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Investigating Routes and Effects of Pesticide Exposure on the Blue Orchard Bee (*Osmia lignaria*)

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INVESTIGATING ROUTES AND EFFECTS OF PESTICIDE EXPOSURE ON THE BLUE ORCHARD BEE (*OSMIA LIGNARIA*)

by

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ABSTRACT

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Osmia lignaria (Megachilidae), commonly known as the blue orchard bee, is an important alternative pollinator of commercial orchards. *Osmia lignaria* are solitary, cavity-nesting bees with a wide distribution across North America. They become active in early spring and only produce one generation a year. The males emerge first and wait for the emergence of the females, one to three days later, so they can copulate. Female *O. lignaria* use soil for nesting substrate to create individual nest cells that are mass provisioned with pollen and nectar within a cylindrical nest. In managed populations, these nests are made of pre-formed wooden tunnels or paper tubes affixed into nest boxes that are mounted in orchards. Females collect pollen and nectar to provision a cell, lay one egg on the provision, then seal the cell with mud with no further contact with her offspring.

The more *O. lignaria* are used in commercial agriculture, the greater the risk of pesticide exposure. In Chapter I, I define the routes of pesticide exposure in solitary, cavity-nesting bees. First, there is exposure through ingestion of pollen and nectar by the larva. Second, is through adult ingestion of nectar and pollen. The third route is through direct contact with plant surfaces or nesting material that is contaminated with pesticides. Lastly, there is potential for transovarial

transmission from mother to offspring. Examples of the various exposure routes and agrochemicals representing different chemical classes are provided and discussed.

In Chapter II, I investigate the impacts of provision type (either apple or almond pollen) and pesticide exposure on developing individual *O. lignaria*. I tested three provision types in laboratory well plates: natal provisions from a managed population at a local apple orchard, and homogenized apple or almond (from a California orchard) pollen. Natal and homogenized provisions were exposed to one of six treatments: the insecticides acetamiprid or dimethoate, a fungicide (boscalid/pyraclostrobin), a mixture of fungicide and acetamiprid, an organosilicone surfactant, or reverse osmosis water (control). How the larval food was provided and how pesticides were incorporated into food revealed that homogenized provision resulted in greater effects on larval development time and survival. Mortality in the homogenized provision was highest when inoculated with acetamiprid, especially for almond pollen provisions.

In Chapter III, I investigate the impacts of pesticide sprays on adult foraging behavior with a field cage study. A treatment of either water (control), fungicide (boscalid/pyraclostrobin), neonicotinoid (acetamiprid), or a mixture of the two chemicals was applied to one side of the forage in each cage. The other halves were treated with water to provide a choice in forage for the bees. Overall, *O. lignaria* did not nest, a possible result of the hot and humid conditions in southern Mississippi, where the experiment was conducted. However, other bee species, such as *Apis mellifera*, were observed foraging outside of the field cages under these same environmental conditions. Mortality for *O. lignaria* was high, and bee foraging was reduced when flowers were sprayed with acetamiprid. Bee mortality in cages with fungicide-treated flowers was low, but female bees appeared to exhibit hyperactive behavior compared to bees on flowers sprayed with water alone.

(143 pages)

PUBLIC ABSTRACT

Investigating Routes and Effects of Pesticide Exposure on the Blue Orchard Bee (*Osmia lignaria*)

Andi M. Kopit

With native pollinator species on the decline and the honey bee (*Apis mellifera* L.) industry suffering, it is imperative that we understand the impacts of agricultural practices on pollinators. The blue orchard bee, *Osmia lignaria* (Megachilidae), is an important alternative pollinator of commercial orchards. *Osmia lignaria* are solitary, cavity nesting bees with a wide distribution across North America. This species and other solitary, cavity nesting bees experience different routes of pesticide exposure than social pollinators, such as colony-dwelling bumble bees and honey bees. Chapter I focuses on routes of pesticide exposure experienced by cavity-nesting bees, incorporating the relative importance of environmental contamination due to pesticide chemical properties. Exposure routes described are larval ingestion, adult ingestion, contact, and transovarial transmission. In Chapter II, to investigate the effect of pesticides on solitary, cavity nesting bee larvae and develop a methodology for larval pesticide testing, a laboratory bioassay was conducted using *O. lignaria*. Two pollen types (apple and almond), two provision compositions (homogenized and intact natal), and four agrochemicals (acetamiprid, boscalid/pyraclostrobin, organosilicone, and dimethoate) were delivered at different doses and examined for effects on larval development times and mortality before larvae began to spin cocoons. Mortality varied by provision type and treatment. All larvae survived to cocoon initiation when only water (control) was added to provisions of all types. When the intact natal provision was used, there was no or low mortality across agrochemical treatments. Mortality in the homogenized provision was highest when acetamiprid was the treatment, especially for

provisions made from almond pollen. In the third chapter, the impacts of pesticide sprays on adult *O. lignaria* foraging behavior was investigated with a field cage study conducted in Poplarville, MS. The fungicide caused hyperactive behavior with low mortality, whereas individuals exposed to the insecticide showed signs of stress and experienced high mortality rates.

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This document is dedicated to the memory of my grandfather, Harold Kopit, D.V.M.

Andi M. Kopit

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CHAPTER I

ROUTES OF PESTICIDE EXPOSURE

IN SOLITARY, CAVITY-NESTING BEES[1]

Abstract

Declines of pollinator health and their populations continue to be commercial and ecological concerns. Agricultural practices, such as the use of agrochemicals, are among factors attributed to honey bee (*Apis mellifera* L. Hymenoptera: Apidae) population losses and are also known to have negative effects on populations of managed non-*Apis* pollinators. Although pesticide registration routinely requires evaluation of impacts on honey bees, studies of this social species may not reveal important pesticide exposure routes where managed, solitary bees are commonly used. Studies of solitary bees offer additional bee models that are practical from the aspect of availability, known rearing protocols, and the ability to assess effects at the individual level without confounding factors associated with colony living. In addition to understanding bees, it is further important to understand how pesticide characteristics determine their environmental whereabouts and persistence. Considering our research expertise in advancing the management of solitary bees for crop pollination, this forum focuses on routes of pesticide exposure experienced by cavity-nesting bees, incorporating the relative importance of environmental contamination due to pesticide chemical behaviors. Exposure routes described are larval ingestion, adult ingestion, contact, and transovarial transmission. Published research reports of effects of several pesticides on solitary bees are reviewed to exemplify each exposure route. We highlight how certain pesticide risks are particularly important under circumstances related to the cavity nesters.

Key words: alfalfa leafcutting bees, insecticides, mason bees, pesticides, pollinators, sublethal impacts

[1] Andi M. Kopit and Theresa L. Pitts-Singer

Relevance and Rationale

Meeting the demand for healthy honey bee (*Apis mellifera* L., Hymenoptera: Apidae) populations for large commercial pollination events has been particularly challenging since colony collapse disorder (CCD) was recognized in 2006 (vanEngelsdorp et al. 2009). According to a 2016 report, winter colony losses were at 28%, which followed a summer loss also reported to be 28% (Steinhauer et al. 2016). Concerns over CCD and other major stressors contributing to chronic honey bee losses have been elicited by bee researchers and the media. Such concerns also have highlighted and strengthened the global recognition of perils for all pollinators. Nonetheless, it is difficult to document pollinator declines, in part due to the paucity of baseline data for wild bees that are not used in managed systems (Klein et al. 2003, Goulson et al. 2015). Causes of pollinator declines include singular and interacting stress factors: habitat loss, nutritional deficiencies, and exposure to pests, pathogens, and pesticides.

In response to the importance and complexity of solving a multifaceted bee health dilemma, the research community has been actively focusing on one of the most scrutinized and debated impact factors, which is bee exposure to chemical pesticides. Most academic and government agency studies to date only have considered pesticide effects on honey bees (e.g., Kubik et al. 1999, Wu et al. 2011, DeGrandi-Hoffman et al. 2013, USEPA 2014, Cutler and Scott-Dupree 2014, Berenbaum 2016, Fisher et al. 2017), although new attention has been given to some species of non-*Apis* bees (EFSA 2013, APVMA 2015, Biddinger and Rajotte 2015, Godfray et al. 2014, 2015; Jin et al. 2015, Lundin et al. 2015), of which there are at least 20,000 species globally (Michener 2000). Goals of new efforts address the ability to assure pollinator health, abundance, and conservation, and to mitigate factors that harm or diminish pollinator populations and their habitats. As a result, better documentation of needed research actions,

knowledge gaps, regulatory requirements, and suggested paradigms for pesticide risk assessments have begun to emerge (EFSA 2012, 2013, 2014; EMBRAPA 2013, USEPA et al. 2014; White House 2014, 2015).

Whether pesticides are used in cropping systems to control arthropod pests, fungal pathogens, and weeds or in residential areas to control mosquitos or garden and lawn pests, bees are exposed to chemicals in many contexts (Johnson 2015, Hladik et al. 2016). Most non-*Apis* bees are solitary and short-lived with limited foraging ranges and restricted geographic distributions compared to social bees. We are particularly interested in the exposure routes to managed, solitary bees that may experience the agricultural landscape differently than do honey bees. We choose to focus on these bees because of their major current and potential roles in North American and Eurasian agriculture. These bees are cavity-nesting bees in the genera *Megachile* and *Osmia* (Hymenoptera: Megachilidae) that can be easily purchased for crop pollination while they are in diapause, and later incubated to produce mature adults for pollination and nesting in artificial bee tunnels in the fields. These bees have similar exposures as honey bees when they come into direct contact with pesticides during applications or by collecting and feeding on pollen and nectar. But on account of their biology, ecology, physiology, and genetics (Kapheim et al. 2015), they can differ from honey bees in their exposures to pesticides via plant materials, soil, and water, and in their susceptibility to some chemistries and ability to recover from contact or ingestion (e.g., Hooven et al. 2014, Heard et al. 2017). Differences that distinguish solitary lifestyles from social ones necessitate the exploration of potential pesticide impacts that are not considered when studying honey bees. Nesting behavior, habitat locations and types, seasonality, immune responses, and mechanisms of detoxification each may render differential routes, intensities, and effects of pesticide exposure.

This paper describes both the known and probable routes of pesticide exposure in managed, cavity-nesting bee species. We hope to enrich the conversation that defines routes of exposure not only to these bees, but also consequently to wild solitary bees that nest both above and below ground. In a forum style, we address critical components of cavity-nesting bee life histories that may expose them to pesticides that persist in the environment due to key characteristics of pesticides, regardless of when those pesticides were applied for pest, pathogen, and weed control. We deliver the details of four routes of exposure: larval ingestion, adult ingestion, adult contact, and transovarial transmission (Figs. 2-5). For each route for several agrochemicals, we also provide recent examples of studies that reveal effects of pesticides on cavity-nesting bees and techniques for examining them. We discuss the interactions between the specific dangers to cavity-nesting bees due to chemical properties of some pesticides and the ecology and behavior of the bees.

Comparison of Managed Bee Life Histories: Solitary, Cavity-Nesting Bees vs. Social Honey Bees

Solitary, cavity-nesting bees make brood cells in old holes in tree trunks and other woody stems, in reeds, and other various above-ground vacancies that exist naturally, but also readily use artificial tunnels provided by bee managers (Fig. 1A). Commercial tunnels are frequently made of cardboard or wood that are placed in protective shelters. Bees will nest in these shelters *en masse*, creating artificial aggregations (Fig. 1B). Each female is a reproductive individual and builds her own nest, with one bee occupying one cavity at a time in the aggregation (Fig. 1C). Solitary bees use various materials to partition brood cells within the nest, such as soil, cut or masticated plant tissue, resin, or a combination of such materials (Cane et al. 2007). Unlike colonies of honey bees where larvae are fed progressively by workers, solitary bee mothers

create a mass provision in one day or less from pollen and nectar she collects from flowers. She then lays an egg on the provision mass, and a larva develops to adulthood on this sole source of food (Bosch and Kemp 2001) (Fig. 1C). The process is repeated to make multiple nest cells per cavity. Usually, nesting bees live for about 4-6 weeks, and brood spend a year in nests to develop and overwinter before emerging as adults in the next season.

Honey bees live in colonies that may include >20,000 worker bees, seasonal males and a queen. Only the queen can produce new worker daughters who perform all hive tasks including feeding larvae, storing food, and building new nest cells. A new colony is started by the swarming of the old queen plus some of the workers. They identify and move into a new nest site to continue the colony cycle. The daughter queen that remains inherits the old hive and workers, where she continues the colony by producing her own offspring. Therefore, honey bee colonies are perennial and never exhibit a solitary phase (Winston 1987).

The greatest risk to a solitary female is the loss of potential offspring, because she is the sole reproductive entity of her nest. Depending on the timing of her death in the nesting season, only the already completed nest cells will represent her total reproductive output. The loss of nesting bees due to direct sprays or bee handling of contaminated forage may kill adult bees and could lead to a local population decline due to low reproductive success. On the other hand, the sociality of honey bees affords the advantage of the resilience of a superorganism (Johansen and Mayer 1990, Straub et al. 2015). As long as a lethal dose of a pesticide does not penetrate the hive, the loss of some of a colony's workers in the field does not affect the honey bee queen, who can replace worker daughters, if she remains healthy and reproductive, and if the number of workers remains above a critical threshold (Dennis and Kemp 2016).

Chemical Characteristics

The chemical properties of a pesticide are important for a product's ability to contact or penetrate the target pest, and these same properties will also contribute to how and where the pesticide may eventually settle in the environment. Lipophilicity, hydrophilicity, and soil adsorption are three characteristics of agrochemicals that are pertinent to understanding their environmental persistence and potential to facilitate routes of exposure of pesticides to bees freely foraging in an agricultural landscape.

Lipophilicity is a chemical's affinity for lipids. Attraction to lipids allows a pesticide to permeate the cuticular lipid layers of both plants and insects, aiding in the distribution of the desired toxin and its effect on pests. Hydrophilicity is a chemical's affinity for water. It affects the accumulation of the chemical in the environment and its bioavailability for uptake by a plant, allowing some pesticides to act systemically. Systemic pesticides can be distributed throughout the plant as it grows, which means it can be found not only in vegetative material, but also potentially in the pollen and nectar (Godfray et al. 2014, Larson et al. 2015).

Lipophilicity and hydrophilicity of a substance are determined using the octanol:water partition coefficient (K_{ow}). This coefficient describes the distribution of a compound between a lipophilic phase (*n*-octanol) and an aqueous phase of the test system. A lipophilic pesticide has a high K_{ow}, and a hydrophilic chemical has a low K_{ow} (Table 1). K_{ow} also indicates the compound's bioaccumulation potential in animal fats and plant lipids plus its adsorption potential in organic matter of soil (Russel 1995). Pesticides with a high K_{ow} are capable of translaminar movement through plant cuticular lipid layers, which might also move across a bee's lipid layer and into the body through simple cuticular contact during foraging and nesting,

as has been suggested for bumble bee workers exposed to various chitin synthesis inhibitors (Mommaerts et al. 2006).

Soil adsorption, or K_{oc}, is the soil organic carbon:water partitioning coefficient. It indicates a chemical's soil binding propensity. Specifically, this coefficient is the concentration of chemical in soil per concentration of chemical substance in water divided by the percent of organic carbon in the soil. A high value for the K_{oc} of a pesticide means that it is more likely to accumulate in the soil; a low K_{oc} value indicates that the pesticide will move with water and leach out of the soil (Fisk 1995, Klaasen 2007).

Chemical characteristics and their interactions with the environment affect their half-lives, i.e., the time it takes for an amount of a pesticide to be reduced by half from being broken down by environmental factors. In general, one half-life indicates that a pesticide has been broken down to 50% of the original amount, and two half-lives means 25% breakdown, and so forth. The amount of a pesticide applied may increase its half-life as well as repeated applications that add to the amount of chemical in a matrix. Factors that break down pesticides include sunlight, temperature, oxygen, soil composition, pH of soil and water, microbial activity, and metabolism or elimination by the insects themselves (e.g., Cresswell et al. 2014). As environmental factors change, so can the duration of a half-life (National Pesticide Information Center 2017).

Pesticides can immediately enter an ecosystem through such avenues as application sprays, dust in the soil or air from seed treatments (Corn Dust Research Consortium 2015, Tsvetkov et al. 2017, Woodcock et al 2017), additives in irrigation systems, or incidental run off and spray drift beyond intended targets. However, because soil and water are ultimate sinks for pesticides, chemicals can be present in bees' foraging landscapes long before bees are actively

visiting a crop in bloom (Kubik et al. 1999, Larson et al. 2015, Long and Krupke 2016, Tsvetkov et al. 2017, Woodcock et al. 2017). Soil is adsorbent with its hydrophobic domains, and chemicals having high K_{ow} and K_{oc} allows them to cling to the soil and persist in this matrix (Fisk 1995, Klaasen 2007, Palmquist et al. 2012). Water acts as solvent and can displace chemicals from hydrophobic domains of soil. Therefore, water disperses chemicals with low K_{ow} and K_{oc} across the environment or allows them to accumulate in a local water source or move beyond the immediate application area (e.g., run-off).

Major Pesticide Classes and Properties

Organochlorines are very persistent nerve toxins that bioaccumulate, such as dichlorodiphenyltrichloroethane (DDT). After extensive use as an important insecticide, DDT was banned by the U.S. Environmental Protection Agency (USEPA) in the early 1970s, because its pervasive and negative environmental and human impacts were realized (Carson 1962, Heberer and Dünnbier 1999). Currently-used organochlorines also are environmentally persistent due to low water solubility (Saldalgo 2013) (Table 1).

Organophosphates and carbamates are also nerve toxins, but with a different mode of action than the organochlorines (Table 1). Organophosphates were originally developed as nerve gases for use in chemical warfare, and many are now banned due to their high human toxicity. Carbamates, used as insecticides and fungicides, have similar modes of action as organophosphates. Although much less widely used now than when popular from 1950s-1980s, carbamates are still applied as broad-spectrum insecticides that protect large commodity crops (e.g., fruit trees, cotton, vegetable and row crops), and their field use remains a concern for bee safety. Like organophosphates, carbamates can have high vertebrate toxicity. Although some

organophosphates are water soluble and can leach into ground water, other organophosphates as well as carbamates that adhere to soil matter can move into water along with soil sediment (Singh 2012, Saldalgo 2013). However, they are easily degraded in nature and not considered persistent or likely to biomagnify (Saldalgo 2013). Carbamates have high lipophilicity, which facilitates their ability to reach an insect's nervous system simply by crossing the lipid-coated cuticle (Ishaaya and Horowitz 1998).

Pyrethroids are synthetic derivatives of the naturally-occurring pyrethrins from chrysanthemums. They are neurotoxins like organophosphates and carbamates, but they are much less persistent than organochlorines, largely due to degradation mechanisms that are catalyzed by ultraviolet light, water and oxygen (Palmquist et al. 2012, Saldalgo 2013). Pyrethroids might offer a potentially reduced risk insecticide option if the spray occurs at night when bees are not on the crop and if the chemicals are degraded under the morning sun before bees begin their forays into the field. However, many pest insects have developed resistance to this insecticide family (Ishaaya and Horowitz 1998). Pyrethroids also do not biomagnify because of their low soil mobility (i.e., their propensity to adhere to soil particles), which reduces a tendency to leach (Saldalgo 2013).

Neonicotinoids are pesticides that overstimulate insect nerve receptors, which eventually causes paralysis and death. Formulations of this relatively new pesticide family are the most widely used insecticides in the world (Goulson 2013, Lundin et al. 2015). Neonicotinoids currently arouse contentious discussion within and outside of the scientific community because of their widespread use and sometimes conflicting claims of negative effects on bees. They are used as seed, soil, and trunk treatments, are painted onto plants, and are applied as foliar sprays (Saldalgo 2013). They are systemic insecticides, being highly water soluble with a low K_{ow} so

that they are absorbed and stored in plant tissue (Ishaaya and Degheele 1998) and occur in nectar and pollen, all of which are major sources of exposure to bees (Goulson 2013, Godfray et al. 2014, 2015; Botías et al. 2015, Rundlöf et al. 2015, Stewart et al. 2014, David et al. 2016, Long and Krupke 2016, Tsvetkov et al. 2017). Neonicotinoids are also prone to leaching, are moderately persistent in the environment, but do not biomagnify (Saldalgo 2013). Due to their hydrophilicity, common neonicotinoids have been detected in macro-ecosystems, such as wetlands of Canada and the Netherlands where invertebrates and vertebrates alike could be exposed (Hallmann et al. 2014, Main et al. 2014, Samson-Robert et al. 2014, Schaafsma et al. 2015), and in micro-ecosystems, such as in guttation fluid of cantaloupe plants that honey bees may imbibe (Hoffman and Castle 2012, Fairbrother et al. 2014).

Anthranilic diamide insecticides are unique ryanodine receptor modulators. Ryanodine binds to the ryanodine receptor, which locks the calcium channel in a partially open condition. By leading to the loss of calcium regulation, a chewing insect that has ingested a diamide insecticide becomes lethargic or paralyzed, ceases to feed, and eventually dies (Teixeira and Andaloro 2013). Diamides, such as, chlorantraniliprole (Cordova et al. 2006, EPA 2008), are used as foliar sprays and in drip irrigation. Recent widespread global use of diamides raises concerns of insect resistance (Teixeria and Andaloro 2013), and extended use may result in soil accumulation (EPA 2008). Persistence in some environments is mitigated by degradation via hydrolysis, light, leaching and runoff (EPA 2008).

Insect growth regulators (IGRs) and juvenile hormone mimics are biorational (reduced risk) pesticides. They are designed to attack immature insects because they prevent molting by inhibiting chitin synthesis or by mimicking molting hormones at the molecular level by binding with receptors (but being ineffective at gene regulation of ecdysis) (Retnakaran et al. 2003).

Such effects result in a soft exoskeleton, deformed appendages and sexual organs, and incomplete larval and pupal molts. IGRs work slower than the other "knock-down" pesticides, but are more effective at reducing an entire pest population because affected insects never reach the reproductive adult stage. Due to very low water solubility, most IGRs are unlikely to leach through the soil, and some persist in the environment with activity at very low levels (Saldalgo 2013). Furthermore, translaminar movement into plant tissue extends the duration of the efficacy of some IGRs, such as the product novaluron (Cutler and Scott-Dupree 2007).

Fungicides can be divided into classes by their chemical structure or by their mode of action. Such classes include the aniline pyrimidines, sterol biosynthesis inhibitors, and succinate dehydrogenase inhibitors (http://www.frac.info/working-group) (Table 1). Fungicides are widely used in agriculture, and there is recent evidence of their sublethal, and perhaps lethal, impact on bees (Ladurner et al. 2005, 2008; Artz and Pitts-Singer 2015; Fisher et al. 2017). Because they are regarded as safe for bees, these chemicals are sprayed during bloom when bees are present as managed and wild pollinators. Although care is often taken to only spray at night, direct, indirect and synergistic effects on bees have been demonstrated in the field and laboratory (Pettis et al. 2013, Sanchez-Bayo and Goka 2014, Artz and Pitts-Singer 2015, Sgolastra et al. 2016, Fisher et al. 2017). Effects on honey bees include worker mortality (Fisher et al. 2017), possibly through inhibition of detoxification mechanisms (Pillings et al. 1995), and effects on solitary bees include disorientation and dispersal from nest sites (Ladurner et al. 2008, Artz and Pitts-Singer 2015).

Herbicides also are among the pesticides detected in wax and pollen in honey bee hives (Mullin et al. 2010, Johnson et al. 2010). Recently, certain herbicides have been shown to affect the bee carotenoid-retinoid system, which is critical for larval development, bee vision and

antioxidant capacity, and may increase bee foraging activity (Helmer et al. 2015). The herbicide glyphosate has been shown to affect conditional learning and also navigation in honey bees (Herbert et al. 2014, Balbuena et al. 2015). Although sublethal effects of herbicides may affect bee health, we will not be discussing them specifically in this paper.

Routes of Bee Exposure to Pesticides

The accumulation of pesticides in both soil and water, and the presence of contaminated nesting materials and food sources within bee foraging ranges, create conditions under which cavity-nesting bees are particularly vulnerable to many potential sources of contamination and the consequences that follow exposure. How pesticide and bee behaviors interact are discussed in the following routes of pesticide exposure for cavity-nesting bees.

Route 1: Larval Ingestion

The routes that pesticides travel to the limited food stores of solitary bee larvae can be attributed to the intersection of pesticides present in the environment and bee nesting behavior (Fig. 2). A single pollen-nectar mass provision created from naturally-occurring resources is the sole source of food consumed by a larva for development to adulthood. If pollen and/or nectar harbor pesticides through systemic uptake by the plant, from direct topical application, or dust clouds and residuals from planting of pesticide-treated seeds, then there is no mechanism for the larva to avoid ingestion of contaminants (except to cease feeding), and any potential detrimental effects of pesticides on larval survival or later adult fecundity will be suffered. Another means of larval exposure via ingestion may originate from the nest-building material (usually soil or leaves) fashioned by the mother bee into cell linings or partitions. Leaf material may be

contaminated at the surface or internally through translaminar and systemic actions of pesticides. Soil can be contaminated with persistent, soil-bound chemicals that land directly on the soil surface, and also temporarily contaminated by pesticides that move with water deeper into or through the soil matrix. Soil also may be contaminated by agricultural aqueous runoff that contains pesticides (Russel 1995, Klaasen 2007). Pesticide residues in nest cell materials may leach from the material into the soft, wet provision. Because nectar is aqueous and contains water and carbohydrates (sugars) (Cane et al. 2011), and because pollen contains lipids and proteins (Dobson 1988, Roulston and Cane 2000), the nectar in the provision mass could attract agrochemicals with a low K_{ow}, and the pollen could attract chemicals with a high K_{ow}. Therefore, the interface between provision mass and contaminated nest material may allow a slow, passive transference of toxins that a larva will eventually encounter through contact or ingestion.

Studies that focus on the effects of pesticides on bee larvae and how those larvae are exposed remain less common than studies on adult bees (Huntzinger et al. 2008b, Sgolastra et al. 2015). Within the hive, it is difficult to follow individual honey bee larvae through development, and even more difficult to know exactly what larval foods are gathered and processed by workers for progressive feeding of each larva. Individual solitary bee larvae in cavity nests are more amenable than honey bee larvae to studies of contamination of larval food and subsequent effects, but studies of solitary bee larvae of ground-nesting species are lacking, due to the absence of techniques for managing these bees in artificial nests or rearing them in the laboratory so that they can be observed over time.

Route 1 Examples:

A. Huntzinger et al. 2008b: In a laboratory study, *Megachile rotundata* F. (Megachilidae) nest cells were uncapped, and provisions remained intact after being placed into plastic well plates. The provisions were injected with four fungicide formulations (1 µl solution under the egg of each provision) to examine their effects on the fungal pathogen *Ascosphaera aggregata* (Skou) and evaluate non-target effects on bee larvae. Fungal spores contaminate larval provisions, and the fungus develops inside larval guts after being eaten. The resulting lethal fungal disease of larvae is called chalkbrood. Three of the four fungicides reduced *A. aggregata* hyphal growth. Interestingly, the fungicide captan (concenrtration of 700g a.i./liter) was ineffective at controlling *A. aggregata* and was lethal to the bee larvae.

B. Hodgson et al. 2011: Using similar techniques to Huntzinger et al. (2008b), *M. rotundata* provisions were dosed with 0.5-10 times the field rate (745 ml/ha) of the chitin synthesis inhibitor novaluron (Table 1) recommended for control of the seed predator, *Lygus hesperus* Knight (Hemiptera: Miridae). In treated bee cells at all dose rates, *M. rotundata* eggs and early instars suffered very high mortality (>85%) compared to controls (>60%). Such consequences for pollinator reproduction (here and in other examples) raises serious concerns for growers that must rely on commercially managed *M. rotundata* for alfalfa seed production.

C. Pitts-Singer and Barbour 2016: *M. rotundata* exposure to novaluron was also studied in large cages placed over a blooming alfalfa plot in which mother bees made nest cells from leaf pieces that had been sprayed with a hand-held sprayer (at full field rate, 745 ml/ha) with novaluron 7-14 days before nesting commenced. Compared to survival of larvae (average mortality approximately 10%) in cages where no novaluron was ever sprayed, significantly more larvae died as eggs or first instars (average mortality approximately 54-74%) in nests from the cages with novaluron-treated alfalfa. Results suggested the possibility that novaluron-treated

alfalfa leaf pieces used to make cell linings were the source of contaminates that could leach into the larval provision that, when fed upon, interrupted larval development. Because alfalfa flowers wilt within a few days after opening (Carlson 1928), those that had gotten sprayed would have already closed by the time that bees were introduced to cages. Therefore, only newly opened flower would have been present, and the nectar and pollen from flowers present at the time of treatment could not have been the source of novaluron contamination.

D. Abbott et al. 2008, Nicholls et al. 2017: By dosing *Osmia lignaria* Say (Megachilidae) mass provisions (natural and ones made of pulverized honey bee pollen) with the neonicotinoid imidacloprid, and *M. rotundata* provisions with clothianidin, larvae were monitored for lethal and sublethal effects (Abbott et al. 2008). No lethal effects were observed in either species at any concentration tested (range = 3-300 ppm). This outcome was explained by the presumed degradation of the products before enough provision had been consumed to cause an effect. However, one sublethal effect was detected: *O. lignaria* larval development and cocoon spinning took longer at the higher doses of imidacloprid (30-300 ppm). A similar type of study that dosed natural provisions of *O. bicornis* with clothianidin (0-10 ppb) showed no effect on larval development time, overwintering survival, or adult weight (Nicholls et al. 2017).

Route 2: Adult Ingestion

Although adult bee ingestion is a well-established risk assessment parameter for honey bees and bumble bees, some studies also confirm that contaminated adult bee food, nectar and pollen, can have a detrimental impact on solitary bees (Mommaerts et al. 2006, Gill and Raine 2014) (Fig. 3). Active solitary adult bees regularly ingest nectar to maintain their energy, and newly emerged female bees also consume pollen to aid in ovary maturation and egg development

(Cane 2016). Likewise, during the solitary founding phase of bumble bee colony cycles, queen bumble bees also risk exposure to contaminated nectar and pollen that negatively impacts survival, nest initiation, and ovary development (Baron et al. 2017, Wu-Smart and Spivak 2017)

Use of the mandibles and tarsi to manipulate and move soil and leaf material may prove another means of adult pesticide ingestion. When constructing nests, bees such as *M. rotundata* females may incidentally ingest masticated leaf material and plant juices, and mason bees such as *O. lignaria* may ingest water or particles from moist soil. Furthermore, bees groom their bodies, which includes use of mouthparts for cleaning body parts, and they may imbibe contaminants or contaminated materials by performing this behavior. To date, no studies have revealed negative effects of contaminated nesting-building materials on solitary female bees nor quantified the amount of pesticide residues (i.e., pesticides and their metabolites) that may exist on or in nest-building materials for direct or indirect bee exposure. It is not clear to what extent solitary bees encounter pesticides by actively collecting standing water, but honey bee workers collect water to make honey and cool the hive (Gary 1992, Free 1993).

Route 2 Examples:

A. Ladurner et al. 2005: Using a laboratory feeding technique that incorporates a real flower with a false, fillable ampule that replaces the corolla (Ladurner et al. 2003), *O. lignaria* and honey bee adults were offered 10 µl of five different sucrose plus fungicide solutions. The fungicide propiconazole (65.0 µg a.i./liter) was found to be lethally toxic to both bee species, and captan (122.5 µg a.i./liter) also was lethal to *O. lignaria*.

B. Artz and Pits-Singer 2015: A study was performed in cages, and the probable direct ingestion of (rather than contact with) fungicides sprayed at night on blooming forage using a hand-held sprayer (full field rates: iprodione = 2.2 kg/ha, pyraclostrobin + boscalid = 1.6kg/ha)

resulted in a change in bee nesting behavior. Before foraging on the sprayed flowers, nesting *O. lignaria* and *M. rotundata* females had readily oriented to their nesting tunnels in provided bee boards, but the morning after the spray, they appeared to be confused and unable to find their nests. This behavioral change was sublethal, but in an open-field situation, would likely have resulted in bees eventually abandoning their nests, as has been reported anecdotally when managed *O. lignaria* were used in cherry and almond pollination (Ladurner et al. 2008).

C. Peach et al. 1995: Sublethal effects of carbaryl (a carbamate) were evaluated for *M. rotundata* after female adults were fed carbaryl bran bait in honey water or plain wheat bran mixed in honey water. Uniquely-marked females were flown in a greenhouse where white clover was offered as a resource for making nests, which were collected and assessed for revealing reproduction by treatment. There was no effect of treatment on adults, adult nesting behavior, nor progeny survival, size, and sex ratio.

D. Sandrock et al. 2013: Based on field-realistic trace residue amounts, the neonicotinoids thiamethoxam (2.87 μg/kg) and clothianidin (0.45 μg/kg) were mixed into sugar water, and the solutions were offered to *O. bicornis* in the controlled environment of flight cages to examine chronic adult bee exposure. No effect was found on nesting female longevity, but reproduction was significantly affected. In the flight cage with the neonicotinoid treatment, reproduction was decreased, offspring mortality was increased, and sex ratio was more male-biased. However, no pesticide residues were found in larval provisions or adult offspring.

E. Rundlöf et al. 2015, Woodcock 2017: In two studies performed in oilseed rape fields planted with neonicotinoid-treated seeds, reproduction for honey bees, bumble bees, and *O. bicornis* were impaired. *O. bicornis* females that foraged in treated fields produced fewer brood in trap-nests adjacent to treated fields compared to trap-nests at control fields. The mechanisms

by which bee nesting is affected by the presence of residues of insecticides in fields has yet to be discerned.

Route 3: Contact

Physical contact between adult bees and toxins on contaminated resources is the simplest and most direct exposure route assessed for solitary bees (e.g., Ladurner et al. 2005, Huntzinger et al. 2008a, Biddinger et al. 2015) (Fig. 4). Toxins that contact the bee cuticle may penetrate it directly or may pass (actively or passively) into the body through such orifices as spiracular openings or pores. Besides being directly sprayed during pesticide applications, bees can land on or walk about on contaminated surfaces of soil, lawns, flowers, foliage, or artificial nest materials and even water located in treated fields or gardens.

Route 3 Examples:

A. Ladurner et al. 2005: In a study of the effects of five fungicides, an effect was observed immediately after a 1 µl topical dose (or ingestion) (122.5 µg a.i./bee) of captan. *O. lignaria* females exhibited abnormal behaviors, such as inactivity, regurgitation of the ingested sucrose solution, extension of proboscis, abdomen and genitalia. No similar effects were observed for similarly-tested honey bees. The other fungicides had neither acute nor delayed toxic effects on the two bee species.

B. Huntzinger et al. 2008a: Topical doses of the same fungicides used in Huntzinger et al. (2008b) were applied to *M. rotundata* adults. Results showed significantly reduced survival of males treated with captan at 684 g a.i./liter. Female survival was reduced at the lesser amount of 342 g a.i./liter, but inexplicably, not at the higher rate like for males. Other fungicides did not appear to harm the adult bees.

Route 4: Transovarial Transmission

The transovarial transmission of pesticides results when chemicals taken in by the mother bee have a deleterious effect on her offspring, resulting in the suppression of targeted pest populations (Fig. 5). Transovarially transmitted pesticides are ingested by an adult female or they penetrate her cuticle. Although the intended use of these pesticides is to reduce pest insect reproduction and protect a crop, they may also reduce pollinator reproductive success and effect the availability of future pollinators. The direct effect of this route of exposure on reproduction is manifested as low or no survival of eggs or reduced egg production (Ishaaya and Degheele 1998, Mommaerts et al. 2006, Hoffman et al. 2008, Trostanetsky and Kostyukovsky 2008).

Route 4 Examples:

A. Hodgson et al. 2011: *M. rotundata* females were fed a sugar-water + novaluron solution or simply sugar-water in the laboratory. Novalruon was diluted to represent a full field rate (745 ml/ha) in the sugar solution. Females then were allowed to forage on uncontaminated alfalfa for nesting in field cages. Almost all (97%) of the eggs failed to hatch if they were laid by females that fed upon the novaluron-treated solution, while females fed only sugar-water laid many eggs that hatched and survived to full larval development (mortality of 12-20%).

B. Pitts-Singer and Barbour 2016: In a follow-up study to Hodgson et al. (2011), caged *M. rotundata* females foraged on alfalfa that had just been sprayed with novaluron (delivered with a hand-sprayer at full field rate, 745 ml/ha) or that had been sprayed with this same IGR one or two weeks prior to bee presence. Compared to controls (0%), significantly more of the resulting nest cells contained pollen balls with dead eggs (5-26%). A pollen ball is a provision mass with an unhatched egg, or no egg at all (Pitts-Singer 2004). The ovicidal effect may have

been from the mother bees' ingestion of contaminated nectar just after application, or ingestion of chemicals when cutting leaf pieces more than a week post-spray.

Highlights, Areas of Concern, and Research Needs

The routes of exposure that we describe here are certainly not the first to be proposed. However, our scenarios are distinct in their focus on solitary cavity-nesting bees. Other diagrammatic conceptual models heavily emphasize pesticide risks to honey bees, and to a lesser extent to bumble bees, while the few models that depict exposure for other bees offer scant details (Cutler et al. 2014, Purdy 2014, USEPA 2014, Heard et al. 2017). Although current pesticide evaluations for bee safety include ingestion and contact with honey bee adults and larvae, by testing only honey bees as the surrogate for all bees, we achieve an incomplete assessment of pesticide safety for all wild and managed pollinators and are left with many unanswered questions (Johansen and Mayer 1990, Biddinger et al 2013, Arena and Sgolastra 2014, Sanchez-Bayo and Goka 2014).

Our models for solitary bees reveal areas where we lack an understanding of how and at what levels these bees may incur higher exposure risks than honey bees or bumble bees due to differences in nesting, foraging, and social behaviors. A solitary bee may experience different exposure routes, have dissimilar pesticide susceptibility and immune response, and present different or unexpected sublethal symptoms and effects (Sandrock et al. 2013, Arena and Sgolastra 2014, Gill and Raine 2014, Jin et al. 2015). Awareness of the interaction and fate of pesticides in the environment on account of their physical properties will help in formulating hypotheses about the probability and extent of risk in a bee's foraging range and activity portfolio.

Pesticides of most concern for exposure risk to all bees include those that easily contaminate pollen and nectar, affecting both adult and larval stages. Additionally important for solitary bee exposure are those pesticides that are expressed in leaves and are persistent in soils. Not all pesticides are equally relevant in their persistence and movement in the environment, and therefore, their likelihood of coming into contact with bees via the various routes of exposure can be predicted by their chemical properties. Systemic and translaminar pesticides (e.g., neonicotinoids and benzoylureas, respectively) will provide a route of exposure for bees that use vegetative materials in nest construction. Chemicals persistent in the soil (e.g., pyrethroids, spinosyns, anthanilic diamides), can be present year-round in soils collected by orchard bees for use during nesting.

Using products with specific targets, modes of action on immatures only, or low environmental persistence may indeed reduce risk to pollinators in some cases. However, in other cases such as for *M. rotundata* used as a pollinator in alfalfa seed production fields treated for *Lygus* control with an insect growth regulator, the simple act of cutting leaf pieces exposes these bees both topically and orally, which results in all four possible routes of pesticide exposure.

Some government agencies (e.g., United States, European Union, and Australia) are moving towards pesticide evaluations for not only honey bees, but also for bumble bees and some solitary bees (e.g., the European red mason bee, *Osmia bicornis* L. (Haskell and McEwen 1998, EFSA 2014). New techniques and protocols are needed across the globe for making standard assessments on non-*Apis* bees and for performing bioassays that better explore the kinds of exposure routes we describe, especially those that extend beyond the worst case scenarios described for honey bees by USEPA (2014). Expectations of lethal, sublethal, and synergistic

effects need to be based on a thorough understanding of all exposure routes, including the levels of potential contamination in each route under various conditions and how each route contributes to varying amounts of bee exposure through contact, ingestion, transmission and their combinations. Beyond the routes already investigated under current guidelines for honey bees, additional important routes may be realized using an ecosystem approach that examines a representative set of bees to consider situations unique to non-*Apis* wild and managed bees, and how ecosystem services may be disrupted as a consequence (e.g., Stanley et al. 2015). With a robust understanding of routes of pesticide exposure in pollinators, more realistic and effective studies can be conducted to better grasp what direct and indirect factors might lead to pollinator stress, decline, or extinction.

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Tables

Table 1. Examples of modes of action on pests and environmental characteristics of various agricultural insecticide families and fungicide classes

Family/Class	Mode of Action	Active ingredient	Log K_{ow}[a]	Activity in environment[b]
Organochlorine	GABA-gated chloride channel antagonists	Endosulfan	3.83	High persistence
Organophosphate	Acetylcholine esterase inhibitors	Dimethoate	0.78	Low persistence (degradation by microbes), low biomagnification; some with high soil adsorbance; some soluble in water and in runoff
Carbamate	Acetylcholine esterase inhibitors	Carbofuran	2.32	Low persistence (degradation by hydrolysis), low biomagnification
Pyrethroid	Axonic excitoxins (prevent closure of sodium channels)	Bifenthrin	6.00	Quick degradation due to UV, water, and oxygen; environmental residuals mostly absent; high soil adsorbance; lipophilic and insoluble in water
Neonicotinoid	Nicotinic acetylcholine receptor agonists or antagonists	Imidacloprid	0.57	High water solubility; systemic; prone to leach into groundwater; moderately persistent; does not biomagnify

Spinosyn	Nicotinic acetylcholine receptor agonists; metabolite of soil actinomycete (bacteria)	Spinosad	2.80–5.20	Low persistence due to photo- and microdegradation; low leaching potential
Sulfoxaflor	Agonists of acetylcholine receptors, by mimicking action of acetylcholine	Sulfoxaflor	0.80	Hydrophilic; rapidly degraded in soil and water
Pyridinecarboxamide	Molecular target not yet identified; Antifeedant effect due to action of compounds on chordotonal organs, proprioceptive sensory organs present throughout the insect body important in hearing, gravity perception, and fine motor coordination	Flonicamid	0.30	Degrades rapidly in soil; low risk of groundwater contamination
Anthanilic diamide	Modulation of ryanodine receptor to cause calcium channel to remain open leading to lethargy, feeding cessation, and death	Chlorantraniliprole	2.90	Persistent and mobile in terrestrial and aquatic environments; residue accumulation in soil after extended use; degradation by hydrolysis, light, leaching, and runoff
Benzoylurea	Chitin biosynthesis inhibitor, type 0	Novaluron	5.27	Translaminar; lipophilic; low water solubility; strong soil adsorption; low leaching potential; persistent

Juvenile hormone mimic	Juvenile hormone and ecdysone analogues	Fenoxycarb	4.30	Lipophilic
Fungicide1[c]	Aniline pyrimidine: inhibits methionine biosynthesis and secretion of hydrolytic enzymes	Pyrimethanil	2.84	Strong soil adsorption; moderately persistent; possible surface runoff with soil particles
Fungicide2	Sterol biosynthesis inhibitor	Iprodione	3.00	Strong soil adsorption; moderately persistent; possible surface runoff with soil particles
Fungicide3	Succinate dehydrogenase inhibitor	Boscalid, Pyroclostrobin	2.96, 3.99	Strong soil adsorption; highly persistent; possible surface runoff with soil particles

[a]Log K_{ow} values from http://www.pubchem.ncbi.nlm.nih.gov.
[b]Characteristics from the following: Thompson et al. (2000), Cutler and Scott-Dupree (2007), Wightwick et al. (2010), Singh 2012, Saldalgo (2013).
[c]Fungicide classifications: http://www.frac.info/working-group.

Figures

Figure 1. A) An *Osmia lignaria* nest box hanging in an almond tree in a California orchard, with close up of mud-plugged nest tubes. B.) Commercial tunnels are made of cardboard or wood, and bees will nest in them, creating aggregations at protective shelters. C.) Mother bees use pollen and nectar to make mass provisions upon which she lays her eggs.

Figure 2. Larval Ingestion Exposure Route with almond orchard example. Developing larvae ingest 1) contaminated pollen and nectar, 2) contaminated soil or plant material used in nest construction, or 3) pesticides leached from nest partition into provisions. Illustration by James Bradford.

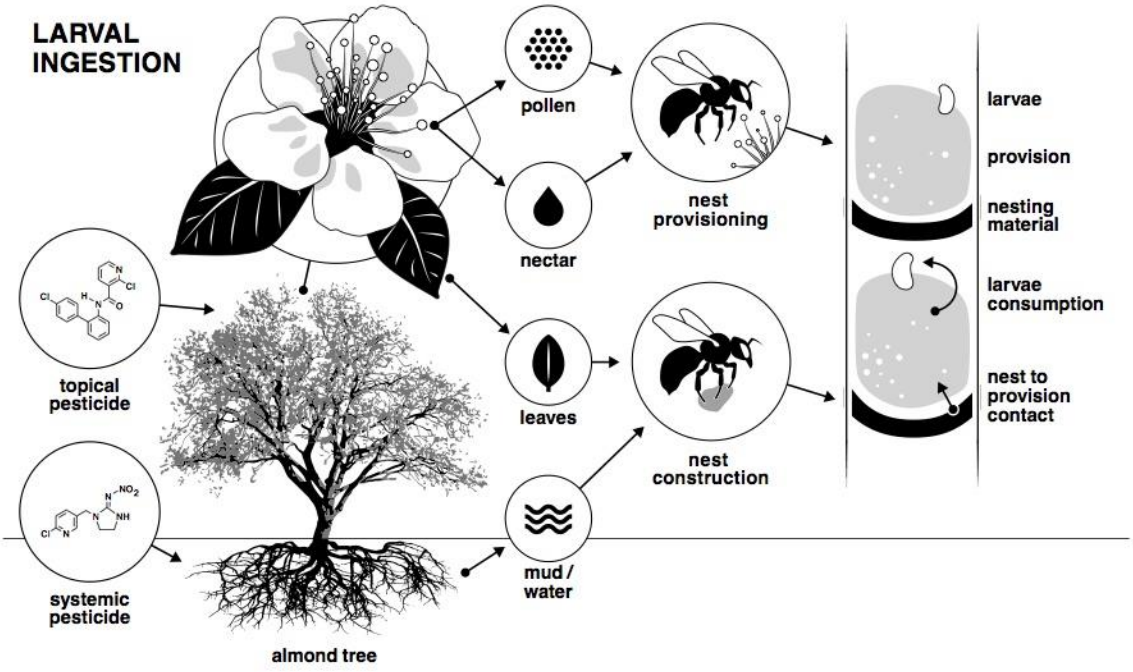


Figure 3. Adult Ingestion Exposure Route with apple orchard example. Adults ingest contaminated 1) nectar and pollen while feeding or provisioning a nest, 2) plant material when cutting or masticating leaves or soil when collecting for nest-building. Illustration by James Bradford.

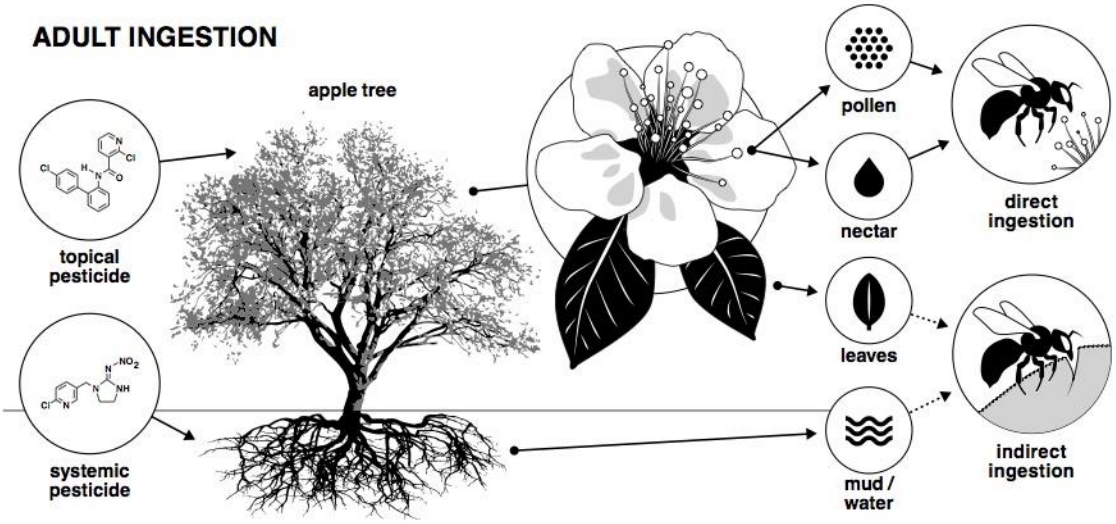


Figure 4. Contact Exposure Route with cherry orchard example. Upon contact, the lipophilic properties of pesticides allow them to enter a bee directly through the cuticle. Illustration by James Bradford.

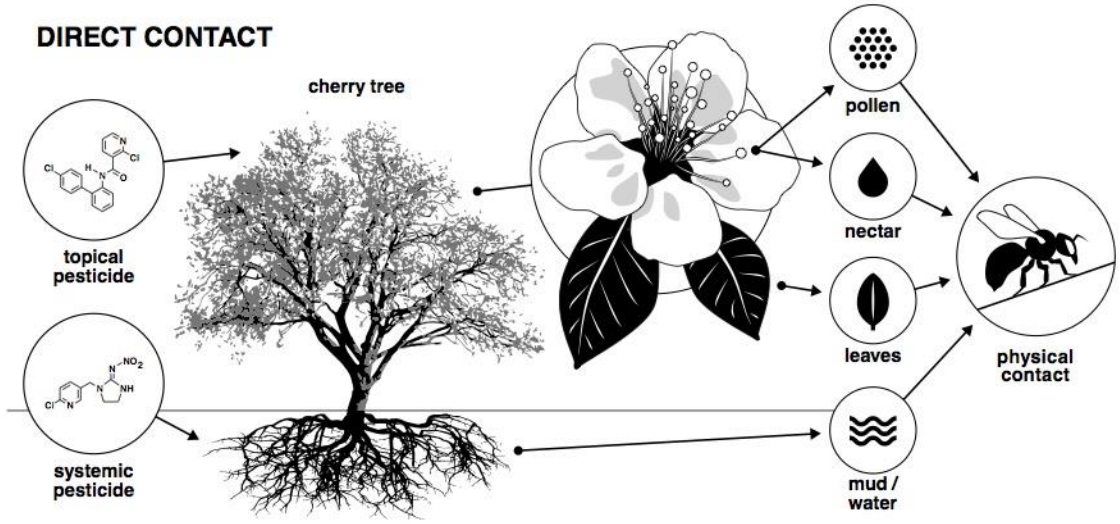
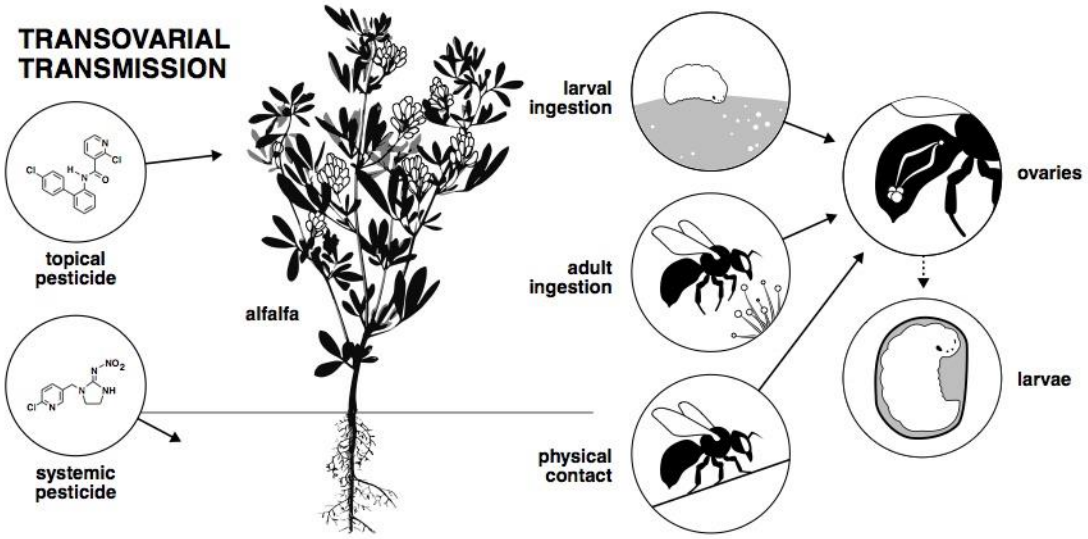


Figure 5. Transovarial Transmission Exposure Route with alfalfa plant example. Pesticides in the mother's system affect (often kill) her eggs, health of her offspring, or reproductive output. Illustration by James Bradford.



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CHAPTER II

EFFECTS OF PROVISION TYPE AND PESTICIDE EXPOSURE ON THE LARVAL DEVELOPMENT OF *OSMIA LIGNARIA* (MEGACHILIDAE)[1]

ABSTRACT

With both native and managed bee species experiencing population declines, understanding the impacts of agricultural practices on developing bees is critical. Delayed larval development could lead to asynchronous emergence, unhealthy and inefficient pollinators, and possibly population decline. Current pesticide risk assessment usually is only performed on honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), adults and larvae, but solitary bees may be differentially exposed to and affected by agrochemicals. We investigated the effect of agrochemicals on developing bee larvae and evaluated a methodology for larval testing using the native solitary bee *Osmia lignaria* Say (Hymenoptera: Megachilidae). Two pollen types (apple and almond), two provision compositions (intact and homogenized), and four agrochemicals (acetamiprid, boscalid/pyraclostrobin, organosilicone, and dimethoate) were delivered at different doses for examination of effects on larval development times and mortality. Statistical analyses only considered the durations of the 2nd to 5th instar and of the 5th instar to cocoon initiation because most bees failed to accomplish cocoon-spinning in artificial cells. Mortality varied by provision type and treatment. All larvae survived to cocoon initiation when only water (control) was added to provisions of all types. When the intact natal provision was used, there was no or low mortality across agrochemical treatments. Mortality in the homogenized provision was highest when acetamiprid was the treatment, especially for provisions made from almond pollen. Optimizing testing methodology for solitary bee exposure to agricultural products is

crucial for properly assessing risks for pollinators and for creating best practices for agricultural systems.

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INTRODUCTION

Native bee species, as well as honey bees, provide important pollination services to flowering plants, including agricultural crops (Kearns et al. 1998, Klein et al. 2007, Kremen et al. 2007, Potts et al. 2010, Gill et al. 2012). Declines in pollinators alongside an increase in pollination demand in agriculture stresses the ability of the honey bee industry to provide cost effective services, which elevates the need for native and alternative pollinators for food production (Kearns et al. 1998, Klein et al. 2007, Potts et al. 2010, Isaacs et al. 2017, Pitts-Singer et al. 2018). When managed bees are necessary for conventional, large-scale cropping systems, such as for almonds and cherries, application of pesticides to protect the crop must be carefully considered when bees are present. For example, approximately 80% of almonds for the world market are grown in California, making them a highly valuable orchard crop (Almond Board of California 2019; CDFA 2020). In order to protect almond flowers, fungicides are applied during almond bloom when bees are foraging and provisioning nests (Bosch and Blas 1994, Artz et al. 2014, Fisher et al. 2017), and insecticides are applied during nut development after bees have been removed. However, depending on the properties of the applied agrochemicals, residues or their metabolites may be present throughout the year in soil, pollen, and nectar (Kopit and Pitts-Singer 2018).

Most studies to date have only considered the effects of pesticides on honey bees (e.g., Kubik et al. 1999, Wu et al. 2011, DeGrandi-Hoffman et al. 2013, Cutler and Scott-Dupree 2014, USEPA, PMRA, and CDPR 2014, Berenbaum 2016, Fisher et al. 2017), but new attention is being paid to non-*Apis* bees, such as bumble bees and some solitary cavity-nesting bees (e.g., Biddinger et al. 2013, EFSA 2013; Elston et al. 2013; Gill and Raine 2014; Godfray et al. 2014, 2015; APVMA 2015; Biddinger and Rajotte 2015; Jin et al. 2015; Lundin et al. 2015; Stanley et

al. 2017). Current pesticide evaluations for bee safety include ingestion and contact assays with honey bee adults and larvae, using this single species as the surrogate for approximately 20,000 species globally (Johansen and Mayer 1990, Michener 2000, Biddinger et al. 2013, Arena and Sgolastra 2014, Sanchez-Bayo and Goka 2014). Government agencies around the world are beginning to evaluate pesticides on bumble bees and some solitary bees (EFSA 2014, Boyle et al. 2019), and pesticide evaluations will need to consider particular bee biology and the properties of pesticides that influence how they move through the bee environment (Kopit and Pitts-Singer 2018; Gierer et al. 2019). Although some studies have employed laboratory bioassays to examine pesticide effects on solitary bee larvae that fed on contaminated provision masses (Huntzinger et al. 2008, Hodgson et al. 2011, Anderson and Harmon-Threatt 2019; Fortuin et al. 2020), techniques were not standardized and may not have appropriately represented how a larva encounters the contaminant that could lead to an acute or chronic exposure. New techniques and protocols are needed for making standard assessments of pesticides on solitary bees and for performing bioassays that better explore the routes of pesticide exposure in natural and agricultural systems. There are four potential routes of pesticide exposure in solitary cavity nesting bees: larval ingestion, adult ingestion, contact, and transovarial transmission (Kopit and Pitts-Singer 2018). For this study, we focus on the route of larval ingestion in *O. lignaria* and how pesticide exposure can impact larvae.

Osmia lignaria is an efficient orchard and berry pollinator (Torchio 1976, Torchio 1982, Bosch and Kemp 2002, Bosch et al. 2006, Pitts-Singer et al. 2018, Andrikopoulos and Cane 2018). This solitary, cavity-nesting bee has a wide distribution across North America. It overwinters as an adult and is active in early spring, producing only one generation a year (Bosch and Kemp 2001). For use of *O. lignaria* as a managed pollinator, nesting cavities are

provided and are made of wood tunnels or paper straws often held in nest boxes as shelters. Shelters protect the nests from rain and sun and can be mounted in orchards or hung from branches, while wild populations use preexisting holes such as abandoned wood boring beetle burrows (Bosch and Kemp 2001, Cane et al. 2007). Males emerge from cocoons 1-3 days ahead of females and wait near nest sites to copulate with newly emerged females. To build nests within a cavity, females gather moist soil to create partitions between individual nest cells and forage for pollen and nectar to make mass provisions (Bosch and Kemp 2001, Cane et al. 2007). One egg is laid on each provision mass, and after making several cells (usually one per day), the female seals the opening of the cavity with a mud plug (Bosch and Kemp 2001, Cane et al. 2007). Once the cell is sealed, no further contact is made between the female and her offspring, unlike honey bees that progressively feed and protect brood in the hive (Michener 2000, Bosch and Kemp 2002). In a commercial orchard, an *O. lignaria* female typically produces 2-4 female cells and 5-8 male cells during her 20-day lifespan. Offspring develop over the summer and become adults before winter (Bosch and Kemp 2000, 2001). In commercial situations, bees are managed from fall to spring. Cocooned adults are left inside natal nests or are extracted from nests so that they can be sorted by size into females and males. Winter storage is usually 4-5°C for a recommended ≥ 180 days for optimal survival and synchronous emergence of bees with orchard bloom (Bosch and Kemp 2001).

For pollinator-dependent crops, honey bees are moved into orchards or fields when bloom is imminent and are removed quickly after bloom ends. Honey bee colonies are transported to pollinate other blooming crops, and the colonies continue brood production through spring and summer. However, a solitary female such as *O. lignaria* must accomplish her lifetime reproduction in several weeks of spring. In almonds and other orchard crops, the bloom

time is shorter than a female's lifespan, but moving bees to new localities disrupts nesting. Therefore, alternative forage (naturally occurring or planted for bees) near the orchards can expand *O. lignaria* nesting time so that the pollinator population can be better sustained (Boyle and Pitts-Singer 2017, Boyle et al. 2020).

Pesticide use on pollinator-dependent crops poses threats beyond acute lethality. Exposure to sublethal pesticide doses affects larval development and adult longevity in honey bees (e.g., Wu et al. 2011, Renzi et al. 2016) and impacts colony success and larval development in bumble bees (e.g., Gill et al. 2012). Without the resilience of the worker-filled colony, solitary bee populations may be more impacted by delayed development and the loss of reproductive females than social bee populations. *Osmia lignaria* females that provision nest cells with contaminated floral resources during crop bloom may be exposing developing larvae to individual or mixtures of agrochemicals in each larva's life-time supply of food (Holloway et al. 2000). Furthermore, usually not addressed in pesticide risk assessments, mixtures of pesticides have been shown to have synergistic effects on pollinators (Pilling et al. 1995, Bingham et al. 2008, Biddinger et al. 2013, David et al. 2016). More specifically, the synergistic effects of insecticide plus fungicide mixes have proven to increase bee toxicity (Pilling et al. 1995, Papaefthimiou and Theophilidis 2001, Biddinger et al. 2013, Wade et al. 2019).

Our study objectives were to assess effects on *O. lignaria* larval survival and development times on account of 1) bee provision preparations and methodology for contaminating with agrochemicals, 2) various doses of agrochemicals in provisions, and 3) potential synergism of an insecticide plus fungicide mixture. Agrochemicals included in this study are two insecticides, a fungicide, a combination of an insecticide and a fungicide, and an adjuvant.

We chose to test the neonicotinoid acetamiprid because its topical application was found to be the least toxic to honey bees and *Osmia cornifrons* Radoszkowski among other neonicotinoids tested, and it was less toxic to *O. cornifrons* than to honey bees (Biddinger et al. 2013, Phan et al. 2020). Additionally, when acetamiprid at LD_{50} (dose that is lethal to 50% of tested individuals) was paired with the fungicide fenbuconazole, which did not have a lethal effect on these bees, the synergistic effect was a five-fold increase in toxicity compared to the insecticide alone (Biddinger et al. 2013). A more lethal insecticide may preclude the ability to assess larval development over time. Dimethoate was the second insecticide chosen because of its common use as a positive reference compound for pesticide testing with honey bees (EFSA 2013).

We chose the fungicide boscalid/pyraclostrobin (BCL/PCSB) because of its widespread use in agricultural systems. The formulation BCL/PCSB is a common carbamate fungicide used in California almond orchards where precipitation during bloom can facilitate fungal diseases such as brown rot (UC IPM 2017). Fungicides are applied to the almond tree buds, and during particularly wet seasons, multiple applications are used to control fungal pathogens (Connell 2002). However, BCL/PCSB may inhibit microbial function that aids in pollen digestion in honey bees (DeGrandi-Hoffman et al. 2017). It also has known sublethal behavioral effects on *O. lignaria* females (Artz et al. 2014, California Department of Pesticide Regulation). Reports of confusion at *O. lignaria* nest sites and loss of females in a conventional almond orchard after BCL/PCSB was sprayed (Ladurner et al. 2008) led to field cages studies in which BCL/PCSB disrupted the ability of *O. lignaria* and *Megachile rotundata* F. (Megachilidae) females to directly return to their own artificial tunnels (Artz and Pitts-Singer 2015).

We also sought to determine if mixing acetamiprid and BCL/PCSB would result in a synergistic or additive effect on bees. There are known effects on honey bees of BCL/PCSB plus some insecticides used in almond orchards (Wade et al. 2019), but this particular combination has not been tested. Lastly, adjuvants are additives to pesticides that aid in the uptake of the active ingredient by plants. They are also used in many cropping systems and are sometimes premixed in pesticide formulations (but labeled as inert ingredients). We chose to test an organosilicone (OSS) because this type of compound can affect honey bee learning and susceptibility to viruses (Mullen et al. 2015, 2016, Fine et al. 2017).

METHODS

Osmia lignaria **Management**

Osmia lignaria adults (in cocoons excised from nests; Watts Solitary Bees, Bothell, WA) were kept in cold storage (4-5°C) until artificial nest cavities were placed in an apple orchard in River Heights, Utah, USA just ahead of bud break. To acquire bee eggs and young larvae for this study required that bees actively nest in provided 49-tunnel wooden nesting blocks with inserted paper straws (7.5 mm dia. tunnel × 15 cm length). We warmed the overwintered bees in an incubator (Percival Scientific, Perry, IA) at 26°C and approx. 40% relative humidity, and after a 2-3 days many males and a few females had emerged from cocoons (Bosch and Kemp 2001). At this time, emerged and about-to-emerged adults were placed in release boxes that were situated in the orchard near provided nesting sites. Bees flew from the boxes and commenced to mate and build nests. Freshly plugged nests within paper straws were collected from the field on 5 May 2016 and 17 May 2016.

To remove provisions and eggs from the nests, longitudinal cuts were made into the straws with razor blades. Paper flaps were pinned back onto a foam board to facilitate collection of provisions and also eggs or 1st instars still inside the chorion (henceforth, "egg" includes egg and 1st instar; older larvae were not used in the study) (Fig. 1).

Provision Types

Three different provision types were used to test effects on larvae due to diet source and consistency: intact provisions made by *O. lignaria* in an apple orchard, homogenized provisions from the same apple orchard, and homogenized *A. mellifera* pollen pellets from an almond orchard. Intact provisions (Fig. 1) were transferred from nests along with the egg directly into the wells of 48-well cell culture plates (inner diam. = 9 mm) (Corning® CellBIND® Multiple Well Plate, Corning, Inc., Glendale, AZ) (Fig. 2) that served as artificial bee cells (similar to Huntzinger et al. 2008, Klinger et al. 2015). These natal provisions were randomly selected from various nests and cells for placement in the well plates; thus, male and female cells from multiple nests were present in each treatment, which meant they were not uniform in size.

The homogenized apple provisions were made by blending many *O. lignaria* provisions in a household coffee grinder until they formed a paste. The homogenized mixture was then partitioned into approximately 0.35 g patties, which was the average weight of natal provisions taken from the same apple orchard. Using a modified 3 mL syringe and a razor blade, the paste was deposited into wells of the 48-well plates (Fig. 2). The homogenized almond provision was made from almond pollen taken from pollen traps on *A. mellifera* hives in a California almond orchard (Wonderful Orchards, California) in March 2016. The pellets were blended with a coffee grinder and mixed by hand for one minute with a sucrose solution (1:1 sucrose in water) until a

paste similar to that of the blended apple provisions was achieved. Just as for the apple homogenate, 0.35 g of paste was deposited into wells of culture plates.

Eggs were transferred from the *O. lignaria* apple orchard nests to the homogenized provision masses. A honey beekeeper grafting tool was used to make depressions in each soft provision mass. To lift an egg, the grafting tool was dipped into commercial (over-the-counter) saline solution so that gentle prodding would move the egg onto the tool tip and into the solution. The solution then helped the egg to slide onto the provision without sticking to the tool itself. Transferred eggs were examined under a dissecting microscope to ensure that eggs were undamaged; damaged eggs were replaced.

Although pathogens can present health and mortality problems in bee rearing studies for which sterilization of larval diet is recommended, pathogens are less pervasive for *O. lignaria* compared to those of another managed solitary bee, *Megachile rotundata* F. (Megachilidae) (Huntzinger et al. 2008, Klinger et al. 2015). Therefore, we did not sterilize provision materials in this study. In addition, sterilization processes can destroy microbiota that potentially contribute to larval nutritional requirements and are important for pollen digestion (e.g., DeGrandi-Hoffman 2017, Dharampal et al. 2019).

Pesticide Exposure and Dosing

Although the orchards that were the origins of pollen and nectar for our experimental larval food were not sprayed with pesticides during bloom, we preserved samples of provisions for chemical evaluation for pesticides that could have confounded our experiment. Samples of each provision type (≥3 g each) were sent to USDA Agricultural Marketing Service, National Science Laboratories, Gastonia, NC in August 2016, and several pesticides were found in both provision sources (Table 1). Pendimethalin, a dinitroaniline herbicide, and 2,4 dimethylphenyl

formamide (DMPF), a non-systemic acaricide, were the most prevalent pesticides in both sources at similar levels. These pesticide contaminants were present at very low levels in all provision sources, and none were the pesticides used in this bioassay. Therefore, it is unlikely our bioassays were affected by their presence.

For the homogenized provisions, agrochemical treatments (acetamiprid, boscalid/pyraclostrobin, dimethoate, the acetamiprid + boscalid/pyraclostrobin mixture, and the organosilicone adjuvant) were added to determine how they impacted larval development. Except for dimethoate, chemical formulations were diluted to provide specific parts per million or billion (ppm or ppb) suspected to be at a level that would result in sublethal effects so that larval development could be assessed (Table 2). RO water was used to create treatment solutions; thus, RO water was added to provisions as the control. The sublethal doses level were at $LD_{12.5}$ LD_{25} and LD_{50} for acetamiprid and BCL/PCSB, and these doses were based on lethal doses (LD_{50}) reported in Johnson et al. (2010) and Johnson (2015) for *A. mellifera* and in Biddinger et al. (2013) for *O. cornifrons* adults. To assess potential synergistic or additive interactions between acetamiprid and BCL/PCSB, the two pesticide solutions were blended, resulting in mixtures with proposed LD_{25} and LD_{50} doses. The dimethoate was added at the oral LD_{100} dose for *A. mellifera* adults (Fiedler 1987, Gough et al. 1994, Ladurner et al 2005, Medrzycki et al. 2013) to achieve the delivery of a greater, possibly lethal larval dose.

Treatment solutions were adjusted with small amounts of water so that each batch of homogenate (8.75 g) received equivalent amounts (130 µl) of treatment solution or water as the control. Each batch of homogenized apple provisions and the homogenized almond provisions produced 25 provisions for the culture plates (0.35 g per provision mass). Each homogenized provision batch and stock solution were mixed thoroughly for one minute by hand using a metal

spatula. Therefore, the appropriate amount of active ingredient was achieved for each provision mass once doled out into wells. For logistic simplicity, each culture plate contained a single treatment and provision type.

For the intact provision masses, the same stock solutions as for the homogenized provisions were used, but only 1-3 µl of any solution was injected into the provision. The choice of the small aliquots of solutions was to resemble the techniques of previous studies (e.g. Huntzinger et al. 2008, Hodgson et al. 2011). However, these other studies based the product dose using application field rate solutions, and solutions were injected on the top of the provision mass next to or under eggs. In this study, treatment solutions were administered to intact natal apple provisions resting in well plates using a 50 mL-micro-syringe with a repeating dispenser (Hamilton Co., Franklin, MA) (Table 3). The dispenser tip was carefully inserted into each provision approximately 3 mm beneath the egg. For the chemicals prepared as $LD_{12.5}$, LD_{25} and LD_{50} dose solutions, 1 µl of the lowest dose stock solution was injected as the treatments for $LD_{12.5}$, 2 µl of the same solution for the LD_{25} treatments, and 3 µl for the LD_{50} treatments. For dimethoate, OSS, and water, only 1 µl of stock solution was added. There were no adjustments to create specific ppm based on a.i. per g of these non-uniform intact provision masses, and the ppm was inherently much less than that applied to homogenized provisions. The exception would be if the solution accumulated at the injection site, then the ppm at that site would be higher than for a similarly sized site in a homogenized provision.

Culture plates were covered with plastic lids to maintain moisture while larvae incubated at settings of 26°C and 40% relative humidity. Daily observations were made to document larval development and survival and to assure that provision masses were neither drying out nor becoming moldy. The stages observed and recorded were egg, 1st instar (inside egg chorion), 2nd

instar (feeding), 5th instar (larva covered in fine hairs and is defecating), initiation of cocoon, cocoon completion, and death. For the daily inspections under a dissecting microscope, all culture plates (Fig. 2) were simultaneously removed from the incubator and kept at laboratory temperature for 60-90 min.

For most larvae reared on all provision types and treatments, we were unable to record the later life stages because, once they began to spin their cocoons, they continued for many days to add silk inside and often outside of their wells, with some failing to complete the cocoon before dying. Presumably, because the wells were larger than an optimally-sized nest cell, the time period to finish cocoons was highly variable and very long, which may have reduced survival of bees before completing the cocoon or surviving to adulthood and overwintering. Indeed, most bees died as prepupae, only a few transitioned to adulthood, and no bees emerged in the spring. Given this discovery, we restricted our subsequent analyses to the pre-cocooned stages of development.

STATISTICAL ANALYSIS

Because many of the larvae failed to complete cocoons (see above), we looked for provision type and treatment effects on the number of days for each larva to develop from 2nd instar to 5th instar and from the beginning of the 5th instar until the larva began to spin a cocoon (cocoon initiation = CI). First, to examine if the provision type affected develop times, we used a generalized linear model (PROC GLIMMIX; SAS 2013) with a normal distribution to compare the provision type effects by examining only the controls (water) for 1) the homogenized apple and the intact apple provision types and 2) the homogenized almond and homogenized apple provision types. Then we assessed the treatment effects between 1) the homogenized apple and

the intact apple provision types, 2) the homogenized almond and homogenized apple provision types, and 3) within each provision type. As appropriate, analyses were followed by Tukey multiple comparisons to reveal which treatments were significantly different. Where applicable, the data for homogenized apple provision was limited to only the treatments that also were applied to the homogenized almond provisions (Table 3).

RESULTS

The percentage of bees that lived to initiate a cocoon varied by provision type and treatment (Table 4). All larvae survived to start releasing silk threads to spin their cocoons when only water was added to provisions as the control. Mortality otherwise was lowest when the intact provision was used, where no mortality was observed when the treatments were acetamiprid $LD_{12.5}$, BCL/PCSB $LD_{12.5}$ and LD_{25}, and mixed pesticide LD_{25}. For the homogenized provision, percent mortality was highest when acetamiprid was the treatment, especially for the almond provisions. For the lowest dose of BCL/PCSB, larval mortality only occurred with use of the almond provision. A low percentage of larvae died when the OSS was added, regardless of provision type. Mortality was surprisingly low for provisions treated with dimethoate for which high mortality was expected.

Intact vs Homogenized Apple Provisions

There was no significant difference between intact (n = 48; mean ± SE = 4.15 ± 0.07) and homogenized apple (n = 43; mean ± SE = 4.42 ± 0.13) provisions treated with water controls for the duration of the 2^{nd} - 5^{th} stage ($F = 3.62$, df = 1, 89, $P = 0.06$). However, the duration of the 5^{th} - CI stage was significantly longer in the homogenized provisions (n = 43; mean ± SE = 17.30 ±

0.41) than in the intact provisions (n = 48; mean ± SE = 13.06 ± 0.40) (F = 55.55, df = 1, 89, P < 0.0001) (Figs. 3 and 4).

Examination of all the apple provision treatments (including control) for effects of preparation of the provision, treatment, and their interaction revealed that all factors were significant (Table 5, Figs. 3&4). Pairwise comparisons also revealed the significant treatment differences (Tables S1&S2) within each provision type. Within the intact apple provision type, there was a significant effect of treatment for both the 2nd - 5th stage (F = 169.30, df = 10, 492, P < 0.0001) and the 5th - CI stage (F = 14.56, df = 10, 480, P < 0.0001) (Table S1; Fig. 3). For the 2nd - 5th instar duration, all treatments for the intact apple provisions were significantly longer than water control except for dimethoate and BCL/PCSB $LD_{12.5}$ and LD_{50} treatments (Table S1). Also, the acetamiprid treatments resulted in longer development times for all other treatments except organosilicone and mixed pesticides. For the 5th – CI stage, BCL/PCSB $LD_{12.5}$ treatment in intact provisions resulted in significantly longer development times than all other treatments and water control. The other BCL/PCSB treatments also caused significantly longer development times than all other treatments except for the control (Fig. 3; Table S1).

Within the homogenized apple provisions, there also was a significant effect of treatment for both the 2nd-5th stage (F = 9.65, df = 10, 430, P < 0.0001) and the 5th - CI stage (F = 27.26, df = 10, 407, P < 0.0001) (Fig. 4; Table S2). Considering 2nd - 5th instar development times for larvae on the homogenized apple provisions, significantly shorter times occurred when BCL/PCSB $LD_{12.5}$ and LD_{50} were the treatments compared to all other treatments and control. Also, the mixed pesticides LD_{25} treatment resulted in significantly longer durations than control, dimethoate, organosilicone and mixed pesticides LD_{50} treatments (Fig. 4; Table S2). For 5th - CI development times, all doses of acetamiprid and both doses of mixed pesticides resulted in

significantly shorter development times than for all other treatments and control. Larvae exposed to BCL/PCSB $LD_{12.5}$ and LD_{50} treatments had significantly longer development times compared to those on BCL/PCSB LD_{25}, dimethoate, organosilicone, and control provisions (Fig. 4, Table S2).

The effect of some treatments on life stage durations also significantly differed between the intact and homogenized apple provisions (Figs. 3&4; Table S3). Development times for the 2^{nd}-5^{th} instar were significantly longer on the intact provisions compared to development on homogenized apple provisions when the treatments were acetamiprid (all doses), organosilicone, BCL/PCSB LD_{50}, and mixed pesticides (both doses). Significantly shorter development times occurred for the 5^{th} - CI stage for larvae reared on the intact provisions when treatments were acetamiprid LD_{25}, dimethoate, organosilicone, BCL/PCSB $LD_{12.5}$ and $LD_{25,}$ and mixed pesticides LD_{50} (Table S3).

Homogenized Apple vs Homogenized Almond Provisions

For the two homogenized provision types (n almond = 41; n apple = 43), examination of only the water controls revealed significantly longer development times when reared on the almond provisions for the 2^{nd} - 5^{th} stage (F = 6.23, df = 1, 82, P = 0.015; almond mean ± SE = 4.85 ± 0.12), but similar times for the 5^{th} – CI stage (F = 0.01, df = 1, 82, P = 0.91; almond mean ± SE = 17.22 ± 0.59) compared to the times for larvae reared on homogenized apple provisions. An analysis of all treatments and control, effects of the provision source, treatment, and their interactions showed that all were significant for both developmental periods, except for the source × treatment interaction for the 2^{nd} to 5^{th} instar (Table 6, Figs. 4&5). Just as for the apple homogenized provisions, within the almond provisions, there were significant treatment differences. For the homogenized almond provisions, the effect of treatment was significant for

both the 2nd - 5th stage (F = 6.61, df = 5, 220, P < 0.0001) and the 5th - CI stage (F = 21.18, df = 5, 199, P < 0.0001) (Fig. 5; Table S3). The duration of the 2nd to 5th instar stage was significantly longer when acetamiprid and mixed pesticides were treatments compared to all other treatments and control. For the 5th - CI stage, developmental times were shorter when treatments were acetamiprid, dimethoate, and mixed pesticides (Table S4; Fig, 5).

Significant differences were also found between the homogenized provision types for some treatment effects (Table S5). Compared to larvae reared on homogenized apple provisions, the 2nd-5th instar stage was significantly longer for larvae on almond provisions when treatments were acetamiprid, BCL/PCSB $LD_{12.5}$, and mixed pesticides LD_{25}. Development times for the 5th - CI stage were significantly shorter for larvae on almonds when the treatments were dimethoate and BCL/PCSB $LD_{12.5}$.

DISCUSSION

The interest and perceived need for creating standardized bioassays to determine the pesticide exposure risks and toxic effects on developing solitary bee larvae (Eeraerts et al. 2020) can be met by studies that inform methodology and observable endpoints. This study helps to resolve some questions concerning approaches to bioassay design and appropriateness of experimental protocols. It further exemplifies the efficacy of *Osmia lignaria* as a readily available candidate species for investigations in North America concerning impacts of agrochemicals on solitary bee larval survival and development.

Deciding upon the appropriate techniques for exposing bee larvae to agrochemicals (or other additives for experimental purposes) in larval food is important for creating realistic scenarios for reliable evaluations of lethal and sublethal impacts. Tests of provision source, chemical treatment, and how treatments are applied revealed significant impacts on survivorship

and development time for the two distinct developmental stages we examined. Effects of composition and diet source proved more impactful on larval development times than expected.

We examined the effect of using intact bee provision masses compared to mixing the provisions from the same origins into homogenates. Having the pasty mixes allowed us to give each larva equal food supplies. Our comparison found larvae to have similar development times from the 2nd to 5th instar using intact and homogenized apple compositions, but the time for the 5th instars to begin to spin cocoons was an average of four days longer in the homogenized provisions. The longer development time may be explained by the intentional use of equivalent amounts of provision for each larva. Homogenized, uniformly apportioned provision masses for some offspring may have been smaller or larger than the provision originally made for them. Nesting bees prepare smaller provisions for male offspring than those made for larger female offspring (Tepedino and Torchio 1989, Bosch and Kemp 2001). Relative variation in natural provision masses could occur if nesting females have limited access to floral resources, are limited in their foraging time due to weather conditions or are unequally efficient at provisioning their nests (Sgolastra et al. 2016). Although eggs for our study were randomly taken across nests and positions within nests for transfer to homogenized provision masses, the possible result is that a larva may have fed longer in the 5th instar because more provision was available than cohorts on natural provisions. In fact, Helm et al. (2017) observed that starved *O. lignaria* larvae quickly entered prepupal diapause (signaled by feeding cessation and cocooning) to become small adults, while larvae fed ad libitum continued to eat and became larger adults than larvae raised on naturally-provided provisions. Because all offspring reared in our study died before becoming prepupae or adults whose sex we could determine, we are unable to confirm any mismatches between provision size and bee sex or weight.

Interestingly, we found that *O. lignaria* larvae took on average about one half a day longer to develop from the 2nd to 5th instar on homogenized almond provisions (for the controls only) that were made from honey bee collected almond pollen plus sugar as compared to those composed of the pollen and nectar gathered from apple flowers by an *O. lignaria* female. Such a statistically significant outcome suggests that the nutritional quality of these provisions was unequal in providing what was needed for larval growth. However, the duration of the 5th - CI stage did not differ between the provision types. Evaluation of nutritional quality of the larval food could have been gained from assessment of adult weight or size and female nesting success, as has been performed in other studies (Sedivy et al. 2011, Sgolastra et al. 2017). Unfortunately, the data documenting the time to complete the cocoon, to metamorphose to the pupal and adult stages, and to survive the winter were unobtainable for this study. In part, the size of the wells (11 mm diameter) in the culture plates were apparently too large for the larvae, which ideally need 7.5 mm diameter wells (Tepedino and Torchio 1989, Bosch and Kemp 2001). The larvae continued to spin energetically expensive silk, and their spinning activity sometimes caused the larva to squirm completely out of the well. Ultimately, no bees in our experiment emerged as spring adults.

The mortality observed between provision types was revealing in different ways. The delivery method for the intact apple provisions meant that less chemical was added to each mass. Although it was assumed that once a larva fed from a high local concentration of an injected toxin, the effect would be more severe (or fatal) than when a larva fed on unavoidable, but evenly dosed amounts of toxin. This was not the case, and more larvae reared on intact provisions survived across treatments compared to those on homogenized provisions. Perhaps larvae were able to avoid the injected toxin if it did not interact with the physical and chemical

properties of the provision mass to spread through it. Beyond higher survival, larvae on intact provisions had longer 2nd – 5th stage durations and shorter 5th – CI durations for most of the chemical treatments compared to larvae on homogenized apple provisions. Whether longer or shorter stages mean that larvae are healthier or more likely to reach later life stages and reproduce would require more experimental data.

Although all larvae survived to spin cocoons on the two types of untreated homogenized provisions, comparison of treatment outcomes for these two provision types made from different plant sources showed that larvae were more likely to die before spinning a cocoon if the provision was made from almond pollen and sugar water. Detriment to larvae was most apparent when acetamiprid was mixed into the provision. For larvae that survived this particular treatment, the duration of the 2nd-5th stage was longer, which may indicate that longer feeding times are negative reactions such as reluctance to feed or feeding cessation. On the other hand, honey bee and bumble bee foragers have been shown to preferentially feed from neonicotinoid-laced solutions (Kessler et al. 2015, Arce et al. 2018). Nonetheless, it is possible that both the plant source and the chemicals added contributed to this response.

Pollen source is known to impact *O. lignaria* larval performance when the pollen is from a non-preferred flower family (Williams 2003). However, both almond and apple are exotic species in North America and in Family Rosaceae, albeit different genera. Differences in chemical composition, however, may play a role in nutritive quality for bee larvae. Almond pollen has high concentrations of the potentially toxic cyanogenic glycoside amygdalin (London-Shafir et al. 2003), which may explain the more detrimental larval effects when this pollen is combined with agrochemicals. Such a compound is probably undetected by bees in nectar where it occurs in very low concentration, similar to the inability of bumble bees to detect several other

potentially toxic, naturally occurring compounds in flower nectar (Tiedeken et al. 2014). London-Shafir (2003) suggests that honey bees prefer to visit other flowers co-blooming with almond, assuming equal rewards, to avoid almond nectar. But recent evidence shows that honey bees remain constant visitors in California almond blossoms even when spring blooming flowers are planted alongside orchards (Lundin et al. 2017). Gelsemine is an alkaloid with mammalian toxicity that is avoided by adult *O. lignaria* females but mixing it in provisions had no negative effect on larvae (Elliott et al. 2008). Zygacine, the neurotoxic alkaloid present in death -camas pollen and nectar, was shown to have detrimental effects on *O. lignaria* larvae and adults when ingested and may explain why few pollinators visit death-camas in the field (Cane et al. 2020). The physiological abilities of bees to tolerate toxic compounds is not fully understood, but the combination of plant secondary compounds and a neonicotinoid may have synergistic lethal or sublethal effects on developing bee larvae. Another unaddressed concern is the transmission of pathogens in the presence of agrochemicals from honey bees to *O. lignaria* via the honey bee collected almond pollen used in this study (Klinger et al. 2015, Fine et al. 2017). A more elaborate and equitable experimental design would have been to collect almond and apple provisions made by *O. lignaria,* to have sterilized the provisions, to have stored (frozen) homogenized pollen of each type, and to have transferred *O. lignaria* eggs laid in each orchard type onto each provision type (one in-season fresh provision source and each of the sources having been frozen for use during each almond and apple season).

In general, analyses within provision types for treatment effects revealed longer development times for the 2^{nd} – 5^{th} instar when acetamiprid was all or a part of the treatment (in mixed pesticides). Conversely, the time for the 5^{th} instar to finish eating the provision mass (or cease to feed) before initiating a cocoon was shortest when larvae were exposed to these same

treatments. When BCL/PCSB was present in larval food, the 2nd-5th instar stages were shorter than other treatments, but similar to controls, while 5th – CI durations consistently were some of the longest durations. From suggestive, unpublished data, the presence of BCL/PCSB on flowers increases female *O. lignaria* foraging activity (Chapter 2, this thesis), and, therefore, may increase larval feeding behavior on provisions.

As the call for risk assessment practices for protection of pollinators beyond the honey bee continues (Boyle et al. 2018, Eeraerts et al. 2020), the tools developed for this purpose need to be appropriately representative of the bee's life history and behavior. Adult and larval honey bees and solitary bees can respond differently to pesticides (Biddinger et al. 2013, Uhl et al. 2016, Hayward et al. 2019). The sublethal impact of delays during larval development could lead to asynchronous emergence, unhealthy and inefficient pollinators, and population decline if offspring fail to survive winter diapause. Unlike the eusocial honey bee, *O. lignaria* and other solitary bees do not have the resiliency of the super organism (Johansen and Mayer 1990). This means that each female that dies or is impacted by asynchronous emergence or inability to mate does not reproduce and may contribute to population declines (Johansen and Mayer 1990, Straub et al 2015, Kopit and Pitts-Singer 2018). In honey bees, many individuals may die on foraging forays, but populations are replenished by the queen who continues to lay eggs (Johansen and Mayer 1990, Straub et al 2015, Kopit and Pitts-Singer 2018).

Having a solitary bee test subject for risk assessment is important. Honey bees and solitary bees differ in their routes of exposure (Kopit and Pitts-Singer 2018, Sgolastra et al. 2018). *Osmia lignaria* could be a good solitary bee proxy in risk assessment trials due to their availability and nesting habits that make for easy manipulation compared to other solitary bee species. Developing a bioassay to test agrochemical impacts on pollinators on a large scale can

assist government agencies in determining whether an agricultural product is safe for use in pollinator-dependent agricultural systems. Perfecting larval testing methodology for solitary bees is crucial for properly assessing chemical risks for pollinators and for creating best practices for agricultural systems.

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Tables

Table 1. Agrochemicals detected in the apple and almond provisions used for this study. Analysis performed by USDA AMS Science & Technology Programs Laboratory Approval and Testing Division on 19 August 2016 (N.D. = Not Detected).

		PPB DETECTED IN:	
AGROCHEMICAL	**PESTICIDE CLASS**	**APPLE PROVISION**	**ALMOND PROVISION**
2,4 Dimethylphenyl formamide (DMPF)	non-systemic acaricide	218	140
Pendimethalin	dinitroaniline herbicide	328	334
Chlorpyrifos	organophosphate insecticide	7.2	5.1
Esfenvalerate	pyrethroid insecticide	8.6	8.7
Cyprodinil	anilinopyrimidine fungicides	N.D.	Trace
Oxyfluorfen	diphenyl-ether herbicide	N.D.	11.2

Table 2. Agrochemical treatments and desired lethal dose levels (LD) of active ingredients (AI), and product trade names and other information for an *Osmia lignaria* larval bioassay.

PESTICIDE TREATMENT	ACTIVE INGREDIENT PER PROVISION	TRADE NAME, SOURCE; OTHER INFORMATION
Acetamiprid (Acmd) $LD_{12.5}$	32.3 ppm	Assail 70 WP Insecticide (acetamiprid 70%: United Phosphorous Inc., King of Prussia, PA)
Boscalid/Pyraclostrobin (BCL/PCSB) $LD_{12.5}$	6.25 ppm BCL + 21.65 ppm PCSB	Pristine Fungicide (boscalid 25.2%, pyraclostrobin 12.8%: BASF Corporation, Research Triangle Park, NC)
Mixed Pesticides LD_{25}	3.23 ppm Acmd + 6.25 ppm BCL + 21.65 ppm PCSB	$LD_{12.5}$ AI of each product added together
Dimethoate LD_{100}	0.5ug; based on oral LD_{50} for dimethoate ranges from 0.10 to 0.35 μg AI/ for adult *A. mellifera*	Dimethoate Technical Insecticide (dimethoate 90%: Shivalik Rasayan Limited, New Delhi, India)
Organosilicone (OSS)	40 ppb	Sylgard ® 309 (A Mixture of 3-(3-Hydroxypropyl) Heptamethyltrisiloxane, Ethoxylated Acetate/125997-17-3, Polyethylene Glycol Monallyl Acetate/27252875, Polyethylene Glycol Diacetate/27252831 100%, Wilbur Ellis, Fresno, CA)

Table 3. Agrochemical treatments and provision type combinations for *Osmia lignaria* larval bioassay. The mixed treatment is a mixture of acetamiprid and BCL/PCSB.

AGROCHEMICAL TREATMENT	INTACT APPLE PROVISIONS	HOMOGENIZED APPLE PROVISIONS	HOMOGENIZED ALMOND POLLEN
Acetamiprid $LD_{12.5}$	X	X	X
Acetamiprid LD_{25}	X	X	
Acetamiprid LD_{50}	X	X	
BCL/PCSB $LD_{12.5}$	X	X	X
BCL/PCSB LD_{25}	X	X	
BCL/PCSB LD_{50}	X	X	
Mixed LD_{25}	X	X	X
Mixed LD_{50}	X	X	
OSS	X	X	X
Dimethoate LD_{100}	X	X	X
Water	X	X	X

Table 4.
Total number of *Osmia lignaria* larvae subjected to agrochemicals or water treatments in larval provisions and the percent that failed to reach the cocoon-initiation life stage. Larval provisions are made of intact and homogenized provisions from nest cells made in an apple orchard and homogenized almond pollen plus sugar water.

Treatment	Intact Apple N	Intact Apple	Homogen. Apple N	Homogen. Apple	Homogen. Almond N	Homogen. Almond
Water	48	0	44	0	41	0
Dimethoate LD_{100}	46	2.2%	42	7.1%	42	9.5%
OSS	45	2.2%	41	2.4%	40	2.5%
Acetamiprid $LD_{12.5}$	47	0	42	16.7%	40	60%
Acetamiprid LD_{25}	46	8.7%	42	4.8%		
Acetamiprid LD_{50}	48	6.3%	42	23.8%		
BCL/PCSB $LD_{12.5}$	44	0	39	0	42	4.8%
BCL/PCSB LD_{25}	44	0	40	5.0%		
BCL/PCSB LD_{50}	47	8.5%	41	2.4%		
Mixed LD_{25}	45	0	38	18.4%	40	22.5%
Mixed LD_{50}	48	2.1%	41	2.4%		

Table 5. Results of generalized linear model for effects of pollen type, agrochemical treatments, and their interactions on the duration (days) of two *Osmia lignaria* larval development times, using provisions from an apple orchard. Provision masses were intact or homogenized. Treatments were additions of various agrochemicals to provisions and water was added as a control. 2^{nd}- 5^{th} = 2^{nd} instar to 5^{th} instar; 5^{th} – CI = 5^{th} instar to cocoon initiation.

	Pollen Type			**Treatment**			**Interaction**		
Stage	*F*-value	df	*P*-value	*F*-value	Df	*P*-value	*F*-value	df	*P*-value
2^{nd} – 5^{th}	306.62	1, 920	<0.0001	74.34	10, 920	<0.0001	33.33	10, 920	<0.0001
5^{th} – CI	322.51	1, 883	<0.0001	46.31	10, 883	<0.0001	10.70	10, 883	<0.0001

Table 6. Results of generalized linear model for effects of pollen type, agrochemical treatments, and their interactions on the duration (days) of two *Osmia lignaria* larval development times, using *Apis mellifera*-collected pollen from an almond orchard and *O. lignaria* provisions from an apple orchard that were homogenized. Treatments were additions of various agrochemicals to provisions and water was added as a control. 2^{nd}- 5^{th} = 2^{nd} instar to 5^{th} instar; 5^{th} – CI = 5^{th} instar to cocoon initiation.

	Pollen Type			**Treatment**			**Interaction**		
Stage	*F*-value	Df	*P*-value	*F*-value	Df	*P*-value	*F*-value	df	*P*-value
2^{nd} – 5^{th}	87.12	1, 449	<0.0001	17.25	5, 449	<0.0001	2.81	5, 449	0.016
5^{th} – CI	33.81	1, 420	<0.0001	56.72	5, 420	<0.0001	4.64	5, 420	0.0004

Supplemental Table 1. Significant Tukey's results comparing treatments for intact apple provisions for effects on days to develop from 2nd to 5th instar and from 5th instar to cocoon initiation for *Osmia lignaria* larvae. For 2nd – 5th instar, d.f. = 492; for 5th instar – cocoon initiation, d.f. = 480. Acmd = Acetamiprid; Dimeth = Dimethoate; OSS = organosilicone; BCL/PCSB = Boscalid/Pyraclostrobin; Mix = mixed pesticides.

Pairings for 2nd – 5th Instar	**t-value**	***P*-value**
Acmd $LD_{12.5}$ vs Dimeth	19.03	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB $LD_{12.5}$	17.48	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB LD_{25}	18.41	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB LD_{50}	14.94	<0.0001
Acmd $LD_{12.5}$ vs Water	19.44	<0.0001
Acmd LD_{25} vs Dimeth	18.82	<0.0001
Acmd LD_{25} vs BCL/PCSB $LD_{12.5}$	17.30	<0.0001
Acmd LD_{25} vs BCL/PCSB LD_{25}	18.22	<0.0001
Acmd LD_{25} vs BCL/PCSB LD_{50}	14.78	<0.0001
Acmd LD_{25} vs Water	19.22	<0.0001
Acmd LD_{50} vs Dimeth	19.12	<0.0001
Acmd LD_{50} vs BCL/PCSB $LD_{12.5}$	17.57	<0.0001
Acmd LD_{50} vs BCL/PCSB LD_{25}	18.51	<0.0001
Acmd LD_{50} vs BCL/PCSB LD_{50}	15.01	<0.0001
Acmd LD_{50} vs Water	19.54	<0.0001
Dimeth vs OSS	-19.03	<0.0001
Dimeth vs BCL/PCSB LD_{50}	-3.83	0.0007
Dimeth vs Mix LD_{25}	-16.55	<0.0001
Dimeth vs Mix LD_{50}	-19.03	<0.0001
OSS vs BCL/PCSB $LD_{12.5}$	17.48	<0.0001
OSS vs BCL/PCSB LD_{25}	18.41	<0.0001
OSS vs BCL/PCSB LD_{50}	14.94	<0.0001
OSS vs Water	19.44	<0.0001
BCL/PCSB $LD_{12.5}$ vs Mix LD_{25}	-15.04	<0.0001
BCL/PCSB $LD_{12.5}$ vs Mix LD_{50}	-17.48	<0.0001
BCL/PCSB LD_{25} vs BCL/PCSB LD_{50}	-3.31	0.039
BCL/PCSB LD_{25} vs Mix LD_{25}	-15.96	<0.0001
BCL/PCSB LD_{25} vs Mix LD_{50}	-18.41	<0.0001
BCL/PCSB LD_{50} vs Mix LD_{25}	-12.54	<0.0001
BCL/PCSB LD_{50} vs Mix LD_{50}	-14.94	<0.0001
BCL/PCSB LD_{50} vs Water	3.99	0.004
Mix LD_{25} vs Water	16.92	<0.0001
Mix LD_{50} vs Water	19.44	<0.0001

Pairings for 5th Instar – Cocoon Initiation	**t-value**	***P*-value**
Acmd $LD_{12.5}$ vs BCL/PCSB $LD_{12.5}$	-7.34	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB LD_{25}	-5.39	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB LD_{50}	-5.25	<0.0001
Acmd LD_{25} vs BCL/PCSB $LD_{12.5}$	-7.14	<0.0001
Acmd LD_{25} vs BCL/PCSB LD_{25}	-5.25	<0.0001
Acmd LD_{25} vs BCL/PCSB LD_{50}	-5.11	<0.0001
Acmd LD_{50} vs BCL/PCSB $LD_{12.5}$	-7.27	<0.0001
Acmd LD_{50} vs BCL/PCSB LD_{25}	-5.34	<0.0001

Acmd LD_{50} vs BCL/PCSB LD_{50}	-5.20	<0.0001
Dimeth vs BCL/PCSB $LD_{12.5}$	-6.83	<0.0001
Dimeth vs BCL/PCSB LD_{25}	-4.90	<0.0001
Dimeth vs BCL/PCSB LD_{50}	-4.78	<0.0001
OSS vs BCL/PCSB $LD_{12.5}$	-7.31	<0.0001
OSS vs BCL/PCSB LD_{25}	-5.37	<0.0001
OSS vs BCL/PCSB LD_{50}	-5.22	<0.0001
BCL/PCSB $LD_{12.5}$ vs Mix LD_{50}	7.34	<0.0001
BCL/PCSB $LD_{12.5}$ vs Mix LD_{25}	6.61	<0.0001
BCL/PCSB $LD_{12.5}$ vs Water	5.25	<0.0001
BCL/PCSB LD_{25} vs Mix LD_{25}	4.68	0.0002
BCL/PCSB LD_{25} vs Mix LD_{50}	5.39	<0.0001
BCL/PCSB LD_{25} vs Water	3.29	0.042
BCL/PCSB LD_{50} vs Mix LD_{25}	4.56	0.0003
BCL/PCSB LD_{50} vs Mix LD_{50}	5.25	<0.0001

Supplemental Table 2. Significant Tukey's results comparing treatments for homogenized apple provisions for effects on days to develop from 2nd to 5th instar and from 5th instar to cocoon initiation for *Osmia lignaria* larvae. For 2nd – 5th instar, d.f. = 430; for 5th instar – cocoon initiation, d.f. = 407. Acmd = Acetamiprid; Dimeth = Dimethoate; OSS = organosilicone; BCL/PCSB = Boscalid/Pyraclostrobin; Mix = mixed pesticides.

Pairings for 2nd – 5th Instar	**t-value**	***P*-value**
Acmd $LD_{12.5}$ vs BCL/PCSB $LD_{12.5}$	5.28	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB LD_{50}	5.47	<0.0001
Acmd LD_{25} vs BCL/PCSB $LD_{12.5}$	5.64	<0.0001
Acmd LD_{25} vs BCL/PCSB LD_{50}	5.84	<0.0001
Acmd LD_{50} vs BCL/PCSB $LD_{12.5}$	4.40	0.0007
Acmd LD_{50} vs BCL/PCSB LD_{50}	4.58	0.0003
Dimeth vs BCL/PCSB $LD_{12.5}$	3.33	0.037
Dimeth vs BCL/PCSB LD_{50}	3.49	0.023
Dimeth vs Mix LD_{25}	-3.86	0.006
OSS vs BCL/PCSB $LD_{12.5}$	3.73	0.010
OSS vs BCL/PCSB LD_{50}	3.89	0.005
OSS vs Mix LD_{25}	-3.51	0.021
BCL/PCSB $LD_{12.5}$ vs Mix LD_{25}	-7.01	<0.0001
BCL/PCSB $LD_{12.5}$ vs Mix LD_{50}	-3.73	0.010
BCL/PCSB $LD_{12.5}$ vs Water	-3.35	0.035
BCL/PCSB LD_{25} vs BCL/PCSB $LD_{12.5}$	4.83	0.0001
BCL/PCSB LD_{25} vs BCL/PCSB LD_{50}	5.01	<0.0001
BCL/PCSB LD_{50} vs Mix LD_{25}	-7.22	<0.0001
BCL/PCSB LD_{50} vs Mix LD_{50}	-3.89	0.005
BCL/PCSB LD_{50} vs Water	-3.51	0.021
Mix LD_{25} vs Water	3.96	0.004
Mix LD_{50} vs Mix LD_{25}	-3.51	0.021

Pairings for 5th Instar – Cocoon Initiation	**t-value**	***P*-value**
Acmd $LD_{12.5}$ vs Dimeth	-6.75	<0.0001
Acmd $LD_{12.5}$ vs OSS	-8.43	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB $LD_{12.5}$	-8.56	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB LD_{25}	-10.25	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB LD_{50}	-10.25	<0.0001
Acmd $LD_{12.5}$ vs Water	-6.51	<0.0001
Acmd LD_{25} vs Dimeth	-3.89	0.005
Acmd LD_{25} vs OSS	-5.60	<0.0001
Acmd LD_{25} vs BCL/PCSB $LD_{12.5}$	-7.51	<0.0001
Acmd LD_{25} vs BCL/PCSB LD_{25}	-5.78	<0.0001
Acmd LD_{25} vs BCL/PCSB LD_{50}	-7.48	<0.0001
Acmd LD_{25} vs Water	-3.59	0.016
Acmd LD_{50} vs Dimeth	-5.28	<0.0001
Acmd LD_{50} vs OSS	-6.89	<0.0001
Acmd LD_{50} vs BCL/PCSB $LD_{12.5}$	-8.67	<0.0001
Acmd LD_{50} vs BCL/PCSB LD_{25}	-7.04	<0.0001
Acmd LD_{50} vs BCL/PCSB LD_{50}	-8.65	<0.0001
Acmd LD_{50} vs Water	-5.02	<0.0001
Dimeth vs BCL/PCSB LD_{25}	-3.60	0.016

Dimeth vs BCL/PCSB LD_{50}	-3.52	0.021
Dimeth vs Mix LD_{25}	4.53	0.004
Dimeth vs Mix LD_{50}	3.96	0.0004
OSS vs Mix LD_{25}	6.13	<0.0001
OSS vs Mix LD_{50}	5.67	<0.0001
BCL/PCSB $LD_{12.5}$ vs Mix LD_{25}	7.91	<0.0001
BCL/PCSB $LD_{12.5}$ vs Mix LD_{50}	7.58	<0.0001
BCL/PCSB $LD_{12.5}$ vs Water	4.08	0.003
BCL/PCSB LD_{25} vs Mix LD_{25}	6.29	<0.0001
BCL/PCSB LD_{25} vs Mix LD_{50}	5.84	<0.0001
BCL/PCSB LD_{50} vs Mix LD_{25}	7.88	<0.0001
BCL/PCSB LD_{50} vs Mix LD_{50}	7.55	<0.0001
BCL/PCSB LD_{50} vs Water	4.01	0.004
Mix LD_{25} vs Water	-4.25	0.001
Mix LD_{50} vs Water	-3.65	0.013

Supplemental Table 3. Significant Tukey's results comparing treatment outcomes between intact and homogenized apple provision types for effects on days to develop from 2nd to 5th instar and from 5th instar to cocoon initiation (CI) for *Osmia lignaria* larvae. For 2nd – 5th instar, d.f. = 920; for 5th instar – cocoon initiation, d.f. = 883. Acmd = Acetamiprid; Dimeth = Dimethoate; OSS = organosilicone; BCL/PCSB = Boscalid/Pyraclostrobin; Mix = mixed pesticides.

Treatments for 2nd – 5th Instar **Intact vs Homogenized**	**t-value**	***P*-value**
Acmd $LD_{12.5}$	9.55	<0.0001
Acmd LD_{25}	9.00	<0.0001
Acmd LD_{50}	10.93	<0.0001
OSS	11.63	<0.0001
BCL/PCSB LD_{50}	5.13	<0.0001
Mix LD_{25}	4.98	<0.0002
Mix LD_{50}	11.63	<0.0001

Treatments for 5th Instar – CI **Intact vs Homogenized**	**t-value**	***P*-value**
Acmd LD_{25}	-3.98	0.013
Dimeth LD_{100}	-7.67	<0.0001
OSS	-11.34	<0.0001
BCL/PCSB $LD_{12.5}$	-7.72	<0.0001
BCL/PCSB LD_{25}	-7.07	<0.0001
Mix LD_{50}	-4.00	0.012

Supplemental Table 4. Significant Tukey's results comparing treatments for homogenized almond provisions for effects on days to develop from 2nd to 5th instar and from 5th instar to cocoon initiation for *Osmia lignaria* larvae. For 2nd – 5th instar, d.f. = 220; for 5th instar – cocoon initiation, d.f. = 199. Acmd = Acetamiprid; Dimeth = Dimethoate; OSS = organosilicone; BCL/PCSB = Boscalid/Pyraclostrobin; Mix = mixed pesticides.

Pairings for 2nd Instar – 5th Instar	**t-value**	***P*-value**
Acmd $LD_{12.5}$ vs Dimeth	3.84	0.002
Acmd $LD_{12.5}$ vs OSS	4.11	0.0008
Acmd $LD_{12.5}$ vs BCL/PCSB $LD_{12.5}$	3.65	0.004
Acmd $LD_{12.5}$ vs Water	4.27	0.0004
Dimeth vs Mix LD_{25}	-2.89	0.048
OSS vs Mix LD_{25}	-3.19	0.020
Mix LD_{25} vs Water	3.32	0.014
Pairings for 5th Instar – Cocoon Initiation	**t-value**	***P*-value**
Acmd $LD_{12.5}$ vs 0SS	-6.30	<0.0001
Acmd $LD_{12.5}$ vs BCL/PCSB $LD_{12.5}$	-4.87	<0.0001
Acmd $LD_{12.5}$ vs Water	-4.90	<0.0001
Dimeth vs OSS	-5.69	<0.0001
Dimeth vs BCL/PCSB $LD_{12.5}$	-3.82	0.002
Dimeth vs Water	-3.87	0.002
OSS vs Mix LD_{25}	8.20	<0.0001
BCL/PCSB $LD_{12.5}$ vs Mix LD_{25}	6.44	<0.0001
Mix LD_{25} vs Water	-6.50	<0.0001

Supplemental Table 5. Significant Tukey's results comparing treatment outcomes between homogenized apple and homogenized almond provision types for effects on days to develop from 2nd to 5th instar and from 5th instar to cocoon initiation (CI) for *Osmia lignaria* larvae. For 2nd – 5th instar, d.f. = 449; for 5th instar – cocoon initiation, d.f. = 420. Acmd = Acetamiprid; Dimeth = Dimethoate; OSS = organosilicone; BCL/PCSB = Boscalid/Pyraclostrobin; Mix = mixed pesticides.

Treatments for 2nd – 5th Instar		
Almond vs Apple	**t-value**	***P*-value**
Acmd $LD_{12.5}$	4.84	<0.0001
BCL/PCSB $LD_{12.5}$	6.47	<0.0001
Mix LD_{25}	3.74	0.011

Treatments for 5th Instar – CI		
Almond vs Apple	**t-value**	***P*-value**
Dimeth LD_{100}	-5.02	<0.0001
BCL/PCSB $LD_{12.5}$	-4.35	<0.0001

Figures

Figure 1. *Osmia lignaria* natal nest straw cut longitudinally and pinned opened for transfer into well plates. The individuals can be seen on the provisions. The individuals pictured here are feeding instars and have hatched from their eggs. Only individuals still in the egg stage were used for this study.

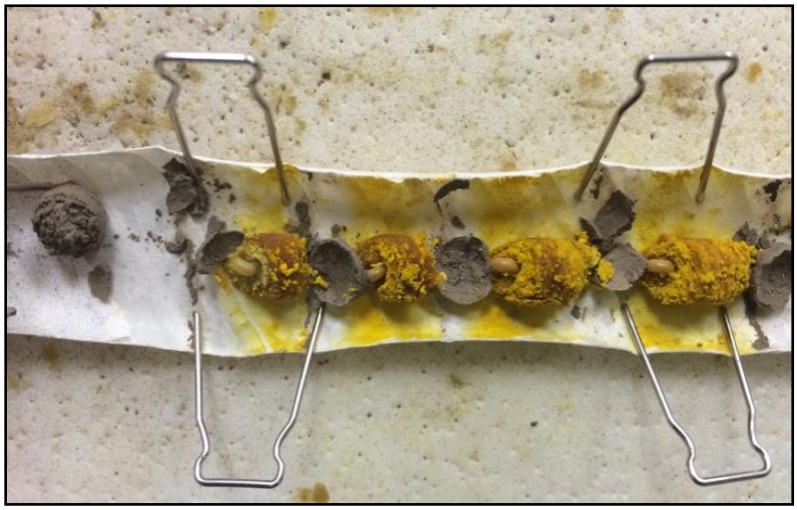


Figure 2. View from under a dissecting microscope, multiple well plate containing *Osmia lignaria* larvae at 5th instar (defecating stage) with some provision masses still being eaten.

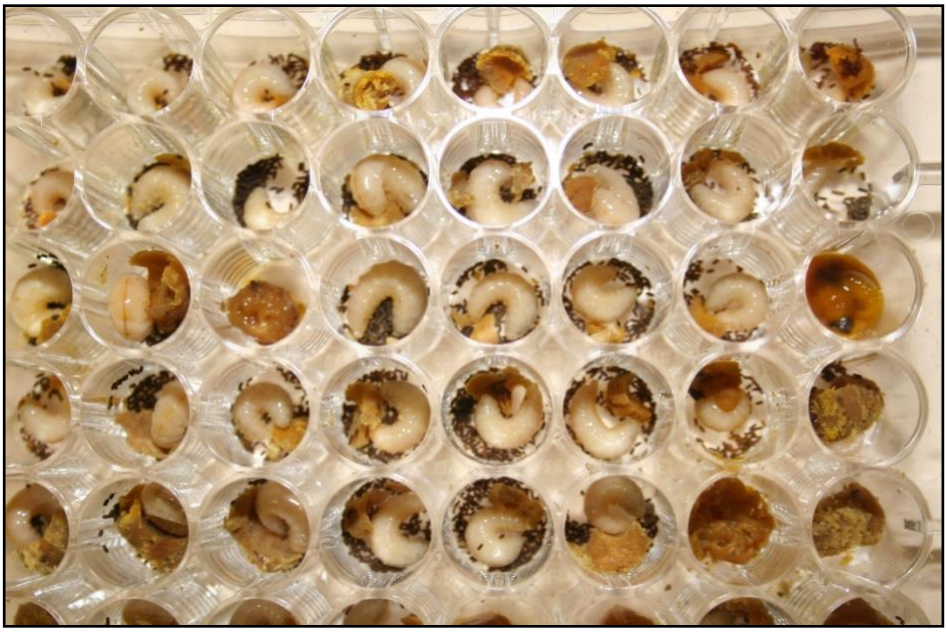


Figure 3. Mean days (± SE) for *Osmia lignaria* larvae reared on intact apple provisions treated with agrochemicals or water to grow from 2nd to 5th instar and from 5 instar to the initiation of cocoon. Different small and capital letters above bars show significant differences at $P < 0.05$ for each respective developmental group.

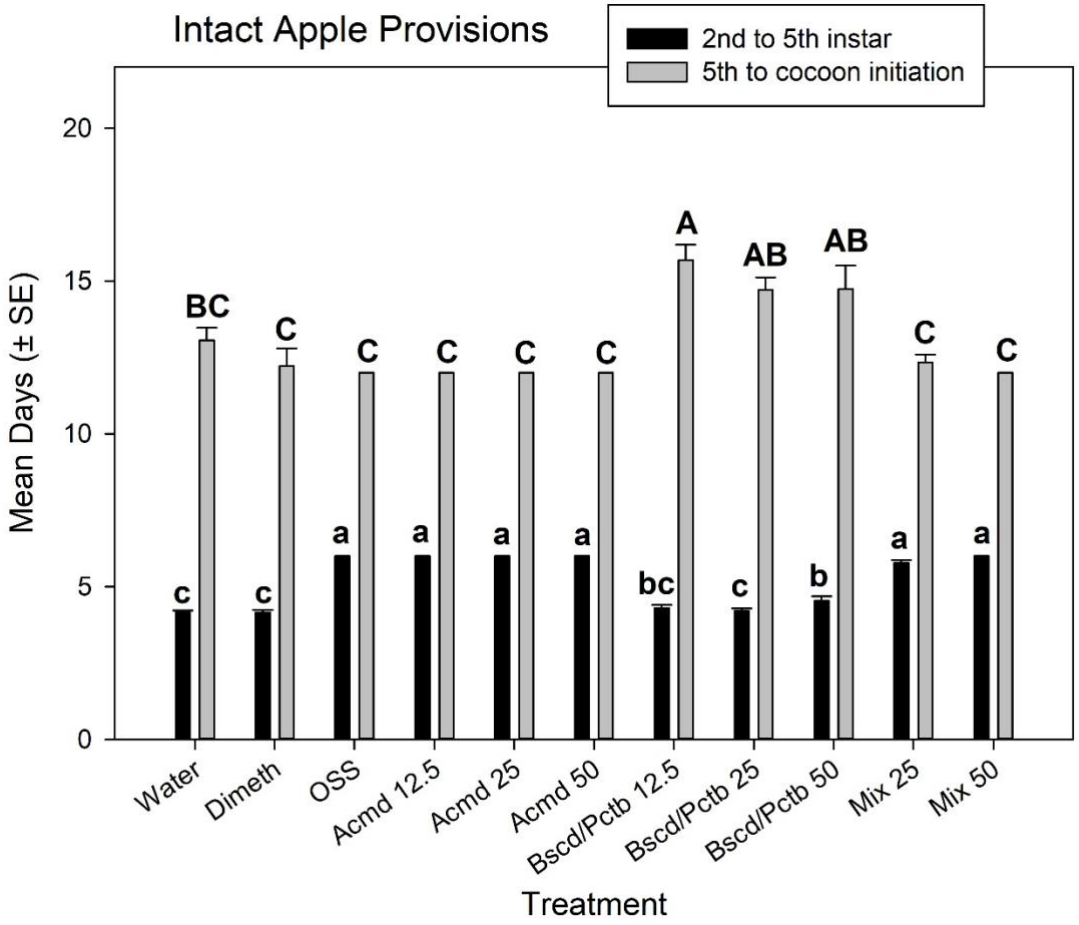


Figure 4. Mean days (± SE) for *Osmia lignaria* larvae reared on homogenized apple provisions treated with agrochemicals or water to grow from 2nd to 5th instar and from 5 instar to the initiation of cocoon. Different letters above black show significant differences within developmental group at $P < 0.05$; absence of letters for grey bars indicates no significant differences.

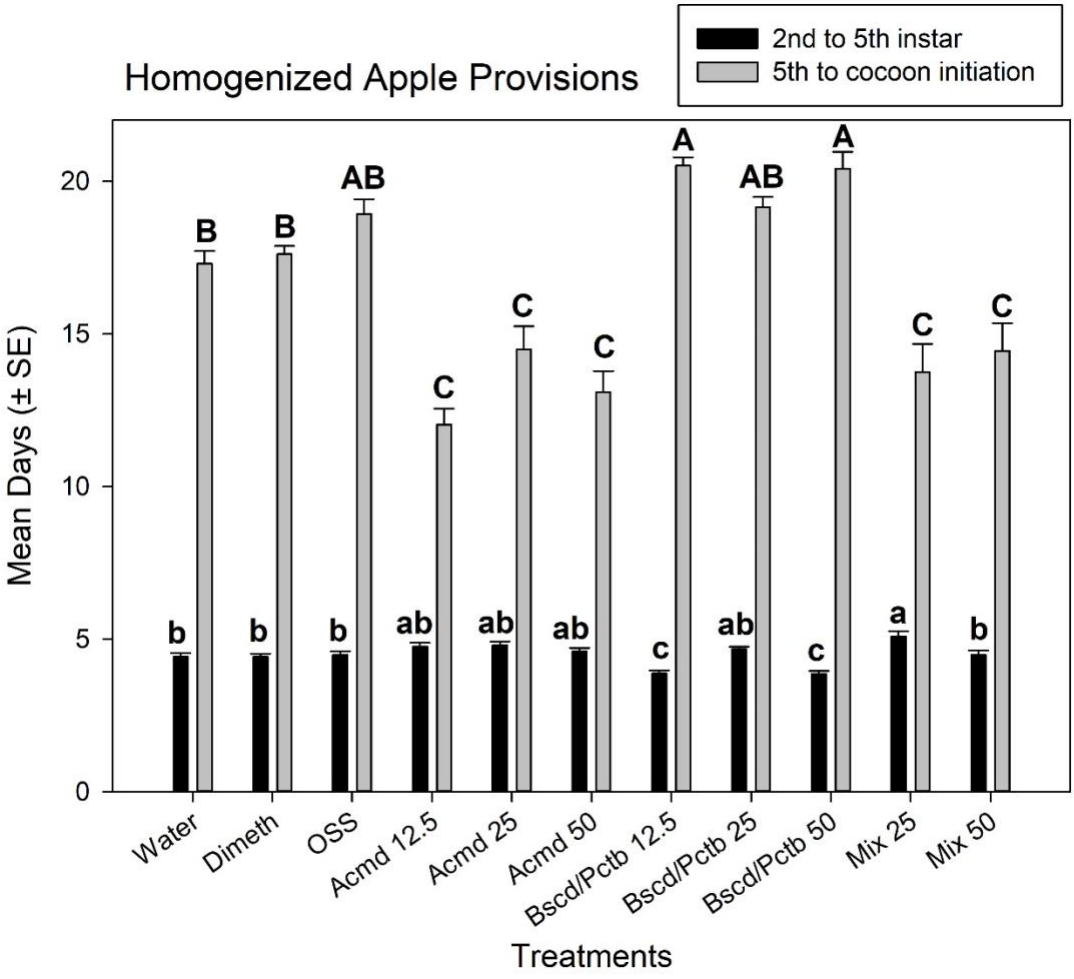
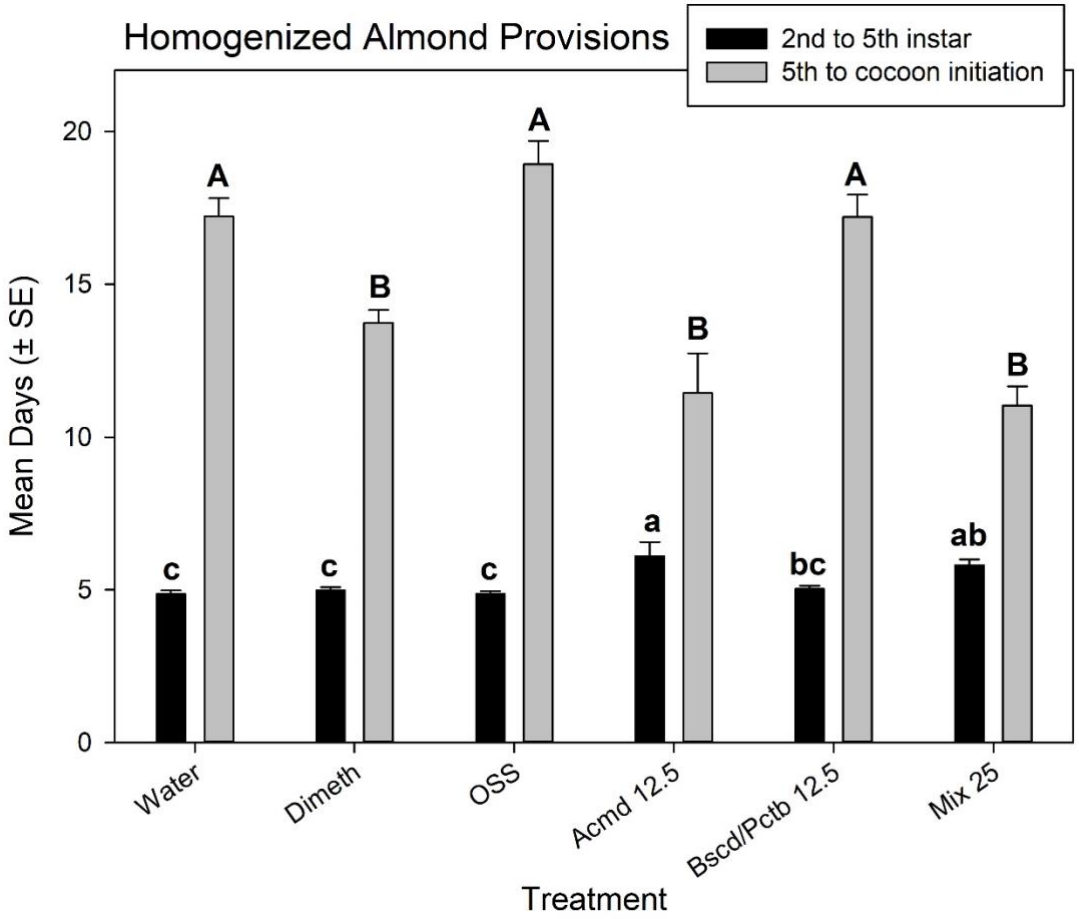


Figure 5. Mean days (± SE) for *Osmia lignaria* larvae reared on homogenized almond provisions treated with agrochemicals or water to grow from 2nd to 5th instar and from 5 instar to the initiation of cocoon. Different letters above black show significant differences within developmental group at $P < 0.05$; absence of letters for grey bars indicates no significant differences.



CHAPTER III

IMPACTS OF PESTICIDES ON THE FORAGING BEHAVIOR OF *OSMIA LIGNARIA* (MEGACHILIDAE)

ABSTRACT

Native and managed pollinator species are declining, making it imperative that the impacts of agricultural practices on pollinators is better understood. *Osmia lignaria* Say (Megachilidae) is becoming an important pollinator of commercial orchards, in particular almond and cherry orchards which bloom in early spring when honey bees are less active. Pesticide sprays are used to mitigate pests and pathogens throughout bloom in commercial almond orchards, exposing *O. lignaria* to pesticides while they forage. This study was conducted to 1) assess a no-choice situation for the effect of pesticides on bee survival and foraging behavior and 2) assess the same parameters under a choice situation. We investigated the fungicide boscalid/pyraclostrobin, the insecticide acetamiprid, and a mixture of the two products, which are known to be used where *Osmia* species are introduced as pollinators and have been shown to have only sublethal effects on bees via oral or contact dosing. A field cage study was conducted at the USDA-ARS Thad Cochran Southern Horticultural Laboratory in Poplarville, Mississippi with individually paint-marked, female *O. lignaria* to assess impacts of pesticide treatments on foraging behavior and mortality. Boscalid/pyraclostrobin caused hyperactive behavior with low mortality whereas individuals exposed to acetamiprid showed signs of stress and high mortality.

INTRODUCTION

Bees pollinate approximately 80% of flowering plants and about 75% of all the fruits and vegetables commercially grown (Gill et al. 2012). With native pollinator species declining and the honey bee (*Apis mellifera* L.; Apidae) industry suffering, it is imperative that we understand the impacts of agricultural practices on pollinators (Kearns et al. 1998, Klein et al. 2007, Potts et al. 2010, Elston et al. 2013). A diet of pollen and nectar as larvae and adults puts bees at risk for pesticide exposure regardless of whether they are part of wild or a managed populations (Sanchez-Bayo 2014, Kopit and Pitts-Singer 2018).

Osmia lignaria Say (Megachilidae), commonly known as the blue orchard bee, is becoming an important pollinator of commercial orchards (Bosch and Kemp 2001, Artz and Pitts-Singer 2015, Boyle and Pitts-Singer 2017, Koh et al. 2017, Pitts-Singer et al. 2018). This solitary bee is native to North America (Bosch and Kemp 2001) and makes a linear series of cells in tunnels or cavities. Females delineate cells with mud partitions and make mass provisions using pollen and nectar, and within each cell, they lay a single egg. *Osmia lignaria* prefers fruit and nut tree flowers and will forage in cloudy, cool weather when other pollinators are less active (Bosch and Kemp 2001). The ability to forage in cool weather makes *O. lignaria* an important wild and managed pollinator for crops that bloom in early spring, such as almonds and cherries in the western United States (Artz and Pitts-Singer 2015, Boyle and Pitts-Singer 2017).

A current agricultural practice is to mix pesticides together in large tanks for higher management efficiency and cost reduction in managed agricultural systems (Houghton 1982). Mixing these agrochemicals together or applying them back-to-back in a field may lead to a synergistic effect occurring between the compounds. Synergism is when the combined effect of

two compounds is greater than the sum of their individual effects. Synergism of pesticides may be intentionally used to increase efficacy on pests that have become resistant to pesticide treatments, such as the use of piperonyl butoxide to increase the efficacy of pyrethroid pesticides in the control of *Tribolium castaneum* (Herbst) larvae or in the fruit fly *Dacus ciliatus* (Loew) (Ishaaya et al. 1983, Maklakov et al. 2001). The synergism of the pesticides may increase the impact on a population compared to when a single pesticide is used alone, thereby reducing the risk of having surviving individuals reproduce and potentially lead to more resistant individuals in future generations (Ishaaya et al. 1983, Young et al. 2005, Bingham et al. 2008).

Mixtures of pesticides have been shown to have a synergistic effect on pollinators, as well as pests (Pilling et al. 1995, Bingham et al. 2008, Biddinger et al. 2013, David et al. 2016). Of particular interest is the synergistic effects of fungicide and insecticide mixtures and the potential increase of toxicity that these treatments have on managed and wild pollinators (Pilling et al. 1995, Papaefthimiou and Theophilidis 2001, Biddinger et al. 2013, Artz and Pitts-Singer 2015). For example, a topical dose study conducted on both *Apis mellifera* and *Osmia cornifrons* (Radoszkowski) showed an increase in mortality when neonicotinoids and fungicides were combined compared to when they were administered separately (Biddinger et al. 2013). The impact of pesticides varied significantly between the two species, although evidence of a synergistic effect was found for both (Biddinger et al. 2013).

The most direct impact of pesticides on bees is death immediately after contact. Other effects may be seen only after chronic exposure. Sublethal effects may occur as reduction of offspring production and survival, lack of colony vigor and queen production in social bees, or changes in foraging or nesting behavior (Gill et al. 2012, Sanchez-Bayo and Goka 2014, Bernauer et al. 2015, Lundin et al. 2015). Depending on the size and capabilities of a bee species,

and depending on the availability of resources, bees can cover a few or many kilometers as their foraging ranges (Bosch and Kemp 2001, Guédot et al. 2006, Greenleaf et al. 2007). In their forays across a landscape, bees make choices to visit resources based on visual and olfactory cues (Guédot et al. 2007, Howell and Alarcón 2007). The effect that pesticides have on the cues used by bees to detect and choose sources of food and nesting materials has not been well demonstrated in the literature. We sought to determine if bees avoid foraging on pesticide-contaminated plants or if their behavior is modified in response to the presence of, or contact with, the contaminants.

We hypothesized that the odor of some non-lethal applications of pesticides may allure or may deter bees from recently sprayed plants (Thompson and Wilkins 2003, Artz and Pitts-Singer 2015). We predicted that blooms of plants sprayed with a fungicide and/or an insecticide would be less frequented than that of plants without a pesticide application. If flowers of a sprayed plant are visited by an individual bee, we predicted its behavior on that flower would be different from the visitation behavior on a flower without the pesticide. We hypothesized that in a no-choice situation, bees that forage on plants in control (i.e., water-treated) field cages would exhibit normal foraging behaviors that included visiting flowers to collect resources used to create mass provisions in nests. We hypothesized that bees that forage on plants in only fungicide-treated or only insecticide-treated cages would be less affected than bees that foraged on plants in cages where a fungicide + insecticide mixture was sprayed due to synergism. Furthermore, we hypothesized that in a choice situation with plants in only one half of the cages being treated, bees would be deterred by the scent of the pesticides and choose to forage on plants on the side of the cage treated only with water. They also may learn to avoid pesticide-treated plants over time if they perceive malicious effects.

This study was conducted to 1) assess a no-choice situation for the effect of pesticides on bee survival and behavior and 2) assess the same parameters under a choice situation. We used one fungicide and one insecticide that are products known to be used where *Osmia* species are introduced as pollinators and that have been shown to have only sublethal, if any, effects on bees via oral or contact dosing (Biddinger et al 2013, Artz and Pitts-Singer 2015).

METHODS

Field Site and Setup

A field cage study was conducted in March 2016 at the USDA-ARS Thad Cochran Southern Horticultural Laboratory in Poplarville, Mississippi. Canola (*Brassica rapa*, spring type) was planted in October and November 2015 so that flowers would bloom to serve as the floral resource for bees in the 2016 field season. This plant was chosen due to its fast growth and use as a cover crop in the eastern United States. Canola is also a valid floral resource for *O. lignaria* and in a pollen choice analysis, 10% of *O. lignaria* nest provisions contained pollen from brassicaceous flowers (Bosch and Kemp 2001, Kraemer and Favi 2005, Cane 2006). Although other flowers may be preferred by *O. lignaria*, they will readily use canola flowers in a field cage (TLP, pers. Obs.). Ten Lumite field cages (6 m × 6 m × 2 m) (BioQuip, Rancho Dominguez, CA) were erected over the field of canola. A path was made down the center of each cage by mowing the canola. The path divided the forage into two halves within the cage and provided easy access to areas of observations during the trial. A wooden nesting block with 28 tunnels (14 cm deep with paper straw inserts 7.5 cm in diameter) was mounted about 1.5 m above the ground to the center post of each cage so that the open ends of the tunnels were facing southeast.

Local weather data for was acquired using the University of Utah's MesoWest weather database (Mesowest 2019). The weather station closest to the research field was the George R Carr Memorial Air Field in Bogalusa, Louisiana.

Pesticide Treatments

The formulation boscalid/pyraclostrobin is a carbamate fungicide often used in U.S. almond-growing regions where precipitation during bloom can facilitate fungal diseases such as brown rot that affects almond bloom (Artz and Pitts-Singer 2015). Brown rot is controlled with fungicide sprays that are applied to the buds, and during particularly wet seasons, multiple applications of pesticides are applied to control the fungal pathogen during bloom (Connell 2002). Boscalid/pyraclostrobin caused confusion in *O. lignaria* and *Megachile rotundata* F. (Megachilidae) females provisioning nests at artificial nest sites in studies conducted in field cages (Artz and Pitts-Singer 2015). The neonicotinoid acetamiprid was chosen because it has been shown to cause lower rates of mortality and have less of a detrimental synergistic effect on *O. cornifrons* when combined with a fungicide than other neonicotinoid pesticides (Biddinger et al. 2013, EFSA 2016). Due to previous research, we decided to look at the impacts and interactions of boscalid/pyraclostrobin and acetamiprid on the foraging behavior of *O. lignaria*.

Bee Maintenance

Osmia lignaria were obtained from a commercial pollination service (Watt's Solitary Bees, Bothell, WA) in their overwintering stage (cocooned adults) and kept in cold storage (4-5°C) until early March 2016 when they were incubated at 25°C to initiate adult emergence from cocoons. Emerged bees (males and females) were maintain in a laboratory benchtop screened container (0.6 m × 0.9 m × 1.2 m) and provided with sugar-water for 4-5 days to allow for feeding and mating until enough bees had emerged for releasing into field cages. Females were

marked on the thorax with unique enamel paint colors so that individuals could be identified while foraging in field cages.

Validation of Pesticide Effects, No-Choice Test:

To gather baseline data for general bee activity and nesting success, we assessed the impacts of individual pesticides on *O. lignaria* nesting and foraging behavior with no choice of forage. Two treatments were applied to canola plants in two field cages: one cage received the boscalid/pyraclostrobin treatment, and one cage received the acetamiprid treatment. Two cages treated with only water were used as controls for both the no-choice test and the choice test due to limited cages. Pesticide formulations were mixed in water at the recommended full field rates for almonds, adjusted for the area within the cages (Table 1); no adjuvants were used. Pesticides were applied to the blooming canola 24 hours before bees were released in the cages.

After 4-5 days in the laboratory, 12 uniquely paint-marked, presumably mated *O. lignaria* females were released into each field cage on 4 March 2016. The individuals were allowed to forage and nest until no flowers remained in the cages (5 days). Nesting and foraging activities were recorded (see Data Collection section below). The females and nest blocks were removed and taken back to the laboratory to document any nest cells that could not be seen in the field and females were freezer killed.

Pesticide Detection and Effects, Choice Test:

To assess whether bees have an awareness of pesticide sprays in making a choice of where to forage, eight more cages were setup over the canola field as previously described. Here, half of the canola in each cage was randomly sprayed with one of four treatments: water (control), boscalid/pyraclostrobin (fungicide), acetamiprid (insecticide), or a combination of boscalid/pyraclostrobin and acetamiprid (mix). Pesticide formulations were mixed in water at

full almond field rates adjusted for the area within the cages (Table 1); no adjuvants were used. The other side of each cage was treated with only water. Each treatment was randomly assigned to two of the eight cages, thus creating two experimental replicates. The left and right position of the treatment was alternated between the two replicates and pesticides were applied as previously explained.

Data Collection:

Behavioral observations were conducted each morning between 0800 and 1200 CDST from 5 March to 9 March. Daily observations along two 6 m long transects were performed to assess the number of bees foraging on canola on each side of the cage in a 60 second time period in both the choice and no-choice cages. Then, individual bee observations were made along the same transects to record their behavior to determine flower visitation rate per female (number of flowers per bee per min) and flower handling duration (seconds per flower per bee). These flower visitation observations were made for 15 min per cage per day until no more forage remained in the cages. Also, video recordings at the nest block were taken during the time to observe foraging in all cages (simultaneously in all cages) to document any bee nesting activity. Due to the low numbers of active bees and/or lack of data replication and high mortality for some treatments, no statistical analyses were performed. Data were pooled by treatment due to the paucity of bees observed overall.

RESULTS

Throughout the duration of both experiments, bees were never seen or recorded constructing nests, although they were observed resting in the entrance of the tunnels and on the

face of the nesting block. Temperatures ranged from 7.2°C to 28.3°C (Fig. 1) with an average high of 24.2°C, and relative humidity ranged from 25% to 100% (Fig. 2) with an average high of 96.6%. High humidity and warm temperatures may be factors impacting the result of no nest construction, but see Discussion.

Validation of Pesticide Effects, No-Choice Test:

Application of acetamiprid resulted in 100% mortality and therefore, no flower visitation or handling time data was collected (Table 2). The cage treated with only boscalid/pyraclostrobin had more active bees compared to the other cages, with bees observed flying around the cage and spending little time on the flowers. The average number of flowers visited per female in the fungicide only cage was 2.2 per minute, with an average of 7.8 seconds spent per flower. With only 8.3% mortality (Table 2), the fungicide-only treatment had one of the lowest mortality rates out of all of the treatments for the no-choice trial and when compared to the choice trials (see below). The data for water (control) cages are reported with the choice test results. There was no detectable difference between the number of bees counted on each side of the field cage during the daily transects conducted in the no-choice treatments (Tables 3 and 4).

Pesticide Detection and Effects, Choice Test:

There were no differences between the treated and untreated sides or the right side and left side of each cage. In other words, the number of bees seen on the treated and untreated sides in the same cage on average were the same. However, for the fungicide|water cages, more bees were observed during the transect observations (4.2 bees on the treated side and 4.4 on the water side) compared to the other treatments, including the water|water cages (2.9 bees and 3.9 bees) (Table 4). Similar to the fungicide only treatment (no-choice), the fungicide|water treatment had the lowest mortality rate of 8.3% (Table 2). The water|water cages had the second lowest

mortality rate and the second highest number of bees observed during transect observations, with 21 out of 24 bees observed and a 12.5% mortality rate. In the insecticide|water cages, only 7 out of the 24 released females were observed during the experiment with nearly 71% mortality overall (Table 2), which mirrors the 100% mortality seen in the insecticide cage in the no-choice test. Eight of the 24 released bees were observed in the mix|water treatments with a 66.7% mortality rate (Table 2). Increased grooming and inactivity at the nest blocks, on the walls of cages, and on the forage was observed in the insecticide|water and mix|water cages, but the behavior was not observed in the cages with water alone or fungicide|water treatments (personal observations made by A.K. and field technicians).

Flower Visitation and Handling Results for Choice test:

Average flower visitation rate was highest in the fungicide|water cages with 5.3 flowers visited per bee per minute regardless of the side of the cage (Fig. 3). Cages treated with insecticide|water had the lowest flower visitation rate with nearly equal numbers of bees seen on the insecticide-treated and water-treated sides of the cages (Fig. 3). Mean flower handling time was shorter in cages with the fungicide|water treatment and the water|water treatment than in cages with the insecticide|water and mix|water treatments (Fig. 4). Fungicide|water and water|water cages had similar flower handling times regardless of the side of the cages. In the insecticide|water cage, bees spent less time per flower on the insecticide-treated side of the cages than on the water-treated side (Fig. 4). Bees in the mix|water cages spent an average of about half the time per flower on the mix-treated side of the cage compared to the water side (Fig. 4).

DISCUSSION

Environment may have been a major factor in the outcome and limitations of this experiment's interpretation. *Osmia lignaria* has two subspecies: *O. lignaria lignaria* found in the eastern United States and *O. lignaria propinqua* found in the western United States. So far, only *O. lignaria propinqua* has been successfully used as a managed pollinator (Bosch and Kemp 2001). *Osmia lignaria propinqua*, which forage in early spring in the western United States and will fly in temperatures as low as 12°C, were used for this experiment (Bosch and Kemp 2001). Kemp and Bosch showed that temperature impacts *O. lignaria* development and that different populations are regionally adapted to different temperatures (Kemp and Bosch 2005). In our study environment, the average high temperature was 24.2°C, and the average relative humidity during the study was 96.6 %. Moving western bees to the eastern U.S. with a distinctly hotter and more humid environment may have proven too stressful for this western species. The high humidity may have also allowed the pesticides to remain more aqueous than they would have been in a drier climate, making the pesticides more readily available for adsorption through the cuticle of the bees via contact and creating another route of pesticide exposure aside from ingestion of pollen and nectar (Kopit and Pitts-Singer 2018). To better tease apart these particular variables, *O. lignaria lignaria* should be trapped and used in the eastern United States or *O. lignaria propinqua* should be used for studies only in more amenable climates, such as in southern areas of certain western states (e.g., southern California), where a crop can be managed to bloom in early spring when *O. lignaria propinqua* naturally fly.

It is important to note that our study had limited replication and so interpretation of these findings is also limited. However, there were some specific effects that were distinct and also supported by other research. The hypothesis for the no-choice situation stated that bees that

forage in the control cages would exhibit normal foraging behaviors, yet no nesting was observed in any of the cages regardless of treatment, which implies that no normal foraging behavior was observed in this study. Overall there was no nesting, which indicates these bees were under pronounced stress or simply oppressed by the environment, since *O. lignaria* readily nest in field cages in western locations (Artz and Pitts-Singer 2015). It was hypothesized that bees that foraged on plants in fungicide-treated and insecticide-treated cages would be less affected than bees that foraged on plants in cages treated with a mixture of fungicide and insecticide due to synergism between the two pesticides. However, there was no evidence of synergism occurring between boscalid/pyraclostrobin and acetamiprid, and instead we detected what appeared to be a mitigating effect. More bees survived when the insecticide was mixed with the fungicide compared to when bees were exposed to the insecticide alone. We hypothesized that in a choice situation, bees would be deterred by the scent of the pesticides and choose to forage on the side of the cage treated only with water, but there was no detectable difference between sides. To better answer the questions posed for this experiment, more field cages for increased replication and longer lasting bloom are needed under favorable conditions to gain a better picture of how pesticides impact *O. lignaria* behavior. If the cages were too small to allow *O. lignaria* to detect a difference in forage because of being confined, then perhaps a laboratory Y-tube assay would be a better way to reduce variables and determine what pesticide odors *O. lignaria* can detect, and then pair these findings with a no-choice field cage study with more replicates to assess the impacts of the pesticides on foraging and nesting behavior.

Observations of bee behavior on flowers with fungicide residues revealed high levels of activity, which is reflected in higher visitation rate (Fig. 3) but shorter handling time (Fig. 4). The bees spent little to no time collecting pollen or nectar despite interacting with flowers. The

flower visitation rate and flower handling time that occurred in the presence of fungicide-treated forage could be described as hyperactive foraging behavior. The impact this type of behavior could have on the provisioning of nests could be detrimental and lead to inefficient pollination of crops and poor bee reproduction. Or perhaps, this hyperactivity is a boon to farmers and increases the pollination services of the bees if adequate pollen amounts are transferred between flowers. Further research is needed to assess the effectiveness of "hyperactive" pollinators and the impacts on fecundity of exposed individuals.

This study of *O. lignaria* in the presence of pesticides showed that acetamiprid, which is reported as somewhat "safe" for pollinators, was not safe in this particular environment (Biddinger et al. 2013, EFSA 2016). Neonicotinoids are nicotinic acetylcholine receptor agonists or antagonists and are water soluble (Kopit and Pitts-Singer 2018). All individuals in the no-choice cage treated with the insecticide acetamiprid were found dead on the ground inside the cage. 100% mortality in this cage meant that no foraging observations could be made. Neurological effects were observed for several bees and similar seizure-like movements were seen in a laboratory dose study on developing *O. lignaria* larvae exposed to provisions also treated with acetamiprid. The mandibles of treated individual larvae were opened and closed spasmodically (Kopit et al. in prep, CHAPTER 2). In the cages treated with acetamiprid and water (insecticide|water), where bees had a foraging choice, there was still high mortality, intensive grooming behavior, and minimal foraging. Bees in these cages were seen chewing up canola flower petals, perhaps showing signs of stress such as dehydration. Although bees in the insecticide|water cages visited on average about 2 flowers per minute, most of the time that the bees were on the flowers was spent grooming, not collecting pollen and nectar. Despite bees spending the most time per flower in the insecticide/water cages, there was little to no collection

of floral resources. Individual bees may have spent more time on flowers in the insecticide|water and insecticide treated cages because they were experiencing sublethal, harmful effects of the pesticides.

CONCLUSION

We were unable to clearly determine if fungicides and/or insecticides deter floral visitation of *O. lignaria.* However, boscalid/pyraclostrobin appeared to impact *O. lignaria* foraging behavior by inducing hyperactivity. An increase in hyperactivity may be a boon to crop pollination, although we do not know the explicit or long-term implications of hyperactive bees. Hyperactivity may deplete the female's fat reserves and cause her to be less reproductive or less successful at maturing and laying eggs. Therefore, hyperactivity may result in less fecund females that lead to a decline in future generations.

In a hot and humid environment, the neonicotinoid acetamiprid appeared to be more detrimental to *O. lignaria* than in laboratory or other field situations (Biddinger et al. 2013, EFSA 2016). Increased moisture and humidity may have made the neonicotinoid more readily available for trans-cuticular absorption or ingestion. More extensive research is needed to better understand the effects of agrochemicals on *O. lignaria* foraging behavior under various conditions and different environments. Performing laboratory assays, such as y-tube tests and dose studies, to tease apart attraction, repellence, and changes in normal behaviors (e.g., foraging and nesting) in conjunction with more semi-field cage studies are needed to gain a more complete picture of the impacts of pesticide sprays on the foraging behavior of *O. lignaria*.

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TABLES

Table 1. For each treatment, field rates of pesticides used in field cages in Poplarville, MS. Pesticide formulations were mixed in water; no adjuvants were added.

Treatment	**Dosage Per Acre (= Almond Rate)**
Boscalid/pyraclostrobin	14.5 oz in 10 gal of water
Acetamiprid	4.1 oz in 10 gal of water
Boscalid/pyraclostrobin & acetamiprid	14.5 & 4.1 oz respectively in 10 gal of water
Water	10 gal of water

Table 2. Count and percent mortality of *Osmia lignaria* females for each treatment in no choice and choice trials. The insecticide and fungicide no choice treatments only had 1 replicate each, so the number of released bees is only half (n = 12) of the other treatments (n = 24).

Treatment	**No. bees survived / No. bees released**	**Mortality rate (%)**
Fungicide only	11/12	8.3
Fungicide|Water	22/24	8.3
Insecticide only	0/12	100
Insecticide|Water	7/24	70.8
Mix|Water	8/24	66.7
Water|Water	21/24	12.5

Table 3. Average number of *O. lignaria* females observed during the 60 second observations across transects for right and left sides of each field cage.

Treatment (Right side|Left side)	**Right Side**	**Left Side**
Fungicide|Water	4.4	4.3
Water|Fungicide	4.5	4
Insecticide|Water	0.2	0.4
Water|Insecticide	0.3	0.3
Mix|Water	1.8	1.1
Water|Mix	1.1	1.9
Water|Water	2.8	3.1
Water|Water	3.1	4.6
Fungicide|Fungicide	4.5	5.4
Insecticide|Insecticide	0	0

Table 4. Average number of *O. lignaria* females observed during 60 second observations across transects for entire study for each choice treatment. The data for both cages of each treatment were pooled, and for each side of the cage was averaged (total number of individuals observed on treated sides / number of observation events, and total number of individuals observed on water treated sides / number of observation events).

Treatment	**Pesticide Side**	**Water Side**
Fungicide|Water	4.2	4.4
Insecticide|Water	0.3	0.3
Mix|Water	1.8	1.1
Water|Water	2.9 (right)	3.9 (left)

FIGURES

Figure 1. Maximum and minimum temperature for each day during the duration of the 2016 field study. Data attained from the George R Carr Memorial Air Field weather station in Bogalusa, Louisiana using Utah State University's MesoWest website (https://mesowest.utah.edu/cgibin/droman/mesomap.cgi?state=LA&rawsflag=3).

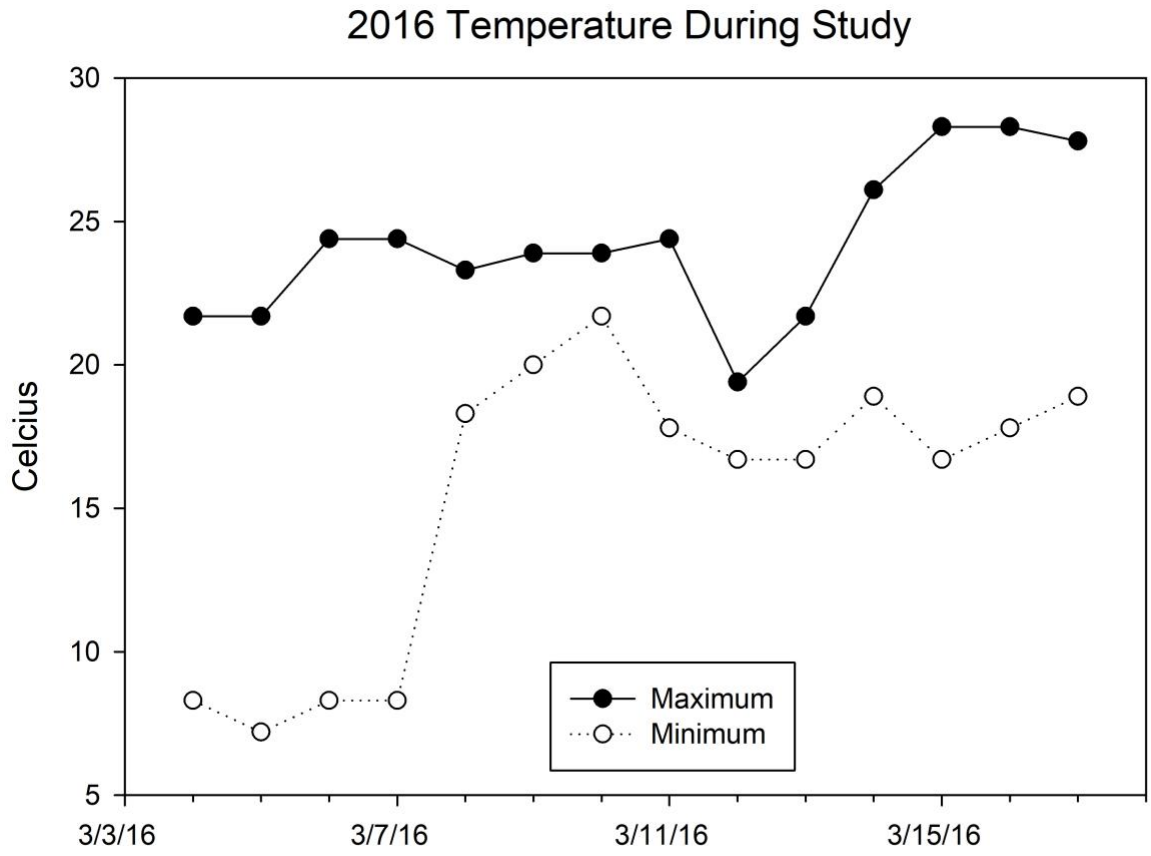


Figure 2. Maximum and minimum percent relative humidity for each day during the duration of the 2016 field study. Data attained from the George R Carr Memorial Air Field weather station in Bogalusa, Louisiana using Utah State University's MesoWest website (https://mesowest.utah.edu/cgi-bin/droman/mesomap.cgi?state=LA&rawsflag=3).

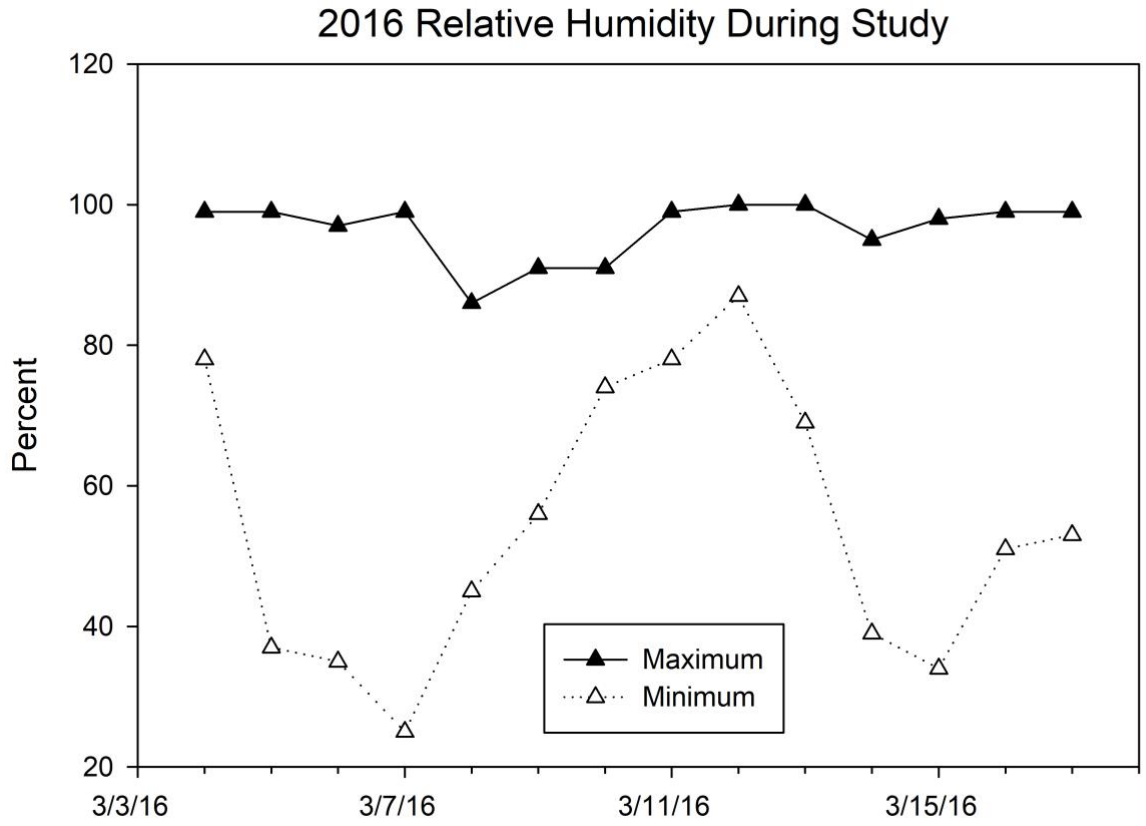


Figure 3. Mean flower visitation by *Osmia lignaria* females in field cages during timed observation periods for foraging choice experiment. All blue bars represent data for the side of the cage with water; other colors are for pesticide treatments.

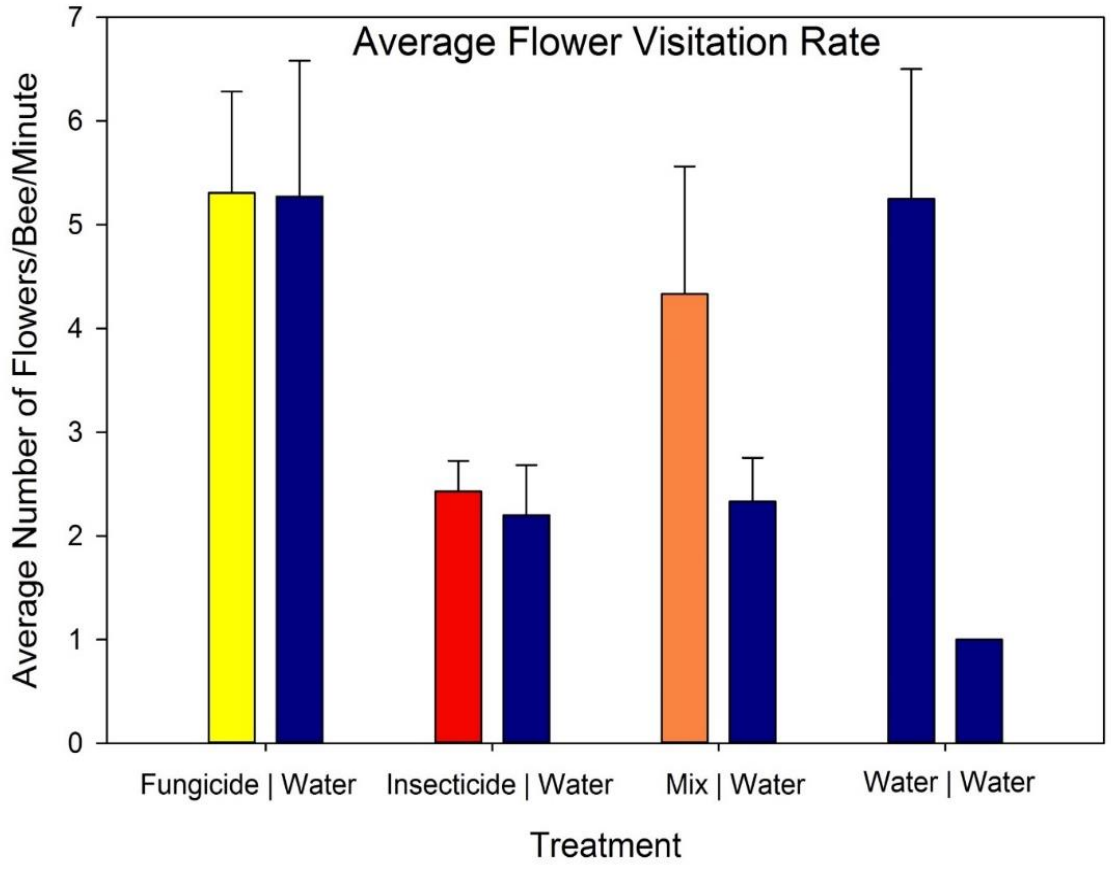
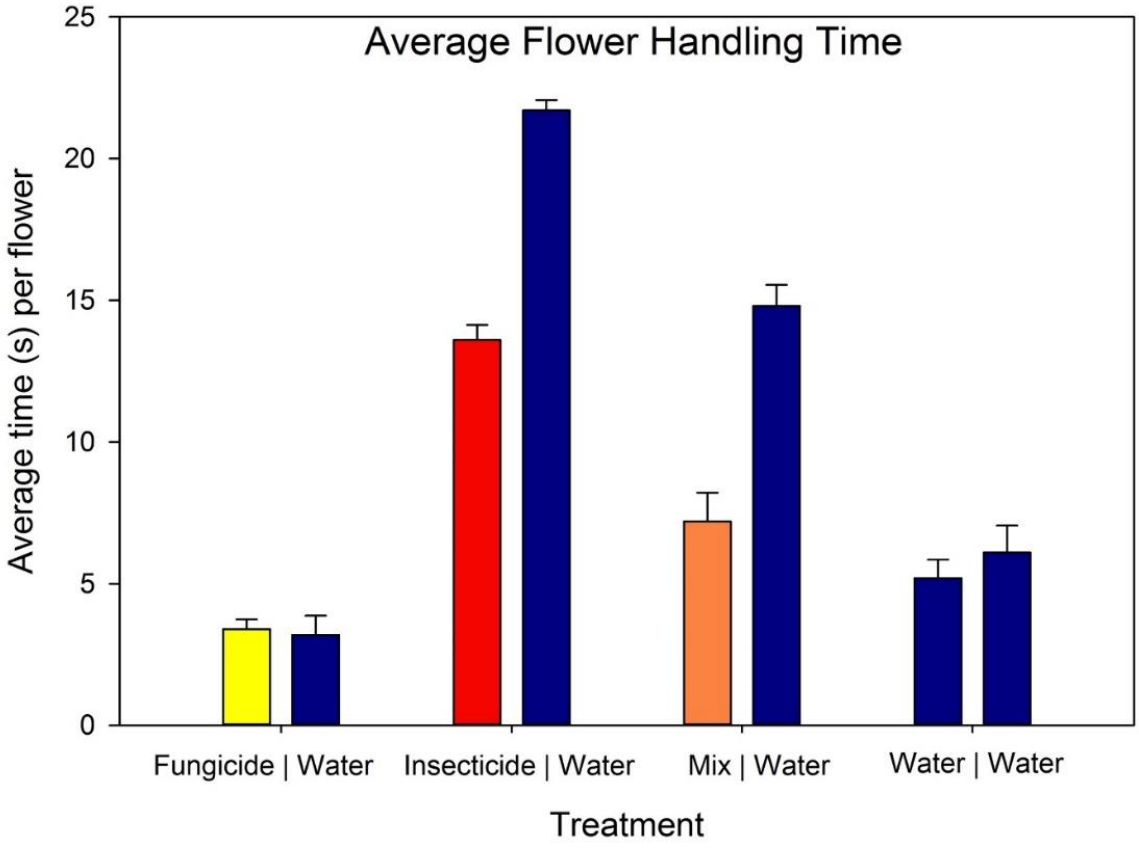


Figure 4. Mean flower handling time by *Osmia lignaria* females in field cages during timed observation periods for the foraging choice experiment. All blue bars represent data for the side of the cage treated with water; other colors are for pesticide treatments.



CONCLUSION

Investigating Routes and Effects of Pesticide Exposure on the Blue Orchard Bee (*Osmia lignaria*)

Andi M. Kopit

This thesis has defined the routes of potential pesticide exposure in solitary, cavity-nesting bees and begins to explore the gaps in our knowledge of pesticide impacts on *Osmia lignaria*. The routes of pesticide exposure experienced by cavity-nesting bees are larval ingestion, adult ingestion, contact, and transovarial transmission. The laboratory bioassay and field cage study conducted with *O. lignaria* just begins to scratch the surface of addressing the question of how non-*Apis* bees are impacted by agrochemicals, not only as adults, but also as developing larvae. Using *O. lignaria* as a solitary, cavity-nesting bee proxy for larval pesticide testing will provide a better picture of the issues faced with pollinator declines. Understanding how agrochemicals effect *O. lignaria* foraging behavior and development will help us understand the impacts of agricultural pest management practices on managed and wild bee populations.