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The Effects of Pesticide Exposure and Diet on the Health, Reproduction, and Behavior of Mason Bees (Osmia spp.)

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The Effects of Pesticide Exposure and Diet on the Health, Reproduction, and Behavior of Mason Bees (*Osmia* spp.)

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Abstract

Several mason bees (*Osmia* spp.), such as *Osmia lignaria* and *Osmia cornifrons*, are efficient and valuable pollinators in orchards. They can be used to supplement honey bee (*Apis mellifera*) pollination, to improve yields and reduce costs compared to *A. mellifera* alone. *Osmia* spp. encounter many stressors in their environment, which can negatively impact their health and pollination ability. These stressors include pesticide use and habitat loss due to agricultural expansion. *Apis mellifera* are often used as surrogates for pesticide risk assessment, but different bee species can vary widely in their response to stressors. Therefore, research into the impact of stressors on solitary bees is needed to create mitigation strategies and better protect the health of bee communities. This study assessed the lethal and sublethal impacts of pesticides on *Osmia* spp. health, physiology, and ability, and the effects of floral diversity on bee activity and fecundity.

As pest insects become more resistant to commonly applied pesticides, new active ingredients and formulations are produced to provide effective pest control. Two recently released systemic insecticides, flupyradifurone and sulfoxaflor, can be used to combat neonicotinoid-resistant pests, but may pose a risk to *Osmia* spp. We exposed four bee species, *A. mellifera*, *O. lignaria*, *O. cornifrons*, and *Osmia californica*, to both insecticides and measured toxicity, as LD_{50} values, and survival over 96 hours. *Apis mellifera* was the least sensitive to both insecticides, followed by *O. cornifrons*. *Osmia lignaria* and *O. californica* were the most sensitive. Female *Osmia* bees were more sensitive than males of the same species.

The sublethal impacts of flupyradifurone and sulfoxaflor on *Osmia* spp. health were measured, using their detoxification enzyme expression, feeding preferences, flight and foraging ability, and gut microbial communities after exposure. Additionally, *O. cornifrons* were exposed to the commonly used garden pesticides, glyphosate, chlorothalonil, and spinosad, and their gut bacterial communities were compared. Flupyradifurone, but not sulfoxaflor, increased the expression of P450 enzymes in *O. lignaria* females. Flupyradifurone also caused impaired

mobility and foraging activity of *O. lignaria* females at 24 hours following exposure. *Osmia lignaria* females showed some avoidance of food contaminated with higher concentrations of flupyradifurone, but overall *O. lignaria* did not avoid sulfoxaflor or flupyradifurone. The effects of the tested pesticides on *O. cornifrons* gut bacterial community composition and diversity were minimal.

Finally, we raised *O. lignaria* in field cages planted with either a wildflower mix or a buckwheat monoculture and measured the effects on bee activity, fecundity, and soil health. The different planting types had little impact on soil nutrient content and the soil microbiome. *Osmia lignaria*, however, were more active and were successfully able to reproduce in the wildflower mix groups, whereas bees in the buckwheat groups had lower activity and produced no offspring. This work demonstrates the risk of insecticides to *Osmia* spp. health and pollination ability and the importance of an adequate and varied diet for bee fecundity.

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Chapter 1:

Introduction

Agricultural Expansion

Beginning in the late 1950s, many countries shifted to using high-yielding varieties of crops, produced through selective breeding. This began the “Green Revolution,” in which global initiatives were put in place to increase agricultural production through high-yielding crops, chemical fertilizers, synthetic pesticides, irrigation, and increased mechanization (Alexander et al., 2015; Evenson & Gollin, 2003a, 2003b; Tilman et al., 2011). Since the beginning of this revolution, population growth and dietary changes have continued to increase agricultural demands, particularly for intensive agricultural production systems (Alexander et al., 2015). High-income countries tend to have greater demands and import a higher percentage of their agricultural goods, which has put pressure on lower-income countries to provide these goods and to expand their agricultural land use (Weinzettel et al., 2013). This expansion of agricultural production has caused several benefits, particularly for consumers, who experience lower food prices and access to a higher calorie diet (Evenson & Gollin, 2003a, 2003b; Keyzer et al., 2005). For growers, however, the results have been more mixed. Some growers have been able to increase their production to compensate for the falling prices, whereas others have experienced heavy income losses (Evenson & Gollin, 2003a, 2003b). For example, in Tanzania, a study found that the majority of production growth was undertaken by larger-scale farmers, who were more likely to have a higher income. Lower income houses and smaller-scale farmers experienced fewer benefits to their income and nutrition from the agricultural expansion (Pauw & Thurlow, 2011).

There are also concerns about the environmental impacts that agricultural expansion has had and will continue to have on native ecosystems and ecosystem services, including the impacts on bee populations and the pollination service provided by them. Many of these concerns arise from the changes to land usage caused by increased agricultural production.

Yield increases due to high-yielding crop varieties, mechanization, and agrochemicals have met some of the rising demand for agricultural goods, but there has still been an increase in land used for agriculture in the past few decades (Foley et al., 2005). Grazing and pasture lands for livestock occupy the greatest portion of agricultural land and have seen the greatest expansion in area. In 2005, pasture land alone took up over 3.3 billion ha (33 million km^2) worldwide, which is about 25% of the global land surface (Alexander et al., 2015; Asner et al., 2004). Crops for human consumption take up a more modest ~800-900 million ha globally (Alexander et al., 2015). In total, about 40% of the planet's land is used for agriculture as pasture land, crops for human use and consumption, and crops for feeding livestock (Asner et al., 2004; Ramankutty & Foley, 1999). From 1961-2011, pasture land use increased by 298 million ha, a faster rate of growth than for crops used for human consumption, which increased 140 million ha, and crops for livestock feed, which increased 139 million ha (Alexander et al., 2015). Going back further, the expansion of grazing and pasture land has been more extreme, with a 600% increase in area over the past 3 centuries (Asner et al., 2004). The increasing demand for animal products, in particular, has caused the majority of the conversion of land from natural habitat into agricultural lands, with ~30% of temperate deciduous broadleaf forest, ~56% temperate evergreen broadleaf forests, and ~10% tropical evergreen broadleaf forests cleared and converted into grazing land (Asner et al., 2004). With the loss of these habitats, there can also be a decline in the ecosystem services that they provide. Many natural areas support a diversity of living organisms, which can contribute to carbon storage, water filtration, and pollination (Johnson et al., 2014; Millennium Ecosystem Assessment, 2005).

Importance of Pollinators

As this agricultural intensification and expansion has occurred, the usage of animal-pollinated crops and the need for insect pollinators have increased, as well. Since the late 1950s, the percentage of agricultural crops dependent on animal pollinators has increased

300% (Aizen & Harder, 2009). Unfortunately, despite the rising need for pollinators, many of the more intensive agricultural practices can cause population decreases and negatively affect pollination services. In many regions, particularly countries in the Americas and Asia, there has been an expansion in the use of pollinator-dependent oilseed crops, but an overall decrease in agricultural diversity, which can negatively impact pollinator populations (Aizen et al., 2019).

The need for pollinators has been especially notable in California, which has seen an expansion of almond growing. By 2017, California almond orchards covered over 380,000 ha and produced around 81% of global almonds (US Department of Agriculture, 2017). Almonds are heavily dependent on bee pollination, especially by western honey bees (*Apis mellifera* L., Hymenoptera: Apidae) (Wade et al., 2019). By 1973, however, California pollinators alone were unable to meet the rising demand for almond pollination. This led to *A. mellifera* hives being brought in from other states and rented for the almond blooming season (Degrandi-Hoffman et al., 2019; Rucker et al., 2012). Since the 1970s, this practice has only become more common. In 2017, California almond growers rented 1.48 million *A. mellifera* colonies, spending over $253 million to insure the adequate pollination of their crops (US Department of Agriculture, 2017; Wade et al., 2019). In recent years, almond growers have also used the native blue orchard bee (*Osmia lignaria* Say, Hymenoptera: Megachilidae) to supplement *A. mellifera* pollination (Boyle et al., 2020; Pitts-Singer et al., 2018).

Bees and other animal pollinators are also important for the stability of wild ecosystems. It is estimated that animals pollinate over 85% of wild angiosperms globally (Chagnon et al., 2015; Ollerton et al., 2011). The focus on pollination services has primarily been in regard to crop plants, but animal pollinators undoubtedly contribute a vital service to wild flowers and to many native ecosystems.

Pollinators make a significant contribution to the human diet, as well. They are estimated to contribute 15-30% of human food, both through the direct pollination of edible crops and through pollinating crops, such as alfalfa and clover, that feed livestock (McGregor, 1976). Of

the 115 leading global food crops, 87 of them benefit from animal pollination and 13 of them are dependent on animal pollinators for reproduction (Klein et al., 2007). This contribution is especially high for vegetable, fruit, and oil crops (Gallai et al., 2009). Animal pollinators increase the diversity of the human diet and as such, they play a role in combating the “hidden hunger” that many people face, in which they receive enough calories, but have nutrient deficiencies due to not eating an adequate variety of foods (Chagnon et al., 2015; Muthayya et al., 2013). Pollinator-dependent crops are responsible for providing a large portion of nutrients and minerals to human diets. The majority of edible fats and oils come from insect-pollinated plants, as do many essential nutrients, including vitamin C, vitamin A, and lycopene (Eilers et al., 2011; McGregor, 1976). Many of the B vitamins are present in grains and starches that do not rely on animal pollination. However, the majority of these vitamins are lost during the processing of these crops into refined starches like white rice and white flour. Because of the popularity of refined starches in many world regions, beans and greens, crops which do benefit from animal pollination, have become important sources of B vitamins (Eilers et al., 2011).

Many crops also show improved yields and quality when animal pollinators are present, which can improve profits for growers (Chagnon et al., 2015; Klein et al., 2007). The price value of pollination can be difficult to estimate, though most agree that pollinators contribute billions of dollars annually to global crop values. One study estimated that 9.5% of the total global agricultural production was due to animal pollinators in 2005, amounting to around 153 billion euros (over 182 billion USD) (Gallai et al., 2009). The majority of this contribution likely comes from *A. mellifera*, which are easily managed and active for much of the year. Wild bees, which in North America consist of bumble bees (*Bombus* spp., Hymenoptera: Apidae) and solitary bees, also contribute a great deal to crop pollination. In the United States, their contribution was estimated as ~$3.06 billion annually (Losey & Vaughan, 2006).

Pollinator Diversity

Over 100,000 animal species are thought to play a role in pollination. Of these, there are some vertebrate species, mostly hummingbirds and nectar-feeding bats, but the vast majority of animal pollinators are insects (Abrol, 2012; Buzato et al., 2000; Nathan et al., 2005). Around a third of all pollinators are bees (Ollerton et al., 2011; Sánchez-Bayo & Wyckhuys, 2019), which makes bee species of particular importance for research and conservation. There is an incredible diversity of bees globally, with over 25,000 species identified (Michener, 2000). In North America, there are around 4,000 species of native bees, of which 54 are *Bombus* spp. and over 3,900 are solitary bees (Spivak et al., 2011; Winter et al., 2006). Most research into insect pollinator populations and services has focused on *A. mellifera*, which is an easily managed crop pollinator. However, it is not a native species in North America, having been imported from Europe in 1622 (Sheppard, 1989). Although *A. mellifera* is a prolific pollinator and producer of honey (Morse & Calderone, 2000), having a diversity of bee species, and especially native bee species, in an area can greatly enhance the pollination of both crops and wild angiosperms (Bispo dos Santos et al., 2009; Hoehn et al., 2008; Javorek et al., 2002; Kremen et al., 2004).

Non-*Apis* species of bees can be more efficient pollinators of certain flowering plants than *A. mellifera*, especially in areas where *A. mellifera* are not native. One reason for this is that many species of solitary bees, stingless bees, and bumble bees are capable of buzz pollination, whereas *A. mellifera* are not (Bispo dos Santos et al., 2009). Buzz pollination is performed by these bees through the vibration of their indirect flight muscles in order to shake pollen loose from the anthers of certain flowers (Buchmann, 1983; Buchmann & Hurley, 1978; De Luca & Vallejo-Marín, 2013). While most flowers release their pollen passively, some restrict their pollen release through small openings at the apical end of their anthers (Buchmann, 1983; Buchmann & Hurley, 1978). There are estimated to be around 15,000-20,000 angiosperm species that restrict their pollen release and that require shaking, either from wind or buzz

pollination, in order to release their pollen (Buchmann, 1983). Such plants include crops like eggplants, tomatoes, potatoes, cranberries, and blueberries (Bispo dos Santos et al., 2009; Buchmann, 1983; De Luca & Vallejo-Marín, 2013; Javorek et al., 2002). *Apis mellifera* can be less efficient for pollinating crops that restrict their pollen release. Eggplants, for example, had the best fruit and seed set when visited by *Bombus* spp. (Lowenstein et al., 2015). Orchards of sweet cherries had higher yields when they were pollinated by *O. lignaria* than when pollinated by *A. mellifera* (Bosch et al., 2006). Likewise, greenhouse-grown tomatoes that were pollinated by the stingless bee, *Melipona quadrifasciata* le Peletier, produced more fruit, larger and heavier fruit, and more seeds, than those pollinated by *A. mellifera* (Bispo dos Santos et al., 2009). In lowbush blueberry (*Vaccinium angustifolium* Aiton), *A. mellifera* showed a lower pollen deposition rate than *Bombus* spp., mining bees (*Andrena* spp.), sweat bees (*Halictus* spp.), and alfalfa leafcutter bees (*Megachile rotundata* Fabricius, Hymenoptera: Megachilidae). Compared to *Bombus* spp. queens and *Andrena* spp., the pollen deposition was 4 times less in *A. mellifera* (Javorek et al., 2002).

There are also benefits in having higher species richness of pollinating bees in an area, both to wildflowers and crop plants. Pollinator communities tend to be more robust if they are comprised of a diversity of species. If one species is lost from the area, others would be able to perform the required pollination services, which can mitigate some of the negative effects of losing a species (Dobson et al., 2006; Kremen et al., 2002). Different bee species can also be active at different times of the day and the year, so more species in an area can help ensure a longer and more thorough period of pollination. Certain crops, including many fruit trees, have a brief bloom period of 2-3 weeks and require an active and robust pollinator community for cross-pollination during this time (Bosch & Kemp, 2001). Varying body sizes and structures of different bees also make certain species more capable of pollinating specific shapes of flowers (Hoehn et al., 2008). Improved pollination with higher species richness was observed in radish (*Raphanus sativus* L.) crops in Switzerland, which showed an increased fruit set when pollinated by a mix of

bee species. Researchers observed that the social bees, solitary bees, and hoverflies (Diptera: Syrphidae) tended to visit flowers at different times of day, which may have improved pollination (Albrecht et al., 2012). Higher pollinator species richness also improved seed production in pumpkins (Hoehn et al., 2008) and purple coneflowers (*Echinacea purpurea* L.) (Lowenstein et al., 2015).

Unfortunately, despite the importance of non-*Apis* bees, the assessment of their populations and health has largely been lacking (Cameron et al., 2011). In North America, research on bee population declines has focused on the non-native *A. mellifera*, and to a lesser extent, *Bombus* spp., but there is little monitoring of the populations of wild, solitary bees. In Europe, there has been more assessment of solitary bees, and the studies show a concerning decline in the ranges and populations of several solitary bee species (Biesmeijer et al., 2006; Fitzpatrick et al., 2006; Rasmont et al., 2005). Additionally, *Apis mellifera* is often used as a surrogate for other bee species in pesticide risk assessments (Thompson & Pamminger, 2019), even though solitary bees and other wild bees can have very different responses to pesticide exposures than *A. mellifera* (Hayward et al., 2019). As such, more information is needed on native North American bees to assess their populations, ranges, and responses to pesticides, in order to make informed decisions about current agricultural practices to promote pollinator health and species richness.

Bee Population Decline

Globally, most of the research into pollinator population loss has been concentrated in Europe and North America, leaving a lack of information available in other world regions (Freitas et al., 2009; Gemmill-Herren et al., 2014). In North America, surveys of bumble bee abundance, ranges, and species richness show concerning trends of decline (Cameron et al., 2011; Colla & Packer, 2008; Kerr et al., 2015; Thomson, 2016). One study observed eight *Bombus* spp. over a 20-year period and found four of the eight species to be experiencing severe range declines.

Two of these declining species, *Bombus occidentalis* and *Bombus pensylvanicus*, formerly had some of the broadest geographic ranges of North American bumble bees. The researchers also noted that some declining species were becoming so rare that they were difficult to find and include in surveys and studies (Cameron et al., 2011). Another survey throughout the eastern United States and Canada compared bumble bees caught in 2004-2006 to those caught at the same site in 1971-1973. Three of the *Bombus* spp. that had previously been found in the 1970s were missing from the 2000s survey, while no new species were found in 2000s that had not been seen in the earlier survey. They also noted a significant shift in the community composition of the bumble bees, with four species increasing and four species declining in relative abundance since the 1970s (Colla & Packer, 2008). Several studies in the midwestern and northeastern United States have compared bumble bees ranges based on museum collections over the past 100-150 years and found declining ranges and abundances of several *Bombus* spp., as well as local extirpations of some species (Bartomeus et al., 2013; Grixti et al., 2009; Jacobson et al., 2018). These surveys indicate a decline in the abundance, diversity, and species evenness of many native North American bumble bees.

There ise less information on the populations of solitary bees in North America. In Illinois, a comparison of forest bee species from the present to historical data sets over 120 years, found around 50% of previously observed bee species had become locally extirpated. Specialist feeders, parasites, and cavity-nesters were more likely to become extirpated in the area (Burkle et al., 2013). A survey of *Osmia* spp. in the Mid-Atlantic region of the United States found that the six native species observed had declining catch rates from 2003-2017, while the two introduced species, *Osmia cornifrons* Radoszkowski and *Osmia taurus* Smith, had stable or increasing catch rates respectively (LeCroy et al., 2020). In Pennsylvania, a 3-year survey of wild bees in apple orchards found overall declines in diversity and abundance. The majority of species, which included members of the Families Apidae, Megachilidae, Halictidae, and Andrenidae, showed decreasing abundance over the years (Turley et al., 2022). In Europe,

where the majority of solitary bee population surveys have been done, there have been declines in the populations of many wild non-*Apis* bee species, including both solitary bees and bumble bees. In western and central Europe, many bees in the Bombini tribe, which includes *Bombus* spp. and cuckoo bees (*Psithyrus* spp.), have declined in range since the early 1990s (Kosior et al., 2007). In Ireland, an estimated 3% of their native species have become extirpated and 41% are threatened or endangered (Fitzpatrick et al., 2006). In Holland and the United Kingdom, there have been losses of non-*Apis* bee diversity. Species that are specialist feeders, in particular, tend to be in decline and the pollinator community composition is becoming more dominated by a smaller number of species (Biesmeijer et al., 2006). In Belgium and France, bees in the families Apidae, Anthophoridae, and Megachilidae had many species in decline (around 58%, 55%, and 25% respectively) (Rasmont et al., 2005). The declines in bumble bee and solitary bee populations and ranges coincide with the growing concerns about global decreases in insect population and biomass (Hallmann et al., 2017; Sánchez-Bayo & Wyckhuys, 2019). There is an unfortunate lack of empirical information on insect declines, other than a few flagship species, such as *A. mellifera*, *Bombus* spp., and monarch butterflies (*Danaus plexippus* L.) (Vogel, 2017). However, in Germany, a 27-year survey at several natural sites, found over a 75% decrease in flying insect biomass since 1989 (Hallmann et al., 2017), but there is little long-term information on insect biomass changes globally.

Several drivers have been proposed to explain the population and range losses of wild bees in North America and Europe, including habitat loss, agrochemical use, parasites and pathogens, climate change, competition for resources, and an interaction of these factors (Belsky & Joshi, 2019; Cameron et al., 2011; Evison et al., 2012; Goulson et al., 2015; Kerr et al., 2015; Le Féon et al., 2010; Thomson, 2016). Understanding the contribution that each of these factors makes to pollinator population decline and how they can interact with each other can better help agricultural schemes prevent and ameliorate these ill effects.

Habitat Loss

Agricultural intensification has been the primary driver of habitat loss (Asner et al., 2004; Foley et al., 2005), but increasing urbanization has also contributed, especially as the human population rises globally and demographics continue to shift from rural to urban (Darrel Jenerette & Potere, 2010). Both of these drivers have caused many habitat types to shrink and become more fragmented. In the United States, the tallgrass prairies have been some of the most affected habitats, with a loss of around 82-99% of the prairies from 1830-1994 (F. Samson & Knopf, 1994). The tallgrass prairies that do remain tend to be small and unable to support the amount of biodiversity that they once did (Samson et al., 2004). Worldwide, around 56% of temperate evergreen broadleaf forest and around 30% of temperate deciduous broadleaf forest have been converted into rangelands for livestock. Tropical forests are currently experiencing heavy deforestation in the Amazon basin, Congo, and southeast Asia (Asner et al., 2004). The clearing and conversion of these habitats has reduced global forest cover and caused many forests to become small and fragmented, with over 70% of forest cover now lying within 1 km of the forest edge (Haddad et al., 2015). Habitat loss can have a profound effect on the local biodiversity. As habitats decrease in surface area, they can support fewer species and a lower abundance of organisms (Haddad et al., 2015; Horváth et al., 2019). The surrounding landscape also has a great influence on the biodiversity of a habitat patch. Fragmented, isolated habitats tend to support less species richness than connected habitats of equivalent surface area (Horváth et al., 2019). Native bee species of North America require large enough habitats to provide adequate floral resources and nesting sites.

Wild bees may be more at risk due to habitat loss and scarcity of floral resources than managed *A. mellifera*, as beekeepers can supplement food for *A. mellifera* (Evans et al., 2018). All bees, however, depend on an adequate diet of pollen and nectar for their health and functioning. There is a great variety in the quality and nutrient content of different flower pollens, with a range of 2.5-61% protein content, which can affect bee health (Roulston et al., 2000). In

A. mellifera, high quality pollen caused the upregulation of certain genes and pathways involved in metabolism, nutrient sensing, and antimicrobial activity (Mao et al., 2013). Bumble bees visited flower species that offer higher quality pollen, with a higher protein content, when those flowers are available (Hanley et al., 2008). Solitary bees, as well, can benefit from a high-quality diet with higher protein content in the pollen. The larvae of the solitary sweat bee, *Lasioglossum zephyrus* Smith, grew larger when fed a diet of protein-rich pollen than one of protein-poor pollen (Roulston & Cane, 2002). Both social and solitary bees of different species can benefit from pollen with a high nutrient content. Habitat loss, which can reduce the diversity of flowers in an area, can also affect the type of pollen available to bees and offer them a poorer selection of pollen to choose from.

The body size of different bee species can also influence the amount of pollen and nectar they need. Larger-bodied bees have a higher pollen requirement, and as such can be more vulnerable to a lack of flower diversity and floral abundance (Müller et al., 2006). In a survey of native bees in the northeastern United States, bee species with larger body sizes tended to decline in relative abundance compared to smaller species (Bartomeus et al., 2013). As well as the amount of food required by adult bees, the breadth of their diet can also relate to their vulnerability to habitat loss. Oligolectic, or specialist feeders, feed on only one or a few closely related species of flowers (Zayed et al., 2005). Specialist feeders tend to be more efficient and less prone to antagonistic adaptations of flowers (Habermannová et al., 2013). Polylectic, or generalist feeders feed on a wider variety of flowers (Zayed et al., 2005). They may not be as efficient, but they often have more resources available to them and can spend less time in acquiring food (Habermannová et al., 2013). They also tend to have higher genetic variation and therefore be more robust to environmental changes (Zayed et al., 2005).

Bees collect pollen and nectar, not only for their own consumption, but also for the feeding and provisioning of their larvae. Like *A. mellifera*, *Bombus* spp. are social insects. Their colonies include a worker caste of nonreproductive females, which are responsible for feeding

the larvae. Some bumble bees, such as *Bombus pascuorum* Scopoli and *Bombus hortorum* L., will store pollen and nectar and allow larval bees to access it as needed. Others, such as *Bombus terrestris* L. and *Bombus terricola* Kirby will directly feed each larva with regurgitated pollen, nectar, and glandular secretions (Pereboom et al., 2003). *Bombus terrestris* workers are even able to distinguish between the cuticular chemical odors of starving and well-fed larvae. The workers will feed larvae that give off this “hunger signal” more often than larvae that smell well-fed (Boer & Duchateau, 2006).

Solitary bees differ from social bees in that each female is reproductively capable and, with the exception of cleptoparasitic species, must provision her own offspring with pollen and nectar (Straka & Bogusch, 2007). Their adult lifespan is generally shorter, so they do not directly feed their larvae, but rather leave behind a food provision in the nest (Batra, 1984; Torchio, 1989). Plentiful floral resources are required by solitary bee females at the time that they are provisioning their nests. In the European orchard bee (*Osmia cornuta* Latreille), larvae that were fed with a larger provision grew to a larger adult body weight (Bosch & Vicens, 2002). Species can vary in the number of flowers they must visit to provision one larva. In one study, the number of floral visits ranged from 0.9 visits for the mining bee, *Andrena marginata* Fabricius, to over 1,000 visits for *Megachile parietina* Geoffroy. Of the species observed, 85% of them required over 30 flowers to feed one larva (Müller et al., 2006). Most solitary bee females will provision from 10-30 larvae (Müller et al., 2006; Torchio, 1989), and so can greatly benefit from having readily available flowers near their nesting sites.

The homogenization of landscapes from diverse natural habitats into agricultural and urban areas can also reduce the availability of nesting sites. Different species of bees have differing nesting site preferences. *Bombus* spp. often nest in subterranean cavities, found cavities, and grassy tufts (Kells & Goulson, 2003; Williams et al., 2014). Some *Bombus* spp. tend to prefer open terrains, like fields and prairies, whereas others prefer forest and field boundaries (Svensson et al., 2000). They do not build their own nests, but rather rely on the

availability of rodent dens, hollow trees, and tussock patches in natural and semi-natural habitats (Williams et al., 2014). There are also species-specific preferences for other factors, such as the level of shelter provided, the amount of direct sunlight, and the soil drainage around the nesting site (Kells & Goulson, 2003). For solitary bees, there is an even greater degree of variability in nesting site preferences. Solitary bees are often divided into ground-nesting and tunnel-nesting species. Ground-nesting bees comprise the majority of bee species and vary in their preferences for moisture level, grain size, and compaction of their nesting site soils (Cane, 1991). Ground-nesting bees can benefit from undisturbed nesting sites, as tilling practices can negatively impact the adult emergence of offspring (Ullmann et al., 2016). Tunnel-nesting solitary bees often make use of found holes, such as old beetle tunnels and hollow reeds, or chew their own nest tunnels, in the case of carpenter bees (*Xylocopa* spp.) (Batra, 1984). Many then line their nest tubes and create partitions using mud, leaf pulp, resin, and other materials (Batra, 1984; Levin, 1966; Torchio, 1989). With such a variety of preferences for nesting locations and materials, the diversity of bee species can greatly benefit from having a variety of habitat types. Artificial nests can increase bee populations in some areas, but they are generally more effective for tunnel-nesting bees than for bumbles bees and ground-nesting bees (Bortolotti et al., 2016).

For many bee species, floral resources must also be in close proximity to a nesting site for the nesting site to be considered suitable (Davis et al., 2017). Bumble bees generally have greater foraging distances than solitary bees, though this can vary by species. Some, like the large carder bee (*Bombus muscorum* L.) prefer to forage within 500 m of their nest. Farther flying species, like *B. terrestris* will often travel over 1000 m, though they are unlikely to fly more than 1800 m away from nest sites (Walther-Hellwig & Frankl, 2000). Solitary bees often stay within 600-700 m from their nest, though some individuals may venture further if there are no preferred floral resources nearby (Gathmann & Tscharntke, 2002; Zurbuchen et al., 2010).

Larger-bodied solitary bees often have larger foraging ranges, but even large species like *Hoplitis adunca* Panzer tend to stay within 700 m of the nest (Zurbuchen et al., 2010).

Natural and semi-natural habitats interwoven into agricultural landscapes have been shown to improve pollinator abundance, species richness, and pollination service in many regions. One study found that fruit set, pollinator visitation, and species richness all tended to decrease when there were no natural or semi-natural lands in the surrounding area (Garibaldi et al., 2011). A higher percentage of natural habitat within 1 km of almond fields improved the fruit set, bee species richness, and bee visitation in the field (Klein et al., 2013). Organic watermelon fields near natural areas could be fully pollinated by wild bees, whereas farms without close natural areas required *A. mellifera* to supplement the pollination (Kremen et al., 2002). A study in Costa Rica examined coffee crops and pollinator diversity along distance gradients from natural riparian and forest habitats. They found that coffee yields, bee species richness and visitation, and pollen deposition were all higher the closer they were to the natural habitat patches. Within 300 m of the forest patches, a mix of stingless bees (Hymenoptera: Meliponini), solitary bees, and *A. mellifera* acted as coffee pollinators, but further than that and plants relied almost entirely on *A. mellifera* (Ricketts et al., 2004).

Given the importance of natural habitats and proper foraging for native bees, there have been some efforts to mitigate the damaging effects of habitat loss on pollinators. One strategy has been to supplement agricultural landscapes with wildflower plantings. The presence of wildflowers planted alongside highbush blueberry fields increased the abundance of both wild bees and syrphid fly pollinators and improved the yield of the blueberries. Fruit set, berry weight, and number of mature seeds per berry all improved with wildflower plantings and higher native pollinator abundance (Blaauw & Isaacs, 2014). Wildflower mixes that were tested in Florida, Michigan, and California all attracted a greater abundance and species richness of wild bees compared to unmanaged weedy plots (Williams et al., 2015). With these wildflower provisioning schemes, however, it is important to note that the floral preferences of native bees can vary by

region and species (Gresty et al., 2018; Williams et al., 2015). There is also the question of whether to focus on attracting generalist feeders (polylectic) or specialists (oligolectic) (Belsky & Joshi, 2019). More research is needed to create the best wildflower mixes in different regions (Williams et al., 2015). Field margins, such as hedgerows and shelterbelts, can offer another opportunity for incorporating natural habitat into agricultural landscapes (Rands & Whitney, 2010). Conserving remaining areas of natural habitat can help to benefit local pollinator health and pollination services in agricultural landscapes (Garibaldi et al., 2011; Klein et al., 2013; Kremen et al., 2004; Ricketts et al., 2004).

Cities can harbor a great deal of pollinator diversity, especially if foraging and nesting sites are available in the urban environments (Baldock et al., 2015; Hall et al., 2017; Sirohi et al., 2015). There is a great potential for adding pollinator habitat into otherwise unused spaces, such as green roofs, powerline easements, and roadside verges (Colla et al., 2009; Eldegard et al., 2017; Hopwood, 2008; Noordijk et al., 2009; Russell et al., 2005). Conventionally, powerline easements and roadsides are managed by frequently mowing and applying herbicides to control weeds. However, unmowed and relatively undisturbed areas can support many cavity-nesting and ground-nesting bees (Russell et al., 2005). Residential spaces can also support pollinator diversity simply by mowing lawns less frequently, as several common lawn “weeds,” like dandelions and clovers, can provide forage for pollinators (Lerman et al., 2018). These urban conservation schemes can benefit a variety of invertebrate life, though Turo and Gardiner (2019) note that cooperation with the local human community is key for any urban pollinator management scheme. As such, urban bee habitats need to work well for bees and local human residents alike (Turo & Gardiner, 2019).

Research into the floral preferences of bee pollinators and the health benefits of different planting types can help further improve the mitigation strategies and recommendations for growers and gardeners. This can help promote robust pollinator communities in agricultural, urban, and residential areas.

Pesticide Use

Most bee species are considered beneficial insects, especially in agricultural landscapes, and are not often the intended targets of pesticide use. Many nontarget species, however, can be exposed to pesticides and other agrochemicals present in the environment. Bees have been the subject of much of this concern for nontarget exposure. *Apis mellifera* are the most often studied in toxicity testing, followed by other *Apis* spp., *Bombus* spp., and solitary bees. Of the solitary bee species, those that are commercially managed, such as *M. rotundata* and *O. lignaria*, have been the most studied (Pisa et al., 2015). There is still a great deal of information to be learned about the effects of pesticides on these non-*Apis* bees in North America.

Current pesticide regulations and requirements for registration can help limit the risk of exposure for beneficial pollinators, however, improvements to risk assessment could better protect a wider variety of pollinator species. The time scales of tests are often shorter than the usage period of the pesticide, with little testing of environmental retention and accumulation over time (Mancini et al., 2019). Much of the testing is focused on mortality, rather than sublethal effects. These sublethal effects can be devastating to pollinators, however. *Megachile rotundata*, for example, showed signs of paralysis after exposure to 0.5-40 ng ai bee^{-1} imidacloprid, even when mortality during the tested time period was lower (Ansell et al., 2021). Monitoring and regulation after a pesticide has been approved is also rare (Mancini et al., 2019). However, unexpected environmental consequences can occur, such as the impact of DDT on bird eggshell strength (Grier, 1982).

For practical reasons, a limited number of surrogate species are used to assess the impact of the pesticides. Often, *A. mellifera* adult workers are used as surrogates for other pollinators (EFSA, 2013; Thompson & Pamminger, 2019). Pesticide sensitivity and likelihood of exposure can vary by species, age, and sex of bees, however. *Osmia lignaria* females were more sensitive to contact exposure to neonicotinoids than *A. mellifera*, but less sensitive to

pyrethroids (Peterson et al., 2021). Similarly, the neonicotinoid, thiacloprid, and the butenolide, flupyradifurone, had low contact toxicity for *A. mellifera*, *B. terrestris*, and *Osmia bicornis* L., but high toxicity for *M. rotundata* (Hayward et al., 2019). These trends are not always consistent even within pesticides classes and bee genera, however. For contact toxicity to two neonicotinoid insecticides, *O. cornifrons* were more sensitive to acetamiprid than *A. mellifera*, but *A. mellifera* was more sensitive to imidacloprid (Biddinger et al., 2013). *Apis mellifera* can have similar pesticide sensitivities to other bee species for certain pesticides (Heard et al., 2017), but not all. Several potential solutions have been proposed, such as using toxicity by weight, adding more solitary bee species, including chronic exposure and sublethal effects testing, and implementing more monitoring of the impact of pesticides after approval (Mancini et al., 2019; Thompson & Pamminger, 2019). These proposals can be costly, however, so research is needed into the most effective solutions.

Routes of Exposure

Bees and other nontarget insects can be exposed to pesticides in several ways. Bees that are out foraging during spraying can suffer from direct contact exposure, which can have a more adverse effect on bee health than residual exposure. *Apis mellifera* and the stingless bee, *Hypotrigona respolii* Magretti, both experienced higher mortality when directly sprayed with a glyphosate-based herbicide or when exposed to freshly sprayed residues, compared to those exposed to dried herbicide residues (Abraham et al., 2018). Several insecticides, including the neonicotinoids, clothianidin and imidacloprid, and the bacterial toxin, spinosad, had medium to high toxicity when directly sprayed on *A. mellifera*, but little to no effects on bee mortality with residual exposure (Bailey et al., 2005). Foliar spraying of plants during bloom times, when bees are out foraging, can therefore pose a threat to bee health.

With systemic pesticides, such as the neonicotinoid class of insecticides, another concern is the presence of pesticide residues in pollen and nectar, which the bees collect and

feed on. Such systemics are often applied to plant seeds and then translocate from the seed to other plant tissue through the phloem and xylem (Bromilow et al., 1990; van der Sluijs et al., 2013). Flower pollen collected from the anthers of both treated and untreated maize and soybean plants were shown to have residues of the neonicotinoid clothianidin. The treated plants had higher levels, of up to 3.9 ppb, than the untreated, but there were also small amounts of clothianidin residues in untreated fields, suggesting some environmental contamination (Krupke et al., 2012). For the butenolide flupyradifurone, pollen and nectar residues varied by crop and type of application. Levels tended to be higher following a foliar spray than drench or seed treatments. Blueberry plants following a foliar spray showed the highest residue concentrations in pollen, up to 67.6 ppm (67.6 mg ai kg^{-1}), followed by apples with up to 26.2 ppm (26.2 mg ai kg^{-1}). Nectar residues were lower than pollen residues in most crops (Glaberman & White, 2014). Apples sprayed with another new systemic insecticide, sulfoxaflor showed no detectable residues in nectar, but an average of 80 ppb in pollen (Heller et al., 2020). Cotton plants treated with drip irrigation of sulfoxaflor contained residues up 39 ppb in pollen and 13.8 ppb in nectar (Jiang et al., 2020). Levels of several pesticides were found in *A. mellifera* stored pollen, as well. The levels varied, as influenced by the timing of spraying and the bee hive proximity to treated fields, but could reach high concentrations. One of the hives near the treated fields contained 88 ppb of clothianidin in their pollen stores (Krupke et al., 2012). Timing of spraying, agricultural practices, and environmental conditions, such as heat and moisture, can all affect residue levels (Dively & Kamel, 2012). Contaminated food may affect adult bees and larvae differently, as larvae tend to consume more pollen than adults, and adults of many species eat primarily nectar (Cane, 2016).

For seed treated plants, pesticides can spread systemically, but residues can also be released into the air during planting. Vacuum planters cause some of the seed coating to chip off and mix with the dust kicked up by the planter. The seed coating can then settle on top of the field and spread to adjacent fields and nearby water sources (Samson-Robert et al., 2014; Xue

et al., 2015). The planter's exhaust can contain high levels of systemic insecticides like clothianidin and thiamethoxam (Krupke et al., 2012). Systemic pesticides can accumulate in water sources and reach levels that have been shown to cause adverse health effects to bees. A study in Quebec found neonicotinoid residues in all bodies of water near treated corn fields, with concentrations ranging from 0.01-63 ppb. They noted that higher levels of neonicotinoids were found during the sowing of the corn plants (Samson-Robert et al., 2014). Another survey in Ontario found neonicotinoid residues in 98% of the bodies of water sampled around treated maize fields, with higher levels during the 5-week period following maize plating. Levels of clothianidin reached up to 43.6 ppb, with a mean of 2.28 ppb (Schaafsma et al., 2015). One of the water sources sampled in a Maryland survey contained 131 ppb of imidacloprid (Johnson & Pettis, 2014). Though the mean concentrations of these studies tend to be below 5 ppb, concentrations following planting can reach high levels.

Nesting material can also become contaminated with pesticides. Commercial *A. mellifera* are often exposed to acaricides in order to treat mite pests. Acaricides like fluvalinate and coumaphos can accumulate in the wax within the hive at high concentrations (Mullin et al., 2010). For *Bombus* spp. and ground-nesting solitary bees, there is concern for contaminated soil. Tunnel-nesting *Osmia* spp., including *O. lignaria* and *Osmia californica* Cresson, will line their nests and create partitions using mud (Cane, 1991; Torchio, 1989), which can contain pesticide residues, especially if in proximity to agricultural fields. Surveyed soil from maize and soybean fields contained clothianidin levels up to 9.6 ppb, imidacloprid up to 7.3 ppb, and the herbicide atrazine up to 52 ppb (Krupke et al., 2012). Soil levels of seed treatment insecticides can also spike to higher levels directly after planting (Schaafsma et al., 2015). Environmental conditions can impact the sensitivity of bee to pesticide residues in soil. *Osmia lignaria* larvae reared with soil containing imidacloprid residues up to 780 ppb were not adversely affected when soil moisture was 20%. Higher moisture content of 40%, however, increased larval mortality over 50% to imidacloprid concentrations of 50-780 ppb (Fortuin et al., 2021).

Levels of pesticides and other toxic synthetic chemicals in the environment can be detected in bees, themselves, as well. One survey of native bees in the western United States found 70% of bees to have at least one pesticide and 48% to have more than one (Hladik et al., 2016). Several of these pesticides have been shown to have deleterious effects on bee health in both lab and field studies (Di Prisco et al., 2013; Fauser-Misslin et al., 2014; Gill et al., 2012; Hayward et al., 2019; Lu et al., 2014; Matsumoto, 2013; Sandrock et al., 2014). Recently, neonicotinoid class insecticides have been widely implicated in bee declines (Belsky & Joshi, 2019; Blacquière et al., 2012; Chagnon et al., 2015; Goulson et al., 2015), though they are not the only class of pesticides toxic to bees.

Solitary bees may be more at risk to stressors than social bees, due to their life history. Social bee reproductive females (queens) spend most of the year in their hive or nest, while the non-reproductive females (workers) go out to forage. Solitary bee females, however, are all reproductive and forage for pollen and nectar. This puts solitary bee reproductive females at higher risk of pesticide exposure than social bee reproductive females (Stoner, 2016). The loss of a solitary bee female will end her reproductive line, whereas the loss of a single worker bee will not (Eeraerts et al., 2020).

Sulfoxaflor

Sulfoxaflor, a novel sulfoximine insecticide manufactured by Corteva Agriscience, was originally registered by the US EPA in May 2013, but drew some concern due to its apparent negative effects on bee health. The registration was overturned by the Ninth Circuit Court of Appeals for this reason, though it was re-registered in 2016 with restrictions, such as prohibiting its used post-bloom on bee-preferred crops (United States Court of Appeals for the Ninth Circuit, 2015; US EPA, 2019). Several of these restrictions were removed in 2019 and several new crops were approved for use. Post-bloom applications on bee-attractive crops were approved when the risk of bee exposure was low (Siviter et al., 2020; US EPA, 2019, 2016).

However, this new, wider approved usage of sulfoxaflor could put pollinators at higher risk of exposure.

The mode of action of sulfoxaflor is somewhat similar to neonicotinoids, with both acting as agonists of the insects' nicotinic acetylcholine receptors (nAChRs) (Sparks et al., 2013). Sulfoxaflor is grouped under the Insecticide Resistance Action Committee (IRAC) Group 4C. Exposed insects can show similar symptoms to excitatory toxins, with tremors, followed by paralysis and death (Watson et al., 2011, 2021). Sulfoxaflor has a unique chemical structure, and has been effectively used against sucking pest insects that are resistant to neonicotinoids (Sparks et al., 2013; US EPA, 2019). It is a systemic insecticide, and as such, levels can be found in pollen and nectar, with higher concentrations observed when sulfoxaflor was applied during flowering (Jiang et al., 2020).

Sulfoxaflor can impact the health of several bee species. Higher concentrations of sulfoxaflor have caused high mortality in *A. mellifera* (Zhu et al., 2017), *B. terrestris* (Siviter et al., 2020), and *O. bicornis* (Boff et al., 2021). Contact exposure with higher field use rates has also caused an increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS) in *A. mellifera*. These molecules can accumulate when they are produced more rapidly than the insect body is able to metabolize or excrete them and can result in cellular damage (Chakrabarti et al., 2020). Lower concentrations, closer to residues that might be found in pollen and nectar, reduced egg laying in *B. terrestris* (Siviter et al., 2020). A semi-field study of *A. mellifera* in flight cages, however, saw no impact of sulfoxaflor and azoxystrobin on foraging activity or colony size up to 50 days after application (Tamburini et al., 2021). Low level, residual exposures often have less negative impact on bee health than fresh sprays, so restrictions to reduce bee exposure during applications can help protect bees.

Flupyradifurone

Flupyradifurone was registered in 2015 by Bayer CropScience LP (US EPA, 2018). It is a butenolide insecticide (IRAC Group 4D) with a similar mode of action to sulfoxaflor and neonicotinoids, also acting as an agonist of insect nAChRs. It has a novel action and can be effective against pests that have developed resistance to neonicotinoids (Hesselbach & Scheiner, 2018). It is a systemic insecticide and can quickly spread throughout the tissues of target plants (Nauen et al., 2015). It has been proposed for use against sucking pests, including stink bugs, whiteflies, and aphids, on a variety of vegetable, cereal, and tree nut crops (Campbell et al., 2016). It is approved for foliar applications on several bee-preferred crops and flowers, including apples, crabapples, and pears, which are attractive to *O. lignaria* and *O. cornifrons* (Batra, 1982; Bosch & Kemp, 2000), and several Asteraceae family flowers, which are attractive to *O. californica* (Cripps & Rust, 1989; Levin, 1966).

Like sulfoxaflor, flupyradifurone has been marketed as an alternative to neonicotinoids, one that poses less of a risk to pollinator health (EFSA, 2015). There have been concerns regarding this claim, however, based on the adverse effects flupyradifurone can have on *A. mellifera* and other pollinators. Acute contact with field use concentrations of the flupyradifurone formulation, Sivanto, caused higher mortality and an increase in reactive oxygen species (ROS), reactive nitrogen species (RNS), and caspase-3 in *A. mellifera* (Chakrabarti et al., 2020). It also seems to have a greater effect on larvae and foragers (older worker bees) than in-hive adult workers (Al Naggar & Baer, 2019; Tosi & Nieh, 2019). At lower doses, closer to residue levels in pollen and nectar, some sublethal effects were observed, including impaired learning and memory, but these low concentrations had little impact on *A. mellifera* mortality (Campbell et al., 2016; Glaberman & White, 2014). Sivanto, manufactured by Bayer CropScience, has been approved for applications during bloom, so it is possible for foraging pollinators to be exposed to higher concentrations, as well (Campbell et al., 2016; Chakrabarti et al., 2020). There has been little examination of the effects of flupyradifurone on non-*Apis* bees, though one study observed

that *M. rotundata* was more susceptible to the insecticide than three other species of bees: *A. mellifera*, *B. terrestris*, and *O. bicornis* (Hayward et al., 2019). Additionally, an EFSA report found flupyradifurone to have moderate to high environmental persistence (EFSA, 2015). Assessing the effect of the insecticide on pollinator health can help mitigate its potential risk.

Mitigation

The effects that these various pesticides can have on pollinators and other beneficial insects is cause for concern, but there are several strategies for mitigating the damage to bee populations and pollination services. Integrated Pest Management (IPM) encourages the usage of multiple techniques for pest control, including mechanical measures (traps and barriers), cultural measures (crop rotations), and biological measures (predators and parasitoids), which can help to reduce the reliance on chemical pesticides (Kogan, 1998). Careful timing of pesticide applications and decreasing spraying during bloom time can also reduce the risk to pollinators (Belsky & Joshi, 2019; Dively & Kamel, 2012). Measures to reduce surface contamination, such as reduced-volume sprayers, can also help lower pesticide residues in the environment (Giles et al., 1992; Xue et al., 2015). Finally, it is important to continue researching these pesticides and how they interact with pollinators and other stressors, in order to make informed decisions about pest control measures.

Pesticide Detoxification in Bees

Exposure to Xenobiotics

When studying pesticide exposure and health effects on bees, it is also important to further the understanding of how insects cope with xenobiotics, including both natural and synthetic toxins. This understanding can further our knowledge of how best to protect beneficial species and also how to deal with pesticide resistance in pest species. Insects have been exposed to toxic compounds long before humans began synthesizing pesticides, so they have

evolved several mechanisms for dealing with this exposure. The most commonly encountered environmental toxins for herbivorous insects and nectar gatherers are the plant secondary metabolites. Each of these compounds are produced by a select group of plants and can have many important functions, including producing floral scents and bright pigment to attract pollinators, preventing microbial growth in fruit, and enhancing fruit flavor to attract animal feeders for seed dispersal (Pichersky & Gang, 2000). Many secondary metabolites also act as anti-feedants and toxins to prevent damage to the plant from herbivorous feeding. Despite this, some specialist insects have adapted resistance to these compounds and may even preferentially feed on plants that produce them (Bennett & Wallsgrove, 1994; Ceja-Navarro et al., 2015). One example of this is the southern armyworm (*Spodoptera eridania* Stoll), which preferentially feeds on plants, like sorghum, barley, and clover, which produce cyanogenic glucosides, a group of secondary metabolite compounds to deter feeding (Brattsten et al., 1983; Scriber, 1978). Similarly, the coffee berry borer (*Hypothenemus hampei* Ferrari) feeds exclusively on coffee beans, which contain high levels of the toxic pseudoalkaloid, caffeine (Ceja-Navarro et al., 2015).

It is not just herbivorous insects interacting with these phytotoxins, however, but pollinators, as well. Several of the plant secondary metabolites can be toxic to pollinating bees. Lupanine, for example, produced by plants in the genus *Lupinus*, reduced drone production in *Bombus* spp. colonies (Arnold et al., 2014). Both nectar and pollen can contain levels of secondary metabolites, though levels in nectar tend to be lower than those in pollen and leaves (Irwin et al., 2014). The interaction between plants, herbivores, and pollinators can drive the evolution of all three groups.

Nectar often contains lower concentrations of toxic compounds than other plant tissues, which can help prevent damage to pollinator health during nectar feeding. This was noted in *Nicotiana* spp., which produce the neurotoxin, nicotine. Outcrossing species, which are reliant on animal pollinators, had lower levels of nicotine in their nectar and other tissues than selfing

species (Adler et al., 2012). In some cases, toxic nectar may have other purposes, such as discouraging nonspecialist pollinators and nectar robbers (Irwin et al. 2014). Plants in the genus *Toxicoscordion* (formerly *Zigadenus*) produce the compound zygacine, which is toxic to the generalist feeding bees, *O. lignaria* and *A. mellifera* (Cane et al., 2004; Hitchcock, 1959). However, the solitary bee, *Andrena astragali* Viereck & Cockerell, which is a *Toxicoscordion* spp. specialist, was less affected by the toxin (Irwin et al., 2014; Tepedino, 1979, 2003). Other hypotheses for the existence of toxic nectar include preventing microbial growth in the nectar and being a side effect of toxin translocation through the plant phloem (Adler, 2000b).

Toxic secondary metabolites can also be present in pollen and at much higher concentrations than those found in nectar (Gosselin et al., 2013; Irwin et al., 2014). Almonds, for example, produce the cyanogenic glycoside, amygdalin, which is present in pollen at concentrations over 280 times higher than the concentrations in nectar (London-Shafir et al., 2003). Pollen has more than one function to a plant, acting as both a reward and attractant for pollinators and as a gamete (Irwin et al., 2014). A higher concentration of secondary metabolites could therefore deter overfeeding on pollen by herbivores and nonspecialist pollinators (Sedivy et al., 2011). Like toxic nectar, allelochemicals in the pollen could promote pollen specialists for the plant (Adler, 2000a; Gosselin et al., 2013). Toxic pollen may also encourage bees and other pollinators to move more frequently from flower to flower, which could improve cross pollination (London-Shafir et al., 2003).

Bee species, even closely related ones, can differ in their response to these secondary metabolites in pollen. Two generalist species, *O. bicornis* and *O. cornuta* differed greatly in their response to *Ranunculus acris* L. pollen, which contains the glycoside, protoanemonin, and *Echium vulgare* L. pollen, which contains pyrrolizidine alkaloids like echimidine. *Osmia bicornis* larvae developed well on the *R. acris* pollen, but had high mortality on *E. vulgare* pollen, whereas *O. cornuta* showed the opposite trend (Sedivy et al., 2011). Bees have several behavioral adaptions to cope with these compounds in their diet. *Apis mellifera* larvae are fed

smaller amounts of pollen than adult bees, and instead are fed secretions from the hypopharyngeal and mandibular glands of nurse bees. Through these secretions, nurse bees are able to dilute some plant secondary metabolites, so that they are present only in low concentrations in the larval diet (Lucchetti et al., 2018). *Apis mellifera* and other generalist feeders will also show avoidance behaviors for plants that produce unfavorable pollen, especially when more favorable diets are available (London-Shafir et al., 2003; Tan et al., 2012). The generalist bumble bee, *Bombus wurflenii* Radoszkowski, for example, avoided feeding on *Aconitum septentrionale* Koelle, which contain potentially toxic alkaloid compounds (lappaconitine, septenine, oreaconine, etc). The specialist bumble bee, *Bombus consobrinus* Dahlbom, however, readily fed on *A. septentrionale*, and contained higher concentrations of the alkaloids in their tissues (Gosselin et al., 2013). Bees may also ameliorate the harmful effects of these compounds through diet mixing. *Osmia cornuta* bees fed a diet entirely comprised of the unfavorable *R. acris* pollen had high larval mortality. When the *R. acris* pollen was mixed 50:50 with the favorable *Sinapis arvenis* L. pollen, however, the larvae experienced little to no effects on their health (Eckhardt et al., 2014). Along with these behavioral adaptations, it is likely that bees also use physiological adaptations to cope with plant secondary metabolites, including producing detoxification enzymes like cytochrome P450s (Gosselin et al., 2013).

These insect interactions with plant secondary metabolites can be highly concentration dependent. For certain phytotoxins, high concentrations may cause harm to insects, whereas lower concentrations may have beneficial effects (Anthony et al., 2015; Züst et al., 2018). Several secondary metabolites, catalpol, thymol, nicotine, and especially anabasine, reduced the parasite load of the microsporidian, *Crithidia bombi*, in the bumble bee, *Bombus impatiens* Cresson (Richardson et al., 2015). Natural levels of anabasine in nectar caused no harm to uninfected bees and reduced parasite loads of infected ones (Anthony et al., 2015), which suggests that low nectar concentrations of certain secondary metabolites may help reduce internal parasites in wild bees.

Mechanisms of Detoxification

Insects have evolved different methods of detoxification and other adaptations to reduce their exposure to harmful xenobiotics. Generally, these methods are divided into behavioral modifications, including toxin avoidance, diet mixing, and food storage, and physiological modifications, including development of a thicker cuticle, toxin sequestration, rapid excretion, target-site mutations, and detoxification enzymes (Després et al., 2007; Irwin et al., 2014; Itoh et al., 2018). For the insect's own detoxification enzymes, which may metabolize xenobiotics, exposure to toxins can cause changes in enzyme expression and mutation to the genes coding for the enzymes. Additionally, many insects have gut bacterial symbionts, which can be involved in the metabolism of both natural toxic allelochemicals and synthetic compounds, like insecticides.

Insects and other animals possess a host of detoxification enzymes to help them cope with exposure to toxic xenobiotics. There are three main families and superfamilies of enzymes that greatly contribute to the metabolism and detoxification of xenobiotics in insects, which are the cytochrome P450 monooxygenases (P450s), glutathione-*S*-transferases (GSTs), and carboxyl/cholinesterases (CCEs) (Douglas, 2015; Itoh et al., 2018; Maiwald et al., 2023; van den Bosch & Welte, 2017). The specific enzymes present in different insect species, as well as the expression of them, can affect their sensitivity to certain pesticides (Haas et al., 2022, 2023; Hayward et al., 2019). Overall, bees tend to have relatively few genes coding for these detoxification enzymes when compared to other insects. *Apis mellifera* had far fewer protein coding genes (~11,000), in general, than the mosquito, *Anopheles gambiae* Giles (~14,000 genes) and the fruit fly, *Drosophila melanogaster* Meigen (~13,500) (Claudianos et al., 2006). Of these coding genes, *A. mellifera* had only around half the number of genes for enzymes involved in detoxification, such as GSTs, P450s, and CCEs. The number of genes for Delta GSTs, Epsilon GSTs, and CYP4s, all of which are associated with insecticide resistance in other insect species, were especially low in the *A. mellifera* (Claudianos et al., 2006). Several

other social bees, including *Bombus huntii* Greene, *B. terrestris*, and *B. impatiens*, and solitary bees, including *M. rotundata* have similar numbers of coding genes to *A. mellifera* (Xu et al., 2013). This low number of detoxification genes could be due to bees' limited exposure to plant defensive chemicals when compared to insect herbivores (Adler, 2000a; Xu et al., 2013).

As well as their own enzymatic activity, many insects rely on bacterial symbionts to aid in detoxification. This can be done either by the symbiont's own enzymatic activity (Boush & Matsumura, 1967; Gangola et al., 2018; Singh & Walker, 2006) or through the symbiont affecting the host insect's gene expression (Rothman et al., 2019; Wu et al., 2020).

By studying the detoxification enzymes and gut microbiota of pollinators, we can enhance our understanding of how pollinators react to pesticide exposure both on a cellular and organismal level.

Study Species

Most of the research on the effects of pesticide exposure to the health and pollination ability of bees has focused on *A. mellifera*, and *Bombus* spp. to a lesser extent, leaving many solitary species under-studied. Many solitary bees, however, can provide a valuable contribution to pollination. The North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa found that both *O. lignaria* and *O. cornifrons* produced a similar or better seed set of several cruciferous plants (*Brassica rapa* L., *Brassica napus* L., and *Sinapis alba* L.) when compared to *A. mellifera* (Abel et al., 2003). *Osmia lignaria* can also be used to supplement *A. mellifera* pollination of almonds in California to improve nut set and reduce costs of importing *A. mellifera* hives (Pitts-Singer et al., 2018). Many other fruit trees and berries, including strawberries, pears, apples, and blueberries, can benefit from *Osmia* spp. pollination, with improved fruit weight and fruit set (Monzón et al., 2004; Torchio, 1985; West & McCutcheon, 2009).

Osmia spp. can be beneficial alongside *A. mellifera* for several reasons. Most species are active in early to mid-spring, when *A. mellifera* colonies are still building up their colony size

after the winter (Batra, 1994; Kline et al., 2023). They have a high rate of contact with the flower stigmas, a high visitation rate, a tendency to move among plants, and a limited flight radius, which helps keep them on the desired crop (Batra, 1994; Calzoni & Speranza, 1998; Monzón et al., 2004; Vicens & Bosch, 2000a). As orchard pollinators, they show high fidelity to Rosaceous flowers. They are also more tolerant of lower temperatures than *A. mellifera* (Vicens & Bosch, 2000b). *Apis mellifera* are vital pollinators and are often used as model organisms to assess pesticide risk to bees, but it is important to also consider native solitary bees, like *O. lignaria*, when studying bee pesticide response.

Osmia lignaria

One important native pollinator and one of the few managed solitary bee species is *O. lignaria* (Bosch & Kemp, 2001). They are widespread across the United States, with two subspecies: *O. lignaria lignaria*, found in the eastern states, and *O. lignaria propinqua*, found in the west. The subspecies are similar in their biology, life cycle, and pollination ability, and have only minimal differences in morphology (Rust, 1974). *Osmia lignaria* bees are generalist feeders and collect pollen and nectar from a variety of fruit trees, wildflowers, and vegetable plants (Cripps & Rust, 1989; Levin, 1966). They are commercially used in agriculture, primarily in fruit and tree nut orchards (Bosch et al., 2006; Pitts-Singer et al., 2018). For a solitary bee, they are relatively commonly studied, though not to the extent of *A. mellifera* or *Bombus* spp.

Osmia lignaria is a tunnel-nesting species that collects mud to create partitions in between their egg cells. Adults are active for about 20 days in the springtime (March-June, depending on the region), during which time they mate and the females construct and provision their nests and lay eggs (Levin, 1966; Rust, 1974; Torchio, 1989). The offspring remain in their tunnel nests as they develop and remain in their cocoons as adults in diapause to overwinter, before emerging the following spring from their cocoons. (Sgolastra et al., 2015; Torchio, 1989).

Osmia cornifrons

Osmia cornifrons is a solitary mason bee and a generalist feeder. They are native to northeastern parts of Asia (Rust, 1974), where they have been managed as orchard pollinators in Japan since the 1930s (Batra, 1982). In the 1970s, they were intentionally introduced to the United States by the USDA-ARS in Maryland for the pollination of apple orchards (Biddinger et al., 2010). Since their introduction, they have become widespread across the northeastern United States and parts of Canada, though they are less tolerant of high temperatures than some native *Osmia* spp., which could limit their use in the southern states (Gutierrez et al., 2023; MacIvor et al., 2022). They are efficient pollinators of many fruits and tree nuts, but there are some concerns of them outcompeting native *Osmia* spp. (LeCroy et al., 2020).

Osmia californica

Osmia californica is a native North American mason bee, though their range is limited to the western United States (Krombein, 1979). Unlike *O. lignaria* and *O. cornifrons*, they are an oligolectic species, with a strong preference for Asteraceae flowers (Cripps & Rust, 1989; Levin, 1966). They are also a tunnel-nesting species, though they used a mix of leaf pulp and mud in their nest construction (Cane et al., 2007). *Osmia californica* bees are also parsivoltine, with some offspring completing development in one year, as is typical of the genus, while others have a delayed emergence and complete development in two years (Torchio & Tepedino, 1982). While not commercially managed, *O. californica* can contribute to the pollination of many wildflowers and garden flowers, as well as providing insights into the toxicity response of oligolectic bees.

Objectives

The primary purpose of this dissertation research was to assess the impacts of pesticide exposure and different diet mixes on *Osmia* spp. health. The main objectives of the project were:

1. Determine the sensitivity of three species of mason bees (*O. lignaria*, *O. cornifrons*, and *O. californica*) and *A. mellifera* to the insecticides, flupyradifurone and sulfoxaflor.
2. Assess the sublethal effects of the insecticides, flupyradifurone or sulfoxaflor, on *Osmia* spp. health and physiology, measured by the following endpoints: P450 enzymatic expression, feeding preference or avoidance behavior, and flight and foraging activity.
3. Characterize the composition and diversity of the gut bacterial communities of *O. cornifrons* males and females after exposure to the commercial insecticides, flupyradifurone or sulfoxaflor, or the common garden pesticides, glyphosate, chlorothalonil, or spinosad.
4. Assess the foraging activity, nesting behavior, and fecundity of *O. lignaria* provided with a diet of a buckwheat monoculture or a wildflower mix.
5. Characterize the composition and diversity of soil bacterial communities and the soil nutrient content in plots planted with a buckwheat monoculture or a wildflower mix.

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Chapter 2:

Toxicity of flupyradifurone and sulfoxaflor insecticides to *Apis mellifera* and different species of *Osmia* bees

Abstract

Mason bees (*Osmia* spp.) are solitary, tunnel-nesting bees. Several species, including the horned-face bee (*Osmia cornifrons*) and the blue orchard bee (*Osmia lignaria*), are commercially managed, primarily for the pollination of fruit trees and tree nuts. They are efficient pollinators and have high pollen fidelity, and so can greatly benefit orchard yields compared to honey bees (*Apis mellifera*) alone. *Apis mellifera* are often used as surrogates for other pollinators during pesticide risk assessment. Other bee species, however, can be more sensitive to certain pesticides, so it is also important to research the impact of novel pesticides on other bee species, such as *O. lignaria*. This study investigated the effect of two recently approved insecticides, sulfoxaflor and flupyradifurone, on the survival of *A. mellifera* and three species of mason bees (*O. lignaria*, *O. cornifrons*, and *Osmia californica*). Bees were orally exposed to sulfoxaflor or flupyradifurone and their survival was measured over four days following exposure. Bee sensitivity to the insecticides varied by species and sex of bee. *Apis mellifera* was the least sensitive, followed by *O. cornifrons*, with *O. lignaria* and *O. californica* as the most sensitive. Male *Osmia* bees were less sensitive than females. These insecticides may pose a higher risk to the health of *Osmia* spp. bees compared to *A. mellifera*.

Introduction

Western honey bees (*Apis mellifera* L., Hymenoptera: Apidae) are the primary commercial pollinators of crops in the United States, due to their large colony sizes, long annual activity period, generalist feeding, and well-known husbandry (Belsky & Joshi, 2019; Thompson & Pamminger, 2019). The pollination service provided by *A. mellifera* and other bee species can help improve crop yields, grower profits, and human nutrition (Chagnon et al., 2015; Gallai et al.,

2009; Klein et al., 2007; McGregor, 1976). Many commercial crops benefit from insect pollination, and several depend on it for reproduction (Klein et al., 2007). For example, almond producers in California alone have spent over $200 million annually to import *A. mellifera* hives and ensure complete pollination of their orchards (US Department of Agriculture, 2017; Wade et al., 2019).

Osmia spp. can be used with *A. mellifera* to supplement the pollination of certain crops. In recent decades *Osmia* spp., such as the blue orchard bee (*Osmia lignaria* Say, Hymenoptera: Megachilidae) and the horn-faced bee (*Osmia cornifrons* Radoszkowski), have been used as effective commercial pollinators of crops, such as apples, almonds, and sweet cherries (Bosch et al., 2006; Bosch & Kemp, 2001; Pitts-Singer et al., 2018; Torchio, 1985). Both *O. lignaria* and *O. cornifrons* produced higher seed production of oilseed rape (*Brassica napus* L.) and white mustard (*Sinapis alba* L.) than *A. mellifera* in a small-scale production system (Abel et al., 2003). *Osmia lignaria* have also been used to supplement almond pollination in California (Pitts-Singer et al., 2018) and to improve apple yields compared to *A. mellifera* pollination alone (Torchio, 1985). Similarly, *O. cornifrons* pollination increased fruit set in cherries (Biddinger et al., 2013), and also produced an equivalent fruit set of highbush blueberry compared to *A. mellifera* and bumble bees (*Bombus* spp., Hymenoptera: Apidae) (West & McCutcheon, 2009). Additionally, many agricultural landscapes and native ecosystems can also benefit from having a greater species diversity of pollinators in an area, rather than just one dominant species (Bispo dos Santos et al., 2009; Hoehn et al., 2008; Kremen et al., 2004).

All bee species encounter stressors in their environment, such as habitat loss, pesticide exposure, diseases, and predation, which can impact their survival, reproduction, and ability to pollinate (Goulson et al., 2015; Sánchez-Bayo & Wyckhuys, 2019). Because of their importance to agriculture, *A. mellifera* are often used in pesticide toxicity assays as surrogates for other bee species (EFSA, 2013; Thompson & Pamminger, 2019; US EPA, 2014). However, different bee species can vary in their sensitivity to pesticides compared to *A. mellifera*. The butenolide

insecticide, flupyradifurone, for example, was much more toxic to the solitary alfalfa leafcutter bee (*Megachile rotundata* Fabricius, Hymenoptera: Megachilidae), compared to *A. mellifera*, *Bombus terrestris* L., or *Osmia bicornis* L. (Hayward et al., 2019). Several neonicotinoid insecticides: imidacloprid, clothianidin, and thiamethoxam were more toxic to *O. lignaria* than *A. mellifera* (Peterson et al., 2021). Because of these species-specific differences in pesticide sensitivity, it is important to assess the toxicity of new pesticides to non-*Apis* bees. Pesticide risk assessment involving different bee families can help provide a more comprehensive analysis on the pesticide risk to bee communities, especially in intensively managed agricultural landscapes and in areas where non-*Apis* bees are commonly managed.

Many pesticides have been investigated as potentially harmful for bee populations. As several pest insects have developed resistance to commonly used neonicotinoid insecticides, the development of novel insecticides can help to combat these resistant pests. Two recently registered insecticides, flupyradifurone and the sulfoximine sulfoxaflor, have been effectively used against neonicotinoid-resistant insects (Watson et al., 2021). Both are nicotinic acetylcholine receptor (nAChR) agonists, and have a similar mode of action to neonicotinoids, though they have a unique chemical structure and novel action (Sparks et al., 2013). Sulfoxaflor was initially registered by the US EPA in May 2013, though this registration was overturned by the Ninth Circuit Court of Appeals due to concerns for its impact on bee health (Siviter et al., 2020; US EPA, 2016). In 2016, it was re-registered, and in 2019, its approved usage was expanded to include more crops and to allow for post-bloom applications on bee-attractive crops (US EPA, 2019, 2016). Both sulfoxaflor and flupyradifurone commercial formulations are approved for usage on *Osmia* spp. preferred crops (Bosch & Kemp, 2000), including pome fruits, stone fruits, and tree nuts, in the case of sulfoxaflor, and pome fruits and dandelions, in the case of flupyradifurone. Both insecticides can have negative health effects on *A. mellifera* and *Bombus* spp. *Apis mellifera* exposed to flupyradifurone had increased adult mortality at higher doses (Chakrabarti et al., 2020; Tosi & Nieh, 2019), impaired learning and memory

(Campbell et al., 2016; Glaberman & White, 2014), and higher larval mortality (Al Naggar & Baer, 2019). Sulfoxaflor also resulted in higher mortality of *A. mellifera* (Zhu et al., 2017), *B. terrestris* (Siviter et al., 2020), and *O. bicornis* (Boff et al., 2021). Both flupyradifurone and sulfoxaflor altered the gut bacterial communities of *A. mellifera* and increased the relative abundance of the opportunistic *Serratia* spp. pathogens (Al Naggar et al., 2022). For *O. bicornis*, exposure to sulfoxaflor and flupyradifurone impaired learning and memory (Arnet, 2022). As these insecticides increase in usage to combat neonicotinoid resistant pests, proper assessment of their impact on non-*Apis* bee health can help inform mitigation strategies and protect solitary bee communities.

In this study, three species of mason bees, *O. lignaria*, *O. cornifrons*, and *Osmia californica* Cresson, and *A. mellifera* were exposed to flupyradifurone and sulfoxaflor. Two of three species, *O. lignaria* and *O. cornifrons*, are commercially managed generalist feeders, and the third, *O. californica*, is an Asteraceae specialist (Cripps & Rust, 1989; Levin, 1966). The three species also differ in their native ranges. *Osmia cornifrons* is a native to eastern Asia that has become widespread in the northeastern United States, following its introduction in the 1970s for apple orchard pollination (Biddinger et al., 2010; MacIvor et al., 2022; Rust, 1974). *Osmia lignaria* is a North American native, found throughout the United States (Rust, 1974), and *O. californica* is a western North American native (Krombein, 1979). *Apis mellifera* is a social bee and generalist feeder, native to Europe, Africa, and western Asia, though it is now commonly found worldwide (Sheppard, 1989). Assessing the risk of these insecticides to solitary bee health and comparing species- and sex-specific sensitivity differences can then inform bee management strategies and help mitigate the potential harm of insecticides to pollinators.

Materials and Methods

Bee Acquisition and Preparation

In March 2021, three species of mason bees (*O. lignaria*, *O. cornifrons*, and *O californica*) were acquired as cocoons (adult bees in winter diapause) from the commercial supplier, Watts Solitary Bees in Bothell, WA. Overwintering cocoons were kept refrigerated at 4°C before experimental use to synchronize emergence. Bee cocoons were sorted by size into males and females and were transferred to small plastic cages in groups of 100. Each cage contained paper towels and cotton rounds soaked in 50:50 w/v organic honey (Nature Nate's Honey Co. in McKinney, TX) solution. Bees were allowed to emerge from cocoons at room temperature (22-23°C) and feed on the honey solution for 24 hours before pesticide exposure. *Apis mellifera* workers were collected from a hive at the University of Arkansas' Milo J. Shult Agricultural Research and Extension Center. The hive was opened and worker bees were collected from the hive frames and brought to the University of Arkansas Insect Toxicology Laboratory. Worker bees were sorted into groups of 15 and immediately used in pesticide assays.

Survival Assay

The two pesticide formulations used in this experiment were Transform® WG by Corteva Agrosciences (50% active ingredient – sulfoxaflor) and Sivanto™ Prime by Bayer CropScience (17.09% active ingredient – flupyradifurone) and were obtained commercially. Pesticide stocks and later dilutions were stored at 4°C for the duration of the experiment. The label field application rates of Sivanto™ Prime are 7-14 fl oz Ac^{-1} with a spray volume of 10 gallons Ac^{-1} and of Transform® WG are 0.75-2.75 oz Ac^{-1} with a spray volume of 10 gallons Ac^{-1} (Chakrabarti et al., 2020). To create the original doses for the *Osmia* spp. exposures, each formulation was diluted to its lowest field use concentration (FUC), according to the label. A tenfold serial dilution was then performed for each pesticide (**Table 2.1**). *Osmia cornifrons*

females were less sensitive to the flupyradifurone and sulfoxaflor doses used in the initial survival assay. With the initial doses, we were unable to properly estimate the LD_{50} values for either pesticide. Because of this, two doses of each insecticide were added (**Table 2.1**). Previous studies on *A. mellifera* have found lower sensitivity to both flupyradifurone and sulfoxaflor (Bell et al., 2020; EFSA, 2015; Glaberman & White, 2014; US EPA, 2013, 2019). Higher doses were used for the oral exposure of *A. mellifera* workers (**Table 2.1**). Fresh pesticide mixtures were mixed weekly throughout the course of the experiment.

For each *Osmia* species, 60 males and 60 females were used per treatment group, except for *O. californica* males. Male *O. californica* emergence from cocoons was lower than expected, so 30-60 males were used per treatment group. For the adjusted doses of the insecticides used for *O. cornifrons*, a total of 30 female bees were used per treatment group. Each bee was orally exposed to 2 µl of either pesticide solution or DI water, in the case of the control groups. Direct feeding with a micropipette was done, placing the pesticide solution directly onto the tongue of each bee (Williams et al., 2013). Following exposure, bees were immediately moved to clean cages, measuring 500 ml with 9.3 cm diameter and 10 cm height, with 5 bees in each cage. They were kept at room temperature (22-23°C) and in natural light. They were provided with new 50:50 w/v organic honey solution daily in a feeder placed inside each of the cages. Survival was recorded at 24, 48, 72, and 96 hours after treatment (HAT). A clean and soft paint brush was used to stroke impaired bees and test for any movement or signs of life.

For *A. mellifera*, 46-60 worker bees were used per treatment group. *Apis mellifera* were fed 2 µl of either pesticide solution or DI water, using the same direct feeding method with a micropipette as was used for the *Osmia* spp. After feeding, *A. mellifera* bees were transferred to clean plastic containers in groups of 15 bees. Each cage contained a plastic tube feeder with a cotton round soaked in 50:50 w/v organic honey solution. *Apis mellifera* were kept in an

incubator at 31-32°C, 50-70% humidity, and a 6/18 (lights on/off) photoperiod. Survival was recorded daily up to 96 HAT and feeder tubes were refilled with honey solution each day.

Data Analysis

A nonparametric Kruskal-Wallis test in JMP Pro 17 (JMP Statistical Discovery LLC in Cary, NC) was used to compare mortality among treatment groups for each species, sex, and day following treatment. A nonparametric comparison using the Dunn Method for joint ranking with Bonferroni corrections test was used to identify significant decreases in survival of each treatment group compared to the control. Two-way (factorial) ANOVA analyses were used to analyze the combined effect of species and treatment for each sex at 48 HAT, the effect of sex and treatment for each species at 48 HAT, and the effect of time and treatment for each species and sex.

A probit analysis to determine the toxicity of each pesticide was performed using PoloPlus (LeOra Software LLC in Parma, MO). Doses were converted to logarithms for the probit analysis. Toxicity, in terms of lethal dose (LD_{50}) values, was calculated for each sex and species of bee at 24, 48, 72, and 96 hours after treatment.

Results

Survival After Acute Exposure

Higher doses of flupyradifurone and sulfoxaflor caused a significant decrease in survival for female *O. lignaria* bees for each recorded day following pesticide exposure (48 HAT: $\chi^2(11) = 122.2$, $p<0.0001$). The highest flupyradifurone dose (2200 ng ai bee^{-1}), equivalent to the lowest field use concentration of the formulation, caused 100% mortality within 3 hours of exposure (**Figures 2.1A** and **2.1C**). Male O. *lignaria* bees also showed a significant decrease in survival following exposure to the highest dose of flupyradifurone and the two highest doses of sulfoxaflor for each day of the experiment (48 HAT: $\chi^2(11) = 103.7$, $p<0.0001$, **Figures 2.1B** and

2.1D). Males were less sensitive to both flupyradifurone and sulfoxaflor compared to the females, though for flupyradifurone exposure, the difference between male and female *O. lignaria* sensitivity was more pronounced (**Figure 2.1**).

High dose exposure to flupyradifurone and sulfoxaflor also significantly decreased survival in *O. cornifrons* (females, 48 HAT: $\chi^2(11) = 105.3$, $p<0.0001$ and males, 48 HAT: $\chi^2(11) = 117.3$, $p<0.0001$). Males were slightly less sensitive to both insecticides, compared to females (**Figure 2.2**).

Female *O. californica* survival was significantly affected by the three highest doses of flupyradifurone and the two highest of sulfoxaflor (48 HAT: $\chi^2(11) = 112.7$, $p<0.0001$, **Figures 2.3A** and **2.3C**). Female bees fed the 2200 ng ai bee^{-1} dose of flupyradifurone were all dead within 24 HAT. Male *O. californica* bees were less sensitive to most pesticide doses, though still significantly affected by the highest doses of flupyradifurone and the highest dose of sulfoxaflor (48 HAT: $\chi^2(11) = 95.9$, $p<0.0001$, **Figures 2.3B** and **2.3D**). Males also experienced 0% survival to the 2200 ng ai bee^{-1} dose of flupyradifurone, like the females.

Apis mellifera workers showed no significant response to flupyradifurone up to the highest dose, 4400 ng ai bee^{-1} (**Figure 2.4A**). This dose corresponds to the highest approved field use concentration according to the pesticide label. Sulfoxaflor, however, did significantly impact worker bee survival (48 HAT: $\chi^2(10) = 29.5$, $p=0.001$). Bees fed the two highest sulfoxaflor doses had 100% mortality by 48 HAT (**Figure 2.4B**).

Species Comparison of Pesticide Sensitivity

Apis mellifera workers used in this study were more tolerant to flupyradifurone exposure than the tested *Osmia* spp. females and had an LD_{50} value over 4400 ng ai bee^{-1} (**Table 2.2**). *Osmia cornifrons* males and females had the lowest sensitivity to flupyradifurone of the tested *Osmia* species, whereas *O. lignaria* and *O. californica* females were more sensitive (**Table 2.2, Figure 2.5A**). Species-specific differences in sensitivity were less pronounced in bees exposed

to sulfoxaflor (**Figure 2.5B**, **Table 2.2**). There were few significant differences in survival at 48 HAT among the three species for each dose of sulfoxaflor. There was also a significant interaction between species and treatment for female ($F=10.99$, $DF=22$, $p<0.0001$) and male bees ($F=12.41$, $DF=22$, $p<0.0001$). Survival among species was similar at lower pesticide doses, but *O. cornifrons* survival was higher compared to *O. lignaria* and *O. californica* at higher doses of flupyradifurone.

Sex Comparison of Osmia Pesticide Sensitivity

As well as species-specific differences in sensitivity to flupyradifurone and sulfoxaflor, there was a significant effect of sex on bee survival and a significant interaction between the effects of sex and treatment for *O. lignaria* and *O. californica* at 48 HAT (*O. lignaria*: $F=14.04$, $DF=11$, $p<0.0001$; *O. californica*: $F=4.5$, $DF=11$, $p<0.0001$), but not *O. cornifrons* ($F=1.72$, $DF=11$, $p=0.067$). Male *Osmia* spp. bees had higher survival than females for most pesticide doses for all three species (**Figure 2.6**). Male *O. cornifrons* and *O. californica* also had higher 48 HAT LD_{50} values than female bees for sulfoxaflor and male *O. cornifrons* also had higher 48 HAT LD_{50} values than female bees for flupyradifurone (**Table 2.2**). Male *O. lignaria* and *O. californica* responses did not always fit the probit model well, but overall male bees were more tolerant of both flupyradifurone and sulfoxaflor than females.

Effect of Time

For most pesticide doses, the greatest drop in survival occurred within 24 HAT (**Figures 2.1-2.4**). *Osmia lignaria* females did experience a significant interaction of time and treatment ($F = 1.83$, $DF = 30$, $p = 0.0049$) and a noticeable drop in survival for the lowest dose of sulfoxaflor at 72 HAT (**Figure 2.1C**). There was no significant interaction of time and treatment for the other species or sexes of bees.

Discussion

The major findings of this study showed that there are species- and sex-specific differences in bee sensitivity to oral sulfoxaflor and flupyradifurone exposure. Male *Osmia* bees were less sensitive than females of the same species to both sulfoxaflor and flupyradifurone. *Apis mellifera* workers were less sensitive to both insecticides compared to female *Osmia* bees. Within the genus *Osmia*, *O. lignaria* and *O. californica* were the most sensitive, whereas *O. cornifrons* was more tolerant. The species-specific sensitivity differences highlight the importance of using non-*Apis* bees in pesticide risk assessment. Because *A. mellifera* can be more tolerant of certain pesticides than solitary bees, they are not always a perfect surrogate for risk assessment. Additional testing of non-*Apis* bees can help inform further management practices and pesticide application decisions.

Lethal dose (LD_{50}) values estimate the dosage that will cause mortality for 50% of individuals of a species or population. The methods of pesticide exposure in this study were similar for all three *Osmia* species and for *A. mellifera*, allowing for more direct comparison of LD_{50} values and sensitivity to the pesticides. Many studies in the literature, however, use the technical grade of the active ingredient (TGAI) or different commercial formulations, which can make direct comparisons of sensitivity difficult. Pesticide adjuvants and solvents in formulations can affect insect sensitivity, compared to the active ingredient acting alone. For example, in a previous study, *A. mellifera* was more sensitive to the TGAI flupyradifurone with an LD_{50} of 1200 ng ai bee^{-1} than they were to the formulation, Sivanto™ SL 200 with an LD_{50} of 3200 ng ai bee^{-1} (EFSA, 2015; Glaberman & White, 2014).

Species-specific differences in pesticide sensitivity were more pronounced in bees that had been exposed to flupyradifurone, whereas sensitivities among the three *Osmia* spp. and *A. mellifera* were more similar following sulfoxaflor exposure. Different bee species can have varying sensitivities to different pesticides. Several studies have compared the difference in solitary bee sensitivity, such as that of *O. bicornis* or *M. rotundata*, to social bees, such as *A.*

mellifera, B. impatiens, or *B. terrestris*. *Osmia bicornis*, a European mason bee in the same subgenus as *O. lignaria* and *O. cornifrons*, was more sensitive to many insecticides, including sulfoxaflor (Azpiazu et al., 2021; Linguadoca et al., 2022), flupyradifurone (Azpiazu et al., 2021), clothianidin (Sgolastra et al., 2017), and thiacloprid-prochloraz mixture (Alkassab et al., 2020), than the social bees*, A. mellifera* and *Bombus* spp. Another study, found that thiacloprid and flupyradifurone had low toxicity for *O. bicornis*, *A. mellifera*, and *B. terrestris*, but high toxicity for *M. rotundata* (Hayward et al., 2019). For comparative studies of sensitivity between *O. lignaria* and *A. mellifera*, it was found that pesticide class can greatly impact species-specific differences in sensitivity. *Osmia lignaria* was more sensitive than *A. mellifera* to neonicotinoid class insecticides (thiamethoxam, imidacloprid, and clothianidin), but less sensitive than *A. mellifera* to pyrethroids (permethrin and bifenthrin) and abamectin (Peterson et al., 2021).

There are few studies comparing differing sensitivities within the *Osmia* genus. Even within the same subgenus, however, as with *O. lignaria* and *O. cornifrons*, sensitivity and survival after exposure can vary greatly by species (**Figure 2.5, Table 2.2**; Arena & Sgolastra, 2014). These differences in sensitivity could affect pollinator population dynamics and species evenness in areas of heavy pesticide use. There is little consistent monitoring of solitary bee populations in the United States, though a recent survey of *Osmia* spp. in West Virginia, Virginia, and Maryland found that from 2003-2017, 6 native *Osmia* spp., including *O. lignaria*, had declining catch rates, while the introduced *O. cornifrons* remained stable (LeCroy et al., 2020). There are many factors that can impact bee populations and interactions, such as competition for nest sites and floral resources, parasites and pathogens, and reduced predation of introduced species (Abel et al., 2003; LeCroy et al., 2020; McQuillan & Hingston, 1999). Pesticide usage may also influence bee communities in agricultural areas, with more tolerant species and populations able to remain stable and more sensitive species experiencing population declines.

Comparisons among different bee species can be difficult, however, due to differences in methodology. For example, many studies on social bees make use of a common feeder with a known concentration of a pesticide. Social bees, often in groups of 5-10 individuals, drink from the common feeder and perform trophallaxis with each other, so it is assumed that each bee receives a similar dose of pesticide (Arena & Sgolastra, 2014; Ladurner et al., 2003). The direct feeding method used in this study, however, helps ensure a precise dose of insecticide for each bee and can be used for both social and solitary bees.

As well as species-specific differences in pesticide sensitivity, there are sex-specific differences, though these can vary by species and insecticide used. In this study, male bee survival was higher than females for most of the tested pesticide doses. Male bees of all species are studied less often than females. Male *Osmia* spp. bees do not contribute to nest building or provisioning, but still have an important role in reproduction and can be valuable pollinators, as well. Additionally, many solitary bees, including *O. lignaria*, have a sex ratio biased towards males (Torchio & Tepedino, 1980), so it is worth including them in pesticide toxicity assays and risk assessment. There is no blanket statement that can be made that solitary bee males are more tolerant to pesticides than females, as sensitivity can vary. Male *O. lignaria* and *O. californica* were less sensitive to sulfoxaflor and flupyradifurone than females in this study. Male *O. cornifrons* were less sensitive to imidacloprid and acetamiprid, though had similar thiamethoxam tolerance compared to females (Phan et al., 2020). In *O. bicornis*, both males and females had similar tolerance to sulfoxaflor (Linguadoca et al., 2022). Alternatively, males can have higher sensitivity than females for certain pesticides. *Megachile rotundata* males were more sensitive to several tested fungicides (Captan, Benlate, Orbit, and Rovral), especially for contact exposure assays (Huntzinger et al., 2008), and male *O. lignaria* were more sensitive to permethrin and abamectin than females (Peterson et al., 2021)

Several factors can influence insect susceptibility to toxins and can contribute to species- and sex-specific differences in sensitivity. Body size can affect sensitivity, with larger-

bodied bees often more tolerant pesticide exposure (Ansell et al., 2021). In *Osmia* spp., however, males have a smaller body length and weight than females. For example *O. bicornis* females are on average 0.97 times heavier than males (Linguadoca et al., 2022), but males of *Osmia* spp. can be more tolerant of certain pesticides than females (Phan et al., 2020). Additionally, *O. cornifrons* were smaller on average than *O. californica* and *O. lignaria* used in this study. Body size may contribute to pesticide sensitivity, but it is not the only predictor of it. Physiology and behavior can also affect bee susceptibility to pesticides. Behaviors, such as feeding avoidance and diet mixing can help bees avoid higher dose exposures to toxic compounds (Eckhardt et al., 2014; London-Shafir et al., 2003; Tan et al., 2012). For oral pesticide bioassays, however, bees are not allowed to forage naturally, and so are less able to diet mix or avoid exposure. The differences in pesticide sensitivities observed among the different species and sexes of bees in this study are therefore likely due to physiological mechanisms. Bee gut microbial symbionts can play a role in detoxification, as has been observed in *A. mellifera* (Wu et al., 2020). Social bees have more consistent core gut microbial communities than solitary bees (Cohen et al., 2020; Kapheim et al., 2021; Kwong & Moran, 2016), which may influence their differential sensitivity to certain pesticides. If the *A. mellifera* core bacteria play a role in flupyradifurone degradation, for example, this could help explain the higher tolerance of *A. mellifera* compared to the solitary *Osmia* spp. Bees and other insects also possess a suite of detoxification enzymes, such as P450 monooxygenases, glutathione-*S*-transferases, and esterases that can reduce their sensitivity to pesticides and other environmental toxins (Landa et al., 1991; Scott, 2008; Wu et al., 2020). Differences in the specific enzyme families, as well as differences in expression of the enzymes, can both influence bee sensitivity (Beadle et al., 2019; Haas et al., 2023; Hayward et al., 2019).

Within the tested *Osmia* spp. females, *O. cornifrons* was more tolerant, especially of flupyradifurone than *O. lignaria* and *O. californica*. These differences in sensitivity cannot be explained by body size, as *Osmia cornifrons* females were smaller on average than *O. lignaria*

and *O. californica* females. Additionally, phylogenetic similarity cannot fully account for the differences among the species. *Osmia cornifrons* and *O. lignaria* share the same subgenus, *Osmia*, whereas *O. californica* is more distant from them, in the subgenus, *Cephalosmia* (Rust, 1974). *Osmia lignaria* and *O. californica* are both North American native species, though O. lignaria has a wider range (Krombein, 1979; Rust, 1974). *Osmia cornifrons*, however, is native to northeastern Asia (Biddinger et al., 2010). As such, *O. cornifrons* may have been exposed to a different variety of wildflowers and a different set of phytotoxins than the North American native *Osmia* spp. This could cause *O. cornifrons* to develope physiological mechanisms, such as specific enzymes, that contribute to the higher tolerance of the species to certain insecticides. Additionally, agricultural areas that heavily use sulfoxaflor and flupyradifurone may unintentionally select for the non-native *O. cornifrons* over the native and more sensitive *O. lignaria*. The use of the pesticides could therefore affect the composition of *Osmia* spp. communities.

Conclusions

The recently released insecticides, flupyradifurone and sulfoxaflor, can pose a risk to *Osmia* spp. health and functioning. The introduced *O. cornifrons* were more tolerant of both insecticides, but flupyradifurone especially, than the native *O. lignaria*. Because both species are used in fruit and tree nut pollination, *O. cornifrons* may be able to maintain more robust populations in areas of intensive insecticide spraying. However, native North American *Osmia* spp. populations, including those of *O. lignaria* and *O. californica*, should also be monitored and protected. Measures to avoid foliar insecticide applications during floral bloom periods and to limit overall pesticide usage by implementing other integrated pest management strategies, such as mechanical and biological control measures, can help reduce environmental pesticide levels and better protect pollinators. Flupyradifurone poses relatively low risk to *A. mellifera*. Growers and gardeners who utilize *Osmia* spp. pollination services, however, should use

caution in applying the insecticide, as the females of the three *Osmia* spp. were much more sensitive to it than *A. mellifera*.

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Table 2.1. Pesticide formulations and doses.

Active Ingredient	Formulation	Dose (ng ai bee^{-1})	Concentration (mg ai L^{-1})
Flupyradifurone	Sivanto™ Prime	0.22*	0.11
		2.2*	1.1
		22*	11
		220*§	110
		440§	220
		1100†§	550
		2200*§	1100‡
		4400†§	2200‡
Sulfoxaflor	Transform® WG	0.056*	0.028
		0.56*	0.28
		5.6*	2.8
		14§	7
		28†§	14
		56*§	28
		280†§	140
		560*§	280‡

*****Original doses for *Osmia* spp.

†Adjusted doses for *O. cornifrons*.

§Doses for *A. mellifera*.

‡Recommended field use concentrations according to the label of the formulation.

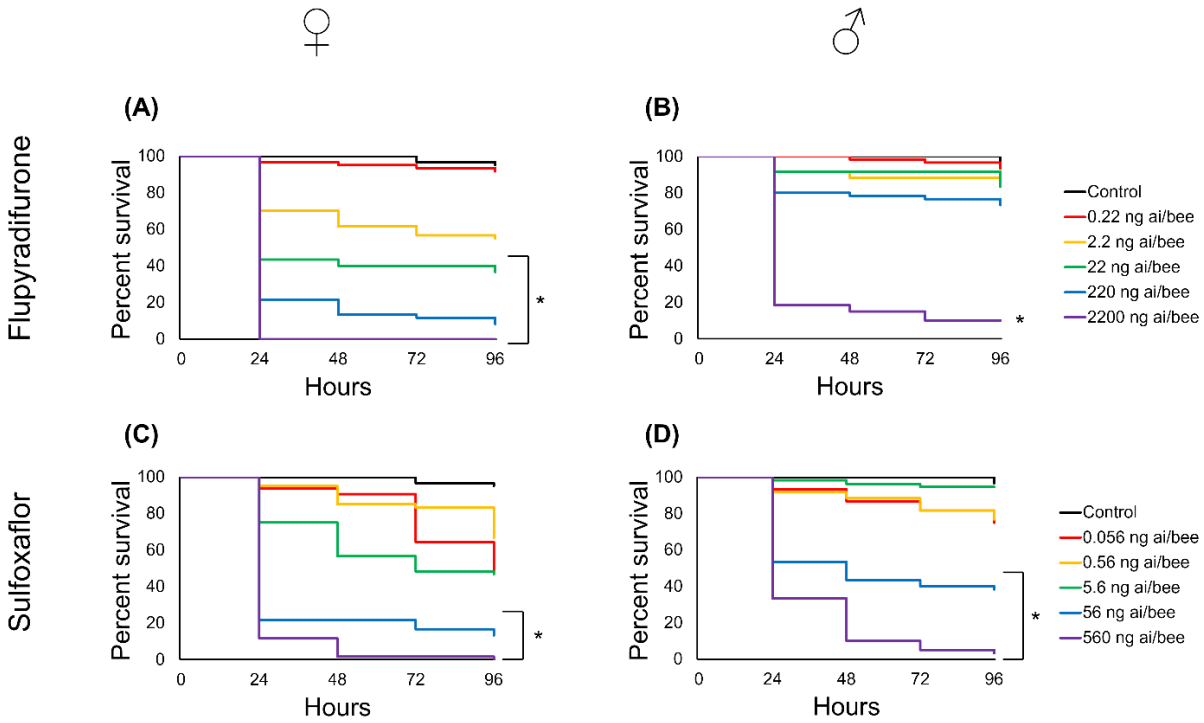

Figure 2.1. Acute oral *O. lignaria* response to flupyradifurone (A – females, B – males) and sulfoxaflor (C-females, D-males). Asterisks represent statistically significant ($p<0.05$) differences in response when compared to the control.

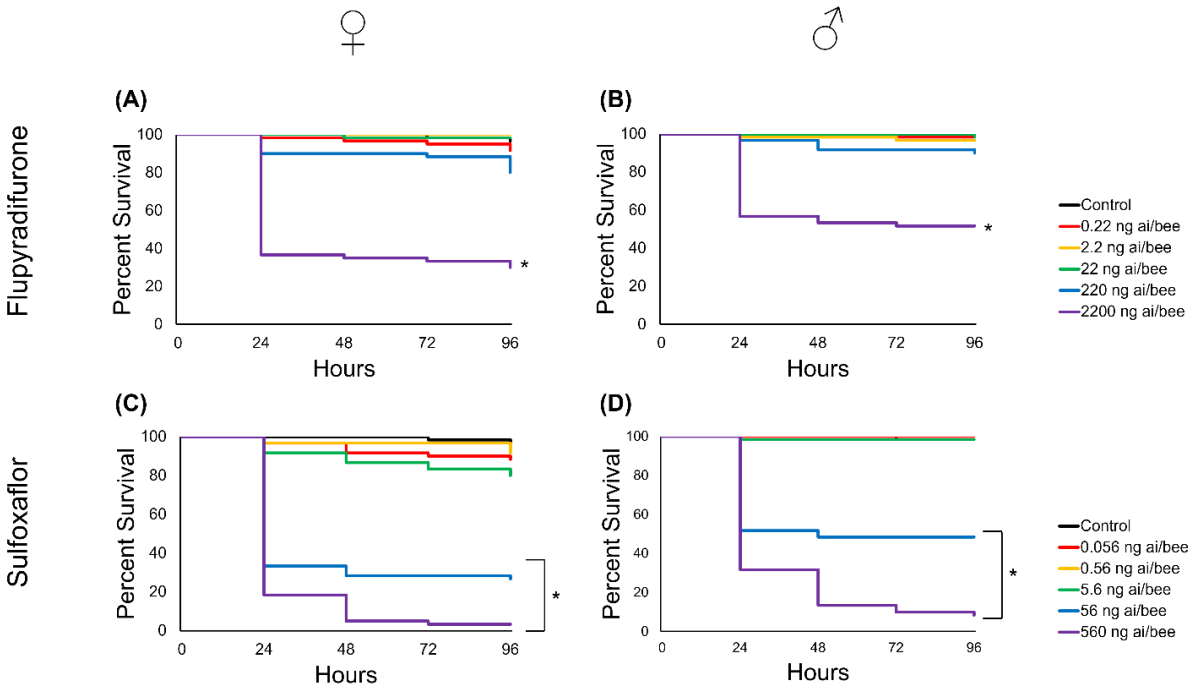

Figure 2.2. Acute oral *O. cornifrons* response to flupyradifurone (A – females, B – males) and sulfoxaflor (C-females, D-males). Asterisks represent statistically significant ($p<0.05$) differences in response when compared to the control.

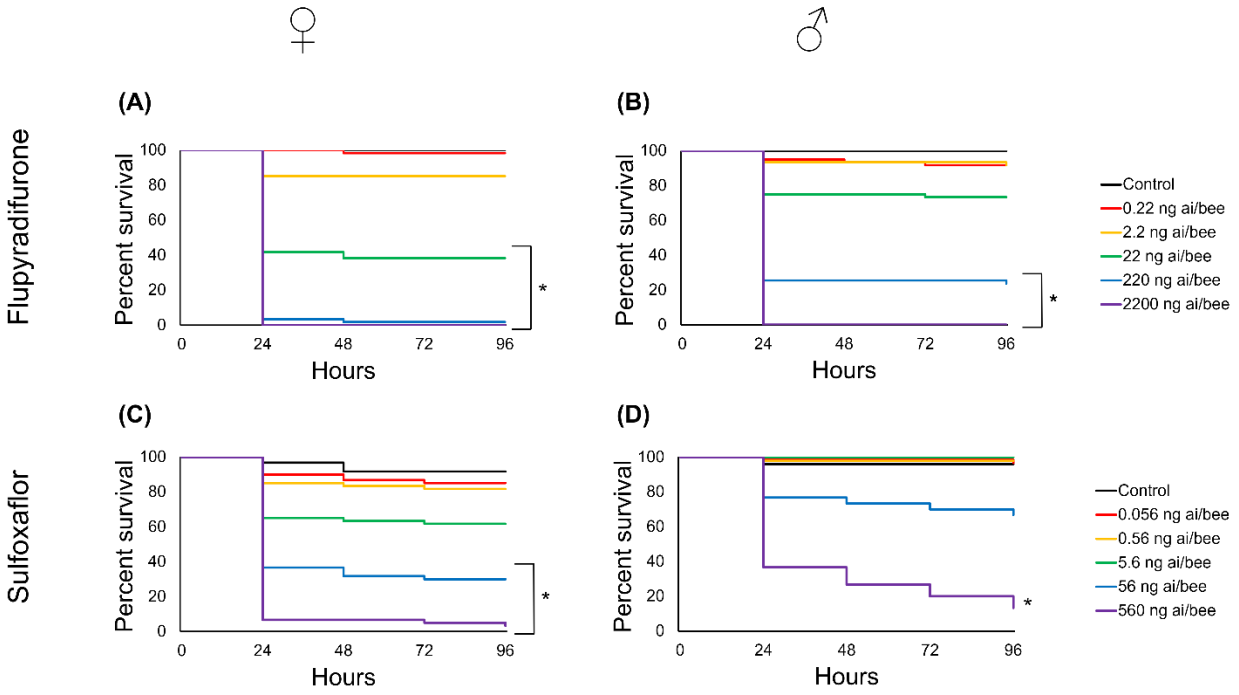

Figure 2.3. Acute oral *O. californica* response to flupyradifurone (A – females, B – males) and sulfoxaflor (C-females, D-males). Asterisks represent statistically significant ($p<0.05$) differences in response when compared to the control.

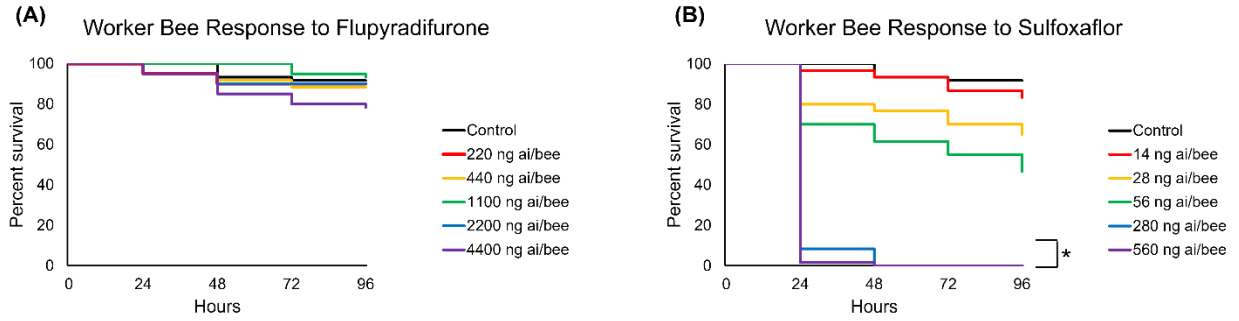

Figure 2.4. Acute oral worker *A. mellifera* response to flupyradifurone (A) and sulfoxaflor (B). Asterisks represent statistically significant ($p<0.05$) differences in response when compared to the control.

Table 2.2. The acute oral toxicity lethal dose of 50% of individuals (LD_{50}) values for males and females of each *Osmia* species used in this study and compared to *A. mellifera* and *Bombus impatiens* from the literature. Lower LD_{50} values indicate greater sensitivity to the formulation. Blank cells in the LD_{50} column indicate a poor fit for the probit model.

Active Ingredient	Formulation	Species	Sex	N	Time (h) of mortality reading	Slope ± SE	LD_{50} (ng ai/bee) (95% confidence interval)	References
Flupyradifurone	TGAI flupyradifurone	*Apis mellifera*	♀ (workers)		48		1200	(EFSA, 2015; Glaberman & White, 2014)
					48		2200	(Bell et al., 2020)
		Bombus impatiens	♀ (workers)		48		>1700	(Mundy-Heisz et al., 2020)
	Sivanto™ SL 200	*Apis mellifera*	♀ (workers)		48		3200	(EFSA, 2015; Glaberman & White, 2014)
	Sivanto™ Prime	*Apis mellifera*	♀ (workers)	360	24		>4400	
					48		>4400	
					72		>4400	
					96		>4400	
		Osmia californica	♀	360	24	1.449 ± 0.154	13.601 (9.428-19.651)	
				359	48	1.542 ± 0.169	12.079 (8.467-17.270)	
					72	1.418 ± 0.148	11.393 (7.867-16.565)	
					96	1.418 ± 0.148	11.393 (7.867-16.565)	
			♂	359	24			
					48			
					72			
					96			
		Osmia cornifrons	♀	360	24	1.405 ± 0.256	1167.399 (717.029-1694.539)	
					48	1.263 ± 0.180	903 (562.944-1359.48)	
					72	1.314 ± 0.215	905.423 (534.281-1359.182)	
					96	1.422 ± 0.268	855.510 (458.837-1279.073)	

Table 2.2 (Cont.)

Active Ingredient	Formulation	Species	Sex	N	Time (h) of mortality reading	Slope ± SE	LD50 (ng ai/bee) (95% confidence interval)	References
Flupyradifurone	Sivanto™ Prime	*Osmia cornifrons*	♂	371	24	1.842 ± 0.491	2742.2 (1861.6-5763.8)	
					48	1.417 ± 0.282	2523.831 (1579.255-5409.271)	
					72	1.495 ± 0.311	2395.209 (1528.102-4878.243)	
					96	1.373 ± 0.306	2442.248 (1501.79-5575.193)	
		Osmia lignaria	♀	360	24	0.876 ± 0.084	14.119 (4.31-44.3)	
					48	0.891 ± 0.086	8.501 (2.920-22.959)	
					72	0.891 ± 0.093	7.938 (1.623-28.117)	
					96	0.930 ± 0.101	6.716 (1.557-20.754)	
			♂	360	24			
					48			
					72			
					96			
Sulfoxaflor	TGAI Sulfoxaflor	*Apis mellifera*	♀ (workers)		48		50	(US EPA, 2013)
					48		146	(US EPA, 2019)
		Bombus impatiens	♀ (workers)		48		19.4	(Mundy-Heisz et al., 2020)
	Closer® SC by Corteva Agriscience	*Apis mellifera*	♀ (workers)		48		51.5	(US EPA, 2019)
		Bombus impatiens	♀ (workers)		48		27	(US EPA, 2019)
	Transform® WG	*Apis mellifera*	♀ (workers)	346	24	2.345 ± 0.214	78.168 (64.469-96.175)	
					48	3.546 ± 0.618	64.542 (37.087-111.242)	
					72	3.113 ± 0.571	57.252 (25.016-96.304)	
					96	2.811 ± 0.458	48.171 (28.015-72.404)	

Table 2.2 (Cont.)

Sulfoxaflor	Transform® WG	*Osmia californica*	♀	360	24	0.878 ± 0.147	19.550 (8.512-35.955)	
					48	0.964 ± 0.155	20.705 (9.343-37.677)	
					72	0.991 ± 0.166	70.804 (30.447-129.512)	
					96	1.060 ± 0.180	70.089 (30.768-125.394)	
			♂	245	24	1.310 ± 0.284	290.986 (157.604-678.413)	
					48	1.466 ± 0.288	198.451 (113.121-380.385)	
					72	1.581 ± 0.294	595.103 (347.317-1055.568)	
					96	1.747 ± 0.315	470.212 (280.065-790.411)	
		Osmia cornifrons	♀	360	24	0.899 ± 0.081	32.451 (5.678-199.194)	
					48	0.906 ± 0.076	16.475 (1.872-134.972)	
					72	1.304 ± 0.142	23.576 (4.31-68.588)	
					96	1.211 ± 0.149	20.933 (2.925-57.82)	
			♂	360	24			
					48	1.479 ± 0.186	75.717 (27.565-193.326)	
					72	1.616 ± 0.199	70.733 (25.829-177.576)	
					96	1.690 ± 0.209	67.82 (27.248-155.498)	
		Osmia lignaria	♀	364	24	0.796 ± 0.074	16.532 (1.419-409.663)	
					48	0.809 ± 0.074	5.671 (1.285-26.979)	
					72			
					96			
			♂	358	24			
					48			
					72			
					96			

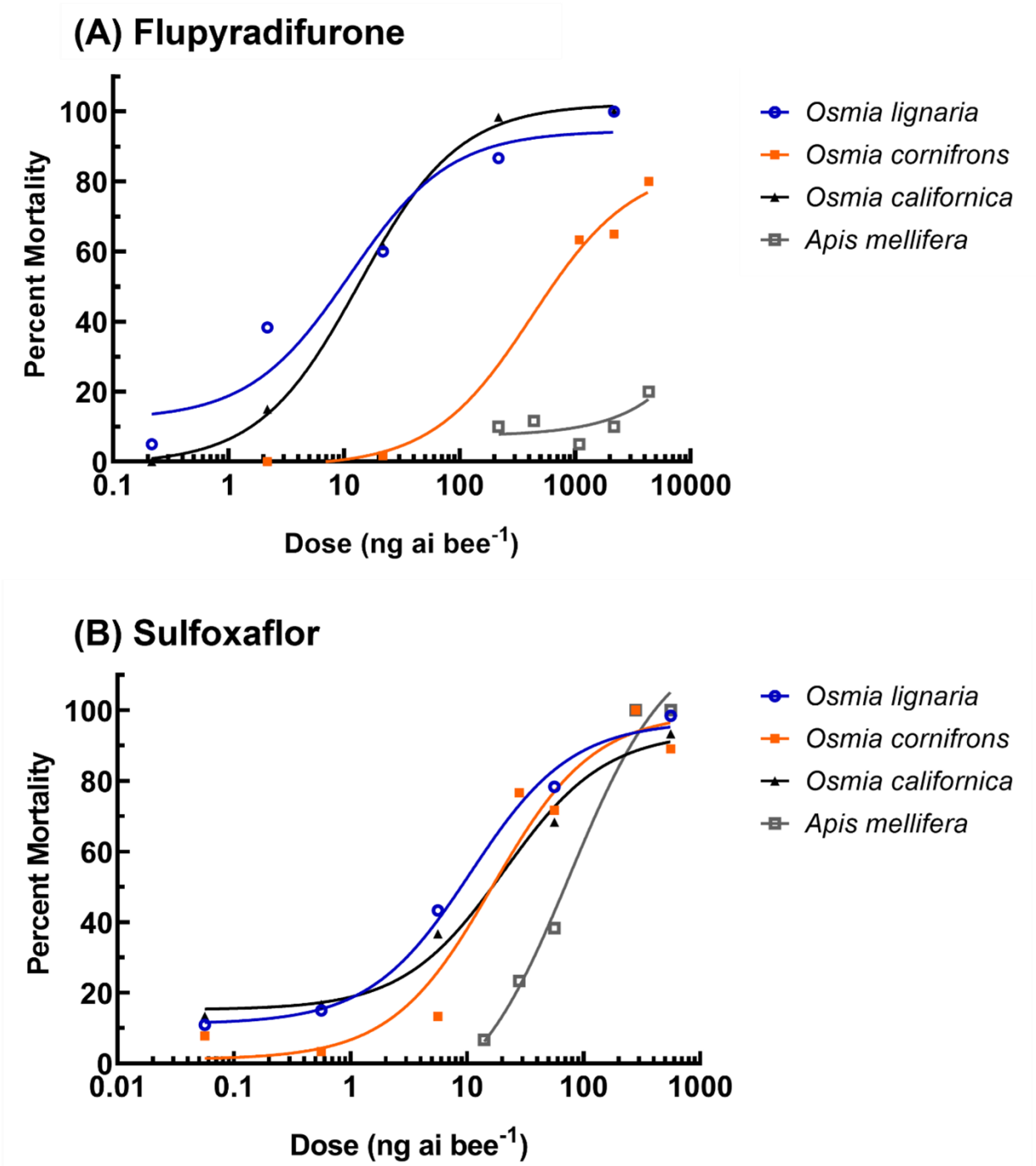

Figure 2.5. Acute oral toxicity responses of *Osmia* spp. and *Apis mellifera* females to flupyradifurone (A) and sulfoxaflor (B) at 48 HAT.

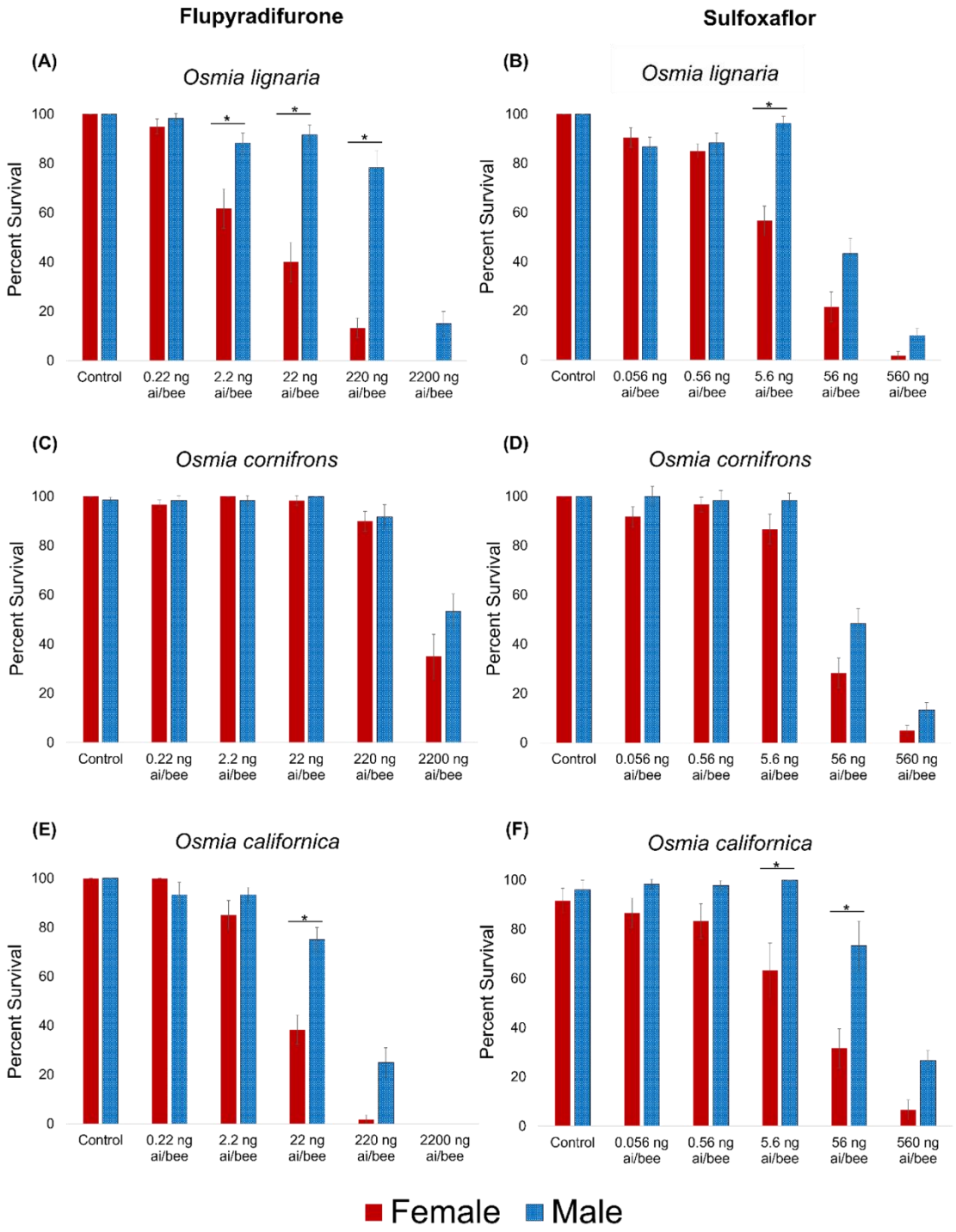

Figure 2.6. Comparison of male and female survival after exposure of three *Osmia* spp. for each pesticide and dosage. Survival was measured 48 hours after treatment.

Chapter 3:

The Sublethal Effects of New Systemic Insecticides on Mason Bee (*Osmia* spp.) Health, Physiology, and Behavior

Abstract

Most pesticide risk assessment for bee pollinators focuses on the impacts on bee survival. Many pesticides, however, can have sublethal effects, even at low doses. These sublethal impacts can include changes to foraging behavior and feeding choice. Physiological changes can occur, as well, such as the upregulation of certain enzymes. Sublethal effects testing can provide a more thorough understanding of the potential risks of pesticides, as well as providing insights into bee mechanisms of coping with toxin exposure. In this study, mason bees (*Osmia* spp.) were orally exposed to two new systemic insecticides, flupyradifurone and sulfoxaflor. The P450 enzyme expression of *Osmia lignaria* females was measured using a luciferin assay. Flupyradifurone exposure resulted in a significant increase in P450 expression, though sulfoxaflor caused no noticeable effect. *Osmia lignaria* males and females were also assessed on feeding preference behavior when presented with pesticide-contaminated food or uncontaminated food. Female bees showed slight avoidance behavior of higher concentrations of flupyradifurone, but overall showed no strong preference or avoidance for pesticide-contaminated food. Finally, *O. lignaria*, *Osmia cornifrons*, and *Osmia californica* males and females were released in flight cages following flupyradifurone exposure and assessed on flying and foraging ability. Flupyradifurone caused the most significant impairment of *O. lignaria* flight and foraging at 24 hours after exposure. Short-term impacts of flupyradifurone were minimal.

Introduction

Pesticide risk assessment on bees often focuses on mortality testing, especially after acute exposure. Lower doses of insecticides, which may not cause significant mortality, can still cause negative effects to bee health and functioning. This can include decreased fecundity,

impaired or altered flying and foraging activity, and changes to bee physiology (Campbell et al., 2016; Chakrabarti et al., 2020; Claus et al., 2021; Glaberman & White, 2014; Morandin et al., 2005). Additionally, bees may cope with sublethal pesticide exposures in multiple ways, including upregulating detoxification enzymes and diet mixing with uncontaminated food (Irwin et al., 2014; Johnson, 2015). Understanding how bees cope with toxin exposure and how sublethal pesticide doses can affect them can contribute to a more complete assessment of pesticide risk beyond mortality-only testing.

Many insects rely on detoxification enzymes to resist the toxic effects of plant phytotoxins and pesticides. There are three main families and superfamilies of enzymes that greatly contribute to the metabolism and detoxification of xenobiotics in insects, which are the cytochrome P450 monooxygenases (P450s), glutathione-*S*-transferases (GSTs), and carboxyl/cholinesterases (CCEs) (Douglas, 2015; Itoh et al., 2018; van den Bosch & Welte, 2017). These enzymes can act in different ways. Some, primarily the P450s and CCEs, change the molecular structure of the xenobiotics and render them non-functional on the insect target sites. Others aid in rapid transport and excretion of the toxins (Berenbaum & Johnson, 2015; Claudianos et al., 2006). The P450s act on a wide range of substrates, including many pesticides, and have been linked to resistant insect populations (Li et al., 2007). They are coded by the gene superfamily Cyt P450 (CYP), which is widely found in plants, animals, bacteria, and fungi (Schuler, 1996). In insects, the CYP4, CYP6, CYP9, and CYP12 families are often used in detoxification (Inceoglu et al., 2009; Li et al., 2007).

Bees, like many insects, can be exposed to toxic compounds while foraging on plants, such as toxic alkaloids in nectar (Adler, 2000b; Adler et al., 2012; Haas et al., 2023). As such, they have evolved mechanisms for degrading these phytotoxins, which can sometimes help them cope with exposure to synthetic pesticides (Haas et al., 2023; Haas & Nauen, 2021; Hayward et al., 2019). Several Hymenopteran P450s, for example, have been linked to the degradation of both naturally occurring phytotoxins and new synthetic compounds (Beadle et

al., 2019; Haas et al., 2023; Haas & Nauen, 2021; Hayward et al., 2019). Bee sensitivity to new pesticide formulations can be impacted by multiple factors, including the number of detoxification enzymes they possess, the specific types of P450s, and the upregulation or downregulation of enzyme expression following exposure.

Overall, bees tend to have relatively few genes coding for these detoxification enzymes when compared to other insects. *Apis mellifera* L. (Hymenoptera: Apidae) had far fewer protein coding genes (~11,000), in general, than the mosquito, *Anopheles gambiae* Giles (~14,000 genes) and the fruit fly, *Drosophila melanogaster* Meigen (~13,500 genes) (Claudianos et al. 2006). For P450 coding genes, specifically, *A. mellifera* have only 46, bumble bees (*Bombus huntii* Greene (Hymenoptera: Apidae), *Bombus terrestris* L., and *Bombus impatiens* Cresson) have 44-50, and the solitary *Megachile rotundata* Fabricius (Hymenoptera: Megachile) have 52, compared to 64 in the mosquito *Aedes aegypti* L. and 85 in *D. melanogaster* (Ahn et al., 2012; Scott, 2008; Xu et al., 2013). This low number of detoxification genes could be due to bees' limited exposure to plant defensive chemicals when compared to other insect herbivores (Adler, 2000a; Adler et al., 2012; Xu et al., 2013). The differences in number of detoxification genes may influence the insects' relative sensitivities to environmental toxins and pesticides (Scott, 2008). However, *A. mellifera* are not necessarily more sensitive to insecticides than other insects, but rather their relative sensitivity varies for different insecticide classes (Hardstone & Scott, 2010).

Other factors likely play a role in the relative sensitivity of bees to toxin exposure, such as the specific types of enzymes they possess and the changes in their enzyme expression following exposure. *Megachile rotundata*, for example, lacks both the CYP9Q and CYP9BU subfamilies of P450 enzymes, which have been implicated in neonicotinoid tolerance in social bees and *Osmia bicornis* L. (Hymenoptera: Megachilidae) (Beadle et al., 2019; Haas & Nauen, 2021; Hayward et al., 2019). *Megachile rotundata* was highly sensitive to the neonicotinoid, thiacloprid, and the butenolide, flupyradifurone, compared to *A. mellifera*, *B. terrestris*, and *O.*

bicornis. Additionally, in vitro, the P450 enzymes of *M. rotundata* were able to metabolize nicotine, but not any of the synthetic pesticides tested (imidacloprid, thiacloprid, flupyradifurone, and tau-fluvalinate) (Hayward et al. 2019). Certain solitary bees may therefore have greater sensitivity to some classes of insecticides due to the differences in their P450 genes.

Exposure to different pesticides can also cause the upregulation of these enzymes, through changes to gene amplification or gene regulation (ffrench-Constant et al., 2004). This has been observed in many insect species: *D. melanogaster* individuals that were resistant to DDT overexpressed the P450 gene, CYP6G1 (Daborn et al. 2002). Swallowtails (*Papilio* spp) resistant to furanocoumarins showed increased expression of two P450s genes, CYP6B1 and CYP6B4 (Cohen et al. 1992). *Apis mellifera* larvae were able to quickly metabolize the botanical insecticide, nicotine, in part due to their upregulation of the P450 genes, CYP6BD1 and CYP9Q1 (du Rand et al. 2017). *Apis mellifera* adult workers exposed to flupyradifurone showed increased expression of two P450 genes, CYPQ3 and CYPD1 (Al Naggar and Baer 2019).

The importance of P450 enzymes in insect detoxification can also be demonstrated when pesticides are mixed with piperonyl butoxide, a P450 inhibitor. When exposed to both imidacloprid (formulation: Advise™) and piperonyl butoxide (P450 inhibitor), *A. mellifera* had higher mortality than imidacloprid exposure alone (Zhu et al., 2017b). Imidacloprid mixed with other enzyme inhibitors, triphenyl phosphate (esterase inhibitor) or diethyl maleate (GST inhibitor), however, did not raise mortality when compared to imidacloprid alone (Zhu et al., 2017b), which would support that P450s are some of the most important enzymes for *A. mellifera* detoxification of neonicotinoids.

There are several methods for measuring insect enzyme expression, which can differ in expense, labor and time requirements, and sensitivity. Derivatives of resorufin, fluorescein, benzopyrene, and especially coumarin are commonly used, though they tend to be expensive and labor intensive (Inceoglu et al., 2009). Recently, commercially available assays (Promega P450-Glo™ assays) using luciferin derivatives have been used to measure the enzyme

expression of mosquitoes (*Culex pipiens* L.) and Colorado potato beetles (*Leptinotarsa decemlineata* Say), and at a lower cost than the traditional coumarin-derivative assays (Inceoglu et al., 2009; Zhu et al., 2016). There have been few tests to evaluate whether these luciferin derivative assays could assess bee enzyme expression, however.

As well as physiological changes, bees can try to cope with toxin exposure through behavioral mechanisms. The risk of pesticides to bee health depends on how sensitive the bees are to the pesticides and how likely they are to be exposed to them. Bees that are out foraging during a foliar application of a pesticide are the most likely to be exposed to higher doses, which can pose the greatest risk to bee survival (Abraham et al., 2018; Bailey et al., 2005). Pesticide residues, however, can also be found in flower pollen and nectar, water sources, and soil (Démares et al., 2022; Dively & Kamel, 2012; Heller et al., 2020; Johnson & Pettis, 2014; Krupke et al., 2012; Mullin et al., 2010; Zawislak et al., 2021), which is used by many *Osmia* spp. in their nest construction (Torchio, 1989). The ability of bees to detect and avoid pesticide-contaminated material can lower their risk of exposure, even when pesticide residues are present in the environment. Several previous studies, however, have found that *A. mellifera* and *B. terrestris* may prefer food sources contaminated with the neonicotinoids, imidacloprid and thiacloprid (Arce et al., 2018; Kessler et al., 2015). A similar study on *B. impatiens* found no preference or avoidance behavior to food sources with imidacloprid, which would indicate that *B. impatiens* is unable to detect imidacloprid in food or is indifferent to its presence (Muth et al., 2020). The feeding preference of bees can affect their likelihood of being exposed to pesticides in the field and should be used to create more effective mitigation strategies for bee pesticide exposure.

Bees are valuable commercial and environmental pollinators, and so the risk assessment of various pesticides for bees should focus on their ability to function as pollinators, rather than only looking at mortality. Lower doses of several pesticides may not kill bees directly, but can cause impairment or other behavioral changes that can affect their pollination

ability. Thiamethoxam exposure, for example, altered the flight duration, distance, and velocity of *A. mellifera*. Exposed bees showed increased activity within 1 hour of exposure, but reduced flying activity after 1-2 days of chronic exposure (Tosi et al., 2017). Similarly, *O. bicornis* that were exposed to sulfoxaflor showed altered foraging behavior, with more time spent on each flower and fewer flower visitations (Boff et al., 2021).

In this study, we investigated the sublethal impacts of the commercial insecticides, flupyradifurone and sulfoxaflor, on *Osmia* spp. To do this, we measured the expression of *Osmia lignaria* female P450s following exposure to the insecticides and assessed the viability of luciferin-derivative assays for measuring *Osmia* spp. P450 expression. We also tested whether *O. lignaria* males and females would show any preference or avoidance behavior for food contaminated with flupyradifurone or sulfoxaflor. Finally, we assessed the flying and foraging behavior of *Osmia* spp. exposed to low doses of flupyradifurone. Measuring bee enzyme expression following pesticide exposure and comparing it to survival data can show how bees cope with certain pesticides. It can also highlight the risks of mixing certain pesticides with enzyme inhibitors. Additionally, flight, foraging, and feeding behavior following pesticide exposure can emphasize the sublethal risks of certain pesticides to create more effective mitigation strategies.

Materials and Methods

Bee Preparation and Pesticide Exposure for Enzyme Assays

Osmia lignaria Say cocoons were obtained from Watts Solitary Bees in Bothell, WA, in March 2022 and 2023. Cocoons were placed in a 4°C refrigerator to synchronize emergence. The cocoons were taken out of the refrigerator and the bees were allowed to emerge at room temperature (22-23°C). They were provided with 50:50 w/v organic honey (Nature Nate's Natural 100% Pure Organic Raw & Unfiltered Honey from Nature Nate’s Honey Co. in McKinney, TX) in DI water solution for 24 hours before pesticide exposure.

At least 36 female bees were used per treatment group to ensure at least 30 live bees at 48 hours after treatment (HAT). Two commercial insecticide formulations were used, Sivanto™ Prime by Bayer CropScience (17.09% active ingredient – flupyradifurone) and Transform® WG by Corteva Agrosciences (50% active ingredient – sulfoxaflor). The treatment groups were: control bees (DI water), lower dose flupyradifurone (0.22 ng ai bee^{-1}), higher dose flupyradifurone (0.97 ng ai bee^{-1}), lower dose sulfoxaflor (0.056 ng ai bee^{-1}), and higher dose sulfoxaflor (0.52 ng ai bee^{-1}). The lower doses of each insecticide corresponded to a ten-thousand-fold dilution from the lowest recommended field use concentration (FUC) according to the insecticide labels. The higher doses of each insecticide corresponded to the LD_{20} values for female *O. lignaria* bees at 48 HAT.

In 2022, female *O. lignaria* bees were orally exposed to 2 µl of pesticide solution or DI water with a micropipette (Williams et al., 2013). Immediately after exposure, bees were transferred to clean plastic, mesh lid cages with paper towels and cotton rounds soaked in 50:50 w/v organic honey solution. Bees were kept at a temperature of 22-23°C and in natural light. Six bees were placed in each cage with 6-7 cages per treatment group. Fresh 50:50 w/v organic honey solution was provided daily. Mortality was measured at 24 and 48 HAT. At 48 HAT, bees were placed in a -80°C freezer until tissue dissections could be done.

Tissue Dissections

Microcentrifuge tubes (1.5 ml) were weighed before and after dissections to measure total collected tissue weights. Dissections were performed under a dissection scope in petri dishes filled with 1X PBS buffer solution. Buffer was replaced and petri dishes were cleaned between each treatment group. For each group, 30 female bees were dissected to have 3 replicates of 10 bees. Heads were removed and discarded. For the female bees, the stingers and venom sacs were removed and discarded. The bee rectum is closely associated to the venom sac, and was discarded, as well, but the Malpighian tubules, ileum, and midgut were

carefully maintained in the bees (Manjon et al., 2018; Zhu et al., 2017a). Then the abdomen and thorax were opened with forceps for each bee and the following tissues were collected: midgut, ileum, Malpighian tubules, ovaries/testes, fat bodies, tracheae, air sacs, and muscles. The tissues were placed in sterile microcentrifuge tubes on ice during the dissections and then moved to the -80°C freezer (protocol modified from Zhu et al., 2016).

Tissue Preparation

A 40 mL cold lysis buffer was made with 50 mM Tris buffer, 1 mM EDTA, 2 mM PMSF, 4 protease inhibitor mini tablets, 2 mM DTT, 100 mM NaCl, and 20% glycerol. The solution was titrated to a final pH of 7.6, cooled on ice for 1 hour, and then stored in the -80°C freezer. The cold lysis buffer was kept for a maximum of 4 days and then remade as needed. Bee tissues and lysis buffer were both kept on ice during use. Cold lysis buffer was added to each microcentrifuge tube of bee tissue, with 1 µl buffer for each mg bee tissue. Micropestles were carefully washed and rinsed with water and then acetone. When dry, the micropestles were used to crush and mix the bee tissues in the cold lysis buffer. A Branson Sonifier 250 sonicator (Emerson Electric Co. in St. Louis, MO) was used to lyse the bee tissue cells, set at 10% duty cycle and output control of 2. Microcentrifuge tubes with tissue were kept on ice during sonication and 5-10 bursts of sonication were done for each tube. Tubes were then visually assessed that they were homogenized and mixed adequately and put through another round of sonication as needed. Following sonication, tubes were placed in a microcentrifuge at 4°C, 14,000 RPM, for 15 min. The supernatant from each sample was collected into a new microcentrifuge tube. 100 µl aliquots of supernatant were placed in 0.5 ml microcentrifuge tubes and dipped in liquid nitrogen to flash freeze. Sample aliquots were placed in the -80°C freezer (protocol modified from Zhu et al., 2016).

Bradford Protein Assays

A Bradford protein assay, using a Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific Inc. in Waltham, MA), was done to check the protein levels present in each sample. In order to create a protein concentration standard curve, bovine serum albumin (BSA) stock solution was serially diluted to the following concentrations: 1.5, 1, 0.75, 0.5, 0.375, 0.25, 0.125, 0.0625, and 0 mg/ml. The BSA dilutions were kept in dark brown microcentrifuge tubes to prevent light degradation. Bee tissue sample aliquots were taken out of the freezer, thawed in room temperature water, and quickly put on ice. Dilutions of 1:10 and 1:100 were made from each sample. Then 4 µl of each BSA standard dilution and tissue sample dilution were added to 3 wells of a clear 96-well plate. 200 µl of 1X Bradford reagent was also added to each well. The plates were incubated for 10 min at room temperature. Then, they were put into a synergy HTX plate reader (Agilent Technologies, Inc. in Santa Clara, CA) and read at 595 nm at room temperature (23.1°C) at 2 min, 5 min, and 10 min. Sample absorbance was analyzed against the BSA dilutions standard curve to determine the total protein concentrations in each sample (protocol modified from Zhu et al., 2016).

Cytochrome P450 Monooxygenase Assays

Two commercially available P450-Glo™ assays (Promega Corporation in Madison, WI) were acquired: a CYP3A4 assay using luciferin-IPA as a substrate and a CYP1A2 assay using luciferin-ME as a substrate. D-luciferin potassium salt was used to create a standard curve and estimate luciferin concentration. Stock concentrations of 8, 1.6, 0.32, and 0.064 µM D-luciferin in DI water were made and would later be further diluted in the well plates to final concentrations of 2, 0.4, 0.08, and 0.016 µM. Stock solutions were kept in dark tubes on ice. Tissue sample aliquots were taken out of the freezer, thawed, and placed on ice. Each standard concentration, sample group, and blank (DI water, cold lysis buffer) was run in 3 wells of a white 96-well plate. 12.5 µl of D-luciferin standard dilution or 12.5 µl sample solution (sample + 1 µl luciferin-ME or

0.133 µl luciferin-IPA) were added to the wells of the plate. The plate was pre-incubated at 37°C for 10 minutes. An NADPH regeneration system (22 µl DI water, 2.5 µl solution A, and 0.5 µl solution B) was added to each well. Then the plates were incubated at room temperature for 30 minutes, in the case of luciferin-ME, or 10 minutes, in the case of luciferin-IPA. Finally, 50 µl of a luciferin detection reagent was added to each well. For the luciferin-ME assays, a reconstitution buffer was added to a lyophilized luciferin detection agent, and for the luciferin-IPA assays, a reconstitution buffer with esterase was added to a lyophilized luciferin detection agent. The plate was mixed for 10 seconds and then sat for 20 minutes at room temperature before the luminescence was measured using a Syngenta HTX plate reader (protocol from Inceoglu et al., 2009; Zhu et al., 2016).

Feeding Preference Study

Four observation cages, made of metal frames and white thin mesh screen and measuring 31 x 31 x 31 cm, were lined with paper towels. Bee feeding dishes were made of aluminum weigh trays (5 cm diameter, 0.5 cm height) with a cotton round inside. Two feeders were placed in each observation cage, spaced 10 cm apart from each other in the front right and front left corners of the cage. 2 mL of 50:50 w/v organic honey solution was added to each feeder and 20 µl of a pesticide solution was added to one feeder in each observation cage. The following pesticides and concentrations were used: high flupyradifurone (110 mg ai L^{-1}), low flupyradifurone (11 mg ai L^{-1}), high sulfoxaflor (280 mg ai L^{-1}), and low sulfoxaflor (2.8 mg ai L^{-1}). Newly emerged *O. lignaria* bees were allowed to feed on 50:50 w/v organic honey solution for 24 hours. Then food and water were withheld from them for 2 hours before experimental use. Bees were transferred in groups of 5 females or 5 males to the observation cages. A total of 4 groups of males and 6 groups of females were run for each pesticide concentration. After release into the cages, bees were observed for 3 hours, and the number and duration of feeding trips were recorded for each feeder. Feeding trips were defined as bee proboscis extension on a

feeder for at least 5 seconds (Arce et al., 2018). Cages were cleaned with water and 70% ethanol between each trial of the experiment. Feeders were replaced between each trial. Additionally, placement of feeders (on the left or right side of the cage) was randomized for each trial to account for bee preference for the side of the cage closest to the windows of the lab.

Flight Studies

The flight studies were performed in the fall of 2022 and summer of 2023 on sunny days with temperatures of 21-24°C and <12 kph wind. *Osmia lignaria*, *Osmia cornifrons* Radoszkowski, and *Osmia californica* Cresson overwintering cocoons were taken out of a 4°C refrigerator and kept at room temperature to allow emergence. They were provided with organic honey and DI water for 24 hours after emergence. Then adult male and female bees were randomly sorted into treatment groups: control (DI water), lower dose flupyradifurone (0.22 ng ai bee^{-1}), and higher dose flupyradifurone (0.97 ng ai bee^{-1}). The formulation Sivanto^{TM} Prime (17.09% ai: flupyradifurone) was used. The lower dose was a ten-thousand-fold dilution from the field use concentration recommended by the formulation label and the higher dose was the LD_{20} value for *O. lignaria* females at 48 hours after treatment. A total of 30 female and 30 male bees were used in each treatment group.

Bees were exposed via direct feeding with a micropipette to 2 µl of pesticide solution or DI water. They were transferred to clean cages, with 5 bees per cage, without food or water for 3 hours. After 3 hours, bees were taken to the Milo J. Shult Agricultural Research and Extension Center. Bee flight cages, measuring 2.5 x 1.5 x 1.5 m had previously been constructed at the site. Cages were made of wooden frames and covered in black aluminum mesh screen. Before the study, the cages were clear cut and potted flowers (*Solidago* sp., *Erigeron* sp., *Coreopsis* sp. hybrid, *Asclepias tuberosa* L.*,* and *Nepeta faassenii* Bergmans) were placed on the western side of the flight cage. A raised platform measuring 33 x 18 cm was placed on the eastern side of the cage. Bees, in groups of 5 individuals, were placed on the raised platform and released.

They were observed for 10 minutes and assessed on the following endpoints: ability to walk, ability to fly, ability to forage, and number of floral visitations. Ability to fly was defined as the ability to maintain flight for at least 5 seconds. Ability to forage and floral visitation were defined as landing on a flower with proboscis extension for at least 5 seconds.

After the 10-minute observation period, the bees were collected and returned to their small lab cages. Following the run of the flight observations, bees were returned to the lab and their survival was measured at 3, 24, and 48 HAT. For *Osmia lignaria* females, additional flight observations were made at 24 and 48 HAT, using the same endpoints.

Statistical Analysis

Differences in relative luminesce units (RLU) were analyzed using an ANOVA and a Tukey HSD multiple comparisons test in JMP Pro 16 (JMP Statistical Discovery LLC in Cary, NC) for each luminescence assay. Pesticide feeding preference was analyzed using a pooled two-sample *t* test for each pesticide dose in JMP Pro 16 to compare the percentage of time and number of feedings spent on the pesticide feeder and non-pesticide feeder for each pesticide concentration and sex of bee. For the flight study, the percentage of live bees able to walk, fly, and forage, the survival percentage, and the number of floral visits were analyzed in JMP Pro 17 using nonparametric Kruskal-Wallis tests and the nonparametric Dunn Method comparisons for joint ranking with Bonferroni corrections to compare treatment groups for each species and sex of *Osmia* bees. Walking, flying, foraging, and floral visits for *O. lignaria* at 24 and 48 HAT were analyzed using an ANOVA, as the assumptions of normality were met.

Results

Cytochrome P450 Enzyme Expression

The commercial P450-Glo™ assays used the substrates, luciferin-ME, which is catalyzed by CYP1A2 enzymes and analogs, and luciferin-IPA, which is catalyzed by CYP3A4

enzymes and analogs. Enzyme activity changes the derivative substrates into luciferin, which can then be mixed with luciferase to produce light, detected as relative luminescence units (RLU). Control bees and bees exposed to sulfoxaflor showed no significant change in relative luminescence units (RLU), and therefore no noticeable change in P450 enzyme expression that could catalyze either the luciferin-ME or luciferin-IPA substrates (**Figure 3.1**). Bees exposed to 0.22 or 0.97 ng ai bee^{-1} flupyradifurone, however, showed significantly higher RLU values. This would suggest that these bees increased expression of CYP1A2 insect analogs, which can successfully catalyze luciferin-ME (**Figure 3.1A**). This was not seen in the luciferin-IPA assay, however, which had no changes to RLU for any treatment group (**Figure 3.1B**).

The luciferin-based assays showed low sensitivity overall, however. A standard curve was created using D-luciferin potassium salt, isolated from fireflies (Coleoptera: Lampyridae), which contains a known concentration of luciferin. The D-luciferin standards were run alongside the bee tissue samples in the luminescence assay. The tissue samples of bees exposed to flupyradifurone were then compared on the standard curve and the amount of luciferin created from enzyme activity on luciferin-ME was estimated (**Figure 3.2**). Although the flupyradifurone-exposed bees showed increased luciferin production and enzyme activity compared to the other treatment groups, the luciferin concentration overall was low compared to the D-luciferin standard (**Figure 3.2**).

Feeding Preference

Osmia lignaria females spent a significantly higher percentage of time feeding on the feeder without pesticides than the one containing the higher flupyradifurone concentration ($t=16.72$, $DF=1$, $p=0.0022$). This shows some avoidance behavior of female *O. lignaria* bees to the pesticide. Lower concentrations, however, went undetected or ignored by female bees, with no difference in time spent on either feeder. There was no significant difference in percentage

feeding time for either concentration of sulfoxaflor or for male bees with either flupyradifurone or sulfoxaflor (**Figure 3.3**).

The number of feeding trips for *O. lignaria* females in the higher concentration flupyradifurone group was also higher on average for the feeder without pesticides, but the difference was not significant. There was no significant difference in number of feeding trips for any other pesticide concentration or sex of *O. lignaria*.

Flight Studies

There were few significant differences in walking ability for living bees at 3 hours after treatment (HAT) for any of the tested species and sexes (**Figures 3.4A** and **3.4C-F**), with the exception of *O. lignaria* males (**Figure 3.4B**). *Osmia lignaria* males exposed to the higher dose of flupyradifurone had a significantly lower percentage of live bees that were able to walk ($\chi^2(2) = 10.17$, $p = 0.0062$), compared to the control bees and bees exposed to the lower dose of flupyradifurone. *Osmia lignaria* and *O. californica* males that were exposed to the higher dosage of flupyradifurone also showed a decreased flying ability, with a lower percentage able to fly at 3 HAT (*O. lignaria*: $\chi^2(2) = 8.12$, $p = 0.0172$; *O. californica* $\chi^2(2) = 10.0$, $p = 0.0067$). No significant differences were observed in the percentage of live bees that were observed landing on flowers during the observation period (**Figure 3.4**).

For foraging activity, the number of floral visits were measured in each treatment group for the three species and both sexes of bees. In *O. cornifrons*, females in the higher dose flupyradifurone group visited a significantly higher number of flowers than the control and lower dose flupyradifurone groups (**Figure 3.5**).

Survival at 3 HAT or 24 HAT was not significantly different for any bees, though *O. lignaria* and *O. californica* males had significantly lower survival in the higher dose flupyradifurone-exposed bees compared to control and lower dose flupyradifurone-exposed bees at 48 HAT (*O. lignaria*: $\chi^2(2) = 7.64$, $p = 0.0219$; *O. californica*: $\chi^2(2) = 9.71$, $p = 0.0078$)

Osmia lignaria females were also observed at 24 and 48 HAT for flying and foraging activity. Though there was no significant difference in the percentage of live bees observed walking, flying, and foraging at 3 HAT, there were differences seen at 24 HAT. The higher dose flupyradifurone-exposed bees had a significantly lower percentage that were able to walk ($F(2) = 6.32$, $p = 0.0102$) and fly ($F(2) = 4.14$, $p = 0.0371$) compared to the control bees at 24 HAT (**Figure 3.6A**). There were no differences in the percentage of bees observed landing on flowers at 24 or 48 HAT. Both the lower and higher dose flupyradifurone-exposed bees had a significantly lower number of floral visits at 24 HAT ($F(2) = 4.46$, $p = 0.0302$; **Figure 3.6C**). However, these significant differences were not observed at 48 HAT (**Figures 3.6B-C**).

Survival of *O. lignaria* females was not significantly different at 3 HAT, 24 HAT, and 48 HAT, though it was noticeably lower in the flupyradifurone-exposed bees, under 90% by 48 HAT (86.67% for lower dose flupyradifurone; 83.33% for higher dose flupyradifurone). Control bee survival stayed above 90%, with a mean of 93.33% at 48 HAT.

Discussion

Osmia lignaria females showed increased P450 enzyme activity following exposure to low doses of flupyradifurone, as was observed in the luciferin-ME assay (**Figure 3.1A**). The commercial P450-Glo™ assay is marketed to measure the activity of CYP1A2, a vertebrate P450 enzyme. Luciferin-ME is a fairly nonselective substrate and can be converted into luciferin by both vertebrate and invertebrate P450 enzymes, including those in the CYP6 family and CYP4 clade (Feyereisen, 2012; Inceoglu et al., 2009; Liu et al., 2019; Zhu et al., 2016). It is likely that the *O. lignaria* CYP6 or CYP4 enzymes were increased in response to flupyradifurone exposure and that they could play a role in bee detoxification of flupyradifurone. *Osmia lignaria* were highly sensitive to flupyradifurone exposure, however, with an LD_{50} value of 5.7 ng ai bee^{-1} for females 48 hours after exposure, compared to *A. mellifera* workers, who had an LD_{50} value over 4400 ng ai bee^{-1} (**Table 2.2**). These differences in sensitivity could be due to differences in

the specific types of enzymes possessed by the different species. *Apis mellifera* possess CYP9Q3 (in the CYP3 clan) enzymes, which have been shown to play a role in flupyradifurone detoxification (Haas et al., 2022). *Osmia* spp. lack this specific enzyme, though they do possess the related CYP9BU enzymes (Beadle et al., 2019; Haas et al., 2022). Though the *O. lignaria* P450 enzymes can increase in expression after exposure to flupyradifurone, their particular P450 enzymes may not fully detoxify flupyradifurone. Because of this, combining flupyradifurone with P450 inhibitors, such as piperonyl butoxide, would likely increase the mortality rate of *O. lignaria* after exposure. As such, these mixes should be used with caution, especially in areas that rely on *Osmia* spp. pollination services.

No increase in enzyme activity was noted for bees exposed to sulfoxaflor in the luciferin-ME assay. Other studies have noted that *A. mellifera* exposed to sulfoxaflor did not show increased expression for other detoxification enzymes, such as esterases and glutathione-*S* transferases (GSTs) (Zhu et al., 2017a). It is possible that the luciferin-ME assay may be too specific or not sensitive enough to measure the P450 enzyme activity of sulfoxaflor-exposed bees, but alternatively, sulfoxaflor may not induce a strong detoxification enzymatic response in bees. The enzymatic response of bees may also be influenced by whether the technical grade of the active ingredient (TGAI) or a formulation of the pesticide is used. Bees can have different survival responses to the TGAI or to formulations containing the same active ingredient (Al Naggar & Baer, 2019; Glaberman & White, 2014; Spruill et al., 2020), and could have different enzymatic responses, as well.

Past studies have used the substrate luciferin-BE, which was acted upon by the vertebrate CYP3A4 enzyme. Luciferin-BE, like luciferin-ME, was not strictly selective and could be used to detect insect P450 activity (Inceoglu et al., 2009; Zhu et al., 2016). At the time of this study, however, the CYP3A4 assay with luciferin-BE was not available, so the CYP3A4 assay with luciferin-IPA was used instead. Luciferin-IPA is a more selective substrate (Cali et al.,

2009). The lack of enzyme expression noted in the luciferin-IPA assays may be due to the substrate being too selective to accurately measure invertebrate P450 activity.

The commercially available luciferin assays offer a more cost effective and time-saving alternative to the traditional coumarin assays for measuring P450 activity (Inceoglu et al., 2009). Though the luciferin assays have been successfully used for several insect species across multiple insect Orders (Inceoglu et al., 2009; Liu et al., 2019; Zhu et al., 2016), they may not be sensitive enough to fully characterize bee enzyme expression without further alterations to the tissue sample preparation. Even in the luciferin-ME assays, the flupyradifurone-exposed bees showed increased enzyme expression, but it was low compared to the D-luciferin standards and compared to other studies on insect P450 expression. Additionally, future studies on the expression of other bee detoxification enzymes, such as esterases and GSTs, could help explain bee sensitivity to certain pesticides.

As well as physiological changes, low concentrations and doses of flupyradifurone and sulfoxaflor resulted in behavioral changes. In the feeding preference study, *Osmia lignaria* females showed some avoidance behavior to higher concentrations of flupyradifurone, but there was no noticeable avoidance of sulfoxaflor at either concentration (**Figure 3.3**). Similar behaviors have been seen in *A. mellifera* forager bees, which avoided sucrose solution that contained 4 mg L^{-1} flupyradifurone and consumed less sucrose after exposure (Wu et al., 2021). Bees that can detect and avoid pesticide-contaminated food can reduce their likelihood of exposure to dangerous pesticide levels. For *O. lignaria* females, however, this avoidance was not seen at the lower concentration of flupyradifurone. *Osmia lignaria* females may be able to avoid higher concentration residues, but residues in floral nectar and pollen tend to be lower, around 4.3 ppm (4.3 mg L^{-1}) in nectar and 21 ppm (21 mg L^{-1}) in pollen (Glaberman & White, 2014). *Osmia lignaria* males and females showed no avoidance of food contaminated with up to 280 mg ai L^{-1} sulfoxaflor, which is higher than typical residues in plant pollen and nectar. In cotton plants treated with 700 g ai ha^{-1} sulfoxaflor via drip irrigation, pollen residues were up to

0.039 ppm and nectar residues were up to 0.0138 ppm (Jiang et al., 2020). Foliar applications may result in a higher risk of contaminated nectar, however, as one study noted that plants with soil applications of sulfoxaflor contained higher concentrations of the less toxic metabolite of sulfoxaflor, X11719474, whereas plants with foliar applications had higher concentrations of sulfoxaflor, itself, in nectar (Zhou et al., 2022). Males and females of *O. lignaria* did not show a preference for food with sulfoxaflor and flupyradifurone, so providing unsprayed and high value food sources, such as floral hedgerows and wildflower strips, alongside commercial orchards may help promote *Osmia* spp. health, allowing them to decrease pesticide exposure by consuming less contaminated pollen and nectar.

Flupyradifurone also impacted *Osmia* spp. foraging activity. At 3 hours after treatment (HAT), few differences were noted for bees' ability to walk, fly, and land on flowers within the 10-minute observation period. Live *O. lignaria* and *O. californica* males exposed to the higher dose of flupyradifurone had a reduced flying ability compared to the control bees. Foraging ability of *O. lignaria* and *O. californica* males also tended to be lower in flupyradifurone-exposed bees, though not significantly so (**Figures 3.4B** and **3.4F**). The number of floral visits was also similar, except for *O. cornifrons* females, which had a significantly higher number of floral visits in higher dose flupyradifurone-exposed bees (**Figure 3.5**). Similar results were seen in *A. mellifera* exposed to the neonicotinoid, thiamethoxam, in a previous study. At 1 HAT, thiamethoxam-exposed *A. mellifera* showed an excitation response, with longer flight times and distances. Chronic exposures over 1-2 days, however, reduced flight distances, speeds, and durations (Tosi et al., 2017). Future studies should look at *O. cornifrons* female bee foraging activity at 24 and 48 HAT to see if the excitation response is reduced over time. For *O. lignaria* females, there was no impact of low-dose flupyradifurone exposure on flight and foraging at 3 HAT, but flupyradifurone exposure did reduce walking ability, flight ability, and the number of floral visits at 24 HAT (**Figures 3.6A** and **3.6C**). These impacts on foraging were not noted at 48 HAT, however. Bees were exposed once and then assessed at 3, 24, and 48 HAT, so it is possible

that the surviving exposed bees were able to recover from the exposure by 48 HAT. Chronic exposure to flupyradifurone could cause more noticeable effects on *O. lignaria* foraging over time.

Several informal observations were made on *Osmia* spp. foraging behavior, as well, and should be used as more formal endpoints in future studies. Time spent per flower should be a metric for further flight and foraging tests. Additionally, at 24 and 48 HAT, exposed *O. lignaria* females showed greater clustering behavior while foraging. Control bees foraging on *Coreopsis* sp. would forage with a maximum of 2 bees per bloom, though more often only 1 bee per bloom. Flupyradifurone-exposed bees would often have 3-5 bees on a bloom.

Additional studies could also look at the impacts of low-dose sulfoxaflor exposure on *O. lignaria*, *O. californica*, and *O. cornifrons* flight and foraging activity. A past study looked at the effect of 50 ppb sulfoxaflor exposure on *O. bicornis* flight behavior, and found that exposed bees had a reduced number of floral visits and a longer amount of time spent on each bloom than control bees over the 5-day study period (Boff et al., 2021). Finally, the flight study focused on the flying and foraging ability of living bees following pesticide exposure. Low-dose exposure to flupyradifurone increased the mortality rate of *Osmia* spp., which could also affect their foraging and pollination ability.

Conclusions

While high concentrations of flupyradifurone and sulfoxaflor pose the highest risk to *Osmia* spp. health, sublethal doses can also impact their activity, physiology, and behavior. Detoxification enzyme inhibitors, such as piperonyl butoxide, may increase the effect of these pesticides and cause higher mortality, and so should be used with caution on bee-preferred flowers. *Osmia lignaria* may avoid higher concentrations of flupyradifurone, but overall did not detect lower concentrations of flupyradifurone or sulfoxaflor. As a generalist feeder, *O. lignaria* often seek out a variety of flower species to forage from, so providing areas of unsprayed flower

mixes could help them to feed on diverse floral resources and reduce pesticide exposure. Low dose exposure to flupyradifurone can also impede *Osmia* spp. mobility and foraging ability, which could limit the pollination service they provide. Chronic exposure may pose the highest risk to bees, as impacts were not often seen until 24 HAT. Measures to limit *Osmia* spp. pesticide exposure, even to lower concentrations, can help ensure their pollination ability in orchards, vegetable gardens, and other ecosystems.

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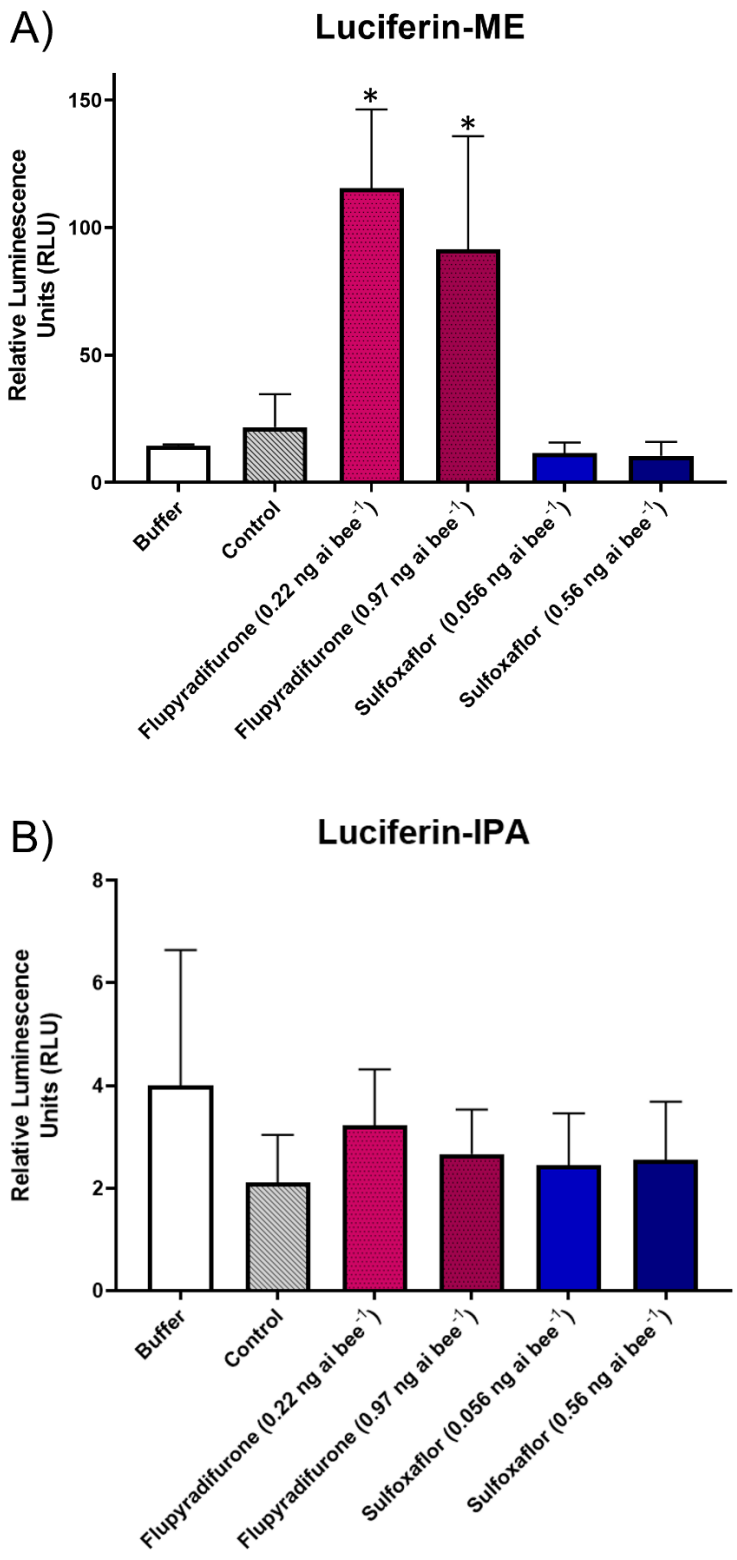

Figure 3.1. Bee tissues were dissected and used following oral exposure to flupyradifurone, sulfoxaflor, or DI water (control). The relative luminescence units (RLU) is shown for bee tissue samples mixed with luciferin-ME (A) and luciferin-IPA (B), as well as the cold lysis buffer alone. Statistically significant ($p<0.05$) differences in RLU are marked with an asterisk (*).

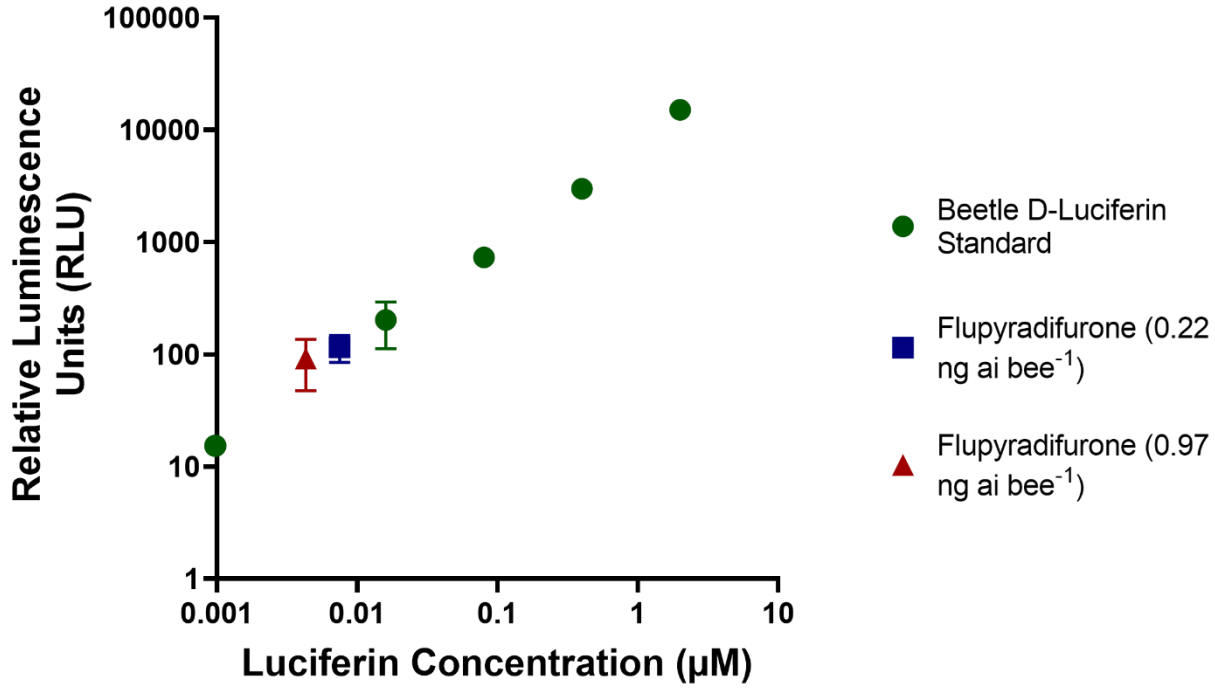

Figure 3.2. The firefly D-luciferin potassium salt standard curve, with known luciferin concentrations (µM), and the estimates of luciferin concentration produced by enzymes in tissues from flupyradifurone-exposed bees.

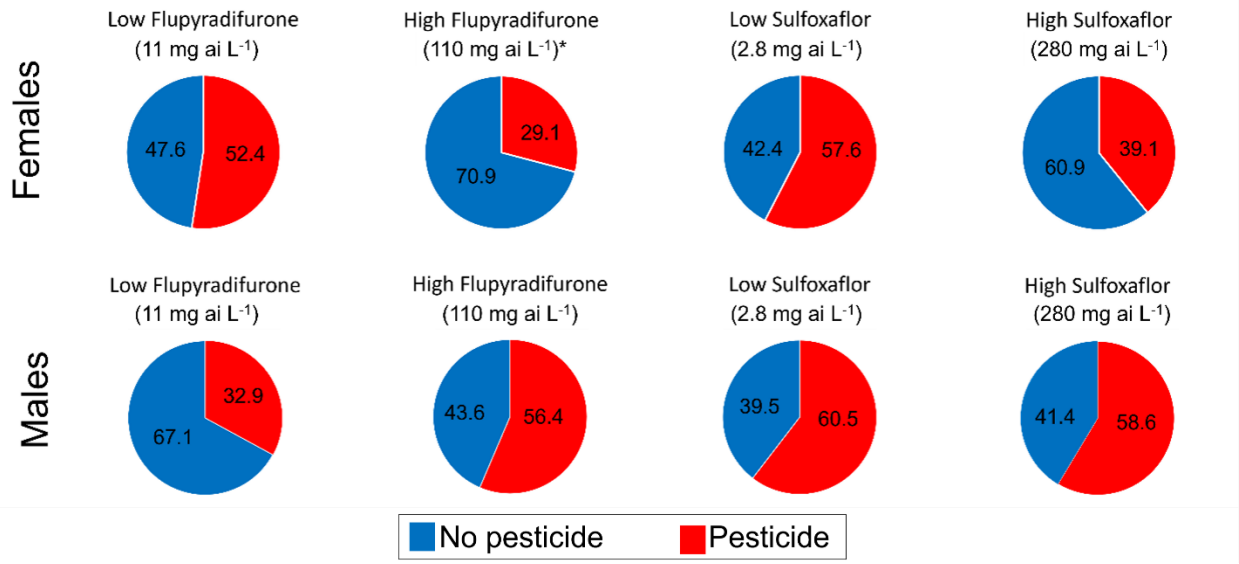

Figure 3.3. Percentage of time spent at pesticide feeder (red) or no pesticide feeder (blue) for male and female *O. lignaria*. Asterisks (*) represent statistically significant difference in feeder preference (p=0.0022).

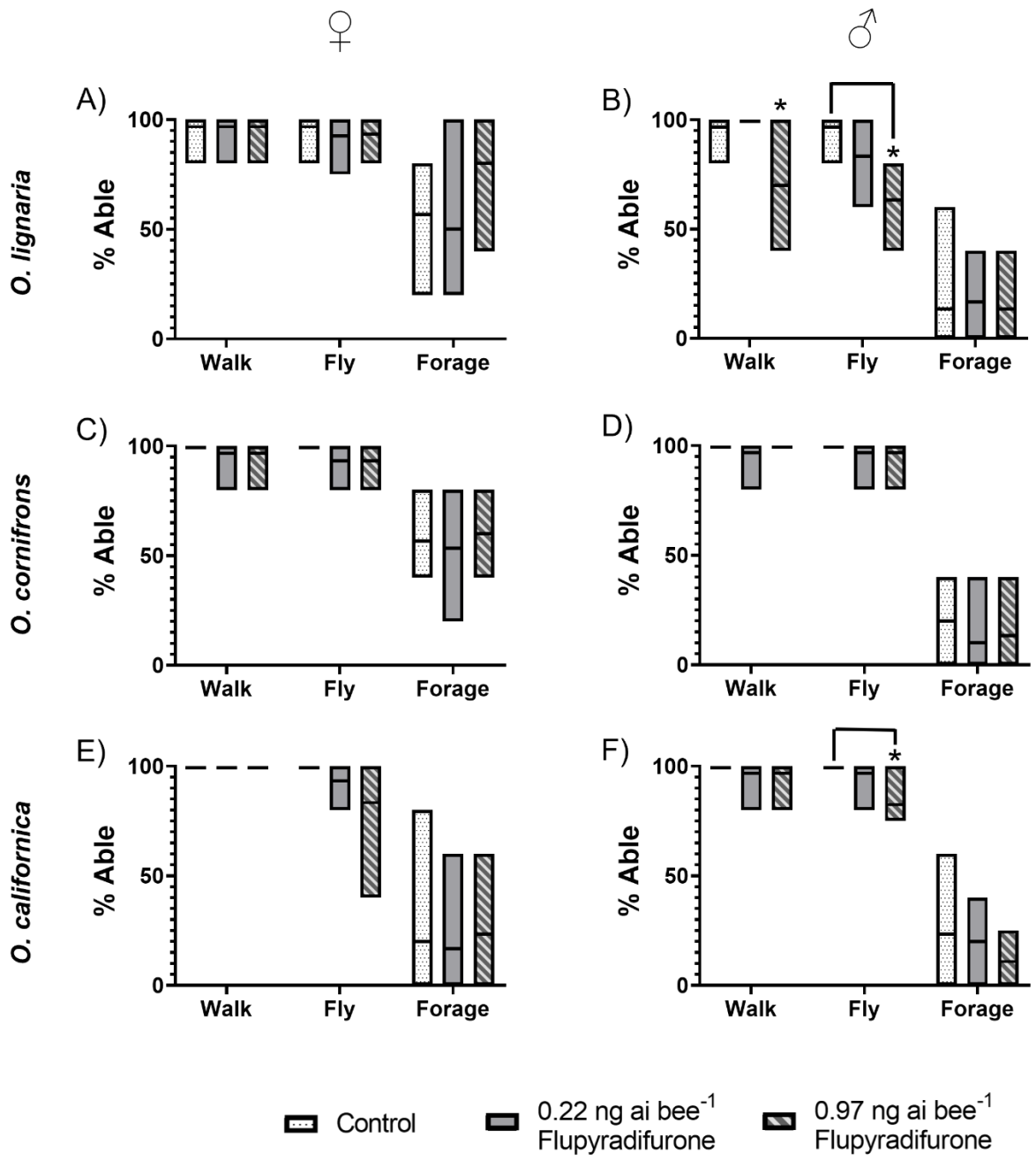

Figure 3.4. The flight and foraging ability of *Osmia* spp. 3 hours following exposure to DI water (control), lower dose flupyradifurone (0.22 ng ai bee^{-1}), or higher dose flupyradifurone (0.97 ng ai bee^{-1}). The percentage of living bees able to walk, fly, and forage within the 10-minute observation period is shown for *O. lignaria* females (A), *O. lignaria* males (B), *O. cornifrons* females (C), *O. cornifrons* males (D), *O. californica* females (E) and *O. californica* males (F). Significant differences ($p<0.05$) in ability (%) are marked with an asterisk (*).

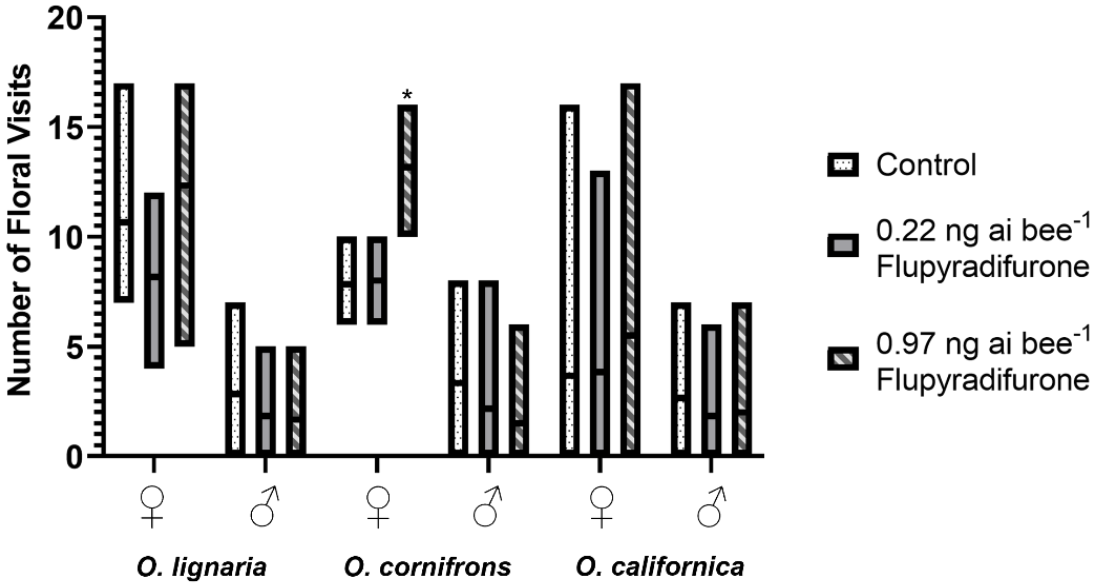

Figure 3.5. The total number of floral visits within the 10-minute observation period is shown for each bee species (*O. lignaria*, *O. cornifrons*, and *O. californica*) and sex. Significant differences ($p<0.05$) in the number of floral visits are marked with an asterisk (*).

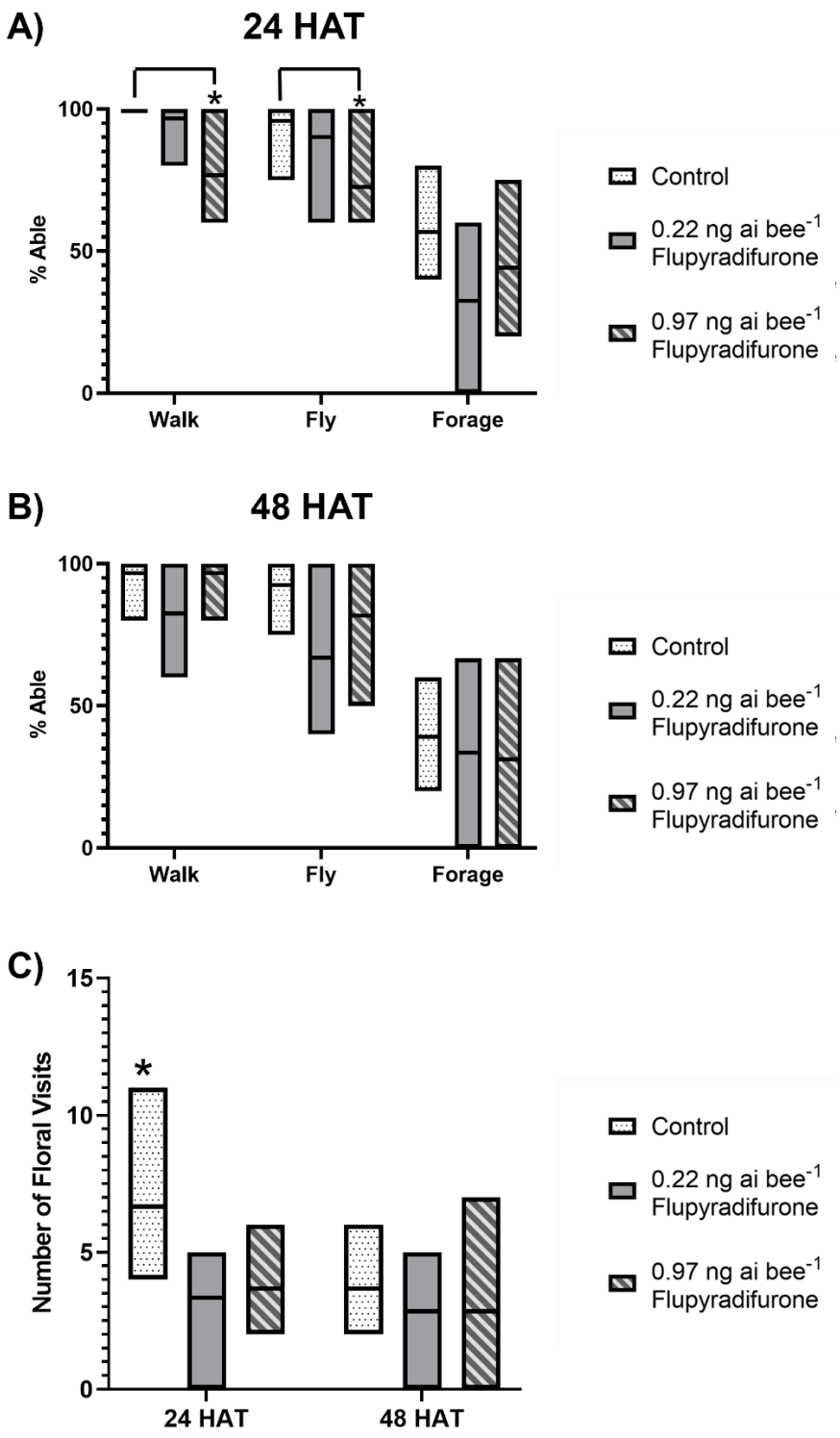

Figure 3.6. The flight and foraging ability of *O. lignaria* females at 24 (A) and 48 (B) hours after treatment (HAT) with DI water (control), lower dose flupyradifurone (0.22 ng ai bee^{-1}), or higher dose flupyradifurone (0.97 ng ai bee^{-1}). The total number of floral visits within the 10-minute observation period (C). Significant differences ($p<0.05$) in floral visits and ability (%) are marked with an asterisk (*).

Chapter 4:

The Effect of Pesticides on the Gut Microbiome of Horned-Face Bees (*Osmia cornifrons*)

Abstract

Many insect species rely on gut bacterial symbionts to aid in food digestion, growth and development, and xenobiotic detoxification. Social honey bees and bumble bees have a relatively well-known core community of gut microbes. For solitary bees, like mason bees (*Osmia* spp.), gut microbial communities tend to be more varied and influenced by the environment. The specific bacteria that they carry in their guts can influence their health and may impact bee-bee and bee-plant interactions via pathogen spread while foraging. Additionally, many pesticides can cause changes to the community composition and diversity of insect gut bacteria. In this study, horned-face bees (*Osmia cornifrons*) were exposed to low doses of pesticides: two synthetic insecticides (flupyradifurone and sulfoxaflor), an organic insecticide (spinosad), a fungicide (chlorothalonil) and an herbicide (glyphosate). Their gut bacterial communities were then sequenced, analyzed, and compared to those of control bees to see if exposure to the pesticides would affect gut bacteria diversity or the prevalence of plant and animal pathogens. The relative abundances of potential pathogens were not significantly affected by pesticide exposure. Sulfoxaflor and chlorothalonil exposure in female bees and spinosad and chlorothalonil exposure in male bees lowered bacterial diversity, though results were only significant in the case of sulfoxaflor. Further testing of *Osmia* spp. gut bacterial communities following pesticide exposure could help highlight trends in diversity.

Introduction

Insects possess many different strains of bacteria in their guts, some of which are pathogenic, some neutral, and some beneficial. The role of the beneficial gut symbionts can be very complex, but they can influence immune response, digestion, growth and development, and detoxification of xenobiotics in the host insect (Engel et al., 2016; Jaffar et al., 2022). The

gut microbiome also contributes to some insects' ability to resist parasite infection. *Bombus* spp. (Hymenoptera: Apidae) with higher gut bacteria diversity, and especially with higher abundance of the four bacterial OTUs (*Apibacter*, *Lactobacillus*, and two *Gilliamella* spp.), had lower parasite loads of *Crithidia bombi* (Mockler et al., 2018). Common bacterial clades in both *Apis mellifera* L. (Hymenoptera: Apidae) and *Bombus* spp. aid in the digestion of complex polysaccharides in the bee hindgut (Lee et al. 2015). *Bombus terrestris* L. treated with both streptomycin and tetracycline antibiotics had high worker mortality (Meeus et al., 2013), supporting the important role of the gut microbiota to *Bombus* spp. health.

Many bacterial gut symbionts also play a role in the metabolism and detoxification of xenobiotics in several insect species. The oriental fruit fly (*Bactrocera dorsalis* Hendel) possesses the gut symbiont, *Citrobacter* sp. (CF-BD), which can degrade organophosphate (OPs) insecticides and increase the resistance of the fruit flies to OPs such as trichlorfon (Cheng et al., 2017). The gut bacterial symbiont, *Bacillus cereus* Frankland & Frankland, was present in *Anopheles albimanus* Wiedemann mosquitos resistant to OPs, but not in susceptible mosquitoes, and seemed to confer OP resistance (Dada et al., 2018). Bean bugs (*Riptortus pedestris* Fabricius) that contained strains of OP-degrading *Burkholderia* spp. symbionts were more resistant to the OP, fenitrothion (Kikuchi et al., 2012). A species of *Enterobacter* increased the resistance of the diamondback moth (*Plutella xylostella* L.) to the OP, chlorpyrifos (Xia et al., 2018). In the bumble bee, *Bombus impatiens* Cresson, individuals who were inoculated with gut microbes from adult bees survived longer than uninoculated bees when exposed to the toxic metalloid, selenate. Two core bacterial species, *Snodgrassella alvi* Kwong & Moran and *Lactobacillus bombicola* Praet, were able to grow in vitro in a selenate treated media, and may possess genes involved in its degradation, which can then provide some protection to the exposed bumble bees (Rothman et al., 2019). For *A. mellifera*, gut bacterial symbionts can aid in resistance to insecticides like the neonicotinoid, clothianidin (El Khoury et al., 2022), and the pyrethroid, deltamethrin (Dong et al., 2022).

For solitary bees, such as *Osmia* spp., however, the gut bacterial communities tend to be more varied among individuals and regions, and tend to be more influenced by exposure to environmental bacteria (Engel et al., 2016; McFrederick & Rehan, 2016; Tuerlings et al., 2023). *Osmia* spp. larvae primarily gain gut bacteria through their pollen provisions and mud partitions within their brood nest (Dharampal et al., 2020). Unlike social bees, who gain their gut microbiome via trophallaxis with nest mates or contact with feces in the hive, solitary bee parents and offspring have no direct contact. Some bacteria from the mother bee's salivary secretions could be present in the provision or mud partitions, but for the most part, mason bee larvae gain bacteria from the pollen and mud in their nest cells (Abrol, 2012; Kueneman et al., 2023; Liu et al., 2023; Voulgari-Kokota, McFrederick, et al., 2019; Voulgari-Kokota et al., 2020). Adult bees can then gain further bacteria while out foraging.

The role of microbes in *Osmia* spp. health is not well understood. There is growing evidence that microbes in the pollen provision may aid in breaking down pollen and increasing its nutritional value (Dharampal et al., 2020, 2022; Kueneman et al., 2023; Voulgari-Kokota, McFrederick, et al., 2019). For the oligolectic mason bee, *Osmia ribifloris* Cockerell (Hymenoptera: Megachilidae), larvae reared on their host plant pollen had greater fitness and development than larvae reared on non-host or sterilized pollen (Dharampal et al., 2020). The microbes present in the host plant pollen can improve larval performance. Even for generalist feeding bees, microbes in pollen can influence health. Bee pathogens, such as *Paenibacillus* sp. and *Bacillus* sp., present in pollen provisions can be associated with higher *Osmia bicornis* L. larval mortality (Voulgari-Kokota et al., 2020). The presence of non-pathogenic bacteria in pollen may help prevent the growth of pathogenic bacteria and may explain the importance of pollen mixing in generalist *Osmia* spp. (Voulgari-Kokota et al., 2020; Williams & Tepedino, 2003). Though these exo-symbionts in pollen have been shown to influence mason bee health, it is not known whether *Osmia* spp. gut endosymbionts play a significant role in the health and functioning of their hosts.

Microbes likely influence the interactions that solitary bees have with flowers and with other bee species. Microbes in floral nectar can alter the floral traits, such as bee-attractive scents, in ways that can influence pollinator visitation (Adler et al., 2021; Vannette, 2020). As such, certain microbes may increase their spread by making host flowers seem more attractive to pollinators. Both social and solitary bees may transmit plant pathogens to plants while foraging, as well. The bacterial pathogen, *Pseudomonas syringae* Van Hall, which can infect a variety of host plants, was able to survive inside an *A. mellifera* hive for at least 2 weeks. *Apis mellifera* collected 2 weeks after exposure to pollen contaminated with *P. syringae* still carried the contaminated pollen grains, which they could unintentionally spread to new host plants (Pattemore et al., 2014). Similarly, *Osmia cornifrons* Radoszkowski larval gut bacterial communities included several potential plant pathogenic taxa, including *Erwinia* spp., *Pantoea* spp., *Ralstonia* spp., and *Pseudomonas* spp. (Kueneman et al., 2023). Kueneman et al. (2023) hypothesized that these potential plant pathogenic bacteria might aid in the breakdown of pollen and increase its nutritional value for the larvae. Flowers may also act as transmission sites for bee-to-bee pathogen spread (Dolezal et al., 2016). The trypanosomatid, *Crithidia bombi*, caused infected *B. impatiens* to defecate more frequently on flowers. Future bees visiting the flowers would then be more likely to be exposed to the pathogen (Figueroa et al., 2019). These interactions among flowers, pollinators, and pathogenic and beneficial microbes can be complex. Research investigating the gut microbial communities of solitary bees and the factors than can affect them may further our understanding of these bee-flower-microbe interactions.

Bee gut microbe communities can be altered by several factors, including diet, aging, and pesticide exposure (Raymann et al., 2017). Fungicides and herbicides, for example, are usually not acutely toxic to bees, but can alter the gut microbial communities and the relative abundances of certain key strains of bacteria (Han et al., 2023; Kakumanu et al., 2016; Motta et al., 2018). Research into the effect of pesticide exposure on bee gut communities have focused on social bees, such as *A. mellifera*, Asian honey bees (*Apis cerana* Fabricius), and *Bombus*

spp. (Al Naggar et al., 2022; Almasri et al., 2022; Han et al., 2023; Helander et al., 2023; Hotchkiss et al., 2022; Kakumanu et al., 2016; Motta et al., 2018; Straw et al., 2023; Wu et al., 2022; Zhang et al., 2022). Though solitary bees lack the consistent core gut endosymbionts, pesticide exposure may alter the prevalence of bee and plant pathogens within the gut. Additionally, changes to gut bacterial communities can indicate pesticide-induced changes to bee physiology. Finally, environmentally-acquired bacteria may still play a role in pesticide metabolism and bee survival.

The primary objective of this study was to examine the impact of sublethal doses of two commercial systemic insecticides (flupyradifurone and sulfoxaflor), an organic insecticide (spinosad), an herbicide (glyphosate), and a fungicide (chlorothalonil) on the gut microbial communities of *O. cornifrons* males and females. The diversity and composition of gut bacteria of exposed and non-exposed bees was compared. This study investigated the overall impact of the pesticides on *O. cornifrons* gut bacterial diversity, as significant changes to gut communities can indicate or contribute to physiological changes of the host bees. Additionally, the prevalence and relative abundance of potential plant and bee pathogens were analyzed. Alterations to pathogen abundance within bees could impair bee health and increase the chance of bees spreading pathogens while foraging.

Materials and Methods

Bee Acquisition and Pesticide Exposure

The overwintering cocoons of *O. cornifrons* were acquired from Watts Solitary Bees in Bothell, WA in Spring 2021, and stored at 4°C to synchronize emergence. Cocoons were taken out of the refrigerator to emerge at room temperature (22-23°C). Newly emerged bees were fed 50:50 w/v organic honey (Nature Nate’s Honey Co. in McKinney, TX) solution for 24 hours following emergence and then randomly divided into treatment groups.

Bees were exposed to three commonly used garden pesticide formulations and two commercial insecticide formulations. The common garden pesticides included the herbicide glyphosate (Roundup® Weed & Grass Concentrate Plus by Bayer CropScience), the fungicide chlorothalonil (Daconil® Fungicide Ready to Use by GardenTech), and the organic insecticide spinosad (Captain Jack's Deadbug Brew® Ready to Use by Bonide). The commercial insecticides included flupyradifurone (Sivanto™ Prime by Bayer CropScience) and sulfoxaflor (Transform WG by Corteva AgriScience). For each pesticide, two dilutions were used (**Table 4.1**). Two control groups, fed DI water, were run alongside the pesticide doses.

Bees were orally exposed to each pesticide dose, or water in the case of control groups, via direct feeding with a micropipette (Williams et al., 2013). A total of 30 female and 30 male bees were used in each treatment group. Immediately after exposure, bees were moved to clean plastic cages with 5 male or 5 female bees per cage. Each cage contained a folded paper towel and a cotton round dipped in 50:50 w/v organic honey solution. Fresh honey solution was provided daily. They were kept at room temperature (22-23°C) and in natural light for 48 hours. Then 8 live bees in each treatment group were placed in a -80°C freezer for storage until dissection.

Sterile Gut Dissections

Two gut tissue replicates were made for each treatment group, with 4 bees in each replicate. The dissection station and dissecting microscope were sterilized with 70% ethanol. Dissections were performed next to a flame in a sterilized petri dish filled with 1X PBS buffer solution. Dissection tools (forceps and small scissors) were sterilized in 90% ethanol and flame before use and between each treatment group and sex. Bees were removed from the freezer and surface sterilized before dissection. They were washed for 30 seconds in 90% ethanol, 30 seconds in 70% ethanol, and then dried for 2 minutes near the flame (Kakumanu et al., 2016). Female bee guts were removed by making a lateral incision down the abdomen and then pulling

the gut out using the stinger. The stinger and venom sack were then separated from the gut. Male bee guts were dissected by opening up the abdomen and pulling a posterior tergite to separate the gut from the thorax. The remaining abdominal exoskeleton was then gently peeled away from the gut to prevent the hindgut from bursting. The guts were placed in a sterile microcentrifuge tube on ice during the dissections and then moved back into the -80°C freezer. Soon after dissections, the guts were shipped to CD Genomics in Shirley, NY for sequencing and analysis.

Sequencing and Bioinformatics Analysis

The following steps were performed by CD Genomics. DNA was extracted from the bee gut tissues. The V3V4 region of the 16S rRNA gene was amplified with PCR, using the primers 341F = CCTAYGGGRBGCASCAG and 806R = GGACTACNNGGGTATCTAAT. PCR products were quantified, mixed, and purified. A paired-end (PE) method on the Illumina platform was used to construct a small fragment library for sequencing. PE 205 sequencing was done for the amplified V3V4 region.

Samples with fewer than 1,000 PE reads were excluded from analysis. The remaining samples underwent quality control and refinement. In QIIME 2 (Caporaso Laboratory, Northern Arizona University in Flagstaff, AZ), the sequences underwent paired reads merging, filtering, and the removal of chimeras. A pre-trained Naïve Bayes classifier and plugin, trained on the Silva 138 99% operational taxonomic units (OTUs), was used to sort the sequences into OTUs and identify the phylogenetic relationships among them.

Rarefaction curves were made to compare number of OTUs per treatment group and to determine the proper sampling depth for comparing alpha diversity metrics among soil microbial communities. Alpha diversity, measured using the Shannon diversity index, was compared among planting groups and sampling times, using a nonparametric Kruskal-Wallis Rank Sum Test in JMP Pro 17 (JMP Statistical Discovery LLC in Cary, NC). Other diversity metrics

(number of phylotypes, ACE, Chao1, and Simpson) were also analyzed using a Kruskal-Wallis Rank Sum Test, in the case of the Simpson index analysis, or an ANOVA, for the other tests, in JMP Pro 17.

Results

Bee Gut Microbiome Community Composition

There were 348 microbial phylotypes found across the 48 bee gut samples. Of these, two were Archaea: *Nitrosopumilus* sp., found in male bees exposed to the lower dose of flupyradifurone and higher dose of sulfoxaflor, and *Woesearchaeales* sp., found in control male samples. The rest of the microbes were all bacteria, with an average of 89 phylotypes per sample. One sample, from female bees exposed to lower dose sulfoxaflor, possessed only one phylotype (**Figure 4.1**), which was likely due to an error in the sample preparation, rather than a true reflection of the gut phylotypes present in the bees.

The most common Phyla present, in order of relative abundance, were Firmacutes (50-70% relative abundance in most samples), Proteobacteria, Bacteroidota, and Actinobacteriota. The overall composition of dominant phyla did not change much based on the pesticide exposure, except for female bees exposed to the lower dose of sulfoxaflor. Their gut communities were almost entirely dominated by Firmacutes (**Figure 4.1**). The most abundant bacterial Classes were Clostridia, Bacilli, Gammaproteobacteria, Bacteroidia, Alphaproteobacteria, and Actinobacteria.

The top 15 most abundant and ubiquitous phylotypes comprised 50-70% relative abundance of most samples (**Figures 4.1** and **4.2**). Female bees exposed to sulfoxaflor were more dominated by *Lactobacillus* sp., had fewer of the top 15 phylotypes present, and a lower relative abundance of “other” bacteria that were not in the top 15 (**Figure 4.1**). Samples with fewer than 1,000 PE reads were excluded from analysis, and while the female sulfoxaflor samples all produced over 1,000 reads, one of the higher dose sulfoxaflor samples and both of

the lower dose sulfoxaflor samples were relatively low, with 1,000-3,000 reads per sample. This could be responsible for the differences in community composition of female sulfoxaflor-exposed bees. Further testing could reveal whether sulfoxaflor can impact the diversity and composition of various bee gut communities.

Several of the taxa in the top 15 most abundant phylotypes have members known to colonize animal guts. These include *Lactobacillus* sp., a genus that is also one of the core *A. mellifera* gut symbionts, *Faecalibacterium* sp., *Ruminococcus* sp. torques group, *Romboutsia* sp., Family Lachnospiraceae sp., *Subdoligranulum* sp., and *Anaerostipes* sp. (**Figures 4.1** and **4.2**). Solitary bees, like *O. cornifrons*, tend to have more varied and environmentally influenced gut communities compared to the social bees. The gut microbes of social bees have clear roles that can influence the health of their hosts, but the influence of gut microbes on solitary bee health is not well established. Their gut communities tend to be more comprised of opportunistic commensals and pathogens.

Solitary bees also have few known bacterial pathogens. *Osmia* spp. can be infected by *Spiroplasma apis* and *Spiroplasma melliferum*, both of which can also infect *A. mellifera* and *Bombus* spp. (Fünfhaus et al., 2018). Neither were present in the *O. cornifrons* gut samples. Several bacterial taxa from the samples have known pathogenic members, though none are known to infect *Osmia* spp. Three phylotypes of Family Enterobacteriaceae were found. Of these, an *Escherichia* or *Shigella* sp. was in the top 15 most abundant taxa. Other genera, including *Stenotrophomonas*, *Mycoplasma*, *Staphylococcus*, and *Bacillus*, include some members that are known animal and human pathogens. *Mycoplasma* spp. use both vertebrate and arthropod hosts, as well. None of these taxa are known to cause disease in *O. cornifrons* or other mason bees.

One potential plant pathogen was found as well, a *Pseudomonas* sp. Some species in the genus, such as *P. syringae*, can cause disease in host plants, whereas others can be helpful for plant growth.

Osmia spp. use mud in their nest building, which may introduce soil bacteria to bee larvae. Common soil bacteria taxa found in the samples included *Acinetobacter* sp., *Stenotrophomonas* sp., and a Family Microbacteriaceae sp. The pollen provision collected by the bee mother can also contain bacteria. Taxa that have been found in floral nectar and were found in the samples included Family Enterobacteriaceae spp., *Stenotrophomonas* sp., *Staphylococcus* sp., and *Pseudomonas* sp. (Álvarez-Pérez et al., 2012; Jacquemyn et al., 2013).

Bee Gut Microbiome Diversity

The diversity of the samples was compared using the number of operational taxonomic units (OTUs) found and the Shannon diversity index values for each sample for the commercial insecticides (**Figure 4.3**) and common garden pesticides (**Figure 4.4**). There was no significant effect of the common garden pesticides on the number of microbial OTUs for males or females. For both males and females, the fungicide chlorothalonil had a noticeably lower number of OTUs compared to the control and other treatment groups. For male bees, the organic insecticide spinosad also had a lower number of OTUs on average, though this was not seen in the female bees (**Figure 4.4A**). Higher replication would likely be needed to see significant differences among the treatment groups, however.

For the commercial insecticides, there was a significant effect of the lower dose sulfoxaflor on the number of OTUs ($F = 2.68$, $DF = 9$, $p = 0.0494$). However, the lower dose sulfoxaflor samples had low numbers of PE reads, which could indicate that the sample preparation process was too sterile for those samples. For female bees, the higher dose sulfoxaflor and higher dose flupyradifurone groups also had noticeably lower numbers of OTUs per sample, though the differences were not significant compared to the control group (**Figure 4.3A**).

Similar trends were seen in the Shannon diversity index values for each treatment group. Sulfoxaflor exposure for female bees had a weakly significant effect on Shannon diversity ($\chi^2(9)$, $p = 0.0688$, **Figure 4.3B**), though again, the low number of reads could indicate some problems with the sample preparation. Flupyradifurone exposure for males and females and sulfoxaflor exposure for males did not affect Shannon diversity.

There was also no significant effect of the common garden pesticides on Shannon diversity (**Figure 4.4B**).

Discussion

Pesticides can vary in their impact on bee gut microbial communities and diversity. *Apis mellifera* exposed to glyphosate, for example, had altered relative abundances of some of their core bacterial phylotypes (Motta et al., 2018). This is not always seen, however, as another study found that glyphosate, difenoconazole, and imidacloprid did not affect the relative abundance of core *A. mellifera* gut bacteria and only altered the abundance of non-core phylotypes (Almasri et al., 2022). *Apis mellifera* bacterial symbionts have also been affected by exposure to the fungicide, chlorothalonil, and the bacterial-derived *Bacillus thuringiensis* (B.t.) toxin (Kakumanu et al., 2016; Steinigeweg et al., 2023; Wu et al., 2022). *Bombus terrestris* gut communities had lower diversity after imidacloprid exposure and changes to the relative abundance of core phylotypes after exposure to flupyradifurone and imidacloprid (Zhang et al., 2022). *Bombus* spp. gut biomes have also had varied responses to glyphosate and to herbicide formulations containing glyphosate. In one study, gut bacterial composition was not affected by glyphosate exposure (Straw et al., 2023). Another, however, found that glyphosate increased bumble bee gut microbial diversity, whereas herbicide formulations containing glyphosate decreased diversity (Helander et al., 2023). The additives within pesticide formulations may also play a role in the pesticide's effect on bee gut symbionts. Additionally, certain bee populations may contain more resistant bacterial strains, and so their gut communities may not be altered

following exposure to certain pesticides. Because of this, bee gut microbial communities can have differing responses to pesticide exposure.

Apis mellifera have consistent core bacterial symbionts, even compared to bumble bees, so changes to community compositions may be more apparent in *A. mellifera* compared to non-*Apis* bees. In this study, *O. cornifrons* gut bacterial communities were not significantly affected by exposure to the common garden pesticides or the commercial insecticides, except for female bees exposed to the lower dose of sulfoxaflor (**Figure 4.3**). The samples from female bees exposed to the lower dose of sulfoxaflor produced a low number of PE reads relative to the other samples, which could indicate an issue during their sample preparation. Because of this, replication is needed to verify these results. Chlorothalonil and spinosad exposure also lowered the alpha diversity of male bee gut communities, though not significantly compared to the control (**Figure 4.4B**). Further replication could help identify whether this was a significant trend in male *O. cornifrons* gut bacterial communities.

By 48 hours after treatment (HAT), the field-realistic doses of glyphosate, chlorothalonil, and spinosad had no impact on *O. cornifrons* male or female survival (**Figure 4.5B**). *Osmia cornifrons* were also much more tolerant of flupyradifurone and sulfoxaflor exposure than other *Osmia* spp., *Osmia lignaria* Say and *Osmia californica* Cresson (**Chapter 2, Figures 2.1-2.3** and **Chapter 4, 4.5A**), though not as tolerant as *A. mellifera* (**Chapter 2, Figures 2.4** and **2.6**). Higher doses of both sulfoxaflor (560 and 56 ng ai bee^{-1}) and flupyradifurone (2200, 220, and 22 ng ai bee^{-1}) resulted in lower survival of *O. cornifrons* females, but lower doses had no noticeable effect. *Osmia cornifrons* was tolerant to low-dose exposure to the commercial insecticides and the common garden pesticides. Because of this, they may not experience physiological changes or impaired health in ways that could affect their gut microbiome after exposure to these pesticides. Future research into the gut microbiome of more sensitive *Osmia* spp. species could help further explain the species-specific differences in pesticide sensitivity.

Bee pathogen prevalence was low across the samples in this study. *Osmia* spp. have been found to harbor *Paenibacillus* sp., the bacterial genus responsible for American foulbrood disease in *A. mellifera* larvae (Voulgari-Kokota et al., 2020). None of the primary bee bacterial pathogens, *Paenibacillus* sp., *Melissococcus plutonius*, or *Spiroplasma* spp. (Fünfhaus et al., 2018) were found in the *O. cornifrons* samples. Several potential animal pathogens were found, including *Stenotrophomonas*, *Mycoplasma*, *Staphylococcus*, and *Bacillus* spp. A *Bacillus* sp. has been linked to higher mortality in *O. bicornis* larvae (Voulgari-Kokota et al., 2020), but it is not known if the particular strains found in *O. cornifrons* can cause clinical disease. Additionally, there were no trends in potential pathogen prevalence based on treatment groups.

Potential plant pathogen prevalence was also low, with only one *Pseudomonas* sp. found. A past study on *O. cornifrons* larvae found a high prevalence and relative abundance of potential plant pathogens, such as *Pseudomonas*, *Ralstonia*, and *Erwinia* spp. (Kueneman et al., 2023). This is not universal across surveyed *Osmia* spp., however (Liu et al., 2023; Lozo et al., 2015; Voulgari-Kokota, Grimmer, et al., 2019), and is likely heavily influenced by region and environmental conditions.

The age and phylogeny of bee hosts can impact their gut bacterial diversity, as well. For *Osmia* spp., adults tend to have higher species richness and more variability in their gut communities than larvae (Kueneman et al., 2023; Liu et al., 2023; Lozo et al., 2015). Larvae remain in a more sheltered environment, inside their nest cells, and so most of their bacterial exposure is from their pollen provision and mud partitions (Abrol, 2012; Kueneman et al., 2023; Liu et al., 2023; Voulgari-Kokota, Grimmer, et al., 2019; Voulgari-Kokota, McFrederick, et al., 2019; Voulgari-Kokota et al., 2020). Adults, however, can be exposed to a wide variety of bacteria while out foraging and finding nest sites. For lab-reared bees, adults may be exposed to more environmental bacteria and to bacteria in their food source, following their emergence from cocoons. Solitary bees also have high species richness and greater community variation compared to social bees. *Bombus terrestris*, for example, were dominated by 10 bacteria

phylotypes, with other bacteria comprising less than 5% of their composition (Straw et al., 2023). For *O. cornifrons*, after the top 15 most abundant phylotypes, the other bacteria make up 30-50% of the community. Similar diversity, richness, and variation is seen in many solitary bees (Tuerlings et al., 2023; Voulgari-Kokota, Grimmer, et al., 2019). Several of the taxa found in *O. cornifrons* guts have also been found in other surveys of *Osmia* spp. (*O. cornuta, O. lignaria*, and *O. excavata*) gut microbial communities, such as bacteria within the genera *Lactobacillus, Acinetobacter*, and *Sodalis*, and the families, Clostridiaceae and Enterobacteriaceae (Cohen et al., 2020; Liu et al., 2023; Lozo et al., 2015). Some taxa found in this study have not been commonly observed in other *Osmia* spp. gut communities, including *Faelibacterium* spp. and *Ruminococcus* spp. The *O. cornifrons* used in this study were lab reared bees, and so may have differed more in their gut bacterial communities than wild, naturally foraging *Osmia* spp. bees.

Conclusions

Pesticides can negatively impact the health and functioning of many bee species and their gut microbial communities. *Osmia cornifrons* is fairly tolerant to certain pesticide exposure, especially compared to other *Osmia* spp. like *O. lignaria* and *O. californica*. The bacterial gut communities of *O. cornifrons* males and females were varied and diverse, as is common for many solitary bees. The 15 most common taxa found in the O. cornifrons samples accounted for 50-70% of the community relative abundance, and most of the samples contained dozen more bacterial phylotypes. The gut bacterial communities were not strongly perturbed by a single exposure to low doses of flupyradifurone and sulfoxaflor or to doses of glyphosate, chlorothalonil, or spinosad. The exception to this was for female bees following lower dose sulfoxaflor exposure, which had an increased relative abundance of *Lactobacillus* sp. and lower diversity overall. High doses of the insecticides, flupyradifurone and sulfoxaflor, can increase mortality of *O. cornifrons* and other *Osmia* spp., and so caution should be taken, especially

when applying foliar sprays of these insecticides, to limit bee exposure and environmental accumulation of the pesticides. The impacts of these pesticides on *O. cornifrons* health may not be related to alterations in bee gut communities, though further testing of sulfoxaflor-exposed female bees and chlorothalonil- and spinosad-exposed male bees is needed.

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Table 4.1. Pesticide formulations and doses.

Active Ingredient	Formulation	Doses (ng ai bee^{-1})	Notes on Dose
Flupyradifurone	Sivanto Prime	0.22	Ten-thousand fold dilution of lowest field use concentration (FUC)
		0.97	LD_{20} value for *O. lignaria*
Sulfoxaflor	Transform WG	0.056	Ten-thousand fold dilution of lowest FUC
		0.52	LD_{20} value for *O. lignaria*
Spinosad	Captain Jack's Deadbug Brew® Ready to Use	0.19	Hundred-fold dilution of the ready to use (RTU) concentration
		19	RTU concentration
Glyphosate	Roundup® Weed & Grass Concentrate Plus	6400	Lowest FUC
		12800	Highest FUC
Chlorothalonil	Daconil® Fungicide Ready to Use	0.021	Hundred-fold dilution of the RTU concentration
		2.1	RTU concentration

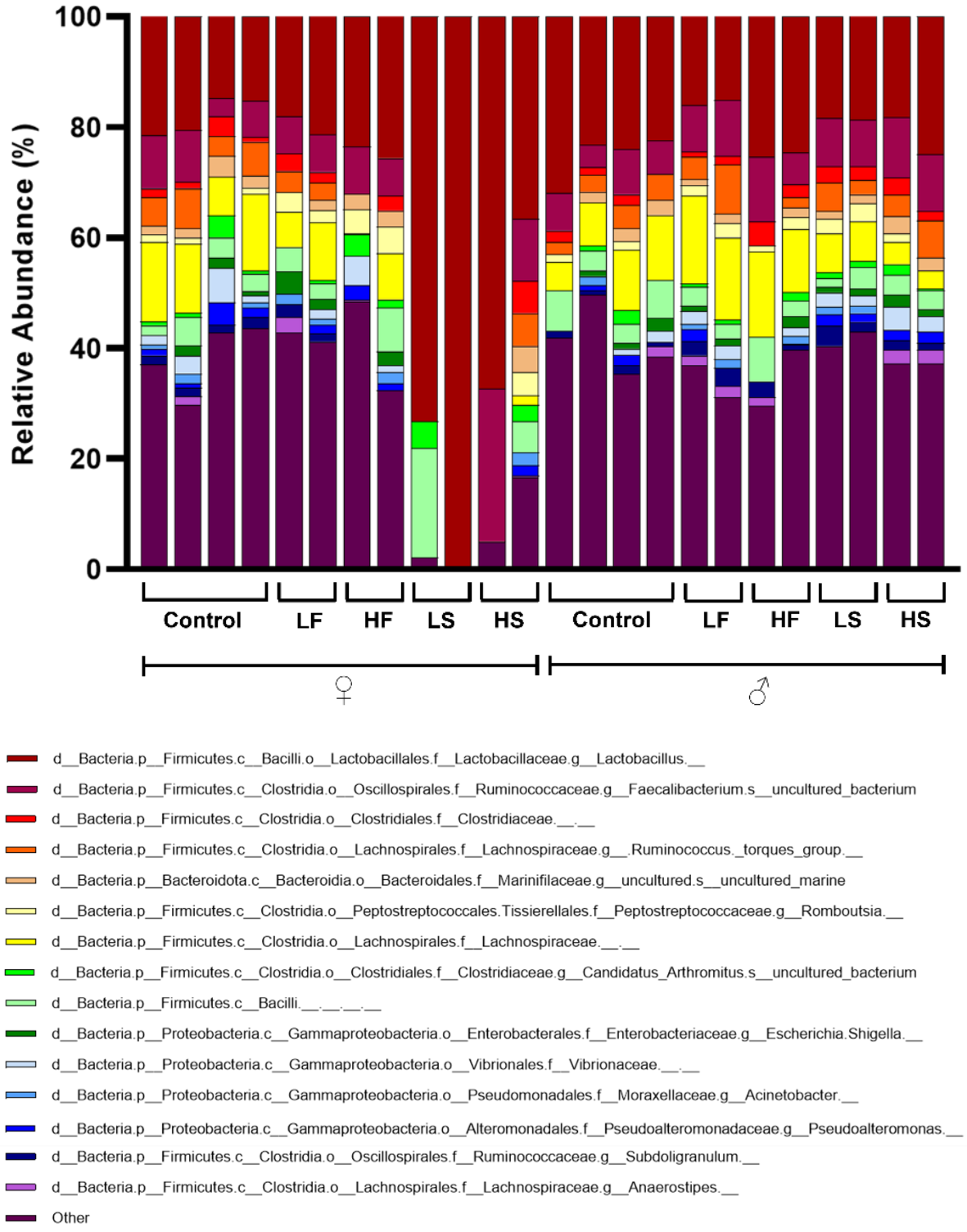

Figure 4.1. The relative abundance of the 15 most abundant and ubiquitous bacterial phylotypes in the guts of *O. cornifrons* females and males 48 hours after exposures to DI water (Control), 0.22 ng ai bee^{-1} flupyradifurone (LF), 0.97 ng ai bee^{-1} flupyradifurone (HF), 0.056 ng ai bee^{-1} sulfoxaflor (LS), or 0.52 ng ai bee^{-1} sulfoxaflor (HS).

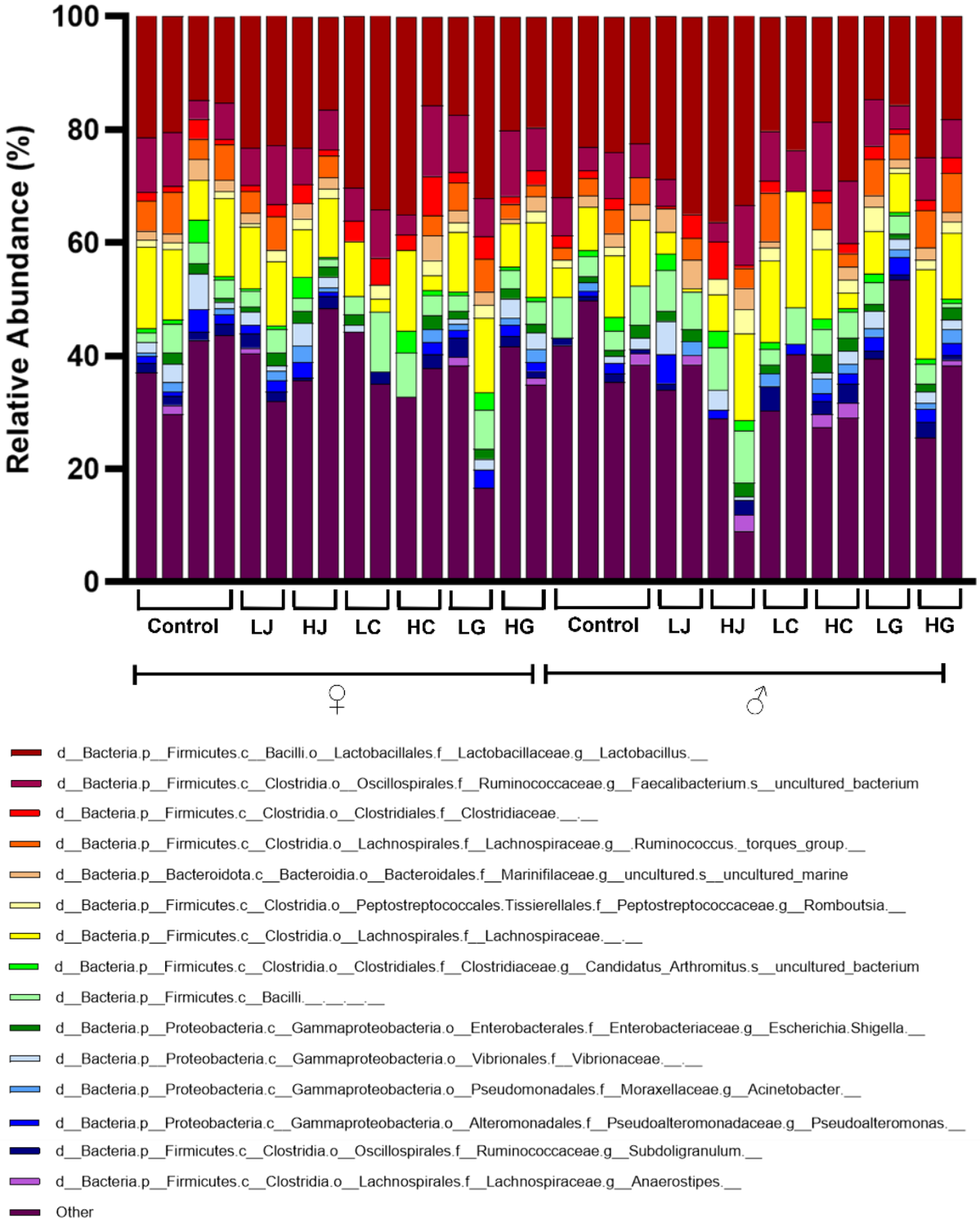

Figure 4.2. The relative abundance of the 15 most abundant and ubiquitous bacterial phylotypes in the guts of *O. cornifrons* females and males 48 hours after exposures to DI water (Control), 0.19 ng ai bee^{-1} spinosad (LJ), 19.0 ng ai bee^{-1} spinosad (HJ), 0.021 ng ai bee^{-1} chlorothalonil (LC), 2.1 ng ai bee^{-1} chlorothalonil (HC), 6400 ng ai bee^{-1} glyphosate (LG), or 12800 ng ai bee^{-1} glyphosate (HG).

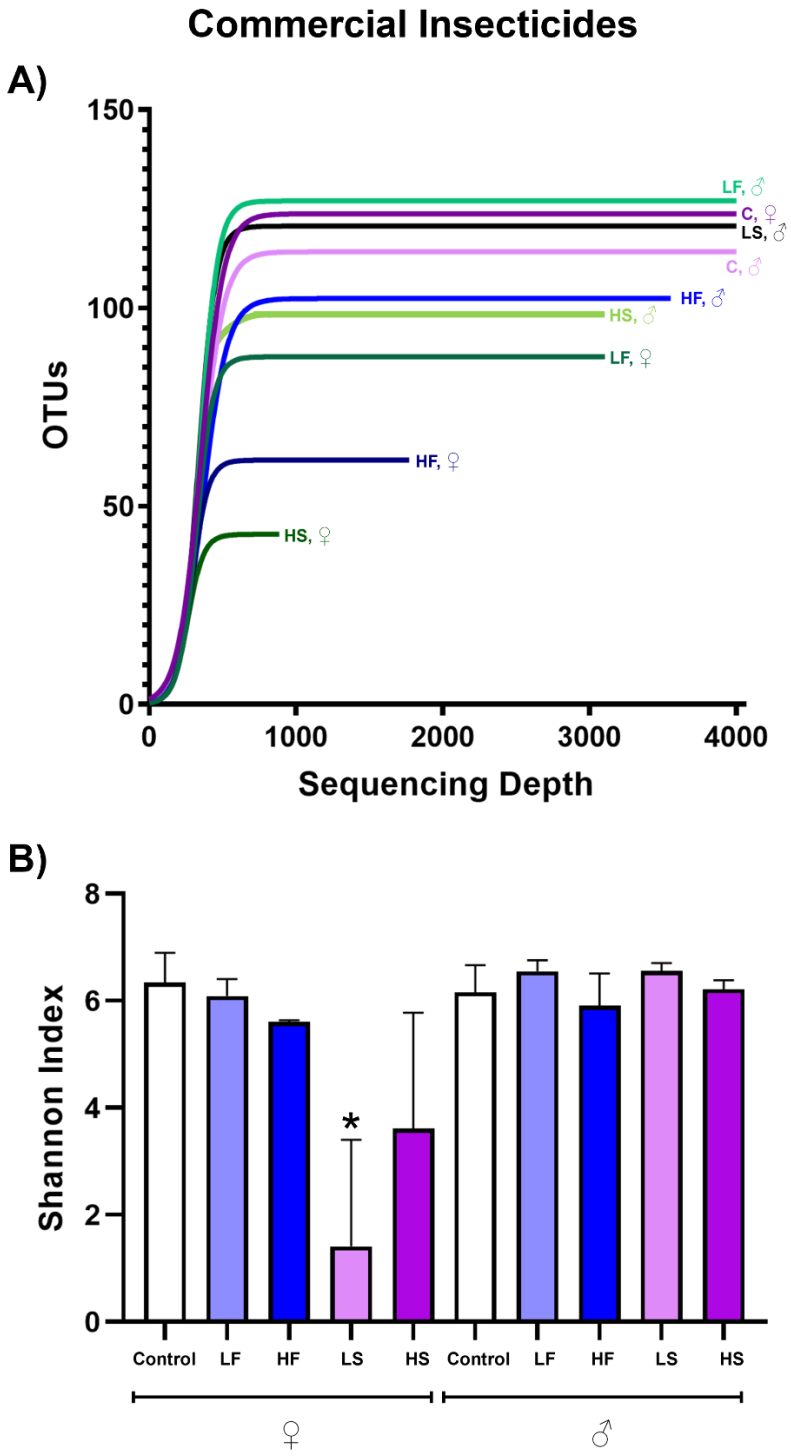

Figure 4.3. Rarefaction curves showing the number of operation taxonomic units (OTUs) by sequencing depth for *O. cornifrons* males and females 48 hours after exposure to commercial insecticides (A). Shannon diversity indexes for bees after exposure (B). Treatment groups were 0.22 ng ai bee^{-1} flupyradifurone (LF), 0.97 ng ai bee^{-1} flupyradifurone (HF), 0.056 ng ai bee^{-1} sulfoxaflor (LS), 0.52 ng ai bee^{-1} sulfoxaflor (HS), or control bees (C). Samples with fewer than 1,000 PE reads were excluded.

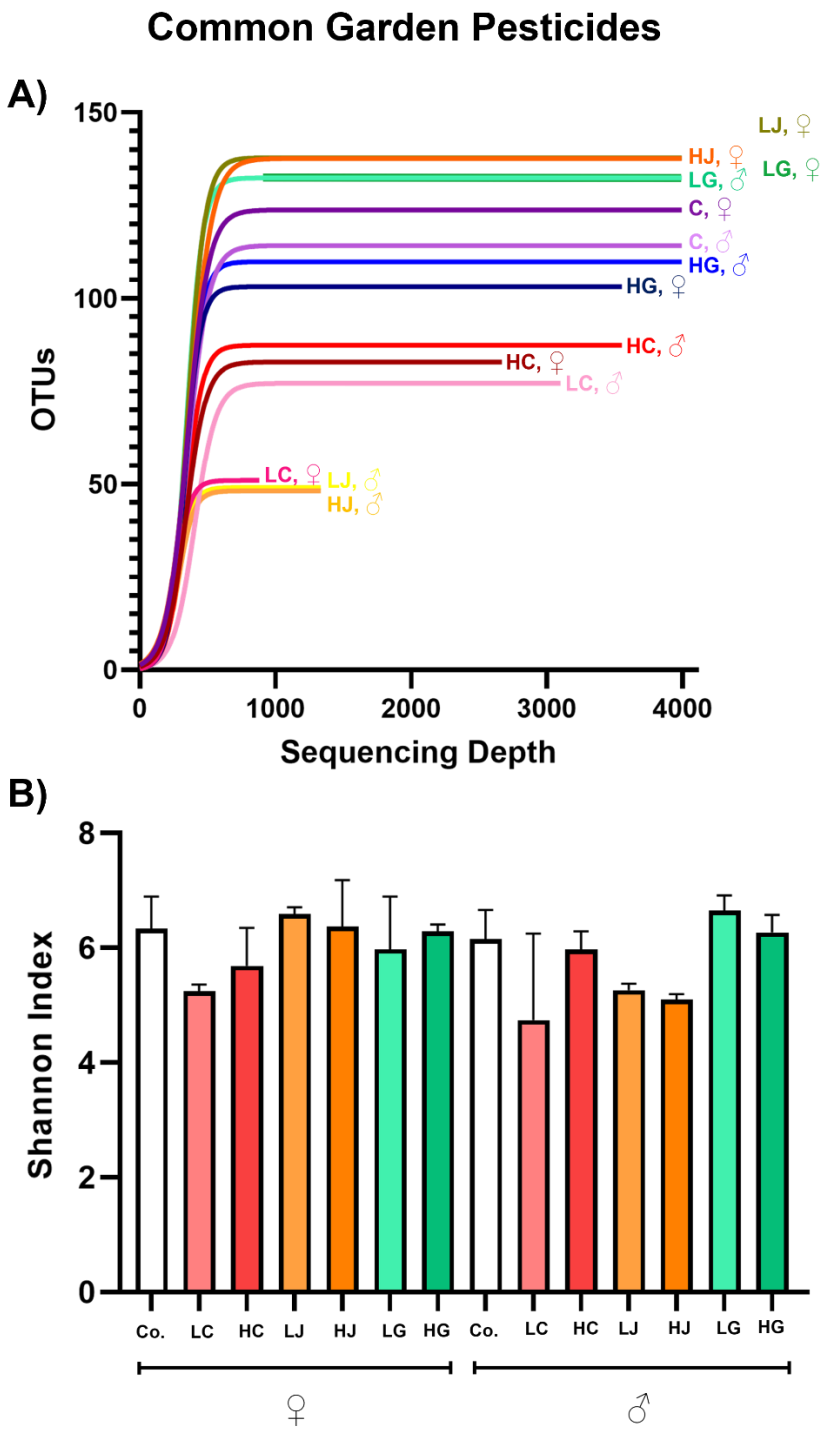

Figure 4.4. Rarefaction curves showing the number of operation taxonomic units (OTUs) by sequencing depth for *O. cornifrons* males and females 48 hours after exposure to common garden pesticides (A) and Shannon diversity indexes for bees after exposure (B). Treatment groups were 0.19 ng ai bee^{-1} spinosad (LJ), 19.0 ng ai bee^{-1} spinosad (HJ), 0.021 ng ai bee^{-1} chlorothalonil (LC), 2.1 ng ai bee^{-1} chlorothalonil (HC), 6400 ng ai bee^{-1} glyphosate (LG), or 12800 ng ai bee^{-1} glyphosate (HG), and control (C/Co). Samples with fewer than 1,000 PE reads were excluded.

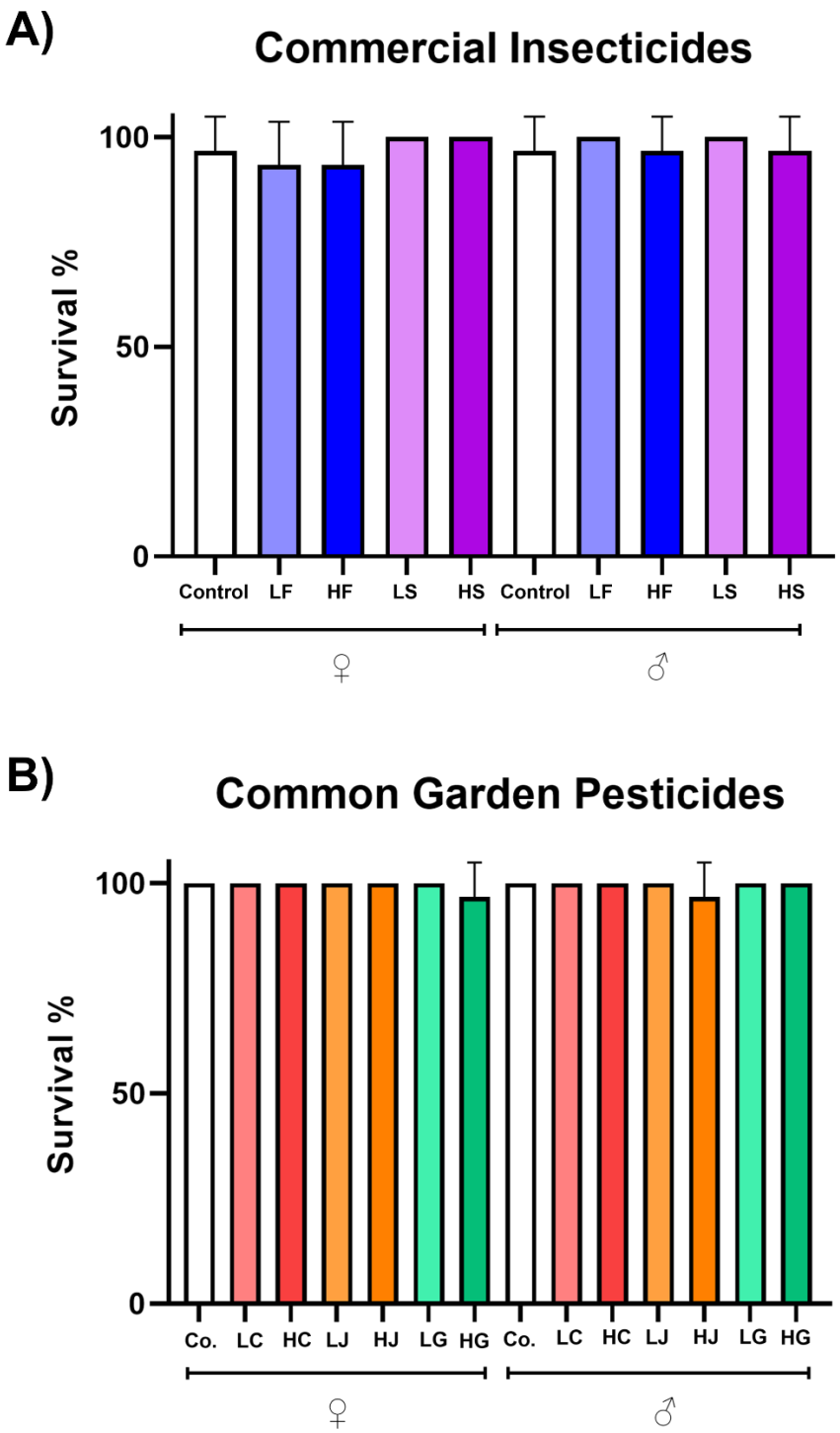

Figure 4.5. The survival of bees after exposure to commercial insecticides (A) or common garden pesticides. Commercial insecticide treatment groups were 0.22 ng ai bee^{-1} flupyradifurone (LF), 0.97 ng ai bee^{-1} flupyradifurone (HF), 0.056 ng ai bee^{-1} sulfoxaflor (LS), 0.52 ng ai bee^{-1} sulfoxaflor (HS), or control bees. Common garden pesticides were were 0.19 ng ai bee^{-1} spinosad (LJ), 19.0 ng ai bee^{-1} spinosad (HJ), 0.021 ng ai bee^{-1} chlorothalonil (LC), 2.1 ng ai bee^{-1} chlorothalonil (HC), 6400 ng ai bee^{-1} glyphosate (LG), or 12800 ng ai bee^{-1} glyphosate (HG), and control (Co).

Chapter 5:

The Effect of Diverse Plant Communities on Blue Orchard Bee (*Osmia lignaria*) Health and Soil Quality

Abstract

Many crop systems use large unbroken monocultures, which may not support a diverse community of bee pollinators. In this study, we used the polylectic mason bee, *Osmia lignaria*, to test the impacts of buckwheat plantings versus wildflower plantings on bee behavior and fecundity. Bees were released in semi-field cages that had been planted with either buckwheat or a wildflower mix. They were observed in their foraging activity, nesting activity, and fecundity (number of offspring produced). Bees in the wildflower mix cages had higher nesting and foraging activity and were able to produce live offspring. Bees in the buckwheat cages had lower foraging activity, minimal nesting activity, and produced no offspring. We also tested the soil nutrient content and bacterial communities in each planting group. Soil nutrition and microbiome compositions were similar across planting groups, though more potential plant-associated nitrogen fixers were present in wildflower plots. Overall, the wildflower plantings improved bee foraging activity and fecundity compared to buckwheat alone, while having a neutral or positive impact on soil nutrition.

Introduction

Modern agriculture often favors unbroken monoculture plantings, which have replaced many diverse natural habitats (Goulson et al., 2015; Rands & Whitney, 2011). The reduction of floral diversity in agricultural areas can greatly impact the local ecosystems, reduce pollinator abundance and species richness, and alter soil properties, such as the pH, nutrient content, and composition of soil microbial communities. Agricultural landscapes often have a lower abundance and diversity of insects compared to natural, semi-natural, and urban environments (Guenat et al., 2019; Samuelson et al., 2018; Theodorou et al., 2020), likely due to the lack of

plant diversity and heavy pesticide use in these areas. Supplementing certain crop monocultures with wildflower planting may help improve pollinator populations and activity in agricultural areas.

Mason bees (*Osmia* spp., Hymenoptera: Megachilidae) are solitary, tunnel-nesting bees that provide a valuable pollination service to many wildflowers and agricultural crops. Several species are commercially managed globally, often for the pollination of fruit trees. These include *Osmia lignaria* Say in North America, *Osmia cornifrons* Radoszkowski in eastern Asia and the northeastern United States, and *Osmia bicornis* L. in Europe (Batra, 1982; Bosch & Kemp, 2002; Gruber et al., 2011; Sedivy & Dorn, 2014). Additionally, many crops can benefit from having a diverse community of pollinators (Albrecht et al., 2012; Hoehn et al., 2008; Kremen et al., 2002; Lowenstein et al., 2015). While much is known about honey bee (*Apis mellifera* L., Hymenoptera: Apidae) management in many crop systems, there is less information available on solitary and other non-*Apis* species. Investigating the nesting site, nesting material, and flower preferences of various non-*Apis* bees can help maintain healthy and effective pollinators for both managed and wild populations.

In the wild, *Osmia* spp. often build their nests in pre-existing tunnels, made from hollow reeds, stems, or abandoned beetle holes (Levin, 1966; Torchio, 1989). For managed *Osmia* spp., artificial nest boxes have been used to promote nesting. These nest boxes can be filled with a variety of tubes, including bamboo, cardboard, glass, and plastic, with varying degrees of success (Levin & Haydak, 1957; Phillips & Klostermeyer, 1978; Wilkaniec & Giejdasz, 2003). *Osmia* spp. also need access to materials for building nest partitions, usually mud and leaves, and clean water (Cane et al., 2007; Torchio, 1989). Although *Osmia* spp. are solitary, many species are gregarious nesters, and can be kept in high densities in artificial nest boxes, as long as there are plentiful floral resources to support them (Artz et al., 2013).

Bees feed on pollen as their primary source of protein and nectar as their source of carbohydrates (Cane, 2016; Hanley et al., 2008; Williams & Tepedino, 2003). The foraging

behavior and efficiency of bees can vary among species. Social *A. mellifera* foragers will often specialize collection on their foraging bouts, gathering nectar or pollen, but not both. *Osmia lignaria*, however, will usually collect pollen and nectar together on each trip from the nest (Tepedino & Parker, 1982; Vicens & Bosch, 2000). *Osmia* spp. females collect pollen and nectar to provision their offspring, as well as for their own consumption (Claus et al., 2021; Torchio, 1989). *Osmia* spp. males only consumer nectar, though they still have high contact with the flower stigmas and can contribute to pollination (Vicens & Bosch, 2000).

As with many bees, *Osmia* spp. require an abundance of flowers to forage from. The amount of food available to foraging female bees can contribute to larger provisions, larger offspring, and higher fecundity (Bosch & Vicens, 2006). *Osmia* spp. males tend to be smaller than females and tend to be reared on a smaller pollen and nectar provision (Bosch, 1994). It has been noted that harsh annual conditions, which limit floral availability, can result in a higher ratio of male:female offspring than in years with plentiful flowers (Bosch & Vicens, 2006). Bees also have specific nutritional requirements that must be met in their diet. *Bombus* spp., for example, are more likely to visit flowers with higher protein concentrations in their pollen (Hanley et al., 2008). Larvae of the sweat bee, *Lassioglossum zephyrus* Smith, developed into larger adults when reared on high-protein pollen than larvae fed an equivalent amount of low-protein pollen (Roulston & Cane, 2002). A diverse diet has been linked to higher immunity, measured as haemocyte concentration, phenoloxidase activity, glucose oxidase activity, and fat body content, in the generalist-feeding *A. mellifera*. The amount of pollen in the diet did not affect these immunocompetence markers, but the number of flower species represented did (Alaux et al., 2010). For other generalist feeders, such as *O. lignaria*, a limited diet diversity could contribute to malnutrition and immunocompromise. *Osmia lignaria* prefer to collect pollen from multiple flower species, even when they must fly farther from their nests to do so (Williams & Tepedino, 2003). By investigating the nesting and feeding preferences of different bee species, mitigation and conservation strategies can be better developed and implemented.

As well as its impact on pollinators, agricultural intensification can impact soil nutrient availability, microbial communities, and productivity (Gaudin et al., 2015; Trivedi et al., 2016). Soil health has been defined in a number of ways over the years, but in broad terms describes the ability of soil to support agricultural production, while at the same time not causing harm to surrounding ecosystems and water supplies. Soil provides multiple environmental functions, including water filtration, improvements to air quality, and encouraging plant growth, and needs to have proper health in order to fulfill these services (Nielsen & Winding, 2002; Trivedi et al., 2016). There are several markers of soil health that can be assessed, including nutrient concentrations, soil microbial community diversity and composition, pH, and soil chemical properties.

Many soil nutrients, including nitrogen, phosphorus, and potassium, are essential for plant growth and can cause recognizable disease when soil is deficient (Shrivastav et al., 2020). The nutrient content in the soil has a great impact on the rate of plant growth and the species of plants that will thrive in a certain soil (Barker & Pilbeam, 2006; Hengl et al., 2017), but can also affect the living organisms within the soil. One study found that the nematode, *Strongyloides stercoralis* Bavay, a parasite of humans, posed a lower threat of infection in high carbon soils (Khieu et al., 2014). Additionally, some nutrients are not required by plants, but too high a concentration can cause harm and reduced yields, as in seen in overly salinized soils (Machado & Serralheiro, 2017). Management practices, such as crop rotation, tilling, and specific plants can impact nutrient content, and monitoring these nutrients can help assess the soil productivity.

Soil microbial communities can also greatly impact plant growth, though many soil phylotypes are not well known. Many collected soil bacteria, for example, do not match a previously recorded genome, and the ecological role of even commonly found phylotypes is poorly understood (Delgado-Baquerizo et al., 2018). The diversity of bacteria phylotypes in soil communities is incredible high, even for samples located close to each other (Delgado-Baquerizo et al., 2018; Ramirez et al., 2014). In spite of the mystery surrounding many soil

microbes, they have been linked to many essential ecosystem functions, including promoting plant growth, nutrient cycling, and preventing plant pathogens and pests from invading (Anith et al., 2004; Babalola, 2010; Bhagwat & Thomas, 1982; Coronado et al., 1995; Delgado-Baquerizo et al., 2018). Some can also be a source for antibiotics and natural insecticides (Salgado et al., 1998). The composition of soil microbial communities is important to human and plant health, as a balanced community can help prevent the overabundance of disease-causing organisms (Wall et al., 2015).

Finally, plants can vary in their preferences for ideal soil pH, though most grow and produce best at a pH between 5.0-8.0 (McCall, 1980). Soil pH can also impact nutrient solubility and microbial diversity (Chu et al., 2010; Fernández & Hoeft, 2012; Lauber et al., 2009). Management practices can impact soil pH, with agricultural soils tending to have a higher pH, though this can vary greatly by region (Trivedi et al., 2016). Monitoring soil pH can be an indicator for changes in management practices and can inform what plants will be able to grow successfully in the area. However, soil pH ranges outside of a crop's ideal range could limit growth and yield.

There have been efforts to ameliorate some of the negative effects of intensive agriculture on soil health. Use of crop rotation and adding mixed plantings have been shown to cause higher returns on nitrogen, phosphorus, and potassium (Wang et al. 2008). Adding hedgerows and shelterbelts around fields can provide better erosion control (Long and Anderson 2010). The interactions between soil microbes, plant growth, and insects can be complex, especially in changing environments, but are necessary for understanding and implementing more sustainable agricultural practices.

In this semi-field study, we compared the impact of a buckwheat monoculture versus two different types of wildflower mixes, on *O. lignaria* activity and fecundity and on soil nutrient content, pH, and microbial communities. The proper management and conservation of *O. lignaria* and other *Osmia* spp. species requires a thorough understanding of their dietary needs.

Additionally, it is important for any wildflower planting schemes to maintain or even improve soil quality around crop lands.

Materials and Methods

Site Preparation and Cage Set-Up

This semi-field study was conducted at the University of Arkansas Milo J. Shult Agricultural Research and Extension Center in Fayetteville, AR. Twelve research plots were selected, measuring 2.5 x 1.5 m, and spaced 2.7-3 m apart. Wood-frame bee cages, measuring 2.5 x 1.5 x 1.5 m, were constructed over each field plot. A sheet of hardware cloth was placed over the top of each cage for added support and then the cages were covered in black aluminum mesh screen (Phifer Incorporated, Tuscaloosa, AL; **Figures 5.1A** and **5.1B**). A 15 cm wide fiberglass mesh "skirt" lined the bottom of the cages and was buried into the soil. The cage frames were constructed from March-May 2020 and the cages were completed February-April 2021.

The research plots were labelled A-L and randomly sorted into 3 planting groups: buckwheat (*Fagopyrum esculentum* Moench; plots A, D, F, and L), honey bee wildflower mix (plots C, E, I, and K), and eastern pollinator wildflower mix (plots B, G, H, and J). The buckwheat seeds were purchased from Main Street Seed and Supply (Bay City, MI) in 2020 and from Orscheln Farm and Home Store (Springdale, AR) in 2021 and 2022. The two wildflower mixes were both ordered from the Sustainable Seed Company (now True Leaf Market in Salt Lake City, UT). Supplementary native blooming flowers were purchased from White River Nursery in Fayetteville, AR to ensure full coverage of the wildflower plots with flowers in bloom (**Table 5.1**). Before the buckwheat and wildflower plots were established in 2020, the area had been a frequently-mowed, but unplanted, grassy field.

In 2020, all plots were manually tilled with a shovel and hoes. Wild flowering plants and grasses were removed from all plots before the seeds were planted. In 2021 and 2022, the

buckwheat plots were tilled as before, but the wildflower plots were allowed to keep growing from the previous year. Weeds, if present, were removed by hand from the wildflower plots.

Seeds were planted using broadcast application in late spring (April-May) of each year. Wildflowers growing wild at the farm, including red clover (*Trifolium pratense* L.) and butterfly weed (*Asclepias tuberosa*), were removed from the buckwheat plots, but allowed to continue growing in the wildflower mix plots. Supplementary native wildflowers from White River Nursery were planted as needed to fill bare spots in the plots. Overall wildflower diversity and abundance was higher in the 2021 season, due to drought and heat waves in the 2022 season.

Each field cage contained a nest box (**Figure 5.1C**), water dish, and sand dish. Nest boxes stands were secured between metal T-posts approximately 3’ off the ground, all facing south. Each of the wooden nest box stands had a plastic roof and awning to prevent rain damage on nest boxes (**Figure 5.1A**). In 2021, two nest boxes were placed in each stand, one with clear plastic straws and one with paper straws lining the nesting tunnels. In 2022, one nest box with white paper straw liners were placed in each stand. Water dishes, made from a plastic bowl filled with clean rocks, were placed beneath the nest box stand in each cage. A dish of river sand, purchased from White River Nursery, was also put in the nest box stands. Blue orchard bees (*Osmia lignaria*) and other *Osmia* spp. use mud to construct partitions and caps in their nest tunnels and have preferences for the soil textures they use (Cane, 1991; Cane et al., 2007). The soil at the farm was primarily clay and silt loam, so providing sand for the bees allowed them to mix soil as needed to their preferred texture.

Bee Acquisition and Release

Osmia lignaria cocoons (adults in winter diapause) were acquired from a commercial supplier (Watts Solitary Bees in Bothell, WA) in March 2021 and 2022. They were kept at 4°C to delay and synchronize emergence. Before release, the cocoons were taken out of the fridge and placed in plastic cups filled with paper towels and cotton rounds soaked in 50:50 w/v

organic honey (Nature Nate's Honey Co. in McKinney, TX) solution, and allowed to emerge at room temperature (22-23°C).

Bees were not released in 2020, as cage construction had not been completed, but were released in June-July 2021 and 2022, to coincide with the bloom period of the planted flowers. In the summer of 2021, 50 female and ~25 males were released into each cage. In 2022, this was lessened slightly to 40 females and 8 males into each cage.

Immediately after their release, the bees were seen mating and beginning to forage on flowers. Over the following weeks, bees were observed for the following behavioral endpoints: number of bees foraging in each cage, number of floral visits within a 2-minute period, floral preferences, number of nest visits within a 2-minute period. Observations were made 2-4 times per week at 3 times of day: morning (9:00-10:00 am), noon (12:00-1:00 pm), and late afternoon (6:00-7:00 pm).

Osmia lignaria live about 20-30 days following their emergence from cocoons, during which time they typically lay 10-30 eggs (Torchio, 1989). In 2021, adult female bees lived and were actively foraging for over a month following release. In 2022, however, the bees died prematurely, before nesting could be completed, likely due to the heat waves and droughts in Arkansas. The nest boxes were then left undisturbed over the autumn and winter to allow the offspring to undergo growth and pupation.

The nest boxes were collected in the spring of 2021, following the 2021 foraging period for the adult bees. The following endpoints were measure for each nest box: number of capped nest tubes, number of nest tubes with mud or pollen residues, total number of brood cells, number of cells with pollen provisions, number of cells with larvae (that did not pupate), and number of cells with cocoons. Insect parasites, parasitoids, or scavengers present in the nest tubes were also collected. Cocoons were removed from the brood cells, weighed, and then placed in clean plastic cages lined with paper towels. They were allowed to emerge at room

temperature (22-23°C). Adult bees that emerged from the cocoons were provided with 50:50 w/v organic honey solution each day and the length of their adult lifespan was measured.

Soil Collection and Analysis

In 2020, 2021, and 2022, pre- and post-planting soil samples were collected for nutrient and pH analysis. Pre-planting samples were taken in early spring, March-April, and post-planting samples were taken in early fall, September, of each year. Samples were taken from each research plot, as well as three samples taken from an adjacent grassy field for comparison. The grassy field was frequently mowed, but never tilled or planted. The soil samples were taken with a 15 cm soil probe with 7 cores taken from each plot and homogenized in a bucket. The samples, which amounted to ~475 mL per plot, were laid out to dry for a week and then sent to the Marianna Soil Test Laboratory in Marianna, AR for analysis. The analysis included the concentrations of phosphorus, potassium, zinc, sulfate-S, nitrate, magnesium, iron, calcium, sodium, manganese, copper, and boron in the soil, as well as the pH and the estimated cation exchange capacity (CEC).

In 2022, soil samples were taken for soil microbial community analysis, as well. The tools: soil probe, bucket, sieve, and work table, were sterilized with 70% ethanol before use and between each planting group. When the 7 soil cores were taken for the nutrient analysis, a small amount, ~56.7 g, was set aside on ice and then transferred to a -80°C freezer for storage until analysis. In March 2023, the soil samples were sent to CD Genomics in Shirley, NY, for DNA extraction, isolation, and sequencing.

The following steps were performed by CD Genomics. DNA was extracted from the soil samples. The V3V4 region of the 16S rRNA gene was amplified with PCR, using the primers 341F = CCTAYGGGRBGCASCAG and 806R = GGACTACNNGGGTATCTAAT. PCR products were quantified, mixed, and purified. A paired-end (PE) method on the Illumina platform was

used to construct a small fragment library for sequencing. PE 205 sequencing was done for the amplified V3V4 region.

Samples with fewer than 1,000 PE reads were excluded from analysis. The remaining samples underwent quality control and refinement. In QIIME 2 (Caporaso Laboratory, Northern Arizona University in Flagstaff, AZ, the sequences underwent paired reads merging, filtering, and the removal of chimeras. A pre-trained Naïve Bayes classifier and plugin, trained on the Silva 138 99% operational taxonomic units (OTUs), was used to sort the sequences into OTUs and identify the phylogenetic relationships among them.

Data Analysis

Differences in bee foraging activity, nesting activity, fecundity endpoints, and post-planting soil nutrient concentrations and pH among treatment groups were compared in JMP Pro 17 (JMP Statistical Discovery LLC in Cary, NC) with a nonparametric Kruskal-Wallis Rank Sum Test for each year of the study. A Dunn's All-Pairs Rank Comparison Test was also done to see significant differences between specific groups. The pre-planting and post-planting soil nutrients were compared for each year of the project using a pooled *t*-test in JMP Pro 17.

A rarefaction curve was made to determine the proper sampling depth for comparing alpha diversity metrics among soil microbial communities. Alpha diversity, measured using the Shannon diversity index, was compared among planting groups and sampling times, using a nonparametric Kruskal-Wallis Rank Sum Test in JMP Pro 17. Other diversity metrics (number of phylotypes, ACE, Chao1, and Simpson) were also analyzed using a Kruskal-Wallis Rank Sum Test, in the case of the Simpson index analysis, or an ANOVA, for the other tests, in JMP Pro 17.

Results

Bee Foraging Activity

The activity period of *O. lignaria* was longer in 2021 (about 4-6 weeks) than in 2022 (about 2 weeks). Compared to the buckwheat planting, bees in the cages planted with the honey wildflower mix and the eastern wildflower mix both had a significantly higher average of floral visits in 2021 ($\chi^2(2) = 31.66$, $p<0.0001$; **Figure 5.2**) and a higher number of total floral visitations throughout the bee activity period (**Table 5.2**). During the 2022 season, wildflower mix plots also had higher average floral visits than in the buckwheat plots, though only the honey bee wildflower mix planting groups had significantly higher average floral visits ($\chi^2(2) = 11.06$, $p=0.04$, **Figure 5.2**). The adult foraging bees also had better longevity in the wildflower planting groups than those in the buckwheat groups for both 2021 and 2022. Bees in the wildflower cages were active for 1-2 weeks after the bees in the buckwheat cages were no longer seen.

Osmia lignaria in the wildflower cages also showed strong feeding preferences for certain wildflowers, including lemon bee balm (*Monarda citriodora* Cervantes), *Rudbeckia hirta* L.*, A. tuberosa, Heliopsis helianthoides* L.*, Coreopsis lanceolata* L.*,* and *Gaillardia aristata* Pursh (**Table 5.2**).

Foraging activity was also higher in the morning observations and lower in the late afternoon, but not significantly so.

Bee Nesting Activity

Female bees nest visits were first observed 7 days after the bees' release into the cages. The first tubes that were capped with mud were observed 14 days after the release. In 2021, the average number of nest visits during the 2-minute observation period was significantly higher for bees in the eastern and honey bee wildflower mix groups than those in the buckwheat groups ($\chi^2(2) = 21.15$, $p<0.0001$). In 2022, nesting activity was low across all planting groups.

Osmia lignaria females in the buckwheat groups were not observed visiting the nest boxes. Those in the wildflower planted cages only had a few observed trips to the nest (**Figure 5.3**). There was no significant difference in nesting trips among planting groups in 2022.

The bees were also provided with both plastic or paper nesting tube liners. The transparent plastic tubes were included in hopes of being able to observe the development of the offspring throughout their life stages. However, the bees did not nest in the plastic tubes when paper was available. The plastic tubes may have retained more humidity and heat than the paper tubes.

Bee Fecundity

Bee fecundity was measured by the following endpoints: number of capped tubes, tubes with mud residue, total number of brood cells, number of pollen provisions (eggs that did not hatch into larvae), number of larvae (that did not pupate), and number of cocoons. Each of the brood cells could either be empty, contain a pollen provision with an unhatched egg, contain a dead larva that never pupated, or contain a cocoon. Offspring and successful nesting were only observed following the 2021 season. Bees in the buckwheat cage had a few observed trips to the nest box, but no brood cells, pollen provisions, or offspring. The honey bee wildflower mix group had a significantly higher number of tubes with mud ($\chi^2(2) = 8.17$, $p = 0.0168$), number of brood cells ($\chi^2(2) = 7.77$, $p = 0.0205$), and pollen provisions ($\chi^2(2) = 7.17$, $p = 0.0278$) compared to the buckwheat planting group (**Figures 5.4B-D**). Both the eastern and honey bee wildflower mix planting groups had a higher number of capped tubes, larvae, and cocoons, though these differences were not significant (**Figures 5.4A** and **5.4E-F**), likely due to the low replication of four plots per planting group. Three of the honey bee wildflower mix cages and one of the eastern wildflower mix cages managed to produce live offspring.

Two types of nest parasites were observed in some of the nest tubes, Dermestid beetle larvae (possibly *Trogoderma* sp.) and also barklice (*Liposceles* sp.). Three cocoons had been

chewed open and were empty due to the beetle larvae feeding. However, most Dermestid beetles are scavengers, so they were likely feeding on bee pupae that had already died. The *Liposceles* sp. were found in cells with mold, which was likely their food source.

A total of 27 cocoons were produced, of which 3 were eaten by the Dermestid larvae, 8 never emerged and 16 successfully emerged as adults. There was a strong male bias for the emerged adult offspring, with 14 males and only 2 females. Seven of the offspring lived over 7 days and three of them lived over 20 days (**Table 5.3**).

In the summer of 2022, the field site in Arkansas, as well as many other areas around the region, experienced an extended drought and heat wave. Wildflower diversity was poor 2022, compared to 2021, and bee health, fecundity, and longevity were poor overall, in all treatment groups.

Soil Nutrient Analysis

The pH of soil samples was significantly different by season for both 2020 ($p=0.0015$) and 2021 ($p=0.0102$), though it became more neutral in late summer compared to spring of 2020 and it became more acidic in late summer of 2021 (**Figure 5.5**). The pH was between pH 6 and 7.5, around neutral, for all planting groups. Phosphorus concentration in the soil decreased later in the year, but this decrease was only significant in 2020 ($p=0.0122$). Potassium concentration also decreased in the post-planting collections, though this was only significant in 2021 ($p=0.0024$). Nitrate (NO_3-N) concentration in the post-planting soil increased both years, but this was only significant in 2021 ($p=0.0369$, **Figure 5.5**).

The post-planting soil pH and nutrient results of the buckwheat, honey bee flower mix, and eastern flower mix groups were then compared to samples taken from an adjacent grassy field to the flower plots (**Figure 5.6**). There was no significant effect of flower planting type on post-planting soil pH for 2020, 2021, or 2022. In all plots, the pH ranged from 6.1-7.4, neutral or

mildly acidic (**Figure 5.6A**). Though plants can vary in their soil pH preferences, most agricultural crops grow well in soil that is within pH 6.2-7.0.

The flower planting groups had no significant effect on post-planting nitrate levels. In 2020, the eastern wildflower planting group and the honey bee flower planting groups had higher nitrate concentration on average, compared to the buckwheat and grassy field groups, but not significantly so. In 2021, the buckwheat plots had a lower average nitrate concentration compared to the other groups, but it was not significant (**Figure 5.6B**).

For each year of the study, phosphorus concentrations were higher on average in the honey bee wildflower mix and eastern wildflower mix plots than the buckwheat and grassy field concentrations, though the differences were not significant (**Figure 5.6C**). Potassium concentrations were similar in each planting group for each year of the study (**Figure 5.6D**).

The honey bee wildflower mix had a significantly higher concentration of zinc than the grassy field in 2021 ($\chi^2(3) = 9.04$, $p = 0.0288$; **Figure 5.6E**). All three of the research planting groups had higher mean zinc concentrations than the grassy field, though not significantly so. The eastern wildflower mix had a significantly higher concentration of boron in 2021 ($\chi^2(3) = 8.04$, $p = 0.0453$; **Figure 5.6F**), sodium in 2020 ($\chi^2(3) = 7.04$; $p=0.0473$) and 2021 ($\chi^2(3) = 10.57$, $p = 0.0143$; **Figure 5.6G**), and sulfate in 2021 ($\chi^2(3) = 10.00$, $p = 0.0186$; **Figure 5.6H**), compared to the grassy field. In other years, however, the boron, sodium, and sulfate concentrations remained similar among planting groups.

Soil Microbiome Composition

The soil samples that produced fewer than 1,000 PE reads were excluded from analysis. From the remaining 15 samples, 148 bacterial phylotypes were found. No single phylotype was found in all samples. The two most abundant, a *Lactobacillus* sp. and *Faecalibacterium* sp., were found in 14 out of the 15 samples. After the 15 most abundant and ubiquitous phylotypes (**Figure 5.7**), one phylotype (a Family Lachnospiraceae sp., NC2004 group) was found at

18.18% relative abundance, but only in one sample (post-planting grassy field “O”). All other phylotypes were present at less than 6% relative abundance in all plots.

The post-planting grassy field “O” sample only possessed 2 bacterial phylotypes, which is likely a reflection of the need for further sampling, rather than a reflection of the true diversity of the grassy field plot.

Excluding the sample from plot “O,” the samples had an average of 52.8 phylotypes per plot. Rarefaction curves were created to determine the proper sequencing depth to assess the number and relative abundance of phylotypes in each sample (**Figure 5.8A**). It is likely that with further sampling efforts, however, the number of phylotypes found in each plot would increase.

The ecological functions of many soil bacteria, including many commonly found taxa, are not well known (Delgado-Baquerizo et al., 2018). Additionally, many of the phylotypes found did not have a close match on GenBank. Several, however, were phylogenetically similar to bacterial taxa with known ecological roles. Members of the genus *Rhizobacter*, for example, are known plant pathogens, which infect a wide range of host plants and cause tumor growth (Caballo-Ponce et al., 2017). A *Rhizobacter* sp. was found in the pre-planting honey bee wildflower mix plot “E.” Few other potential plant pathogens were identified.

Several nitrogen fixing bacteria were also found. Soil nitrogen fixers can be free-living heterotrophs, autotrophic cyanobacteria, or symbionts with other organisms (Stancheva et al., 2013; Wagner, 2011). A *Bacillus* sp. was found in buckwheat, eastern wildflower mix, and grassy field plots, and a *Clostridium* sp. was found in a honey bee wildflower mix plot. Both genera contain free-living nitrogen fixers, but it is not known if the particular phylotypes found in the samples are nitrogen fixers. Similarly, a *Rivularia* sp., a cyanobacterium, was found in a pre-planting buckwheat plot. The most notable nitrogen fixers, however, are those that form symbiotic relationships with plant species, such as legumes (Family: Fabaceae). Legumes were planted in the wildflower plots (**Table 5.1**) and some wild legumes, such as *T. pratense* and *T. repens*, managed to grow in the mowed grassy field. A *Bradyrhizobium* sp. was found in the

grassy field. A Xanthobacteraceae species was found in a pre-planting buckwheat plot and a post-planting eastern wildflower mix plot and a Rhizobiaceae species was found in pre- and post-planting eastern wildflower mix, honey bee wildflower mix, and grassy field plots. Both families Xanthobacteraceae and Rhizobiaceae contain plant-associated nitrogen fixers, though it is unknown if these particular phylotypes are nitrogen fixers. No potential plant-associated nitrogen fixers were found in the post-planting buckwheat plots.

Soil Microbial Diversity

Replication in the study was low, as several of the collected soil samples did not produce an adequate number of PE reads to be included in the analysis. There was no significant difference in the number of bacterial phylotypes found and in the Shannon diversity index values (**Figure 5.8B**) for the plots by planting group and sampling time.

The post-planting buckwheat plot had most community similarity to the grassy field samples (**Figure 5.9**). Overall, bacterial communities across all plots were highly varied. Further sampling could reveal more trends in β-diversity, however.

Discussion

In this study, we compared a buckwheat monoculture to two wildflower mixes, and the impacts each could have on pollinator and soil health. In a semi-field environment, wildflower plantings improved *O. lignaria* foraging activity and fecundity compared to buckwheat. Wildflowers also had either a neutral or positive impact on soil nutrient properties and on the presence of plant-associated nitrogen fixers.

Osmia lignaria is a generalist feeder and foraging bees will fly farther and expend more energy in order to collect pollen from multiple flower species (Williams & Tepedino, 2003). Diet diversity seems particularly important to generalist *Osmia* spp. A similar study on *O. bicornis* found that bees reared on a diverse wildflower diet were more tolerant of the neonicotinoid,

clothianidin, than those reared on an oilseed rape (*Brassica napus* L.) monoculture (Klaus et al., 2021). Female bees in this study also showed strong floral preferences, particularly to *M. citriodora, R. hirta*, and *A. tuberosa*. Diet quality, particularly the protein content of pollen is important to many bee species. While male bees forage solely on nectar, females of the related species, *Osmia californica* Cresson, required pollen for 7-10 days in order to complete their ovary development and begin producing offspring (Cane, 2016). *Osmia lignaria* showed a similar trend of feeding for around 7 days after release into the cages and then beginning to visit the nest boxes. It is likely that they, too, require a source of pollen as adult bees. Female *O. lignaria* in the buckwheat cages showed minor interest in the nest tubes early in the observation period, but failed to construct any nest cells, collect any provisions, or lay any eggs. Buckwheat yields are improved by insect pollination, especially by *A. mellifera* (Bartomeus et al., 2014; Liu et al., 2020). Buckwheat flowers are visited by a wide variety of insects, including many flies, beetles, wasps, ants, and Megachilid bees (Taki et al., 2009). *Apis mellifera* collect pollen and nectar from buckwheat (Alekseyeva & Bureyko, 2000), but the pollen quality and quantity may be inadequate for *O. lignaria* females to complete their ovary development and produce offspring. Other monoculture plantings, such as *B. napus*, apple (*Malus domestica*), or lowbush blueberry (*Vaccinium angustifolium* Aiton), could provide a healthier food source for *O. lignaria*. However, for buckwheat specifically, the addition of some wildflower plantings with bee preferred flowers may improve *Osmia* spp. activity and visitation.

Even in the wildflower planted cages, *O. lignaria* females produced a high ratio of males to females. *Osmia* spp. typically produce more male offspring than females, though the ratios tend to be closer to 2:1 (Rust, 1986; Torchio & Tepedino, 1980). The strong bias of the offspring toward male bees could be explained by unsuccessful mating attempts. Like all bees, *O. lignaria* are haplodiploid. A fertilized egg will result in a female offspring, whereas an unfertilized egg will result in a male. If mating was incomplete or unsuccessful, then the female bees would only be able to produce male offspring. The moisture retention and humidity within some of the nest

tubes may have affected the female brood cells more than the males, and impeded the development of some of the female offspring. *Osmia* spp. lay female eggs toward the back of the nest tube. Males emerge first from their cocoons, and so are placed closer toward the entrance of the tubes (Torchio, 1989). The mold was mostly found toward the back of the tubes, and therefore may have disproportionately affected the female offspring over the males. Finally, floral resource availability can affect the sex ratio of offspring. Female offspring are larger and require bigger pollen and nectar provisions than males, so scarcity of flowers can result in more male offspring (Sedivy & Dorn, 2014; Torchio & Tepedino, 1980). The number of flower blooms available was limited by the cage size, so building larger bee cages with more planted flowers could improve the fecundity of the studied bees.

Additional improvements to the husbandry of *O. lignaria* in this semi-field study could better show the differences in health and reproduction among the treatment groups. As well as increasing the cage size, increasing the replication of field plots could also improve the results. Males are necessary for reproduction, but may impede the female bee foraging. The males coul be aggressive in their mating attempts and were observed knocking female bees off of the flowers. Too few males, however, can result in inadequate mating and could contribute to a higher ratio of male to female offspring. Bees could be mated in a lab setting and then the successfully mated female bees could be released into the cages to improve both female foraging and successful reproduction. The nest boxes could be removed soon after the parent bee foraging period was complete. They could be moved to a cool, dry location, such as a 4°C refrigerator, to help prevent mold growth. Mold growth was also more prevalent in the plastic nest tubes. As the bees strongly preferred the paper nest tubes, only including paper tubes is recommended. Finally, including other monocultures, such as canola or oilseed rape, would be useful. Buckwheat is a practical research plant for Arkansas as it is fast growing and heat-tolerant. Though it is well-liked by *A. mellifera* and visited by a wide variety of insect pollinators,

it may not be an adequate food source for *O. lignaria* without other flowers to supplement their diet.

As well as affecting bee health, land management can impact the soil physical properties, nutrients, and microbial communities. Nitrates are an essential plant nutrient and necessary for plant growth. Nitrate deficiencies can cause many noticeable problems and symptoms in plants, though different plants can vary in their preferred nitrate levels (Olson & Kurtz, 1982; Shrivastav et al., 2020). Some plants, including tomatoes and sweet corn, prefer higher levels of nitrates in soil, and can cause the depletion of soil nitrates over time. Many legumes (Family: Fabaceae), on the other hand, can form symbiotic relationships with nitrogen fixing *Rhizobium* sp. Bacteria and therefore can increase nitrates in soil (Bhagwat & Thomas, 1982). Levels of nitrates can also vary by season and by environmental conditions, such as moisture content in the soil. Post-planting nitrate levels were higher in 2021 than 2020 across all planting groups. They were on average higher in the two wildflower treatments both years and in the grassy field in 2021, compared to the buckwheat plots, though these differences were not significant. Both wildflower mixes included members of the Fabaceae family, which could increase the nitrate content of the soil through symbiotic Rhizobia. There were, however, many species of wildflower in each mix, and of those only four species growing in the wildflower plots were legumes, so the nitrifying effect could be minor. Potassium is another essential nutrient for plant growth (Shrivastav et al., 2020). Plants with potassium deficiencies may be more vulnerable to drought and more susceptible to certain diseases (Amtmann et al., 2008; Aslam et al., 2012). Phosphorus, another plant essential nutrient, is important for plant development and growth rate (Malhotra et al., 2018; Shrivastav et al., 2020). Essential plant macronutrient levels were adequate for the growth of most plants across the planting groups.

Sulfur, in the plant-absorbable form of sulfates, is a plant micronutrient and necessary for building sulfur-containing compounds like glutathione. Levels across the planting groups were consistently around 10-15 ppm. In 2021, the eastern wildflower mix plots had significantly

higher sulfate levels, up to 30 ppm. Many crop plants can deplete soil sulfates over time, so maintaining adequate concentrations can help ensure proper plant growth. Micronutrient deficiencies in soil are rare, except for boron and zinc. The availability of most micronutrients decreases as pH increases, and deficiencies of boron and zinc rarely occur when the soil pH is below 6.5. Zinc levels increased in all planting groups with each year and tended to be lowest in the grassy field plots. A significant difference was only seen in 2021, between the honey bee wildflower mix plots and the grassy field plots (**Figure 5.6E**). Zinc concentrations stayed in the medium-optimum range for most plants of 2.5-8 ppm for all planting groups and years. In 2022, some of the eastern wildflower plots contained above optimum zinc levels of ~9 ppm, though on average they were ~7 ppm. Boron deficiencies (<0.2 ppm) can limit plant growth and excessively high concentrations (> 2 ppm) can be toxic. All plots for all years contained medium concentrations of boron. Finally, sodium is not an essential nutrient for plant growth, but is monitored in soil as high sodium levels can limit plant growth. Levels in all planting groups were low – well below concentrations that have been reported to cause root damage.

Soil microbial communities are incredibly diverse both on a global and regional scale. One survey of soil plots in New York City found few phylotypes shared among samples, even for plots located close to one another (Ramirez et al., 2014). Global location and climate can have great impacts on communities and cause variations in the most abundant taxa present at different sites (Chu et al., 2010; Delgado-Baquerizo et al., 2018; Lauber et al., 2009). Because of this, many of the phylotypes found in this study did not have a close match on GenBank and did not have previous knowledge available on their ecology. Additionally, the impacts of different plantings on soil bacterial communities can take years to become noticeable (Fierer, 2017). In this 3-year study, there were some differences in notable phylotypes among treatment groups, but a longer time scale and more replications would help study these changes. It is important to note, too that several of the top 15 bacterial taxa found in the soil samples were also found in the bee gut sample from the previous chapter (**Figures 4.1-4.2** and **5.7**). The soil samples and

bee gut samples were stored separately, but in the same University of Arkansas laboratory, and were sequenced in the same facility. Because of the similarities in taxa, it is possible that some cross-contamination occurred. The full complexity of soil microbial communities and their ecology is still poorly understood. However, many soil microbes can contribute to plant growth and therefore human health in both positive and negative ways, so it is vital to monitor and further understanding of these bacterial communities and their interactions with plants.

Conclusions

The wildflower mixes were able to improve bee fecundity and foraging activity compared to buckwheat, without negatively impacting soil nutrient concentrations and soil microbial communities. Because agricultural areas can contain a paucity of insects, adding wildflower plantings to agricultural areas, in the forms of hedgerows, personal gardens, and roadside verges, especially those containing bee-preferred flowers, can help support bee populations. These plantings tend to be cost-effective compared to traditional management strategies (Janke et al., 2021) and can ensure decent forage to be available to bees for the entirety of their annual activity period. Using wildflowers to supplement buckwheat plantings in particular may help improve *O. lignaria* populations and foraging activity.

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Figure 5.1. Bee cage and nest box design. Bee cage, planted with wildflower mix, showing the nest box and sand dish (A). The 12 bee cages and experimental plots (B). Close up of a nest box filled with paper nest tubes (C).

Table 5.1. List of flowers in bloom at the time of bee activity in the research cages.

Planting Group	Plot ID	2021 Wildflower List	2022 Wildflower List
Buckwheat	A, D, F, L	*Fagopyrum esculentum*	*Fagopyrum esculentum*
Honey Bee Wildflower Mix	C, E, I, K	*Asclepias tuberosa*[2]*, *Aster oblongifolius*[4]**, *Chamaecrista fasciculata*[†3]*, *Coreopsis lanceolata*[4], *Coreopsis verticillata*[4]**, *Desmodium canadense*[†3]*, *Echinacea purpurea*[2], *Erigeron annuus*[4]*, *Eryngium yuccifolium*[3]*, *Gaillardia aristata*[4], *Heliopsis helianthoides*[4]**, *Heterotheca subaxillaris*[2]*, *Liatris spicata*[4]**, *Monarda citriodora*[2]*, *Oenothera lamarckiana*[1], *Papaver rhoeas*[2], *Rudbeckia fulgida*[3]**, *Rudbeckia hirta*[4]*, *Scrophularia marilandica*[4]**, *Torilis* sp.[3]*, *Trifolium pratense*[†3]*, *Trifolium repens*[†2]*	*Asclepias tuberosa*[2]*, *Coreopsis lanceolata*[1], *Daucus carota*[1]*, *Desmodium canadense*[†3]*, *Erigeron annuus*[3]*, *Eryngium yuccifolium*[2]*, *Gaillardia aristata*[1], *Heliopsis helianthoides*[1]*, *Heterotheca subaxillaris*[3]*, *Lactuca* spp. (likely *serriola*)[1]*, *Monarda citriodora*[2]*, *Rudbeckia hirta*[1]*
Eastern Wildflower Mix	B, G, H, J	*Asclepias tuberosa*[3], *Aster oblongifolius*[4]**, *Buddleia* sp. (hybrid)[4]**, *Centaurea cyanus*[2], *Chamaecrista fasciculata*[†3], *Coreopsis lanceolata*[4], *Coreopsis tinctoria*[3], *Cosmos bipinnatus*[2], *Cosmos sulphureus*[1], *Desmodium canadense*[†2], *Echinacea purpurea*[4], *Erigeron annuus*[4]*, *Eryngium yuccifolium*[3]*, *Gaillardia aristata*[4], *Heterotheca subaxillaris*[3]*, *Medicago lupulina*[†1]*, *Monarda citriodora*[4], *Oenothera lamarckiana*[1]*, *Phlox drummondii*[3], *Plantago lanceolata*[3]*, *Rudbeckia fulgida*[3]**, *Rudbeckia hirta*[4], *Stokesia laevis*[4]**, *Trifolium pratense*[†4]*, *Trifolium repens*[†3]*	*Asclepias tuberosa*[1], *Chamaecrista fasciculata*[†2], *Coreopsis lanceolata*[1], *Desmodium canadense*[†3], *Echinacea purpurea*[2], *Erigeron annuus*[3]*, *Eryngium yuccifolium*[1]*, *Gaillardia aristata*[2], *Heterotheca subaxillaris*[3]*, *Lactuca* spp. (likely *serriola*)[1]*, *Monarda citriodora*[2], *Rudbeckia hirta*[1]

*Wild at farm, not present in seed mix
**Purchased from White River Nursery, not present in seed mix
[1] Present in 1 plot of the group
[2] Present in 2 plots of the group
[3] Present in 3 plots of the group
[4] Present in 4 plots of the group
[†] Legume

Table 5.2. Total number of bee floral visits over the activity and observation period

Planting Mix	**Plot ID**	**Wildflower Species**	**Total Number of Floral Visits**	
			Year- 2021	Year-2022
Buckwheat	A, D, F, L	*Fagopyrum esculentum*	49	25
Honey Bee Wildflower Mix	C, E, I, K	*Asclepias tuberosa*	22	0
		Coreopsis lanceolata	13	1
		Coreopsis verticillata	2	0
		Desmodium canadense	4	2
		Echinacea purpurea	1	0
		Erigeron annuus	2	0
		Eryngium yuccifolium	2	2
		Gaillardia aristata	6	0
		Heliopsis helianthoides	16	4
		Heterotheca subaxillaris	4	14
		Monarda citriodora	74	15
		Papaver rhoeas	1	0
		Rudbeckia hirta	12	2
		Scrophularia marilandica	2	0
		Torilis sp.	1	0
		Cage Total	**162**	**40**
Eastern Wildflower Mix	B, G, H, J	*Asclepias tuberosa*	36	16
		Coreopsis lanceolata	31	3
		Coreopsis tinctoria	2	0
		Cosmos bipinnatus	3	0
		Desmodium canadense	2	2
		Echinacea purpurea	5	3
		Erigeron annuus	3	0
		Gaillardia aristata	20	0
		Heterotheca subaxillaris	0	3
		Medicago lupulina	1	0
		Monarda citriodora	61	5
		Rudbeckia hirta	27	3
		Stokesia laevis	3	0
		Trifolium pratense	5	0
		Trifolium repens	1	0
		Cage Total	**200**	**35**

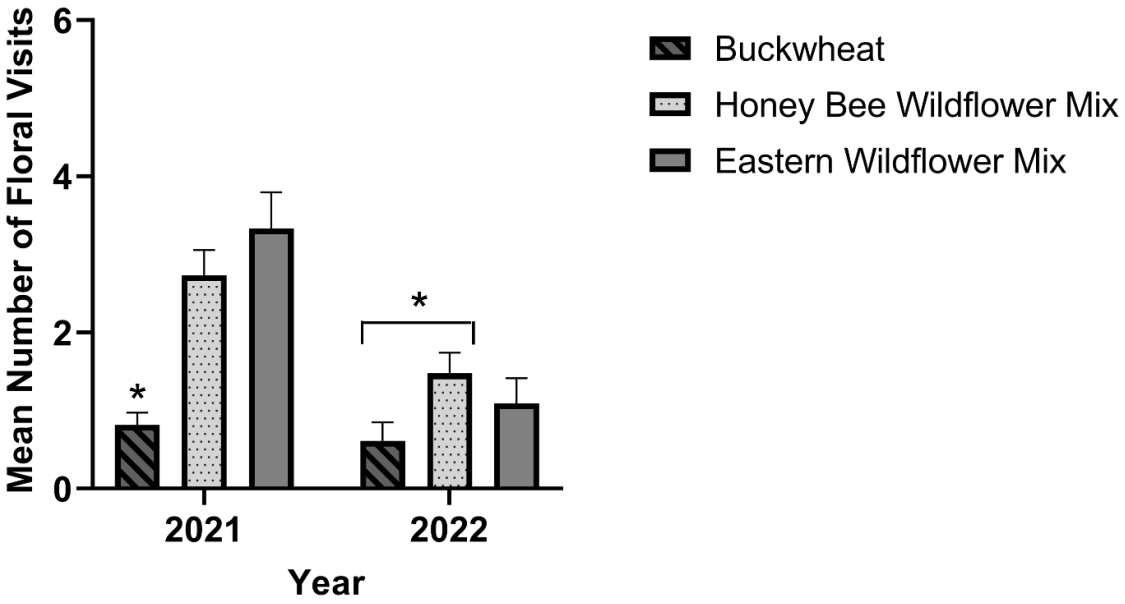

Figure 5.2. The average number of floral visits of the *O. lignaria* bees during 2-minute observation periods for the three flower planting groups: buckwheat, honey bee wildflower mix, and eastern wildflower mix. An asterisk (*) denotes statistically significant difference among planting groups (p≤0.04).

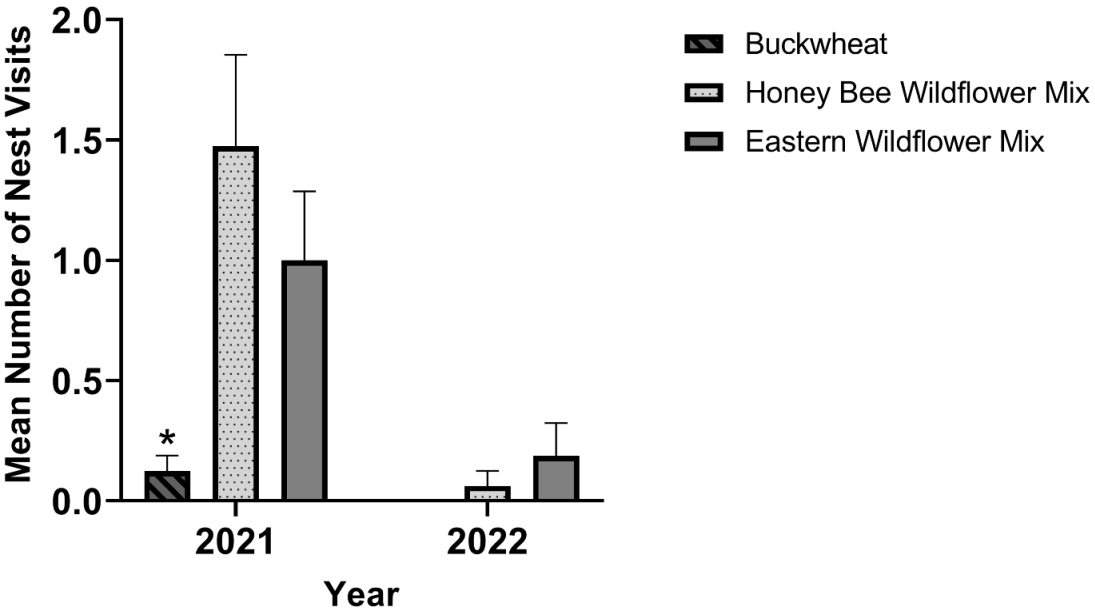

Figure 5.3. The average number of trips to the nest box over 2-minute observation periods for each treatment group. An asterisk (*) denotes a significant difference among planting groups (p<0.0001).

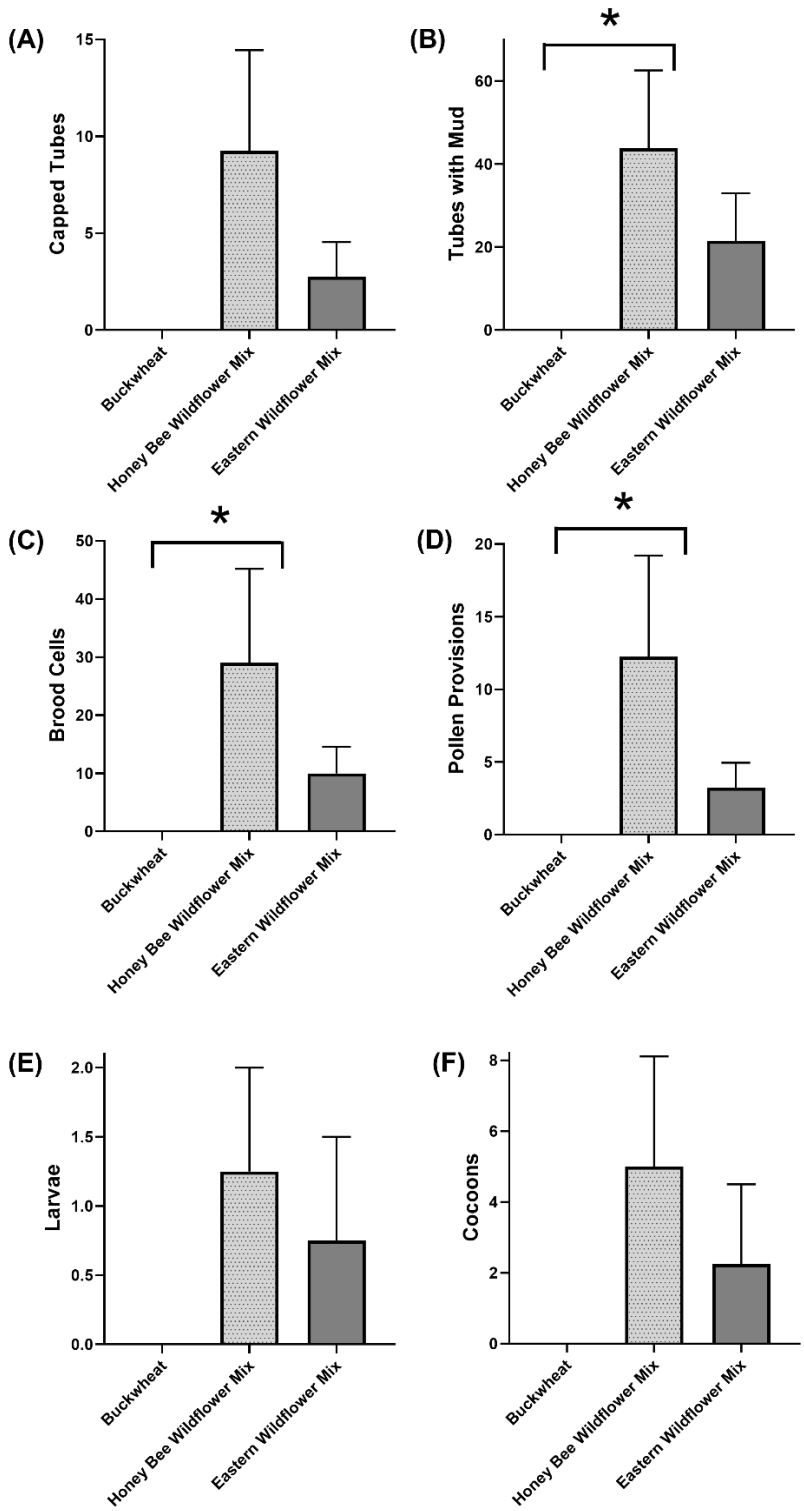

Figure 5.4. The fecundity of blue orchard bees in the different planting groups from the 2021 season, measured as number of capped tubes (A), number of tubes with mud residue (B), numbers of brood cells (C), number of uneaten pollen provisions (D), number of dead larvae (E), and number of cocoons (F). Significant differences between treatment groups are noted with an asterisk (*).

Table 5.3. The cocoon weight, sex, and longevity of bee offspring after the 2021 season.

Planting Group	Cage	Cocoon Weight (mg)	Sex	Bee Longevity After Emergence (days)
Eastern Wildflower Mix	E	61	F	1
		25	-	-
		62	M	3
		52	M	4
		42	M	4
		25	-	-
		37	M	3
		38	M	3
		35	M	3
		25	-	-
		29	M	3
	I	50	M	14
		42	M	9
		8	-	-
	K	11	-	-
		38	M	27
Honey Bee Wildflower Mix	G	42	M	12
		26	-	-
		41	M	24
		44	F	23
		27	M	6
		28	M	8
		11	-	-
		1	-	-

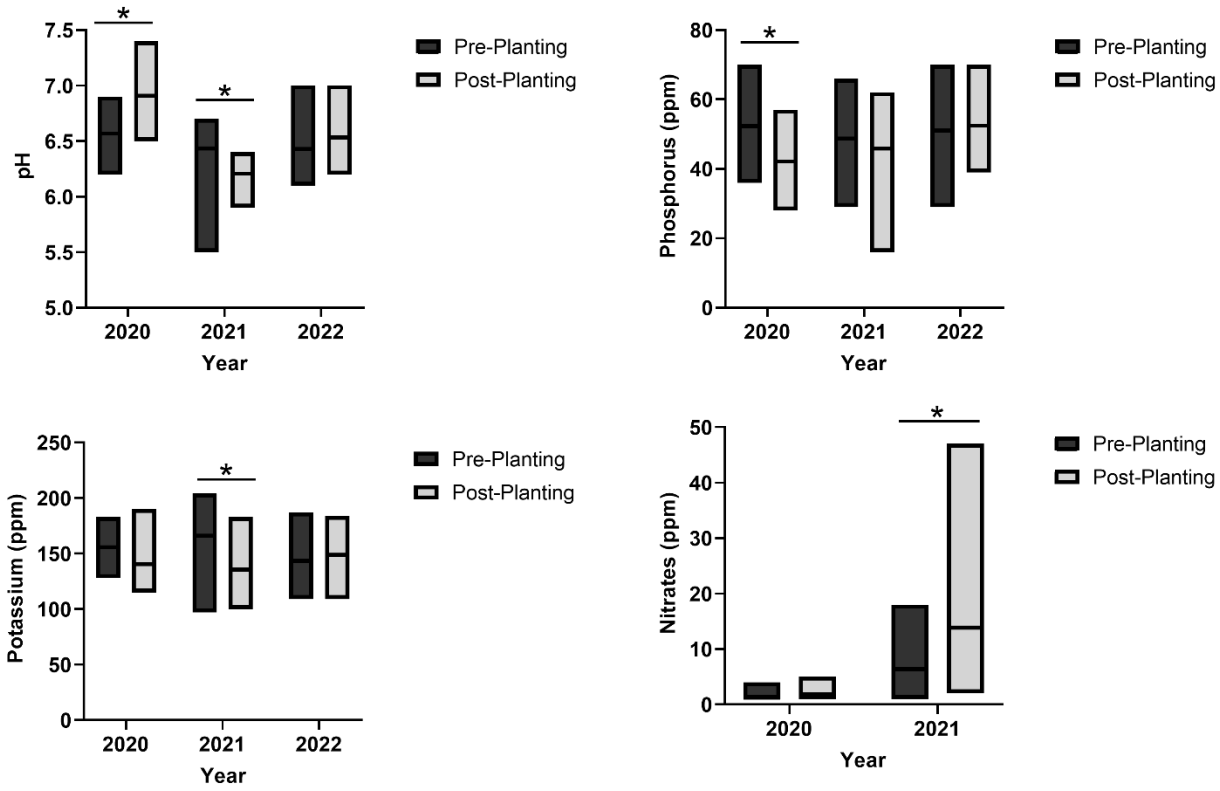

Figure 5.5. The seasonal fluctuations of soil pH (A) and concentrations of phosphorus (B), potassium (C), and nitrates (D), from samples taken before and after floral plantings. The 2021 results show nutrient and pH content after one growing season of buckwheat/wildflowers and the 2022 results after two seasons. An asterisk (*) denotes a significant difference between the pre-planting and post-planting levels.

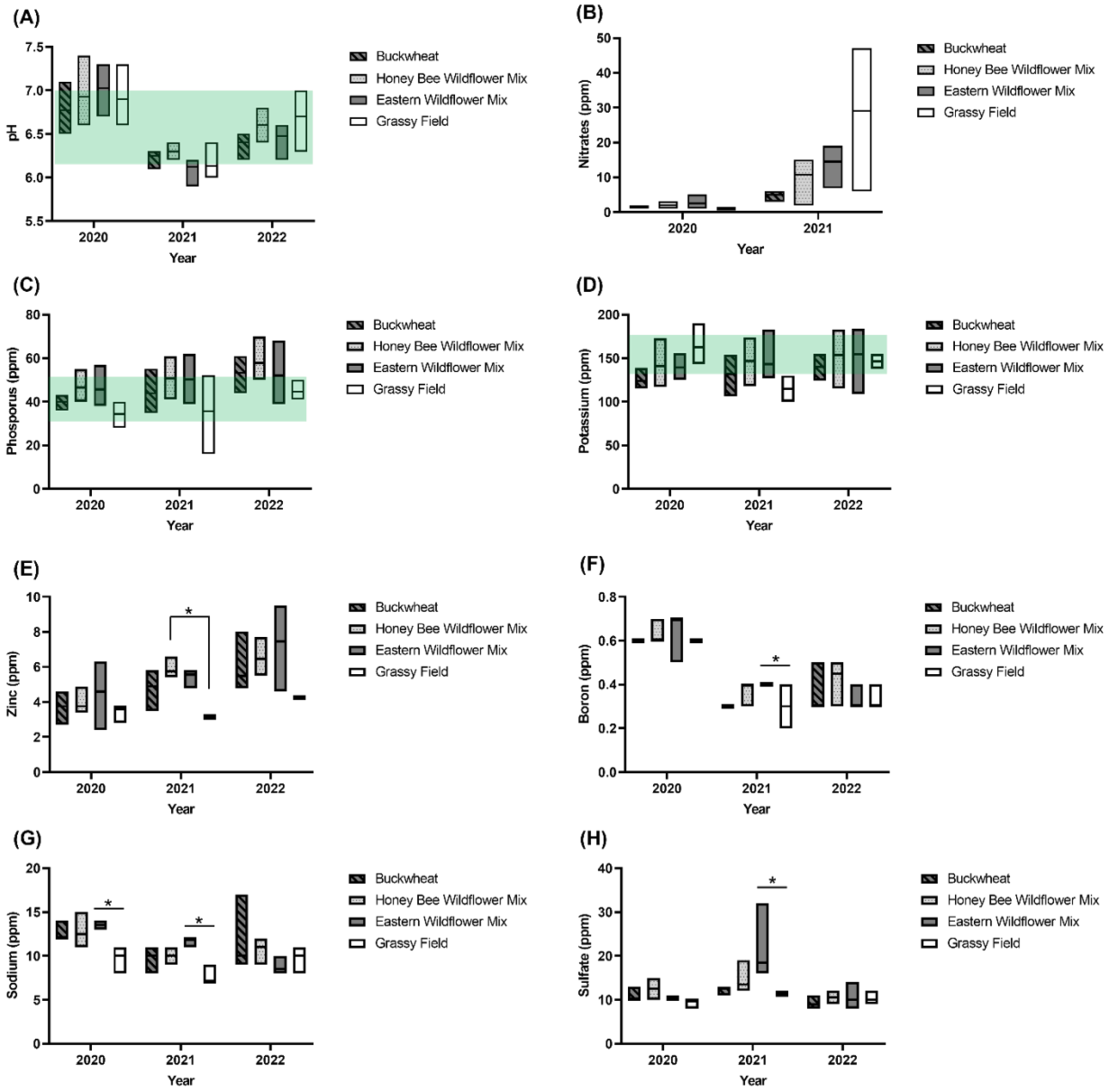

Figure 5.6. The post-planting pH (A) and nutrient concentrations (B-H) for each year and planting group. The optimum pH, phosphorus, and potassium levels for most crops, as determined by the Marianna Soil Test Laboratory, are shown in green (A, C, and D). Significant differences among planting groups are marked with an asterisk (*).

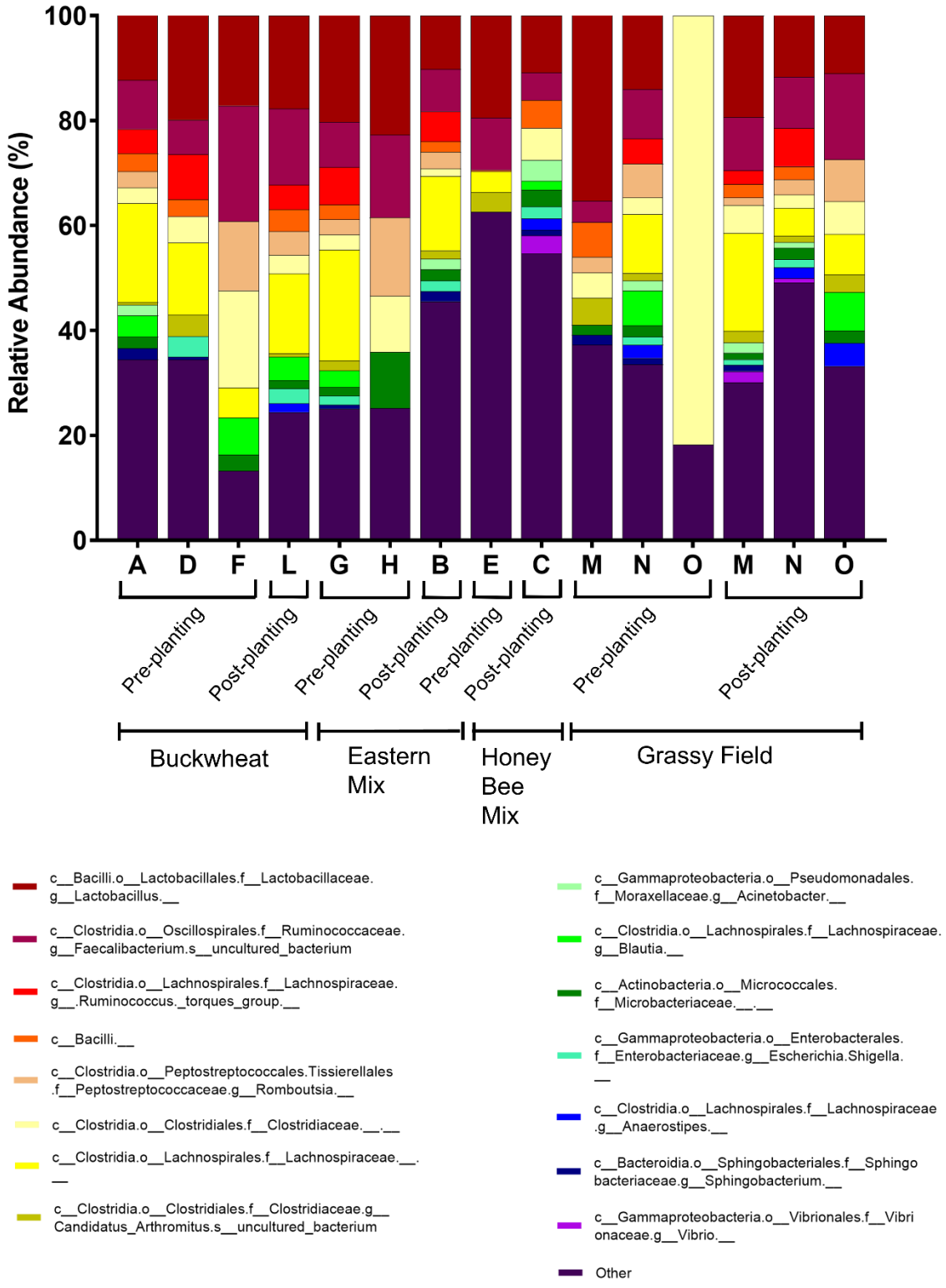

Figure 5.7. The relative abundances of the 15 most abundant and ubiquitous bacterial phylotypes in soil samples taken before and after planting. Soil samples were collected in 2022 after 2 growing seasons. The planting groups were buckwheat, eastern wildflower mix, and honey bee wildflower mix, as well as an unplanted grassy field.

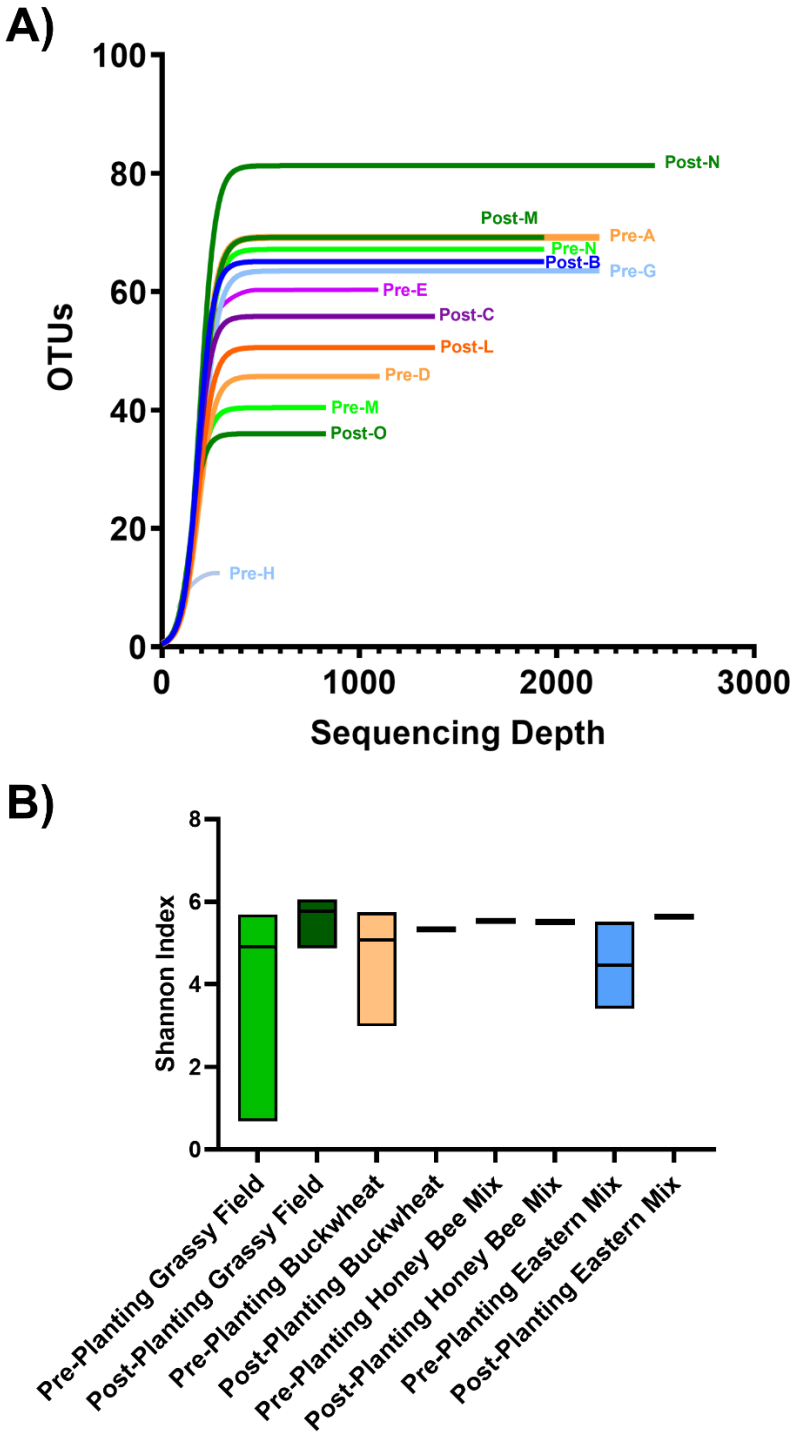

Figure 5.8. Soil was collected pre-planting (Pre) and post-planting (Post) in 2022, after two prior growing seasons. The plots were planted with buckwheat (A, D, F, and L), honey bee wildflower mix (C, E, I, and K), or eastern wildflower mix (B, G, H, and J). Soil samples were also taken from three sites in an adjacent field (M, N, and O). Rarefaction curve showing the number of operational taxonomic units (OTUs) by sequencing depth for each sampled research plot (A). Plots with fewer than 1,000 clear reads were excluded. Shannon diversity index for each planting group (B).

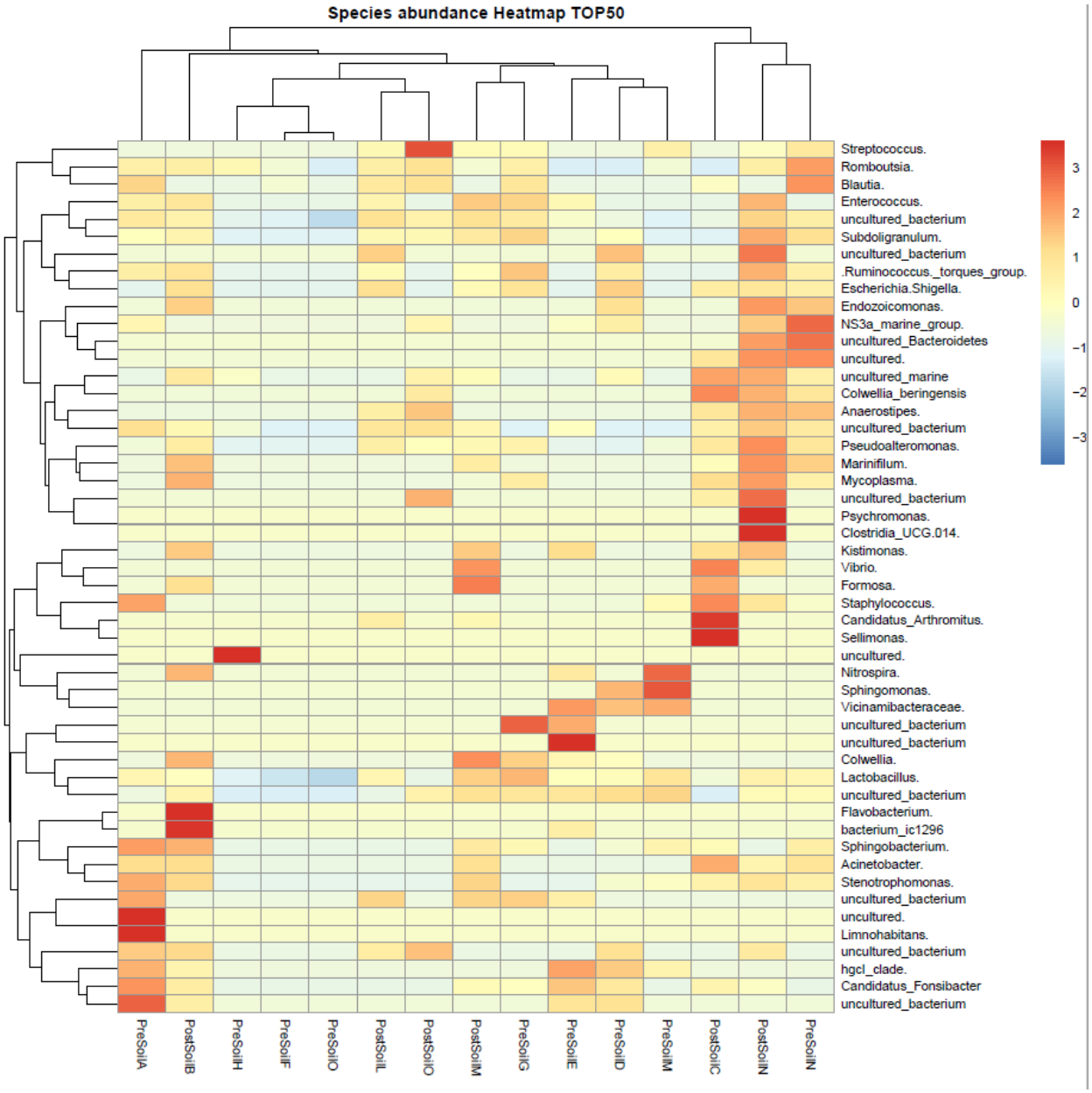

Figure 5.9. Phylotype abundance heat map for bacteria in soil collected pre-planting (Pre) and post-planting (Post) in 2022. The plots were planted with buckwheat (A, D, F, and L), honey bee wildflower mix (C, E, I, and K), or eastern wildflower mix (B, G, H, and J). Soil samples were also taken from three sites in an adjacent field (M, N, and O).

Chapter 6:

Conclusion

This dissertation examined the lethal impacts of insecticides on *Apis mellifera* L. and *Osmia* spp., the sublethal impacts of insecticides on *Osmia* spp. enzyme expression, feeding behavior, foraging activity, and gut microbial communities, and the effects of wildflower plantings versus a buckwheat monoculture on bee and soil health. Most pollinator research has focused on *A. mellifera* health and pollination activity, and so more information has been needed about the factors that impact the health of important solitary bee pollinators. Many *Osmia* spp. are used to pollinate orchard crops, helping to increase the profits of many growers and provide a better quality of fruit for consumers. Because of the vital pollination service to both crops and wildflowers, it is important to investigate potential stressors and their effects on bee health.

After exposure to the novel systemic insecticides, flupyradifurone and sulfoxaflor, *Osmia* spp. bees were more sensitive than *A. mellifera*. *Osmia cornifrons* Radoszkowski was the least sensitive of the tested *Osmia* spp. *Osmia lignaria* Say and *Osmia californica* Cresson were highly sensitive to field-realistic doses of the insecticides. Despite their smaller body size, male *Osmia* bees were less sensitive to both insecticides than females of the same species.

Sulfoxaflor and flupyradifurone also caused some sublethal effects on *Osmia* spp. physiology, behavior, and gut microbial communities. *Osmia lignaria* females exposed to flupyradifurone showed increased P450 enzyme expression. These P450 detoxification enzymes likely help the bees survive low-dose exposure to flupyradifurone, though they may not be able to fully metabolize high doses. Additionally, the use of P450 inhibitors alongside flupyradifurone would likely increase bee mortality. As such, these mixes (of an active ingredient with synergists, such as P450 inhibitors) should be used with extreme caution in areas that utilize *O. lignaria* for pollination. *Osmia lignaria* was also unlikely to detect or indifferent to the presence of sulfoxaflor or flupyradifurone in food sources. Female bees showed some

avoidance of feeders contaminated with higher concentrations of flupyradifurone, but not to sulfoxaflor or lower concentrations of flupyradifurone. Short-term (3 hours after treatment) effects of flupyradifurone on the flight and foraging ability of *Osmia* spp. (*O. lignaria*, *O. cornifrons*, and *O. californica*) were minimal, though *O. cornifrons* females showed an excitation response. At 24 hours after treatment, however, *O. lignaria* mobility and foraging were impaired. Neither flupyradifurone or sulfoxaflor impacted the abundance or prevalence of potential bee or plant pathogens. Sulfoxaflor significantly reduced microbial diversity, though further evaluations are needed to examine the impact of different doses and exposure periods on gut microbial diversity.

Finally, the impacts of different flower plantings on bee and soil health were examined. *Osmia lignaria bees* were released in semi-field cages planted either with buckwheat or with a wildflower mix. Bees were observed on their foraging and nesting activity and their reproduction. Soil samples were also collected before and after planting. The plantings of wildflower mixes improved bee foraging and nesting activity compared to the buckwheat monoculture. Bees feeding on wildflowers were able to successfully produce offspring, whereas those foraging solely on buckwheat did not provision any nest cells or lay any eggs. The wildflower plots also had higher average soil nitrogen content and more potential plant-associated nitrogen fixing bacteria compared to the buckwheat plots.

These studies highlight some of the risks facing *Osmia* spp. pollinators and also provide new insights into potential mitigation schemes. Using caution while applying pesticides, such as limiting applications during floral blooms, especially on bee-preferred crops, can help reduce the risk of *Osmia* spp. exposure. Additionally, adding unsprayed wildflower plantings alongside crop plantings can improve diet diversity and allow bees to diet mix as needed. Such studies on solitary bees are necessary to help protect bee communities and the vital pollination services that they provide.