15	Spinetoram	100

Table 4 (cont.)

Compound	LOD	Compound	LOD	Compound	LOD
Clofentezine	6	Hexachlorobenzene (HCB)	5	Spinosad	15
Clothianidin	15	Hexazinone	10	Spirodiclofen	5
Coumaphos	3	Hexythiazox	15	Spiromesifen	50
Coumaphos oxon	2	Hydroprene	100	Spirotetramat	30
Cyantraniliprole	25	Imazalil	20	Sulfoxaflor	25
Cyazofamid	30	Imidacloprid 5-hydroxy	150	Tebuconazole	15
Cyfluthrin total	10	Imidacloprid olefin	50	Tebufenozide	5
Cypermethrin total	10	Indoxacarb	30	Tebuthiuron	15
Cyphenothrin0	100	Iprodione	50	Tefluthrin	5
Cyprodinil	10	Kresoxim-methyl	10	Tetrachlorvinphos	15
Cyromazine	25	Lindane	10	Tetraconazole	15
DDD p,p'	5	Linuron	15	Tetradifon	5
DDE p,p’	5	Malathion	10	Tetramethrin	30
DDT o,p'	20	Mandipropamide	10	Thiabendazole	5
DDT p,p'	5	Mesotrione	30	Thiacloprid	5
Deltamethrin	50	Metalaxyl	5	THPI	15
Diazinon	15	Metconazole	10	Thymol	50
Diazinon oxon	5	Methamidophos	40	Tolfenpyrad	5
Dichlorvos (DDVP)	15	Methidathion	5	Triadimefon	10
Diclofop-methyl	10	Methiocarb	30	Triadimenol	25
Dicloran	5	Methomyl	25	Tribufos (DEF)	10
Dicofol	5	Methoprene	80	Triflumizole	40
Dieldrin	10	Methoxyfenozide	5	Trifluralin	5
Diflubenzuron	5	MGK-264	25	Triticonazole	30
Dimethenamid	10	MGK-326	30	Vinclozolin	5

LOD = lower limit of detection

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Chapter IV:

RFID-based Automated Monitoring of Honey Bee Colonies Exposed to Chronic Sublethal Levels of Imidacloprid

Abstract

In the course of plant pollination and nectar collection, foraging honey bees are potentially exposed to numerous hazardous pesticide compounds, through contact with residues or through ingestions of contaminated nectar, pollen or water. Exposure to pesticides is often not acutely toxic, but sublethal effects on the health or behavior of individual bees can contribute to the weakening or failure of a whole colony. Historically, the ability to monitor individual honey bees in a free-flying colony over a long duration has been limited to tedious human surveillance of marked bees at a hive entrance or in a glass observation hive. Recent advances in miniature electronics now allow researchers to autonomously monitor many aspects of a hive's conditions, as well as the record the activities of multiple individual bees over a long period. In this study we tagged individual honey bees with RFID microchips to record their activity level over their entire adult lives. Bees fed sucrose syrup exhibited a normal mean adult lifespan of 26.3 days. Bees fed sucrose syrup with 20 ppb imidacloprid exhibited delayed transition to foraging behavior, with a mean lifespan of 45.8 days, or 1.7 times longer than untreated bees. The improvement of techniques for autonomous and continuous monitoring of large numbers of honey bees in field conditions will improve researchers' ability to conduct more meaningful field studies in the area of honey bee ecotoxicology.

Introduction

Honey bees are among the most widely studied organisms on the planet, due to their importance as agricultural pollinators, their historical and cultural significance, and their utility as producers of multiple useful substances, such as honey, beeswax, and propolis. Increased losses of honey bee colonies have highlighted concerns over crop pollination and food security (Edwards et al. 2018, Fikadu 2019). These losses have been attributed to numerous interacting factors, such as habitat loss, pathogens, parasites, and environmental toxins (Marshman et al. 2019), but the effects of chronic sublethal exposure to agricultural pesticides remains a primary concern for researchers and the beekeeping industry (Steinhauer and Saegerman 2021).

Worker honey bees potentially encounter a wide range of pesticide chemicals while foraging outside of their hive for pollen and nectar. Neonicotinoid pesticides, as a class, are the most widely used chemicals in modern agriculture (Lu *et al*. 2018). These compounds have received intense scrutiny for their perceived ecological effects, with particular emphasis on their impacts on honey bees and other pollinators (Gill et al. 2012, Goulson et al. 2015, Heller et al. 2020, Pisa et al. 2015, Sánchez-Bayo 2014, Sgolastra et al. 2020, Siviter et al. 2021, Tsetkov et al. 2017, Woodcock et al. 2017).

Neonicotinoid pesticides function by targeting nicotinic acetylcholine receptors in insects' central nervous systems, overstimulating these receptor sites, ultimately blocking their function and leading to insect death (Tomizawa and Casida 2003). Honey bees rely on sophisticated adaptive learning and memory processing functions for successful foraging at great distances from their hives (Klein et al. 2019, Menzel 2012), and thus interference with their nervous function could have significant negative effects on the neural processes.

Honey bees are at once both specialists and generalists in the duties they perform within a colony. Each worker is capable of performing all duties of hive maintenance during its life, but tends to perform only a single highly specialized task at any given moment. The particular tasks in which a worker bee engages will be determined by a combination of her age, her physiological state and the overall needs of her colony. Winston (1995) summarized the observations of numerous researchers, and concluded that the temporal caste system of honey bee polyethism is a flexible system, within which worker bees will usually follow a general progression of duties from nest cleaning to foraging as they age, but individuals can adapt their activities as required by the needs of the colony. Differences in gene expression may also play a role in division of labor, with some genetic lines of bees demonstrating variability in the frequency of performing of specific tasks (Calderone and Page 1988).

Foraging is the most demanding task a worker bee will undertake, both energetically and cognitively. It is also a risky endeavor, which exposes bees to multiple dangers outside the hive. By engaging in this task only near the end of its life, an individual worker conceivably contributes more time and effort to the maintenance of the hive and the well-being of her colony.

A natural aged worker has a mean expected survivorship of seven days once foraging duties are assumed (Dukas 2008). This short foraging career is not surprising, given the many dangers honey bees face on repeated, risky long-range flights outside the hive. However, as associative learning of nectar gathering improves with experience (Sigg et al. 1997), honey bees continue to become more efficient and productive foragers as their lifespans exceed this average (Dukas and Visscher 1994). Even still, foraging workers experience an expected exponential increase in mortality as they age (Dukas 2008). Physiological senescence of flight muscles and

physical deterioration of wings contribute to older bees slowing down, and thus are more susceptible to predation (Visscher and Dukas 1994).

Besides outright loss of foragers, numerous debilitating factors have been identified that can stress the health and productivity of bee hives, fostering precocious foraging. These include exposure to pesticides as well as other stressors such as parasites and pathogens, and poor nutrition. Sublethal exposure to pesticides has been identified as causing reduced in-hive activities, precocious foraging, and reduced learning and cognition in foragers (Desneux et al. 2007, Klein et al. 2017, Muth et al. 2019, Siviter et al. 2021, Piiroinen and Goula 2016, Thompson et al. 2003). Over a prolonged period, the response of a bee colony to one or more sub-lethal stressors, singly or in combination, can perpetuate a sequence of events that hasten the failure of an entire colony.

As the subjects of many scientific studies, a great deal is known about honey bee biology and behavior. Many investigations have relied on time-consuming manual observations of individually marked bees in glass-walled observation hives, or by using mark-recapture techniques to monitor or predict bee activities (Galindo-Cardona et al. 2015, Dukas and Visscher 1994, Greenleaf et al. 2007, Hagler and Jackson 2001, Koeniger et al. 2005, Ratnieks and Shackleton 2015, Visscher and Seeley 1982). New technologies have recently been developed that allow for automated monitoring of bee colony conditions (Meikle et al. 2008, Meikle and Holst 2015, Potamitis et al. 2019) and even continuous monitoring of a single honey bee's activities (He et al. 2016, Henry et al. 2012, Riley et al. 2005). These methods can allow investigators to accurately observe and record the fates of multiple individual hive members simultaneously over a long time period using QR codes (Crall et al. 2015, Mégret et al. 2019) or

radio frequency identification (RFID) tags (Ayup et al. 2021, Colin et al. 2019, Perry et al. 2015, Schneider et al. 2012, Streit et al. 2003).

Observations of a honey bee colony's daily flight activity level can be an indicator of overall colony health. Specifically, the age at which adult bees begin foraging, the number of foraging trips undertaken, the length of time for individual trips, and survivorship of individual foragers can indicate overall stress or health problems within a honey bee hive. In general, when colonies are under stress, the life spans of individual bees are often shortened, and the reduced cohort of older foragers initiates premature foraging in younger bees. These precocious foragers are, in turn, less successful and efficient at their tasks, further perpetuating the situation, and increasing the likelihood of eventual colony failure if stressful conditions persist.

Extensive monitoring of an individual bee's activities has historically been difficult and laborious, relying on tedious human observation of marked bees in an observation hive, or at the entrance to bee hives. Automated continuous monitoring of multiple individual bees throughout their adult lives could reveal subtle differences in the health and behavior of bees exposed to sub-lethal levels of pesticides. In this context, the main objective of this study was to determine the utility of radio-frequency identification (RFID) technology in monitoring the activity of foraging bees. By using RFID readers at the entrances to honey bee colonies, we attempted to monitor the movements of tagged free-flying foragers under semi-field conditions, to observe differences in the activity levels of bees fed sucrose syrup with those of bees that were fed 20 ppb imidacloprid in sucrose syrup, to determine if exposure would significantly altered honey bee behavior. The findings of this study can help in refining RFID-based automated monitoring systems used in bee biology and ecotoxicology studies.

Materials and Methods

Observations of daily honey bee flight activity were recorded using small, lightweight RFID tags to identify individual worker bees. Monitoring equipment was mounted at bee hive entrances to automatically scan tagged honey bees exiting and entering the hives, to record the frequency and duration of outside flight activities.

Bee Hives

Two identical honey bee colonies were set up in a commercial honey producing apiary in Pulaski County, Arkansas, using standard five-frame nucleus hives. Entrances to the hives were divided and modified to restrict honey bee travel, and to mount monitoring equipment. Each hive was equipped with two bee escapes (HD- 665, Mann Lake Ltd., Hackensack, MN), which were modified to allow bees to travel through them in only one direction, either in or out (Fig. 3). These also narrowed the size of the hive entrance, allowing only a single bee to pass through at one time, under the reader wand. A clear pane of glass was placed above the entry passage. The underside of the glass was coated with Fluon® polymer (Insect-a-Slip Barrier, Bioquip Products, Inc. CA) to encourage bees to walk in an upright orientation below the reader. A laser reader wand (PharmaSeq, Princeton, NJ) was positioned above each bee escape (2 per hive), to detect tagged bees passing beneath, with the laser beam oriented just above the restricted entrance or exit. The red laser light was considered to have little or no effect on honey bees, because of bees' low visual sensitivity to light in the red wavelengths (Lunau et al. 2011). A protective cover housed the readers and kept the electronics dry.

Each hive was equipped with a second 5-frame hive body on top, which had a solid wood floor. This floor was cut to hold a 1 quart (0.95 L) jar feeder (FD-103 Mann Lake Ltd.,

Hackensack, MN), which provided *ad libitum* syrup to the bees below, while preventing robbing by bees from outside the hive. In each hive, the floor of the upper box also included a one-way bee escape, which allowed bees to travel down into the hive below. This allowed for tagged bees to be introduced to the hive without disturbing the colony, but did not allow bees to travel upward and into the space in the upper box.

Honey Bee Colonies

Honey bee nucleus colonies were created by dividing an established bee colony. Each of the resulting splits received approximately 5,000 adult worker bees, with two frames of capped brood, and a new queen. To reduce genetic variability, queen bees were sisters from the same cohort, obtained from a local breeder, but were allowed to mate openly prior to their introduction to test hives. These queens were introduced, in protective cages, into the experimental hives within 24 hours of setting them up. Colonies were not provided with any frames of honey, pollen or open brood, in order to forage for pollen, and to more readily accept provided syrup.

Treatments

One colony ("Control") was fed 1:1 sucrose syrup, *ad libitum*. Syrup also contained 5 ml per liter of Honey-B-Healthy (Honey-B-Healthy, Inc., Cumberland, MD), a blend of wintergreen and lemongrass essential oils, commonly used by beekeepers as a feeding stimulant, also used here to disguise any repellant taste of treatment material. The other colony ("Treatment") was fed the same syrup with the addition of 20 ppb imidacloprid. Syrup was prepared by adding 0.70093 µL of technical grade imidacloprid (Admire Pro, 42.8% AI) to 15L (3.96 gallons) of prepared sucrose syrup. The concentration of imidacloprid detected in nectar from field studies has usually varied from 0.07 to 11.2 ppb (Cresswell 2011, Gooley and Gooley 2020), but has

been reported as high as 80 ppb in some fruit and vegetable crops, depending on method of application (Dively and Kamel 2012). A level of 5 ppb is often used as a standard "field realistic" concentration. A concentration of 20 ppb was chosen in order to increase any dose-dependent response to ingestion, but still remaining within reported LD_{50} values that range from 3.8-81 ppb (Fairbrother et al 2014). Also, our previous work showed no significant differences in brood production or syrup consumed at 20 ppb (Meikle et al 2016). Higher concentrations of imidacloprid, however, have sometimes been suggested to cause lower feeding rates (Cresswell 2011, Meikle 2016).

Bee colonies were supplied with syrup for three weeks prior to tagging and data collection. Colonies were allowed to forage outside the hive to provide pollen, but the seasonal nectar flow had ended, and the area was experiencing dry conditions with limited floral bloom, which encouraged bees to feed on provided syrup. Flight activities of tagged bees were monitored for the duration of their adult lives. Bees were considered dead after their last recorded entry/exit from the hive. Hives were monitored for 1 week after no bees were detected. Tagged bees' longevity was inferred from the date they were collected as newly emerged adults until they were no longer detected, but did not include the 21 days as a larva or pupa.

Monitoring Technology

Individual worker honey bees were fitted with photo-activated RFID microchip tags (p-Chips®, PharmaSeq, Inc., Princeton, NJ). Each tag was approximately 0.5 mm^2, 0.1 mm thick, and weighed approximately 85µg each, which is less than 0.6% of an average load carried by a foraging worker (Winston 1995). These tags contained a tiny integrated circuit with a unique serial number. The tags themselves carried no onboard power source, but when illuminated by a red laser beam from a reader wand, a tag received sufficient power to briefly broadcast its serial

number through a variable magnetic field. The tip of the reader wand contained an antenna coil that could reliably read this broadcast signal if within 8 mm of the tag.

Tagging Procedure

Newly emerged adult worker bees (0-24 hours old) can be recognized visually by the lighter appearance of their body hairs (Fig. 1). These young bees were collected individually from honey combs using soft forceps, to avoid injuring bees, and placed in an escape-proof container. Bees were then anesthetized by placing them in a refrigerator at 4°C (40°F) for 30-40 minutes. When bees were removed from refrigeration, but container remained on ice during the tagging procedure, to keep them torpid. Individual bees were situated into a cardboard cradle to keep them upright. A wooden toothpick was used to place a tiny drop of glue (Elmer's E616 Super Glue) on the thorax of the bee. Another slightly wet toothpick was used to pick up and place two microchip tags onto the bee's thorax (Fig. 2). Two tags were used per bee to increase the likelihood of at least one of the tags being detected by the laser wand reader. The tagged bee was then scanned with a reader wand to ensure that both tags were functional. The serial numbers of both microchip tags were associated with a unique name for each bee, so that a particular bee could be identified when either tag was scanned. The tagged bee was replaced in a cool container to remain immobile while the glue dried. After bees were tagged, they were allowed to warm and then were returned to their bee hive. Tagging procedure took less than three minutes per bee. Microchip tags were not expected to be recovered after the death of bees.

Introducing bees

Initially 21 bees were tagged and introduced into the Control Hive, and 20 bees were tagged and introduced into the Treatment Hive on the same date (July 29). After 24 hours, 12

tagged bees were found dead in the Treatment Hive. One of the bees had become stuck in the bee-escape, trapping the rest in the introduction chamber in the top of the hive, where these newly-emerged adults died, likely of starvation. Another 14 newly emerged bees were tagged and introduced into the hive to replace the dead ones on August 6.

Data collection

Each time a tagged bee passed under a reader wand, when exiting or entering the hive, the laser provided sufficient energy to power the microchip tag, which briefly broadcast a radio signal with a unique serial number identifying that chip. Laser reader wands were powered by a USB connection to a nearby computer, which also recorded the date, time and a tag's unique serial number each time a chip was activated. Data were continually appended to a simple text file on the computer, which could be read or manipulated as a spreadsheet. Because replacement bees were tagged on a different day, longevity was calculated form the date tagged, regardless of when they were introduced into their hive.

Results

The mean period that tagged adult bees were actively detected in the untreated control hive was 26.3 ± 6.6 (SD) days, with a range of 9.4 to 33.7 days. Bees that received 20 ppb imidacloprid in syrup were active for a mean of 45.9 ± 9.8 (SD) days, with a range of 27.8 to 58.4 days). An independent t-test was conducted to compare the longevity of treated and untreated workers. Treated bees remained active for a significantly longer time period than untreated bees ($t(33)=7.02$, $P<0.001$). Figure 4 summarizes the proportion of honey bees that remained active in each hive on each day during the study.

Of the 21 tagged bees introduced into the Control Hive, two were never recorded by the reader wands and were removed from the study. The normal age for workers to transition to foraging duties is approximately 21 days. The mean age for Control bees to begin orientation flights was 18.7 ± 6.6 (SD) days. After 21 days in the hive, 3 of these 19 remaining tagged workers (16%) had been scanned at least once, but had since disappeared, leaving 84% still active. Within another week (day 28) only 11 tagged bees (58%) remained active in the Control Hive. By the fifth week (day 35) all tagged bees in the Control Hive had ceased to be detected.

In the Treatment Hive, 22 workers were tagged and introduced. Of these bees, 6 were never scanned by a reader wand, and were removed from the study, leaving 16 active bees in the Treatment hive. The mean age for Treatment bees to begin orientation flights was 23.2 ± 7.4 (SD) days. There was no significant difference in the mean age at which Control and Treatment bees began orientation flights ($t(33)=1.86$, $P=0.724$). On day 21, 100% of the tagged bees were still present in the Treatment Hive. A week later, (day 28) 15 tagged bees (94%) remained active. By day 35, 88% remained; on day 42, 56% remained; on day 49, 44% remained; on day 56, 19% remained. As of day 59, no more activity was detected in the Treatment Hive, although the hive was monitored for an additional week with no subsequent detections of any tagged bees (Fig. 4).

Tagged honey bees were recorded entering or exiting hives a total of 1183 times. Often a tagged bee was scanned multiple times than within a few seconds. When these duplicate detections were excluded, by discounting all subsequent scans of the same bee within 30 seconds, bees were recorded on 807 unique occasions (344 in Control Hive, 463 in Treatment Hive). Figure 5 shows all unique detection events by time of day and age of bee (days since emergence and tagging).

During the study (August 6-October 1, 2021), sunrise progressed from 6:19 a.m. to 7:03 a.m.; sunset progressed form 6:45 p.m. to 8:11 p.m. The majority of the tagged bees' movements in and out of hives were detected during daylight hours (82% in Control Hive, 97% in Treatment Hive). Figure 6 summarizes the total detected activity of both treatment groups by time of day. Treatment bees were less active during the morning (18.2% of detected activity between 6:00 am and 1:00 p.m.) than during the afternoon (75.8% of activity between 1:00 p.m. and 8:00 pm). Control bees, however, were more consistently detected each hour during daylight hours (40.6% of activity between 6:00 a.m. and 1:00 p.m.; 39.1% of activity between 1:00 p.m. and 8:00 pm).

Discussion

Tagged honey bees in our study were only detected when they entered or exited from a hive, passing under a reader wand. If we use cessation of detection as a proxy for mortality, we can infer their longevity from the data collected,

Mortality is naturally high among foraging worker bees, which is why only the oldest bees are generally sent out to forage. Breed et al. (1990) described wing wear as a key trait of experienced foraging honey bees, and Carter (1992) also suggested that wing wear was a significant factor responsible for increased mortality in older foraging bumble bees. Dukas (2008) concluded that predation was the most significant cause of forager bee mortality, but that physiological senescence in aging workers made them slower and weaker, and thus easier targets for predators. It's likely that all of these are responsible for rapid forager mortality.

A naturally aged worker has a mean foraging span of approximately 7 days once they reached this stage in their colony's division of labor. The mean age at which bees begin foraging has been estimated to be around 21 days. This suggests a mean life adult life expectancy for honey bees to be approximately 28 days.

The tagged bees in the Control Hive had a recorded mean adult lifespan of 26.35 ± 6.59 (SD) days. Of these active bees, 11% died before reaching this presumed foraging age of 21 days. Of the 89% that did survive past 21 days, their mean lifespan was 28.8 ± 2.3 (SD) days, and thus had a presumed foraging career lasting about 8 days, similar to Dukas and Visscher (1994). However, the bees in the Treatment Hive began to disappear only after day 27, and continued to do so at a slower rate than the Control Hive bees, with an average a recorded adult lifespan of 45.86 ± 9.76 (SD) days.

Winston and Punnett (1982) reported a correlation between the size of a colony and the age at which workers begin foraging, with smaller colonies beginning foraging at earlier ages (around 20 days), but with the onset of foraging delayed as colony population increased. Both of our colonies were small, and our control colony showed normal forager age development, while the treated colony demonstrated delayed foraging behavior.

Colin et al. (2019) conducted observations similar to ours, while feeding bees 5 ppb imidacloprid, and found that exposed honey bees began foraging 15% younger, performed 28% fewer foraging trips, and died 1.2 times faster than control bees. Exposure to 1.5 ppb of imidacloprid was found to reduce the number of foraging flights and significantly increased the duration of foraging flights (Schneider et al. 2012). Another study found that bumble bees fed 1 ppb imidacloprid demonstrated reduced foraging motivation and fewer flower visits (Lämsä et al. 2018). Honey bee colonies are complex entities, with numerous feedback loops and

interacting variables. A larger controlled study, in a single location feeding multiple concentrations, could eliminate some variability and highlight dose-dependent responses to imidacloprid.

Some bees were able to enter and exit without being scanned by lasers each time. Due to some missing data, we were unable to accurately count the total number or duration of foraging trips, but were still able to estimate longevity and most active times of day from the data collected. Our bees exposed to a dose of 20 ppb imidacloprid appeared to begin foraging at a later age, and survived longer. Their longevity may have been directly associated with their reduced activity level caused by exposure to this neonicotinoid. Lower activity level may have reduced wear on their wings, reduced physiological stress, and lowered their exposure to predation. However, future research in this direction would be needed to evaluate these assumptions.

The tagged bees in the Treatment Hive were detected moving in and out mainly during daylight hours, while Control Hive bees were detected throughout the day as well as during the night. During hot weather, a portion of a colony's workers may routinely spend the night on the outside of their hive. This behavior, termed "bearding," is thought to be a response to overcrowded, overheating or poorly ventilated hives, and believed to help maintain an optimal brood nest temperature (Hamdan 2010). Bees were observed bearding on the Control Hive during hot weather, but very little of this behavior was observed on the Treatment Hive. Internal hive temperatures were not monitored during the study. Tackenberg et al (2020) determined that neonicotinoid exposure disrupted circadian rhythms in honey bees, thought to regulate important behaviors such as foraging and orientation and navigations well as sleep cycles. The lack of bearding could have been due to a difference in population size, and therefore internal hive

temperature. Neither hive was opened during the experiment, to minimize disturbing the bees, and to avoid letting tagged bees in or out without passing under tag readers, and therefore population was not monitored.

Imidacloprid ingestion may have lowered the treated bees overall activity levels, as suggested by their comparatively longer lifespans, which could have affected their tendency for temperature regulation, which could affect a colony's ability to overwinter in a cold climate.

Pitfalls of RFID-Based Methodology and Recommendations for Improving Automated Monitoring System

Timing is an important factor to consider while initiating RFID-based experiments to assess the effect of pesticide exposure on forager honey bees from treated syrup. Our previous work (Meikle et al. 2016) suggested that honey bees prefer fresh forage if available, but will consume syrup when flowers are not abundant. During the study year, we experienced a wet summer conditions around our experimental apiary, which kept flowers in bloom later than usual. The bees in our experimental hives began consuming syrup only towards the end of June. They needed to drink treated syrup for a minimum of 3 weeks prior to being tagged to ensure that every bee in the Treatment Hive had potentially been exposed to imidacloprid since its larval stage. Treated syrup was fed beginning June 27 and continued through September 30. Control Hive received untreated syrup during the same time period. When bees began consuming syrup, the local area was experiencing a natural seasonal nectar dearth, and so foraging activities were reduced. Internal feeders were necessary to prevent contamination of Control Hive bees. Feeding inside of the hive may have altered bees' behavior, reducing natural foraging activities

since bees did not have to leave the hive to get food, but this should have been reflected in both Control and Treatment hives. By the time bees were feeding significantly on syrup, pollen bearing plants were generally less available in the landscape, which typically causes queen honey bees to reducing brood production. This may have also have reduced foraging activity, because the presence of brood tends to encourage bees to forage.

Bees often paused momentarily before passing through bee escapes, or struggled briefly to push apart the prongs of the bee escapes to pass through them. This sometimes resulted in the same tag being read multiple times, or both tags being read consecutively. This required the data to be sorted and cleaned up to remove multiple simultaneous detections.

Bee escapes are designed to allow bees to pass in only one direction (in or out). Ideally this forces bees to use separate gates to exit and reenter the hive. By recording when an individual bee left the hive and when she returned, we should be able to calculate the total elapsed time of each foraging trip. However, bees were sometimes observed to force their way through the gates in the wrong direction, resulting in incorrect data associated with their entrance or exit. Bees appeared more likely to try to return through the exit gate than to exit through the entry gate. This may be due to the bees' orientation on the exit as their only perceived entry point. Also, bees produce a pheromone from their tarsal glands as they walk. The buildup of this "footprint" scent near the exit may have served as a cue for the bees to try to reenter at this same point.

Despite the restricted entrances to the bee hives, honey bees were sometimes able to enter or exit without being detected, resulting in missing data. All bees had to traverse through narrow passages, beneath the laser scanners. However, some bees were observed to pass through scanners without activating the microchip tags every time. Laser beams were narrow (1.5 mm

diameter), and tags were very small (0.5 μm^2), thus bees were sometimes able to pass without the laser activating the RFID microchip. Bees were also observed to walk through gates upside down, bypassing the laser. A coating of Fluon® was applied to the underside of the glass pane to prevent bees from walking upside-down, but was not always effective. The constant movement of bees under the glass appeared to clean off the layer over time, allowing bees to walk on its lower surface. As a result, not every passage of bees through the entry/exit gates was logged, and data collection was incomplete.

Because we were not able to record every entry or exit, the precise duration of each foraging flight could not be calculated. Also, the total number of foraging flights made by individual bees, per day or in total over its lifetime, could not be accurately calculated. Survivorship and longevity of honey bees could be inferred by subtracting the date a newly emerged adult bee was collected and tagged from the final date on which that individual was scanned, although it is possible for a bee to have remained alive for some time after its final detection.

Failure to read tags is the main problem with using RFID technology to monitor honey bees. Errors in data collection have also been reported by other researchers using RFID tags to monitor honey bee hives (Ai and Takahashi 2021, Ohashi et al. 2010, Robinson et al. 2009, Susanto et al. 2018, Tenczar et al. 2014). Larger RFID tags are available, but are more likely to interfere with honey bee flight capability.

Restricting travel at hive exits creates a bottleneck for bees trying enter or leave the hive, potentially affecting their behavior. Bees struggling to exit were often crowded by other bees attempting to do the same, which may have affected tag reading. At times, bees attempting to enter through the wrong gate would prevent another bee from exiting in the appropriate

direction. Additional hive entrances could alleviate this bottleneck, but each would need to be fitted with another tag reader, significantly increasing the cost of each experimental hive. The ability to simultaneously monitor and record the activities of large numbers of honey bees remains an obstacle to the use of RFID tags in full-sized colonies in field studies. However, developing accurate, inexpensive and efficient methods to do so will improve our ability to collect more meaningful and relevant data on honey bee behavior and toxicology under true field conditions.

Fig 1. Newly emerged worker bee and older worker. A newly emerged worker bee (top) can be easily identified by their visual appearance. They typically have lighter colored body hairs, which will begin to darken after about 24 hours. An older worker (bottom) has darker hairs, and typically lose the hairs on their dorsal thorax from constant movement in and out of cells.

Fig 2. Worker bee tagged with RFID microchip. Newly emerged worker honey bees were collected when fewer than 24 hours old, anesthetized with cold, and tagged with two RFID microchips to monitor their activity and longevity. Tags are lightweight and not expected to interfere with honey bee flight capability. Chips were not expected to be recovered after the death of the tagged honey bee.

Fig. 3. Modified Bee hive entrances. Hives were fitted with bee escapes that allowed passage of bees in only one direction. Reader wands were positioned above these to scan tagged bees entering or exiting bee hives. A pane of clear glass allowed the transmission of the laser beam, but was thin enough to cause no interference with the radio transmission.

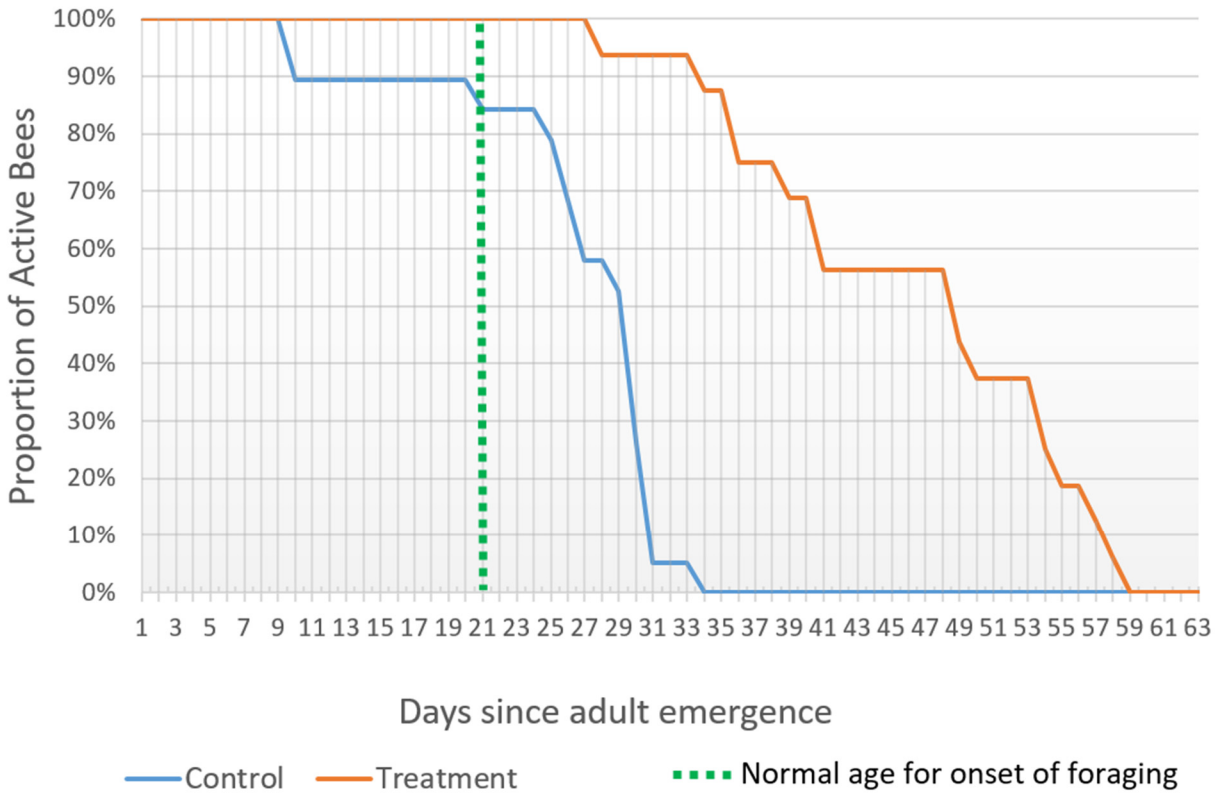

Fig 4. Proportion of tagged honey bees detected on each day of experiment. Hives were monitored continuously until no tagged bees were detected for one week.

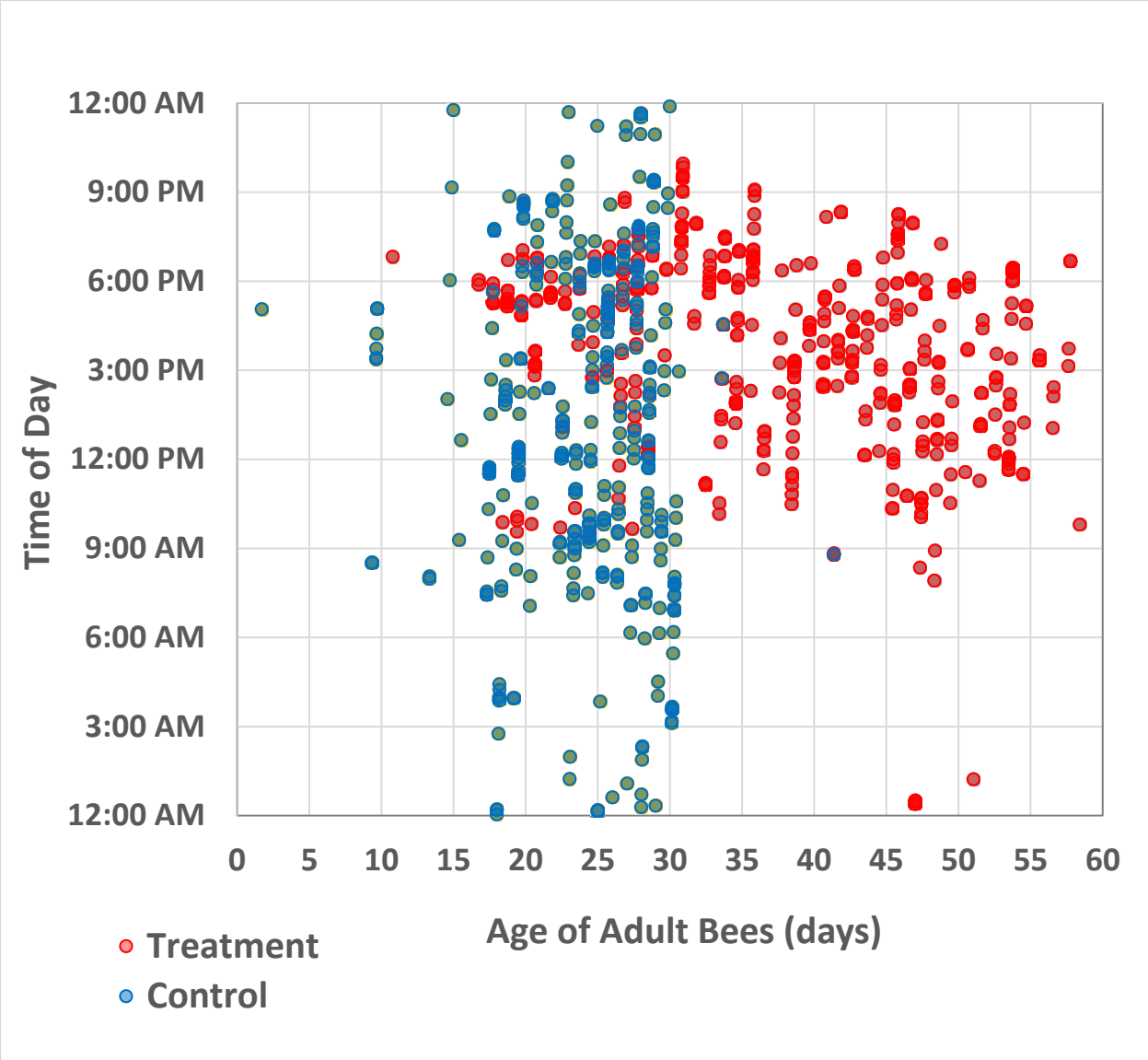

Fig 5. Date and time of day of unique detections of honey bee RFID tags.

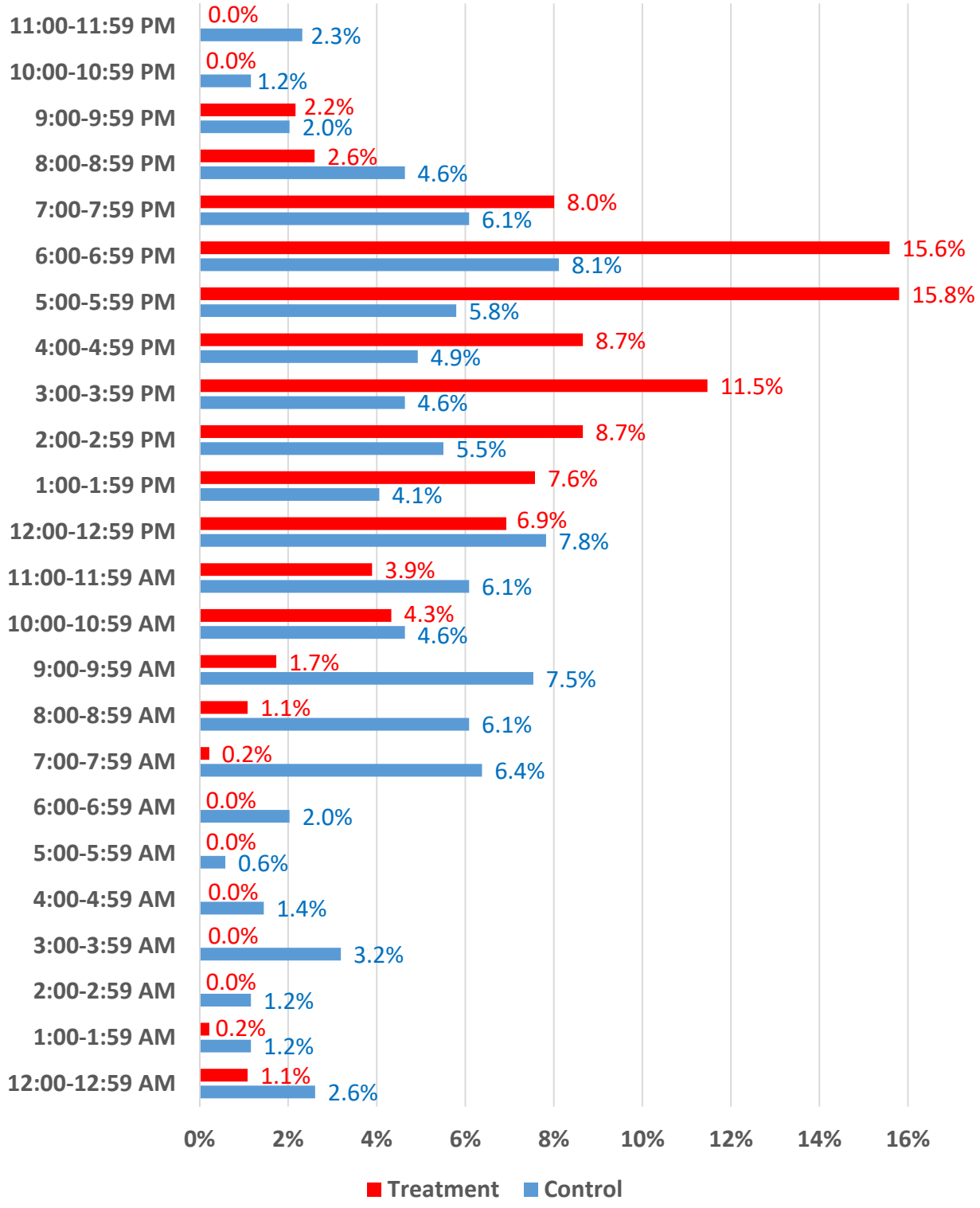

Fig. 6. Proportion of honey bee activity by time of day for each treatment group.

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Chapter V:

Conclusion

Bees are keystone species, providing essential ecological services of plant pollination, and thus enhancing the production of angiosperm seeds, nuts and fruits. Although physically small in size, their beneficial effects on the ecosystem are immense. By ensuring successful plant reproduction and continuity bees are critical to maintaining terrestrial food webs. Bee pollination in crops is also an enormous contribution to the human food sustainability and the agricultural economy (Aizen et al. 2008, Jordan et al. 2021, Klein et al. 2007). The western honey bee, *Apis mellifera*, has been introduced around the world, and remains the most frequently utilized pollinator in agriculture as well as one of the most frequent floral visitors in many natural habitats (Aslan et al. 2016, Saunders et al. 2021).

Honey bees and other pollinators are currently facing significant declines in abundance and diversity in many parts of the world, and for a variety of reasons (Angelella et al. 2021, Choate et al. 2018, Hamblin et al. 2018, Steinhauer et al. 2018). One of the primary factors affecting bee declines is the loss of natural habitat that provides balanced nutrition and suitable nesting sites (Goulson et al. 2015, Kline and Joshi 2020, Olynyk et al. 2021). Intensely managed urban, recreational, and agricultural landscapes may offer poorer nutrition, and may be unable to support healthy, diverse pollinator communities. Global trade and the continual transportation of honey bees, for sale or for pollination rental, has led to a cosmopolitan distribution of exotic parasites and pathogens, which combine to significantly impact bee health (Boncristiani et al. 2020, Goulson et al. 2015). Novel environmental toxins from pollution and pesticide use also affect bee health (Grassl et al. 2018, Mullin et al. 2010, Traynor et al. 2021). While many pesticide compounds can be acutely toxic to bees, exposure to sub-lethal concentrations are more

common, and affect bee health in numerous ways. Many pesticides are neurological toxins, which impair learning and memory processing in bees (Colin et al. 2020, Iqbal et al. 2019, Ludicke and Nieh 2020). These compounds also impair immune system response, making bees more susceptible to pathogens (O'Neal et al. 2018, Pettis et al. 2012). Bees that are nutritionally stressed are at even greater risk when impacted by multiple factors simultaneously (Dolezal and Toth 2018, Negri et al. 2019, Ulutaş and Özkirim 2018). Of particular interest has been the neonicotinoid compound imidacloprid, which is used as both a seed treatment for crops as well as a foliar-applied insecticide, and one of the most commonly used pesticide products in agriculture worldwide. Neonicotinoids have been implicated as a significant factor in honey bee declines (Cresswell et al. 2012, Mason et al. 2013, Sgolastra et al. 2020). We investigated the role of neonicotinoids used in midsouth agriculture on honey bees by (1) characterizing the landscape surrounding bee colonies, and the pesticides applied to it during a growing season; (2) investigating the naturally foraged diet of honey bee colonies in terms of plant taxa and pesticide residue exposure chronologically throughout the season in this agricultural setting; and (3) examining the effects of known a known concentration of a neonicotinoid compound on honey bee behavior by monitoring individual worker bees throughout their lives.

In order to investigate the effects of an agricultural environment on honey bee heath, the landscape surrounding an apiary was quantified in terms of land use (crop and non-crop), and a survey was conducted to determine the number of insecticide compounds applied to the landscape within foraging range of the bee colonies. Samples of bee hive products (bees, beeswax, honey, and pollen) were analyzed for pesticide contamination. The study found that approximately 80% of the landscape was under cultivation with 5 commodity crops (soybeans, corn, cotton, rice and grain sorghum). Residues from beeswax, honey, pollen and bees indicated

varying levels of 26 pesticide product residues, but all were mostly below acutely toxic concentrations (LD_{50}) for honey bees. No neonicotinoid compounds were detected in any of the samples in two years, despite being applied as seed treatments or foliar applications to more than 60% of the landscape.

Once inside the hive, pollen and nectar are mixed and stored with other resources, making it difficult to assess potential sources of contamination. Beeswax also readily absorbs lipophilic compounds, but the origin of these compounds is difficult to determine. Collecting pollen loads from returning honey bees, however, allows for the collection of discrete sampling at specific intervals and at specific locations, creating a chronological record of plant taxa bees visit by choice, and of pesticide residues to which they have been exposed. To investigate the role of pollen from seed-treated crops as a contaminant of honey bee diet, colonies were monitored from mid-March, (before seed-treated crops were planted) through the end of August (after crops had ceased blooming). This study found only trace levels (below the 1 ppb limit of detection) of imidacloprid and thiamethoxam (both neonicotinoids) on one occasion each, and only early in the growing season. This was likely associated with contaminated dusts from planting treated seeds (Krupke and Long 2015), and is particularly associated with corn planting (Greatti et al. 2003, Krupke et al. 2012, Pistorius et al. 2009), which is much less of a risk to bees in the midsouth than in northern corn belt growing region. Other insecticides and fungicides were also detected, but rarely at levels of concern. The analyses detected at least one or more herbicide on every sample date throughout the season, often at levels above published LD_{50} for honey bees. This has implications for bee health at both acute and sublethal concentrations (Abou-Shaara 2018, Balbuena et al. 2015, Belsky and Joshi 2020, Faita et al. 2018, Motta and Moran 2020, Jumarie et al. 2017). Bees in the agricultural area encountered potentially toxic

levels of pesticides throughout the season, in pollen from both crops and non-crop plants. Bees in the urban area were also exposed to toxic levels of insecticides on several occasions, but not at concentrations approaching acute toxicity. Simultaneous exposure to multiple pesticides occurred at both sites on multiple occasions. These results underscore the need to conserve areas of habitat and forage for both wild and managed pollinators within both agricultural and urban landscapes. Palynological data such this can help rank the relative importance of flowering plants in terms of bee-attractiveness. And collection of season-long data can help in the selection of plants to include in pollinator planting mixes to ensure consistent season-long availability of nutrition.

Although the previous studies failed to detect quantifiable concentrations of neonicotinoids from in-hive sampling or in bee-collected pollen, and no colony mortality was observed, the possibility of sublethal exposure to neonicotinoids still exists (Sandrock et al. 2014, Shi et al. 2017). Published LD_{50} concentrations for neonicotinoids are be below the lower limit of detection for standard screening methods (Zawislak et al. 2021). Many studies that have experimentally tested the effects of neonicotinoid exposure on honey bees have observed bees for only a short time in a laboratory setting (Carreck and Ratnieks 2014), or evaluated the colony unit in terms of weight or brood area (Alburaki et al. 2017, Negi et al. 2021, Meikle et al. 2016). The resulting body of literature has been contradictory in determining the effects of neonicotinoids on honey bees, and knowledge gaps remain (Flores et al. 2021, Lundin et al. 2015, Walters 2016). The results of controlled laboratory studies or semi-field studies are rarely equivalent to those conducted under field conditions with entire bee colonies. It is necessary to be able to monitor effects of exposure to individual honey bees *in situ*, in a free-flying colony with limited disturbance. Historically, the ability to monitor individual honey bees in a colony

over a long duration has been limited to tedious human surveillance of marked bees at a hive entrance or in a glass observation hive (Galindo-Cardona et al. 2015, Ratnieks and Shackleton 2015). To elucidate the effects of exposure to a known concentration of one neonicotinoid compound (imidacloprid), individual honey bees were tagged with RFID microchips to record their activity level over their entire adult lives. Bees fed on syrup with 20 ppb imidacloprid exhibited delayed transition to foraging behavior had a mean lifespan 1.7 times longer than bees fed on sucrose syrup alone. The improvement of these techniques for autonomous and continuous monitoring of large numbers of honey bees in field conditions will improve researchers' ability to conduct more meaningful field studies in the area of honey bee ecotoxicology.

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