Sublethal Behavioral and Physiological Effects of an Agricultural Fungicide on Honey Bees

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved May 2023 by the
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ARIZONA STATE UNIVERSITY

August 2023

ABSTRACT

Pollinator populations globally have declined at concerning rates in recent years, which is problematic given that roughly a third of all food production depends on them. Managed honey bee colony losses in particular have alarmed beekeepers and scientists, especially in the United States. Widespread agrochemical use has been implicated as one of the major causes of these colony losses. While the lethal effects of agrochemicals often receive the most attention, sublethal effects can occur at lower doses and can substantially weaken colonies over time. Impaired associative learning ability is a sublethal effect of a number of agrochemicals, and is particularly concerning, as it may hinder the abilities of bees to forage for food or find their way back to the colony. Here, I focus on the fungicide Pristine® (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin), which is sprayed on honey bee-pollinated crops during bloom and is known to poison bee mitochondria at ppm levels. First, I show that Pristine® impairs performance on an associative learning assay in the laboratory. Next, I show that Pristine® alters carbohydrate absorption in honey bees, providing a possible mechanism underlying this impaired learning performance. Finally, I demonstrate that Pristine® interacts with high temperatures to induce homing failure in exposed bees. My results raise concerns that this common fungicide may not be safe for pollinators and will be relevant to policymakers as they make decisions surrounding the regulation of fungicide use in agriculture.

ACKNOWLEDGMENTS

I would like to acknowledge the excellent ASU undergraduate students who helped me collect data, including Elise Chester, Leon Dilly, Maya Fortier, Samantha Jacobs, Jessalynn Macias, Shreya Nandy, and Daniela Soto. They provided critical support that ensured these experiments ran smoothly and successfully.

I would also like to thank the members of the Social Insect Research Group (SIRG) who provided a great sense of community during my time at ASU. In particular, I would like to acknowledge Chelsea Cook, Adrian Fisher, Jordan Glass, Meredith Johnson, Colin Lynch, and Cahit Ozturk, who all provided crucial advice and support regarding experimental design, data collection, and analysis.

I would like to thank my committee members, Gloria DeGrandi-Hoffman, Dale DeNardo, and Stephen Pratt for their helpful feedback during our meetings. I would also like to express my gratitude for all of the guidance, patience, and flexibility provided by my co-advisors, Jon Harrison and Brian Smith.

Finally, I would like to thank my family, Vicki, Kent, and Erica DesJardins. I could not have finished this PhD with out them. They pushed me to keep going through the most challenging times and I will always be grateful for their support.

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CHAPTER 1

THE EFFECTS OF ANTHROPOGENIC TOXINS ON HONEY BEE LEARNING: RESEARCH TRENDS AND SIGNIFICANCE

ABSTRACT

Managed honey bees are experiencing high rates of colony loss, in part due to widespread exposure to agrochemicals and other environmental toxins. The ability to learn about relevant environmental stimuli is an important skill necessary for foraging and navigation, although it is sometimes impaired in bees that have been exposed to toxins or other stressors. Here, I review the effects of anthropogenic toxins (which I divide into five major classes: insecticides, acaricides, biopesticides, other agrochemicals, and other toxins) on learning performance in European honey bees. I discuss the general trends of these studies, including that neurotoxic insecticides are overwhelmingly the most well-studied, and that most studies focus on acute exposure of individual, adult bees to a single toxin. Protocols for "field-relevant" exposure vary widely among labs, and I make suggestions to aid in the standardization of future studies. I review the relevance of learning studies for toxicological risk assessment, concluding that they are valuable tools for assessing sublethal behavioral effects of toxins. Their inclusion in risk assessment studies would be an improvement over current procedures, which focus largely on lethality.

INTRODUCTION

Insect pollinator populations are in decline globally (Potts et al., 2010). Colony loss rates for managed honey bees are also high both in the United States (Bruckner et al., 2023) and worldwide (Gray et al., 2022). These losses are happening simultaneously with a global increase in the demand for pollination services (Aizen et al., 2019). There is evidence that the production of certain fruit crops (such as apples, blueberries, and cherries) in the United States is already being limited by a lack of pollinators (Reilly et al., 2020). Future pollinator losses are expected to have devastating economic consequences (Lippert et al., 2021), with fruit, vegetable, and stimulant crops being especially vulnerable (Gallai et al., 2009).

Pollinators face a variety of threats, but the three most commonly implicated as causes of population declines include poor nutrition, parasites and pathogens, and agrochemicals (Goulson et al., 2015). These factors can interact to increase risk of individual or colony die-off (Mayack et al., 2022). This review will focus on the effects of agrochemicals and other environmental toxins on honey bees, a major area of concern in recent years (Johnson, 2015). Honey bees in the United States have been exposed to increasingly toxic levels of agrochemicals over the past 30 years (Douglas et al., 2020). Large varieties of pesticides are often found in hive products and bee-collected pollen (Mullin et al., 2010; Ostiguy et al., 2019). In some cases, large amounts of agrochemicals in hives have correlated with colony mortality (Traynor et al., 2016, 2021).

While agrochemicals and other toxins can be lethal for bees exposed to high enough doses, sublethal effects can occur at lower doses and cause substantial harm. Sublethal effects are defined as harmful physiological, developmental, or behavioral effects that occur in individuals that have survived exposure to a toxin (Desneux et al., 2006). Physiologically, toxins can impair immune responses to pathogens (Glavinic et al., 2019) and damage various organs (Catae et al., 2014; Faita et al., 2018; Zaluski et al., 2017). Developmental sublethal effects can include reduced egg eclosion (Fine, 2020), impaired egg and sperm viability (Milone & Tarpy, 2021), reduced queen emergence (Ricke et al., 2021), and general developmental delays (B. Li et al., 2022). Behavioral effects include altered locomotion (Aliouane et al., 2009; Delkash-Roudsari et al., 2020; Luo et al., 2021), impaired nestmate recognition (Cappa et al., 2019), and altered waggle dancing (Eiri & Nieh, 2012). This review will focus on impaired learning ability, another popular measure of behavioral sublethal effects (Siviter et al., 2018).

Learning has been tested in honey bees using a variety of methods; however, the proboscis extension reflex (PER) paradigm is the most popular for ecotoxicology studies. It is a form of classical Pavlovian conditioning (Rescorla, 1988) which utilizes the natural proboscis extension reflex of the honey bee—that is, a bee will reflexively extend its proboscis when a sugar solution is touched to its antennae (Bitterman et al., 1983; Takeda, 1961). In a typical experiment, an odor is used as a conditioned stimulus (CS) while sugar water solution acts as the unconditioned stimulus (US). Over a series of trials, a bee is exposed to the odor, and then the sugar solution is immediately brought to its antennae, eliciting the PER. A bee that has successfully learned the association will extend its proboscis in response to the odor alone, before the sugar solution is delivered (Figure 1.1A). Testing groups of individuals over a series of trials generates learning curves, which can then be compared across treatment groups (such as bees exposed to varying doses of a toxin; see Figure 1.1B for an example) (Matsumoto et al., 2012; Smith & Burden, 2014). In addition to the acquisition phase, memory is often tested at various time points post-conditioning (1 hour, 24 hours, 48 hours, etc.) by conducting one or two trials where the odor is presented without the reinforcing sugar solution. This technique has been adapted in countless ways to address a variety of topics, ranging from the cellular and molecular bases of learning and memory to the ecological associations between flowers and pollinators (Giurfa & Sandoz, 2012). The PER paradigm can be adapted to measure visual (Hori et al., 2006) and tactile (Scheiner et al., 1999) associative learning, as well as habituation (Braun & Bicker, 1992).

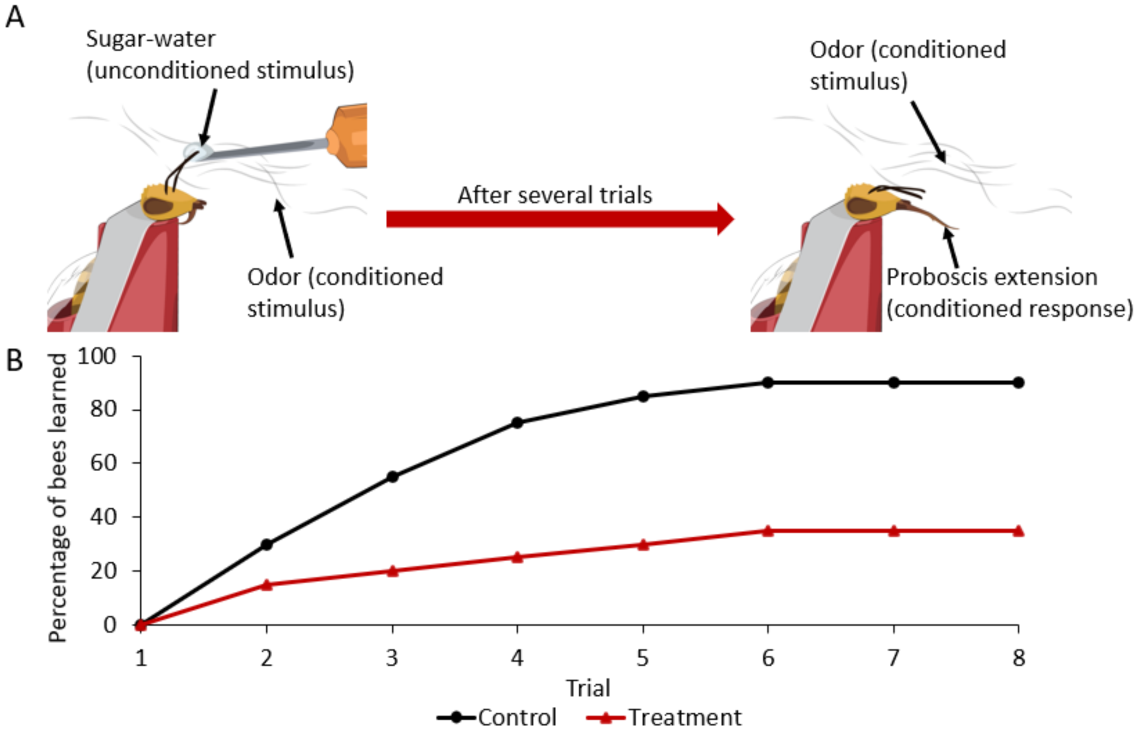


Figure 1.1A. Schematic showing the steps of PER. Over a series of trials, bees are exposed to an odor, and then a sugar water solution is immediately touched to their antennae. Bees that successfully learn the association will extend their proboscis in response to the odor alone, without any exposure to sugar. ***B.*** Hypothetical acquisition curve for a PER experiment comparing a control group and a group exposed to a toxin. The line represents the percentage of bees showing a learned response (proboscis extension prior to sugar water presentation) for each trial. In this case, the control group achieves a higher learning rate than the treatment group, suggesting that the toxin is negatively impacting learning performance.

Scope of the study. In this review, I focus on European honey bee (*Apis mellifera*) studies that involve “anthropogenic” toxins, which are present in the environment at higher levels than normal because of human activity. I focus on European honey bees because of their importance to pollination and because they have been subject to the greatest number of studies, enabling comparisons across toxin types. These compounds can be categorized into five major groups: insecticides, acaricides, biopesticides, other agrochemicals, and other toxins (see Figure 1.2 for a summary of the effects of each class). I focus on compounds mixed in with the unconditioned stimulus, applied topically, given orally, or injected, or, in the case of air pollutants, mixed in with the conditioned stimulus. The majority of these studies use the PER paradigm. I also cover studies that utilize other methods for testing associative learning such as T-mazes, shuttle boxes, and free-flying assays.

In each section, I note the studies that found effects at field-relevant toxin levels. As I discuss in the “General Trends” section below, the term “field-relevant” can be interpreted in a variety of different ways, but for the purposes of this review, I defer to the authors’ judgement regarding whether their dose and exposure protocol qualify as something bees could realistically be exposed to when foraging in the field.

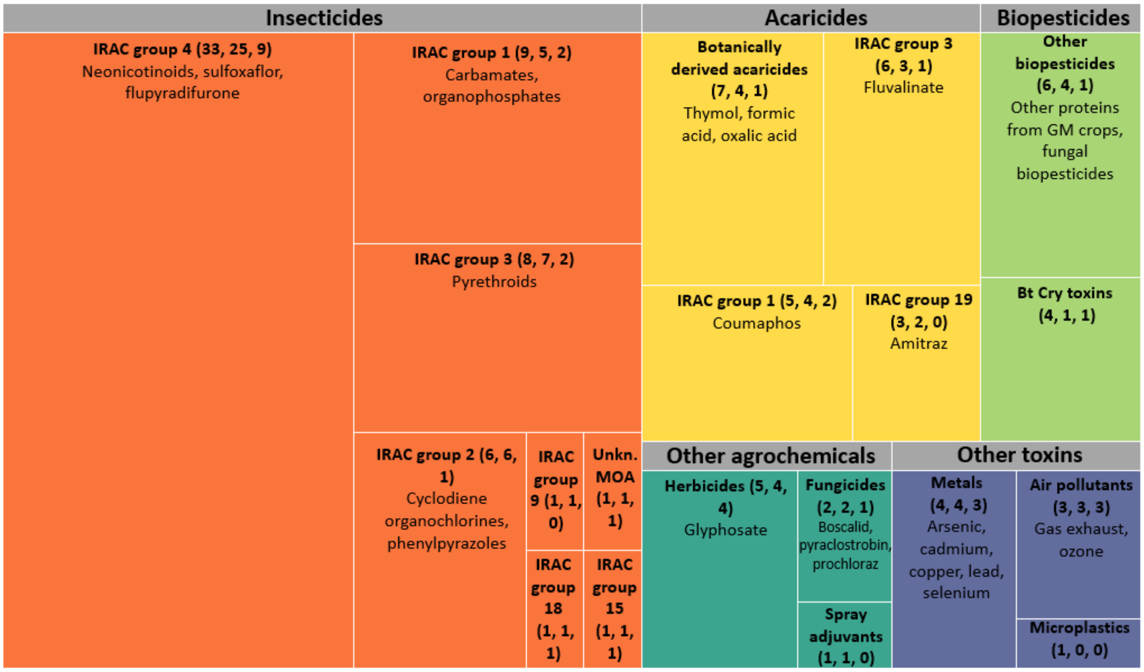


Figure 1.2. Breakdown of studies devoted to each class of toxin. Of the three numbers in parentheses for each class, the first is the number of papers that examined the effects of that toxin class on honey bee learning (also proportional to the size of the box), the second is the number of papers that reported negative effects, and the third is the number that reported negative effects at field-relevant (according to the study's authors) levels. Insecticides are classified based on Insecticide Resistance Action Committee (IRAC) mode of action groups. Studies focused on multiple toxins from different classes are counted more than once.

EFFECTS OF ANTHROPOGENIC TOXINS ON LEARNING

Insecticides. Insecticides are pesticides designed specifically to kill insect pests in households and agriculture. Insecticides are sprayed on bee-pollinated crops and are often found at high levels in hive food stores (Johnson, 2015; Mullin et al., 2010; Ostiguy et al., 2019; Traynor et al., 2021). There are 32 major groups of insecticides classified according to their mode of action (Insecticide Resistance Action Committee [IRAC], 2022). Of these 32 groups, nine have been tested for their effects on honey bee learning. Of these nine groups, six have modes of action related to insect nerves or muscles, two are insect growth regulators, and one affects the midgut (IRAC Group 11, which for the purposes of this review is included in the “Biopesticides” section). Neonicotinoids (IRAC Group 4) and imidacloprid in particular, have been the focus of many studies on honey bee learning. In this section, I first summarize the studies concerning imidacloprid and other neonicotinoids, and then broaden the scope to include other classes of insecticides.

The effects of imidacloprid (neonicotinoid, IRAC Group 4, nicotinic acetylcholine receptor competitive modulators) on honey bee learning and memory are by far the most-well-documented. The majority of PER studies report some negative effect of imidacloprid on learning and/or memory (Decourtye et al., 2003, 2004a, 2004b,; Z. Li et al., 2019; Schwartz et al., 2021), including effects at field-relevant doses (Mengoni Goñalons & Farina, 2015, 2018; Mustard et al., 2020; Wright et al., 2015; Yang et al., 2012). A few studies show no effects of imidacloprid alone (Karahan et al., 2015; Williamson et al., 2013; Williamson & Wright, 2013). The presence and magnitude of the negative effect can vary based on season, with summer bees being more prone to experiencing negative effects than winter bees, (Decourtye et al., 2003) and bee age, with young adult bees being more negatively affected than older bees (Mengoni Goñalons & Farina, 2015). Field-relevant larval exposure to imidacloprid also impairs PER learning (Yang et al., 2012). Other forms of learning, including habituation (Guez et al., 2001; Lambin et al., 2001) and free-flying associative learning (Decourtye et al., 2004b) are altered by imidacloprid exposure. Imidacloprid impairs bee performance in an aversive shuttle box assay (Delkash-Roudsari et al., 2020) and in an aversive paradigm that simulates predation (Zhang & Nieh, 2015). Some studies also show physiological alterations associated with impaired learning, including changes in cytochrome oxidase activity (Decourtye et al., 2004a) and gene transcription (Z. Li et al., 2019) in the brain.

Other neonicotinoids, including acetamiprid (Thany et al., 2015), clothianidin (Mustard et al., 2020; Piiroinen & Goulson, 2016; Tison et al., 2019), dinotefuran (Mustard et al., 2020), thiacloprid (Begna & Jung, 2021; Tison et al., 2017), and thiamethoxam (Aliouane et al., 2009; Mustard et al., 2020; Papach et al., 2017; Wright et al., 2015) negatively affect PER learning and/or memory, including some compounds at field-relevant levels (Mustard et al., 2020; Piiroinen & Goulson, 2016; Wright et al., 2015). A smaller number of studies report no effects of acetamiprid (Aliouane et al., 2009; El Hassani et al., 2008), clothianidin (Alkassab & Kirchner, 2016), and thiamethoxam (El Hassani et al., 2008) on PER learning. In addition to these PER studies, Ludicke and Nieh (2020) report negative effects of a field-relevant dose of thiamethoxam on a T-maze learning task in which bees choose between sections of the maze illuminated with different colored lights for a food reward. Newer-generation pesticide groups including sulfoximines and butenolides have the same basic mechanism of action as neonicotinoids. Fewer studies have examined the effects of these chemicals on honey bee PER learning, although one study reports a negative effect of sulfoxaflor (Cartereau et al., 2022), while another finds no effect (Siviter et al., 2019). Two studies report negative effects of flupyradifurone (H. Bell et al., 2020; Hesselbach & Scheiner, 2018), one at field-relevant levels (H. Bell et al., 2020).

Insecticides from a number of other classes that target the insect nervous system produce negative effects in PER learning assays. This includes carbamates (IRAC Group 1, acetylcholinesterase inhibitors) (Abramson et al., 1999; Weick & Thorn, 2002), organophosphates (IRAC Group 1, acetylcholinesterase inhibitors) (Z. Li et al., 2017; Urlacher et al., 2016), cyclodiene organochlorines (IRAC Group 2, GABA-gated chloride channel blockers) (Abramson et al., 1999; Decourtye et al., 2005), phenylpyrazoles (IRAC Group 2, GABA-gated chloride channel blockers) (Aliouane et al., 2009; Decourtye et al., 2005; El Hassani et al., 2005, 2009), pyrethroids (IRAC Group 3, sodium channel modulators) (Abramson et al., 1999; Decourtye et al., 2005; Liao et al., 2018; Mamood & Waller, 1990; Ramirez-Romero et al., 2005; Taylor et al., 1987; Thany et al., 2015), and pyridine azomethine derivatives (IRAC Group 9, chordotonal organ TRPV channel modulators) (Abramson et al., 2012). Most of these classes have reports of negative effects at field-relevant levels as well, including carbamates (Abramson et al., 1999), organophosphates (Urlacher et al., 2016), cyclodiene organochlorines (Abramson et al., 1999), and pyrethroids (Abramson et al., 1999; Ramirez-Romero et al., 2005). One study reports a positive effect of injection of the organophosphate trichlorfon on PER learning (Shapira et al., 2001). Additionally, fipronil (phenylpyrazole) produces negative effects on PER learning with a tactile instead of olfactory stimulus (Bernadou et al., 2009). Ethion (organophosphate) impairs learning performance in an aversive shuttle box assay (Delkash-Roudsari et al., 2020).

Only a few non-neuroactive or unknown mechanism of action (MOA) insecticides have been tested for effects on honey bee learning. The insect growth regulators diflubenzuron (IRAC Group 15, inhibitors of chitin biosynthesis affecting CHS1) and tebufenozide (IRAC Group 18, ecdysone receptor agonists) both produce negative effects on PER learning at field-relevant levels (Abramson et al., 2004). Additionally, a field-relevant dose of dicofol (unknown MOA) produces negative effects on PER learning (Stone et al., 1997).

Acaricides. Acaricides are compounds used to control arachnid pests (mites and ticks). Although their mechanisms of action overlap considerably with insecticides, I discuss them separately here because they are widely used directly within honey bee colonies to control the parasitic mite *Varroa destructor*, and thus are often the most commonly-found chemicals in samples of wax, bee bread, and honey (Johnson, 2015; Mullin et al., 2010; Ostiguy et al., 2019; Traynor et al., 2021). Here, I discuss the effects of synthetic (e.g., coumaphos, fluvalinate, and amitraz, presented according to IRAC classifications) and botanically derived (e.g., formic acid, oxalic acid, thymol) acaricides.

Many synthetic acaricides produce negative effects on PER learning and/or memory, including coumaphos (IRAC Group 1, acetylcholinesterase inhibitors) (Gashout et al., 2020; Weick & Thorn, 2002; Williamson et al., 2013; Williamson & Wright, 2013), fluvalinate (IRAC Group 3, sodium channel modulators) (Frost et al., 2013; Gashout et al., 2020; Taylor et al., 1987), and amitraz (Begna & Jung, 2021; Gashout et al., 2020), with effects at field-relevant levels reported for coumaphos (Williamson et al., 2013; Williamson & Wright, 2013) and fluvalinate (Frost et al., 2013). A few studies report no effects of fluvalinate (Decourtye et al., 2005) and amitraz (Rix & Cutler, 2017) on PER learning, and one study reports no effect of fluvalinate on visual/aversive shuttle box learning (Colin et al., 2020).

Some botanically derived acaricides also produce negative effects on PER learning, including formic acid (Gashout et al., 2020) and thymol (Bonnafé et al., 2015, 2018), with thymol producing negative effects at field-relevant levels (Bonnafé et al., 2018). One study reports that oxalic acid improves PER learning (S. Schneider et al., 2012). Thymol also alters congruency between olfactory and gustatory stimuli in the PER assay (Chapuy et al., 2019).

Biopesticides. Biopesticides are agricultural pesticides based on living organisms and/or their products. They can be broken into three broad categories: naturally-occurring biochemicals acting through non-toxic mechanisms (botanically derived compounds, essential oils, etc.), microbial entomopathogens (live bacterial or fungal organisms), and plant-incorporated protectants from genetically-engineered plants (most commonly, Cry proteins originally from the bacterium *Bacillus thuringiensis*, Bt). They are often touted as pollinator-friendly alternatives to more traditional, synthetic pesticides, and their use has increased in recent years (Cappa et al., 2022). Here, I focus on Bt Cry toxins first, and then broaden the scope to consider a few other biopesticides.

Bt Cry proteins (IRAC Group 11, microbial disruptors of insect midgut membranes) are used to control lepidopteran and coleopteran pests via the production of lesions in the midgut epithelium. These proteins are expressed in the pollen of transgenic plants, which may be collected by bees (Johnson, 2015; Picard-Nizou et al., 1997). Most studies report no effects of Bt Cry toxins on PER learning (Dai et al., 2012, 2016; Han et al., 2010). The exception to this is Ramirez-Romero et al. (2008), which reports that Cry1AB alters PER extinction at field-relevant levels. Bt Cry toxins also produce no effects on visual T-maze learning (Han et al., 2010) or free-flying associative learning (Ramirez-Romero et al., 2005).

A few other biopesticides have been tested for their effects on honey bee learning. Bioganic®, a commercial formulation marketed for household pest control and composed of a mixture of essential oils, produces negative effects on PER and free-flying associative learning at field-relevant levels (Abramson et al., 2006). Soybean Bowman Birk Inhibitor reduces PER learning, while Kunitz Soybean Trypsin Inhibitor has no effect (Pham-Delègue et al., 2000). Cowpea trypsin inhibitor reduces PER and free-flying associative learning (Picard-Nizou et al., 1997). Hv1a/GNA, a fusion protein containing a calcium channel blocker from spider venom, produces no negative effects on PER learning (Nakasu et al., 2014). *Beauveria bassiana*, an entomopathogenic fungus that infects hosts via cuticle contact and is used in agriculture to control a wide range of pest species, produces no effects on PER associative learning. However, it does alter PER habituation, increasing the number of trials needed for habituation to occur (Carlesso et al., 2020).

Other agrochemicals. Fungicides are pesticides used to control fungal diseases in agriculture. These chemicals are commonly sprayed on bee-pollinated crops, sometimes during bloom, and have been widely found in various in-hive food stores (Johnson, 2015; Mullin et al., 2010; Ostiguy et al., 2019; Traynor et al., 2021). There are 13 major groups of fungicides, targeting a wide range of biochemical processes in fungal cells (Fungicide Resistance Action Committee [FRAC], 2022). Representatives from two out of the 13 groups have been tested for their effects on honey bee learning. The first of these is prochloraz (FRAC code G1, targeting C14-demethylase in sterol biosynthesis), which causes faster extinction of PER learning (Decourtye et al., 2005). A formulation containing two active ingredients meant to interfere with cellular respiration (boscalid, FRAC code C2, targeting succinate dehydrogenase and pyraclostrobin, FRAC code C3, targeting cytochrome bc1 at Qo site) produces negative effects on PER learning and memory at field-relevant levels (DesJardins et al., 2021).

Likewise, herbicides are commonly applied to bee-pollinated crops, and a wide variety of compounds have been found in hive food stores (Johnson, 2015; Mullin et al., 2010; Ostiguy et al., 2019; Traynor et al., 2021). Only one herbicide, glyphosate (HRAC Group 9/inhibition of enolpyruvyl shikimate phosphate synthase), has been tested for effects on honey bee learning, representing one out of 26 major herbicide groups (Herbicide Resistance Action Committee [HRAC], 2022). Glyphosate produces negative effects on PER learning in young adult bees (Mengoni Goñalons & Farina, 2018). It impairs PER learning and memory in foraging-age bees at field-relevant levels (Herbert et al., 2014; Hernández et al., 2021; Luo et al., 2021). One study reports no effect of glyphosate on aversive shuttle box learning (Delkash-Roudsari et al., 2020).

Adjuvants can either be included in pesticide formulations (formulation adjuvants) or added to tank mixes together with pesticides (spray adjuvants). They are added to enhance the efficacy of the active ingredients. Although widely used, they are often assumed to be inert and are rarely tested for possible effects on pollinators, and their presence usually is not tested for in hive food stores (Mullin et al., 2015). While some studies described above did test the effects of whole formulations, I focus here on the one study that tested adjuvants by themselves, conducted by Ciarlo et al. (2012). This study tested multiple compounds from three major adjuvant classes (organosilicones, nonionic surfactants, and crop oil concentrates). Organosilicones have significant negative effects on PER learning, suggesting they may not be safe for pollinators (Mullin et al., 2016). Nonionic surfactants have slight negative effects, and crop oil concentrates produce no effects.

Other toxins. A number of metals produce negative effects on PER learning at field-relevant levels, including selenium (Burden et al., 2016), lead (Monchanin et al., 2021a, 2021b), copper, and arsenic (Monchanin et al., 2021b). Cadmium also produces negative effects at sublethal doses (Z. Li et al., 2022). Learning is most severely impaired when bees are fed a combination of lead, copper, and arsenic, suggesting additive effects (Monchanin et al., 2021b). These metals may be present in the soil due to mining and industrial operations. They are taken up by plants, resulting in contaminated pollen and nectar, which is then collected by bees (Johnson, 2015).

Industrial air pollutants including diesel exhaust (containing carbon monoxide and nitrogen oxide gases) and ozone produce negative effects on PER learning at field-relevant levels. The concerns regarding these chemicals are twofold. First, there is concern that these pollutants could mask floral volatiles used by honey bees to locate food sources when foraging, impairing odor recognition. This can be tested by mixing an air pollutant with the conditioned stimulus in PER learning, and indeed, this produces negative effects (Leonard et al., 2019). There is also concern that honey bees could be exposed to these chemicals outright, producing sublethal effects, possibly including impaired learning. When bees are exposed before conditioning, both diesel exhaust (Reitmayer et al., 2019) and ozone (Démares et al., 2022) produce negative effects on PER learning.

Microplastics are widely present in water and soil, and have been found in honey stores (Alma et al., 2023). One study examined the effects of both acute and chronic consumption of microplastics on PER habituation and associative learning, and it reports no effects (Balzani et al., 2022).

GENERAL TRENDS

Neurotoxic insecticides/acaricides are overwhelmingly the most well-studied. My review found 52 studies focused on compounds known to be toxic to arthropod nervous systems, 9 studies focused on other types of insecticides/acaricides, 10 studies focused on biopesticides, 5 studies on herbicides, 2 studies on fungicides, 1 study on spray adjuvants, 4 studies on metals, 3 studies on air pollutants, and 1 study on microplastics (some studies are counted more than once because they tested compounds from more than one of these classes). Most of the compounds tested produced some sort of negative effect, so importantly, direct neurotoxicity is not a prerequisite for negative effects on learning.

Many studies use acute, individual exposure rather than chronic and/or colony-level exposure. I found 43 studies that used acute, individual exposure (feeding individual bees a known amount of a toxin one time before conditioning or testing). This is compared to 24 studies that fed toxins to groups of adults in cages. In this case, a set concentration was usually provided in a sugar solution *ad libitum* over a period of several days. Eight studies exposed entire colonies to the toxin; this usually involved providing contaminated nectar or pollen over a period of days or weeks and then capturing adult foragers for learning tests. Presumably, each of these approaches comes with benefits and drawbacks. For example, an acute/individual exposure paradigm provides the benefit of being able to control the exact amount of toxin received and the life stage of the individual, while colony-level exposure is presumably more relevant to field conditions.

Most studies look only at active ingredients, rather than formulations. Among agrochemical studies (not including adjuvants or genetically-modified crops), 12 focused on commercial formulations, while 51 focused on active ingredients only. There are likely benefits and drawbacks to both approaches here as well. Focusing on active ingredients only may be prudent because the compounds themselves may become disassociated with their formulations by the time bees are exposed to them (e.g. in contaminated hive food stores). However, it may be short-sighted to completely ignore adjuvants and co-formulants, as these can also impair learning on their own (Ciarlo et al., 2012).

Most studies only focus on adult exposure. I found 2 studies that exposed larvae to toxins (and then tested their learning capacities when they became adults), as opposed to 65 studies that exposed and tested adults and 8 studies that exposed entire colonies (and thus were not specific about which life stage was exposed). Exposure across a combination of life stages may be more relevant to field conditions and can lead to greater impairments than when bees are only exposed as adults (DesJardins et al., 2021); this should be a focus area for future studies.

Some studies looking at the synergistic effects of multiple pesticides on learning have been conducted, but not very many. Some studies have found negative effects of combinations of insecticides and acaricides on PER learning (Begna & Jung, 2021; Colin et al., 2020; Williamson & Wright, 2013). One study found that a mixture of the neonicotinoid insecticide imidacloprid and the herbicide glyphosate impairs PER learning (Mengoni Goñalons & Farina, 2018). One study found that a mixture of four organophosphate insecticides produces no effect on learning (Al Naggar et al., 2015).

Another intriguing area of study has focused on synergistic effects between parasite/pathogen and pesticide exposure. The insecticides flupyradifurone and clothianidin impair PER learning when bees are also exposed to *Nosema cerenae* (H. Bell et al., 2020; Piiroinen & Goulson, 2016), and imidacloprid and *Varroa destructor* interact to produce negative effects on learning (Schwartz et al., 2021). As bees are likely to be exposed to complex mixtures of agrochemicals and other toxins inside hives (Mullin et al., 2010; Ostiguy et al., 2019), there should be an increased focus on testing the effects of field-relevant synergisms.

Methodological details vary widely between labs, which highlights the need to exercise caution when comparing results. Even a relatively "standardized" procedure such as PER (Barascou et al., 2021) is prone to methodological variations that can alter the outcome; previous reviews have raised this concern and called for more standardization across labs (Frost et al., 2012; Matsumoto et al., 2012; Smith & Burden, 2014). Examples of such methodological variations include the number of trials during the acquisition phase, whether differential or absolute conditioning is used, the duration of the intertrial intervals, whether and when memory is tested, colony history (such as past acaricide treatments), the subspecies or genetic strain of honey bees tested, and season during which the experiment was conducted. Given this, it is generally advisable to refrain from directly comparing results among labs (or at least to exercise extreme caution when doing so), and the field should strive to adopt a more standardized set of methods. Previous reviews on the topic (Frost et al., 2012; De Stefano et al., 2014; Matsumoto et al., 2012; Smith & Burden, 2014) offer detailed suggestions for standardizing PER procedures; I suggest referring to those when deciding on which procedures to adopt.

Many studies claim to expose bees to a dose or concentration that is field-relevant, but define that in different ways. I found 16 studies that did not claim to test a dose or concentration bees would be likely to encounter in the real world; these studies usually just picked a sublethal dose/concentration by choosing a set fraction of the LD-50 or LC-50. Thirty-nine studies claimed some degree of field relevance for their chosen dose or concentration, although the definition of that term varied. Some used the dose or concentration recommended by a governing body or manufacturer to control a particular pest. Some chose a dose or concentration that had been previously found in hive food products (e.g. bee bread, honey) or in bee bodies. Some chose a dose or concentration that had been found in nectar or pollen from treated crops. The three air pollutant studies chose concentrations that were likely to be present in polluted air.

When deciding on an exposure protocol that is field-relevant, factors that should be carefully considered include whether to use acute or chronic exposure, whether to expose individuals or groups (in cages or whole colonies), the life stage(s) during which exposure and testing occur, the administration method (oral via nectar or pollen, topical, etc.), and the dose itself. My recommendation for creating a truly field-relevant exposure protocol is to think carefully about methodological details and create an “exposure scenario” in which the combination of variables mimics a situation that bees may encounter in the field. For example, a field-relevant dose could be drawn from a study that measured pesticide residues in treated flowers, nectar, and/or bee-collected pollen (e.g. Graham et al., 2022). In this case, it may make more sense to focus on feeding a single acute dose to individual adult foragers (or a series of doses corresponding to the length of the blooming period), directly mimicking a scenario in which they are foraging on treated crops. If drawing the field-relevant dose from a study that measured levels of toxins in hive food stores such as bee bread (e.g. Mullin et al., 2010; Ostiguy et al., 2019; Traynor et al., 2021), it would make sense to expose whole colonies over a longer period of time, perhaps via contaminated pollen patties, mimicking a scenario in which a generation of bees consumes contaminated food as larvae and young adults. Whatever the protocol, each factor should be explicitly described and justified.

SIGNIFICANCE OF LEARNING STUDIES IN TOXIN RISK ASSESSMENT

Lab-based learning experiments are popular ways to test for behavioral sublethal effects because associative learning is necessary for foraging and navigation, which are critically important for colony function and survival. Bees are central place foragers, which means that they navigate to floral resources outside the colony and bring their products back home (W. Bell, 1990). Successful navigation requires bees to learn sensory cues from the environment and integrate these with motor outputs (Buehlmann et al., 2020). Relevant sensory cues can be either visual or olfactory. Bees start their foraging lives by performing a series of orientation flights around the hive, through which they become extensively familiar with the surrounding visual landmarks (Capaldi & Dyer, 1999). They use these landmarks to navigate home after foraging over long ranges (Pahl et al., 2011). Olfactory cues are used to navigate at shorter ranges to help bees home in on the correct location of either a floral patch or the colony (Chaffiol et al., 2005). As part of the forager recruitment process (which also includes the waggle dance), bees learn floral odors from their nestmates in the hive. These cues help them navigate to the same floral patch during their own trip (Arenas et al., 2007). This information suggests that olfactory and visual learning experiments may be predictive of foraging and navigation behaviors in the field, and indeed, PER learning performance correlates with real-world foraging performance (Cabirol et al., 2018). Bees are also able to transfer knowledge of odors they have learned in the real world to the PER paradigm in the laboratory (Gerber et al., 1996).

Many of the compounds discussed above that impair learning performance in the lab also produce negative effects on foraging and navigation behaviors in the field. Some neonicotinoids reduce the number of foraging trips made by individual foragers (Ohlinger et al., 2022; C. Schneider et al., 2012; Tison et al., 2020). Neonicotinoids can also increase the duration of foraging trips, with treated bees taking longer to return to the hive than controls (C. Schneider et al., 2012; Yang et al., 2008). Neonicotinoids can also impair homing ability, with neonicotinoid-exposed bees returning at lower rates than controls in experiments (Fischer et al., 2014; Henry et al., 2012). The phenylpyrazole insecticide fipronil also decreases foraging trips (Decourtye et al., 2011). Pyrethroid insecticides can also produce negative effects, with deltamethrin impairing homing ability (Van Dame et al., 1995) and fluvalinate altering duration of foraging trips (Colin et al., 2021). In addition to these insecticides, the herbicide glyphosate (Sol Balbuena et al., 2015) increases the average duration of homing flights in treated bees when compared to controls. Ultimately, it has been suggested that bees are so vulnerable to environmental stressors because central place foraging requires relatively advanced cognitive abilities, which can be negatively affected even at low doses of toxins (Klein et al., 2017).

Given that lab-based learning experiments seem to be relevant to bee behaviors in the real world (Henry et al., 2015), the question becomes, should they be used when assessing pesticide risks to bees, and if so, how? There have been calls to include more nuanced testing for sublethal effects of pesticides on bees (Barascou et al., 2021; Decourtye et al., 2013; Fisher, 2021). Learning experiments played a (somewhat indirect) role in the banning of three neonicotinoids in Europe. Initial learning experiments (e.g., Decourtye et al., 2004a) raised concerns that this class of pesticides could be especially harmful to bees. This led to field-based studies on foraging and navigation (e.g., Henry et al., 2012; C. Schneider et al., 2012). It was ultimately these studies that garnered the attention of activists and policymakers and led to a ban on the neonicotinoids imidacloprid, thiamethoxam, and clothianidin in the European Union (Auteri et al., 2017; Sgolastra et al., 2020).

Overall, lab-based learning studies are a relatively easy way to quantify behavioral sublethal effects of environmental toxins in honey bees, as they typically require less resources than field studies and allow for more control over extraneous variables. Despite this, some have called the ecological relevance of these experiments into question (Barascou et al., 2021). Studies that show negative effects of toxins on foraging and navigation behaviors in the field might be more likely to get the attention of policymakers. PER assays would perhaps be most effective if they were incorporated into lower-tier risk assessments with explicitly standardized procedures and field-relevant exposure protocols. This would be an improvement over current lower-tier studies, which mostly focus on LD-50s (Barascou et al., 2021). If PER experiments produce negative effects, then higher tiers could include foraging and/or navigation experiments in the field. This approach should be explored as a possible way to incorporate important behavioral sublethal effects into risk assessment procedures.

CONCLUSION

Roughly 80 studies have examined the effects of anthropogenic toxins on honey bee learning, finding effects of a variety of agrochemicals, biopesticides, metals, and air pollutants. Learning experiments are a relatively easy way to test for behavioral sublethal effects, and can correlate with negative effects on foraging and navigation behaviors in the field. These studies should be further incorporated into procedures for toxin risk assessment, including to assess a more diverse set of toxins for behavioral sublethal effects. Relatively few studies have focused on toxins such as fungicides and metals, although bees may be as likely or even more likely to encounter them as they are insecticides (fungicides are sometimes sprayed during bloom, for example, increasing the probability that foraging bees are exposed). Efforts should also be made to test for synergisms between compounds that are likely to be encountered together in the field.

REFERENCES

Abramson, C. I., Aquino, I. S., Ramalho, F. S., & Price, J. M. (1999). The Effect of Insecticides on Learning in the Africanized Honey Bee (*Apis mellifera* L.). *Archives of Environmental Contamination and Toxicology*, *37*(4), 529–535. https://doi.org/10.1007/s002449900548

Abramson, C. I., Singleton, J. B., Wilson, M. K., Wanderley, P. A., Ramalho, F. S., & Michaluk, L. M. (2006). The Effect of an Organic Pesticide on Mortality and Learning in Africanized Honey Bees (*Apis mellifera* L.) in Brasil. *American Journal of Environmental Sciences*, *2*(1), 33–40. https://doi.org/10.3844/ajessp.2006.33.40

Abramson, C. I., Sokolowski, M. B. C., Brown, E. A., & Pilard, S. (2012). The effect of pymetrozine (Plenum WG-50$^{®}$) on proboscis extension conditioning in honey bees (*Apis mellifera*: Hybrid var. Buckfast). *Ecotoxicology and Environmental Safety*, *78*, 287–295. https://doi.org/10.1016/j.ecoenv.2011.11.038

Abramson, C. I., Squire, J., Sheridan, A., & Mulder, P. G. (2004). The Effect of Insecticides Considered Harmless to Honey Bees (*Apis mellifera*): Proboscis Conditioning Studies by Using the Insect Growth Regulators Tebufenozide and Diflubenzuron. *Environmental Entomology*, *33*(2), 378–388. https://doi.org/10.1603/0046-225X-33.2.378

Aizen, M. A., Aguiar, S., Biesmeijer, J. C., Garibaldi, L. A., Inouye, D. W., Jung, C., Martins, D. J., Medel, R., Morales, C. L., Ngo, H., Pauw, A., Paxton, R. J., Sáez, A., & Seymour, C. L. (2019). Global agricultural productivity is threatened by increasing pollinator dependence without a parallel increase in crop diversification. *Global Change Biology*, *25*(10), 3516–3527. https://doi.org/10.1111/gcb.14736

Al Naggar, Y., Wiseman, S., Sun, J., Cutler, G. C., Aboul-Soud, M., Naiem, E., Mona, M., Seif, A., & Giesy, J. P. (2015). Effects of environmentally-relevant mixtures of four common organophosphorus insecticides on the honey bee (*Apis mellifera* L.). *Journal of Insect Physiology*, *82*, 85–91. https://doi.org/10.1016/j.jinsphys.2015.09.004

Aliouane, Y., El Hassani, A. K., Gary, V., Armengaud, C., Lambin, M., & Gauthier, M. (2009). Subchronic exposure of honeybees to sublethal doses of pesticides: Effects on behavior. *Environmental Toxicology and Chemistry*, *28*(1), 113–122. https://doi.org/10.1897/08-110.1

Alkassab, A. T., & Kirchner, W. H. (2016). Impacts of chronic sublethal exposure to clothianidin on winter honeybees. *Ecotoxicology*, *25*(5), 1000–1010. https://doi.org/10.1007/s10646-016-1657-3

Alma, A. M., de Groot, G. S., & Buteler, M. (2023). Microplastics incorporated by honeybees from food are transferred to honey, wax and larvae. *Environmental Pollution*, *320*, 121078. https://doi.org/10.1016/j.envpol.2023.121078

Arenas, A., Fernández, V. M., & Farina, W. M. (2007). Floral odor learning within the hive affects honeybees' foraging decisions. *Naturwissenschaften*, *94*(3), 218–222. https://doi.org/10.1007/s00114-006-0176-0

Auteri, D., Arena, M., Barmaz, S., Ippolito, A., Linguadoca, A., Molnar, T., Sharp, R., Szentes, C., Vagenende, B., & Verani, A. (2017). Neonicotinoids and bees: The case of the European regulatory risk assessment. *Science of The Total Environment*, *579*, 966–971. https://doi.org/10.1016/j.scitotenv.2016.10.158

Balzani, P., Galeotti, G., Scheggi, S., Masoni, A., Santini, G., & Baracchi, D. (2022). Acute and chronic ingestion of polyethylene (PE) microplastics has mild effects on honey bee health and cognition. *Environmental Pollution*, *305*, 119318. https://doi.org/10.1016/j.envpol.2022.119318

Barascou, L., Brunet, J.-L., Belzunces, L., Decourtye, A., Henry, M., Fourrier, J., Le Conte, Y., & Alaux, C. (2021). Pesticide risk assessment in honeybees: Toward the use of behavioral and reproductive performances as assessment endpoints. *Chemosphere*, *276*, 130134. https://doi.org/10.1016/j.chemosphere.2021.130134

Begna, T., & Jung, C. (2021). Effects of sequential exposures of sub-lethal doses of amitraz and thiacloprid on learning and memory of honey bee foragers, *Apis mellifera*. *Journal of Asia-Pacific Entomology*, *24*(2), 77–83. https://doi.org/10.1016/j.aspen.2021.03.012

Bell, H. C., Montgomery, C. N., Benavides, J. E., & Nieh, J. C. (2020). Effects of *Nosema ceranae* (Dissociodihaplophasida: Nosematidae) and Flupyradifurone on Olfactory Learning in Honey Bees, *Apis mellifera* (Hymenoptera: Apidae). *Journal of Insect Science*, *20*(6), 29. https://doi.org/10.1093/jisesa/ieaa130

Bell, W. J. (1990). Central place foraging. In W. J. Bell (Ed.), *Searching Behaviour: The behavioural ecology of finding resources* (pp. 171–187). Springer Netherlands. https://doi.org/10.1007/978-94-011-3098-1_12

Bernadou, A., Démares, F., Couret-Fauvel, T., Sandoz, J. C., & Gauthier, M. (2009). Effect of fipronil on side-specific antennal tactile learning in the honeybee. *Journal of Insect Physiology*, *55*(12), 1099–1106. https://doi.org/10.1016/j.jinsphys.2009.08.019

Bitterman, M. E., Menzel, R., Fietz, A., & Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Psychology*, *97*(2), 107–119. https://doi.org/10.1037/0735-7036.97.2.107

Bonnafé, E., Alayrangues, J., Hotier, L., Massou, I., Renom, A., Souesme, G., Marty, P., Allaoua, M., Treilhou, M., & Armengaud, C. (2018). Monoterpenoid-based preparations in beehives affect learning, memory, and gene expression in the bee brain. *Environmental Toxicology and Chemistry*, 337–345. https://doi.org/10.1002/etc.3527

Bonnafé, E., Drouard, F., Hotier, L., Carayon, J.-L., Marty, P., Treilhou, M., & Armengaud, C. (2015). Effect of a thymol application on olfactory memory and gene expression levels in the brain of the honeybee *Apis mellifera*. *Environmental Science and Pollution Research*, *22*(11), 8022–8030. https://doi.org/10.1007/s11356-014-2616-2

Braun, G., & Bicker, G. (1992). Habituation of an appetitive reflex in the honeybee. *Journal of Neurophysiology*, *67*(3), 588–598. https://doi.org/10.1152/jn.1992.67.3.588

Bruckner, S., Wilson, M., Aurell, D., Rennich, K., vanEngelsdorp, D., Steinhauer, N., & Williams, G. R. (2023). A national survey of managed honey bee colony losses in the USA: Results from the Bee Informed Partnership for 2017–18, 2018–19, and 2019–20. *Journal of Apicultural Research*, *0*(0), 1–15. https://doi.org/10.1080/00218839.2022.2158586

Buehlmann, C., Mangan, M., & Graham, P. (2020). Multimodal interactions in insect navigation. *Animal Cognition*. https://doi.org/10.1007/s10071-020-01383-2

Burden, C. M., Elmore, C., Hladun, K. R., Trumble, J. T., & Smith, B. H. (2016). Acute exposure to selenium disrupts associative conditioning and long-term memory recall in honey bees (*Apis mellifera*). *Ecotoxicology and Environmental Safety*, *127*, 71–79. https://doi.org/10.1016/j.ecoenv.2015.12.034

Cabirol, A., Cope, A. J., Barron, A. B., & Devaud, J.-M. (2018). Relationship between brain plasticity, learning and foraging performance in honey bees. *PLOS ONE*, *13*(4), e0196749. https://doi.org/10.1371/journal.pone.0196749

Capaldi, E. A., & Dyer, F. C. (1999). The role of orientation flights on homing performance in honeybees. *Journal of Experimental Biology*, *202*(12), 1655–1666. https://doi.org/10.1242/jeb.202.12.1655

Cappa, F., Baracchi, D., & Cervo, R. (2022). Biopesticides and insect pollinators: Detrimental effects, outdated guidelines, and future directions. *Science of The Total Environment*, *837*, 155714. https://doi.org/10.1016/j.scitotenv.2022.155714

Cappa, F., Petrocelli, I., Dani, F. R., Dapporto, L., Giovannini, M., Silva-Castellari, J., Turillazzi, S., & Cervo, R. (2019). Natural biocide disrupts nestmate recognition in honeybees. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-019-38963-3

Carlesso, D., Smargiassi, S., Sassoli, L., Cappa, F., Cervo, R., & Baracchi, D. (2020). Exposure to a biopesticide interferes with sucrose responsiveness and learning in honey bees. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-020-76852-2

Cartereau, A., Pineau, X., Lebreton, J., Mathé-Allainmat, M., Taillebois, E., & Thany, S. H. (2022). Impairments in learning and memory performances associated with nicotinic receptor expression in the honeybee *Apis mellifera* after exposure to a sublethal dose of sulfoxaflor. *PLOS ONE*, *17*(8), e0272514. https://doi.org/10.1371/journal.pone.0272514

Catae, A. F., Roat, T. C., Oliveira, R. A. D., Nocelli, R. C. F., & Malaspina, O. (2014). Cytotoxic effects of thiamethoxam in the midgut and malpighian tubules of Africanized *Apis mellifera* (Hymenoptera: Apidae). *Microscopy Research and Technique*, *77*(4), 274–281. https://doi.org/10.1002/jemt.22339

Chaffiol, A., Laloi, D., & Pham-Delègue, M.-H. (2005). Prior classical olfactory conditioning improves odour-cued flight orientation of honey bees in a wind tunnel. *Journal of Experimental Biology*, *208*(19), 3731–3737. https://doi.org/10.1242/jeb.01796

Chapuy, C., Ribbens, L., Renou, M., Dacher, M., & Armengaud, C. (2019). Thymol Affects Congruency Between Olfactory and Gustatory Stimuli in Bees. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-43614-8

Ciarlo, T. J., Mullin, C. A., Frazier, J. L., & Schmehl, D. R. (2012). Learning Impairment in Honey Bees Caused by Agricultural Spray Adjuvants. *PLOS ONE*, *7*(7), e40848. https://doi.org/10.1371/journal.pone.0040848

Colin, T., Forster, C. C., Westacott, J., Wu, X., Meikle, W. G., & Barron, A. B. (2021). Effects of late miticide treatments on foraging and colony productivity of European honey bees (*Apis mellifera*). *Apidologie*. https://doi.org/10.1007/s13592-020-00837-3

Colin, T., Plath, J. A., Klein, S., Vine, P., Devaud, J.-M., Lihoreau, M., Meikle, W. G., & Barron, A. B. (2020). The miticide thymol in combination with trace levels of the neonicotinoid imidacloprid reduces visual learning performance in honey bees (*Apis mellifera*). *Apidologie*. https://doi.org/10.1007/s13592-020-00737-6

Dai, P.-L., Jia, H.-R., Geng, L.-L., & Diao, Q.-Y. (2016). Bt Toxin Cry1Ie Causes No Negative Effects on Survival, Pollen Consumption, or Olfactory Learning in Worker Honey Bees (Hymenoptera: Apidae). *Journal of Economic Entomology*, *109*(3), 1028–1033. https://doi.org/10.1093/jee/tow088

Dai, P.-L., Zhou, W., Zhang, J., Cui, H.-J., Wang, Q., Jiang, W.-Y., Sun, J.-H., Wu, Y.-Y., & Zhou, T. (2012). Field assessment of Bt *cry1Ah* corn pollen on the survival, development and behavior of *Apis mellifera ligustica*. *Ecotoxicology and Environmental Safety*, *79*, 232–237. https://doi.org/10.1016/j.ecoenv.2012.01.005

De Stefano, L. A., Stepanov, I. I., & Abramson, C. I. (2014). The First Order Transfer Function in the Analysis of Agrochemical Data in Honey Bees (*Apis Mellifera* L.): Proboscis Extension Reflex (PER) Studies. *Insects*, *5*(1), 167–198. https://doi.org/10.3390/insects5010167

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M., & Pham-Delègue, M.-H. (2004a). Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). *Pesticide Biochemistry and Physiology*, *78*(2), 83–92. https://doi.org/10.1016/j.pestbp.2003.10.001

Decourtye, A., Devillers, J., Aupinel, P., Brun, F., Bagnis, C., Fourrier, J., & Gauthier, M. (2011). Honeybee tracking with microchips: A new methodology to measure the effects of pesticides. *Ecotoxicology*, *20*(2), 429–437. https://doi.org/10.1007/s10646-011-0594-4

Decourtye, A., Devillers, J., Cluzeau, S., Charreton, M., & Pham-Delègue, M.-H. (2004b). Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotoxicology and Environmental Safety*, *57*(3), 410–419. https://doi.org/10.1016/j.ecoenv.2003.08.001

Decourtye, A., Devillers, J., Genecque, E., Menach, K. L., Budzinski, H., Cluzeau, S., & Pham-Delègue, M. H. (2005). Comparative Sublethal Toxicity of Nine Pesticides on Olfactory Learning Performances of the Honeybee *Apis mellifera*. *Archives of Environmental Contamination and Toxicology*, *48*(2), 242–250. https://doi.org/10.1007/s00244-003-0262-7

Decourtye, A., Henry, M., & Desneux, N. (2013). Overhaul pesticide testing on bees. *Nature*, *497*(7448), 188–188. https://doi.org/10.1038/497188a

Decourtye, A., Lacassie, E., & Pham-Delègue, M.-H. (2003). Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Management Science*, *59*(3), 269–278. https://doi.org/10.1002/ps.631

Delkash-Roudsari, S., Chicas-Mosier, A. M., Goldansaz, S. H., Talebi-Jahromi, K., Ashouri, A., & Abramson, C. I. (2020). Assessment of lethal and sublethal effects of imidacloprid, ethion, and glyphosate on aversive conditioning, motility, and lifespan in honey bees (*Apis mellifera* L.). *Ecotoxicology and Environmental Safety*, *204*, 111108. https://doi.org/10.1016/j.ecoenv.2020.111108

Démares, F., Gibert, L., Creusot, P., Lapeyre, B., & Proffit, M. (2022). Acute ozone exposure impairs detection of floral odor, learning, and memory of honey bees, through olfactory generalization. *Science of The Total Environment*, *827*, 154342. https://doi.org/10.1016/j.scitotenv.2022.154342

DesJardins, N. S., Fisher, A., Ozturk, C., Fewell, J. H., DeGrandi-Hoffman, G., Harrison, J. F., & Smith, B. H. (2021). A common fungicide, Pristine®, impairs olfactory associative learning performance in honey bees (*Apis mellifera*). *Environmental Pollution*, 117720. https://doi.org/10.1016/j.envpol.2021.117720

Desneux, N., Decourtye, A., & Delpuech, J.-M. (2006). The Sublethal Effects of Pesticides on Beneficial Arthropods. *Annual Review of Entomology*, *52*(1), 81–106. https://doi.org/10.1146/annurev.ento.52.110405.091440

Douglas, M. R., Sponsler, D. B., Lonsdorf, E. V., & Grozinger, C. M. (2020). County-level analysis reveals a rapidly shifting landscape of insecticide hazard to honey bees (*Apis mellifera*) on US farmland. *Scientific Reports*, *10*(1), 1–11. https://doi.org/10.1038/s41598-019-57225-w

Eiri, D. M., & Nieh, J. C. (2012). A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. *Journal of Experimental Biology*, *215*(12), 2022–2029. https://doi.org/10.1242/jeb.068718

El Hassani, A. K., Dacher, M., Gary, V., Lambin, M., Gauthier, M., & Armengaud, C. (2008). Effects of Sublethal Doses of Acetamiprid and Thiamethoxam on the Behavior of the Honeybee (*Apis mellifera*). *Archives of Environmental Contamination and Toxicology*, *54*(4), 653–661. https://doi.org/10.1007/s00244-007-9071-8

El Hassani, A. K., Dacher, M., Gauthier, M., & Armengaud, C. (2005). Effects of sublethal doses of fipronil on the behavior of the honeybee (*Apis mellifera*). *Pharmacology Biochemistry and Behavior*, *82*(1), 30–39. https://doi.org/10.1016/j.pbb.2005.07.008

El Hassani, A. K., Dupuis, J. P., Gauthier, M., & Armengaud, C. (2009). Glutamatergic and GABAergic effects of fipronil on olfactory learning and memory in the honeybee. *Invertebrate Neuroscience*, *9*(2), 91. https://doi.org/10.1007/s10158-009-0092-z

Faita, M. R., Oliveira, E. de M., Alves, V. V., Orth, A. I., & Nodari, R. O. (2018). Changes in hypopharyngeal glands of nurse bees (*Apis mellifera*) induced by pollen-containing sublethal doses of the herbicide Roundup®. *Chemosphere*, *211*, 566–572. https://doi.org/10.1016/j.chemosphere.2018.07.189

Fine, J. D. (2020). Evaluation and comparison of the effects of three insect growth regulators on honey bee queen oviposition and egg eclosion. *Ecotoxicology and Environmental Safety*, *205*, 111142. https://doi.org/10.1016/j.ecoenv.2020.111142

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B., & Menzel, R. (2014). Neonicotinoids Interfere with Specific Components of Navigation in Honeybees. *PLOS ONE*, *9*(3), e91364. https://doi.org/10.1371/journal.pone.0091364

Fisher, A. (2021). Protect pollinators—Reform pesticide regulations. *Nature*, *595*(7866), 172–172. https://doi.org/10.1038/d41586-021-01818-x

Frost, E. H., Shutler, D., & Hillier, N. K. (2012). The proboscis extension reflex to evaluate learning and memory in honeybees (*Apis mellifera*): Some caveats. *Naturwissenschaften*, *99*(9), 677–686. https://doi.org/10.1007/s00114-012-0955-8

Frost, E. H., Shutler, D., & Hillier, N. K. (2013). Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival. *Journal of Experimental Biology*, *216*(15), 2931–2938. https://doi.org/10.1242/jeb.086538

Fungicide Resistance Action Committee. (2022, March). *FRAC Code List©*2022: Fungal control agents sorted by cross-resistance pattern and mode of action (including coding for FRAC Groups on product labels).* https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2022--final.pdf?sfvrsn=b6024e9a_2

Gallai, N., Salles, J.-M., Settele, J., & Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, *68*(3), 810–821. https://doi.org/10.1016/j.ecolecon.2008.06.014

Gashout, H. A., Guzman-Novoa, E., Goodwin, P. H., & Correa-Benítez, A. (2020). Impact of sublethal exposure to synthetic and natural acaricides on honey bee (*Apis mellifera*) memory and expression of genes related to memory. *Journal of Insect Physiology*, *121*, 104014. https://doi.org/10.1016/j.jinsphys.2020.104014

Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalksy, O., Wüstenberg, D., & Menzel, R. (1996). Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Animal Behaviour*, *52*(6), 1079–1085. https://doi.org/10.1006/anbe.1996.0255

Giurfa, M., & Sandoz, J.-C. (2012). Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learning & Memory*, *19*(2), 54–66. https://doi.org/10.1101/lm.024711.111

Glavinic, U., Tesovnik, T., Stevanovic, J., Zorc, M., Cizelj, I., Stanimirovic, Z., & Narat, M. (2019). Response of adult honey bees treated in larval stage with prochloraz to infection with *Nosema ceranae*. *PeerJ*, *7*, e6325. https://doi.org/10.7717/peerj.6325

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957. https://doi.org/10.1126/science.1255957

Graham, K. K., Milbrath, M. O., Zhang, Y., Baert, N., McArt, S., & Isaacs, R. (2022). Pesticide risk to managed bees during blueberry pollination is primarily driven by off-farm exposures. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-11156-1

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Bugeja Douglas, A., Cadahía, L., Charrière, J.-D., Chlebo, R., Coffey, M. F., Cornelissen, B., Costa, C. A. da, Danneels, E., Danihlík, J., Dobrescu, C., Evans, G., Fedoriak, M., Forsythe, I., Gregorc, A., … Brodschneider, R. (2022). Honey bee colony loss rates in 37 countries using the COLOSS survey for winter 2019–2020: The combined effects of operation size, migration and queen replacement. *Journal of Apicultural Research*, *0*(0), 1–7. https://doi.org/10.1080/00218839.2022.2113329

Guez, D., Suchail, S., Gauthier, M., Maleszka, R., & Belzunces, L. P. (2001). Contrasting Effects of Imidacloprid on Habituation in 7- and 8-Day-Old Honeybees (*Apis mellifera*). *Neurobiology of Learning and Memory*, *76*(2), 183–191. https://doi.org/10.1006/nlme.2000.3995

Han, P., Niu, C.-Y., Lei, C.-L., Cui, J.-J., & Desneux, N. (2010). Use of an innovative T-tube maze assay and the proboscis extension response assay to assess sublethal effects of GM products and pesticides on learning capacity of the honey bee *Apis mellifera* L. *Ecotoxicology*, *19*(8), 1612–1619. https://doi.org/10.1007/s10646-010-0546-4

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., & Decourtye, A. (2012). A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. *Science*, *336*(6079), 348–350. https://doi.org/10.1126/science.1215039

Henry, M., Cerrutti, N., Aupinel, P., Decourtye, A., Gayrard, M., Odoux, J.-F., Pissard, A., Rüger, C., & Bretagnolle, V. (2015). Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1819), 20152110. https://doi.org/10.1098/rspb.2015.2110

Herbert, L. T., Vázquez, D. E., Arenas, A., & Farina, W. M. (2014). Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour. *Journal of Experimental Biology*, *217*(19), 3457–3464. https://doi.org/10.1242/jeb.109520

Herbicide Resistance Action Committee. (2022, January 6). *HRAC Mode of Action Classification 2022.* https://hracglobal.com/files/HRAC_MOA_Poster_January_6_2022.pdf

Hernández, J., Riveros, A. J., & Amaya-Márquez, M. (2021). Sublethal doses of glyphosate impair olfactory memory retention, but not learning in the honey bee (*Apis mellifera scutellata*). *Journal of Insect Conservation*, *25*(4), 683–694. https://doi.org/10.1007/s10841-021-00335-6

Hesselbach, H., & Scheiner, R. (2018). Effects of the novel pesticide flupyradifurone (Sivanto) on honeybee taste and cognition. *Scientific Reports*, *8*(1), 1–8. https://doi.org/10.1038/s41598-018-23200-0

Hori, S., Takeuchi, H., Arikawa, K., Kinoshita, M., Ichikawa, N., Sasaki, M., & Kubo, T. (2006). Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L. *Journal of Comparative Physiology A*, *192*(7), 691–700. https://doi.org/10.1007/s00359-005-0091-4

Insecticide Resistance Action Committee. (2022, December). *Mode of Action Classification*. https://irac-online.org/documents/moa-brochure/

Johnson, R. M. (2015). Honey Bee Toxicology. *Annual Review of Entomology*, *60*(1), 415–434. https://doi.org/10.1146/annurev-ento-011613-162005

Karahan, A., Çakmak, I., Hranitz, J. M., Karaca, I., & Wells, H. (2015). Sublethal imidacloprid effects on honey bee flower choices when foraging. *Ecotoxicology*, *24*(9), 2017–2025. https://doi.org/10.1007/s10646-015-1537-2

Klein, S., Cabirol, A., Devaud, J.-M., Barron, A. B., & Lihoreau, M. (2017). Why Bees Are So Vulnerable to Environmental Stressors. *Trends in Ecology & Evolution*, *32*(4), 268–278. https://doi.org/10.1016/j.tree.2016.12.009

Lambin, M., Armengaud, C., Raymond, S., & Gauthier, M. (2001). Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. *Archives of Insect Biochemistry and Physiology*, *48*(3), 129–134. https://doi.org/10.1002/arch.1065

Leonard, R. J., Vergoz, V., Proschogo, N., McArthur, C., & Hochuli, D. F. (2019). Petrol exhaust pollution impairs honey bee learning and memory. *Oikos*, *128*(2), 264–273. https://doi.org/10.1111/oik.05405

Li, B., Ke, L., Li, A.-R., Diao, Q.-Y., Wang, Q., & Liu, Y.-J. (2022). Exposure of Larvae to Sublethal Thiacloprid Delays Bee Development and Affects Transcriptional Responses of Newly Emerged Honey Bees. *Frontiers in Insect Science*, *2*. https://www.frontiersin.org/article/10.3389/finsc.2022.844957

Li, Z., Li, M., Huang, J., Ma, C., Xiao, L., Huang, Q., Zhao, Y., Nie, H., & Su, S. (2017). Effects of Sublethal Concentrations of Chlorpyrifos on Olfactory Learning and Memory Performances in Two Bee Species, *Apis mellifera* and *Apis cerana*. *Sociobiology*, *64*(2), 174–181. https://doi.org/10.13102/sociobiology.v64i2.1385

Li, Z., Qiu, Y., Li, J., Wan, K., Nie, H., & Su, S. (2022). Chronic Cadmium Exposure Induces Impaired Olfactory Learning and Altered Brain Gene Expression in Honey Bees (*Apis mellifera*). *Insects*, *13*(11), Article 11. https://doi.org/10.3390/insects13110988

Li, Z., Yu, T., Chen, Y., Heerman, M., He, J., Huang, J., Nie, H., & Su, S. (2019). Brain transcriptome of honey bees (*Apis mellifera*) exhibiting impaired olfactory learning induced by a sublethal dose of imidacloprid. *Pesticide Biochemistry and Physiology*, *156*, 36–43. https://doi.org/10.1016/j.pestbp.2019.02.001

Liao, C., He, X., Wang, Z., Barron, A. B., Zhang, B., Zeng, Z., & Wu, X. (2018). Short-Term Exposure to Lambda-Cyhalothrin Negatively Affects the Survival and Memory-Related Characteristics of Worker Bees *Apis mellifera*. *Archives of Environmental Contamination and Toxicology*, *75*(1), 59–65. https://doi.org/10.1007/s00244-018-0514-1

Lippert, C., Feuerbacher, A., & Narjes, M. (2021). Revisiting the economic valuation of agricultural losses due to large-scale changes in pollinator populations. *Ecological Economics*, *180*, 106860. https://doi.org/10.1016/j.ecolecon.2020.106860

Ludicke, J. C., & Nieh, J. C. (2020). Thiamethoxam impairs honey bee visual learning, alters decision times, and increases abnormal behaviors. *Ecotoxicology and Environmental Safety*, *193*, 110367. https://doi.org/10.1016/j.ecoenv.2020.110367

Luo, Q.-H., Gao, J., Guo, Y., Liu, C., Ma, Y.-Z., Zhou, Z.-Y., Dai, P.-L., Hou, C.-S., Wu, Y.-Y., & Diao, Q.-Y. (2021). Effects of a commercially formulated glyphosate solutions at recommended concentrations on honeybee (*Apis mellifera* L.) behaviours. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-020-80445-4

Mamood, A. N., & Waller, G. D. (1990). Recovery of learning responses by honeybees following a sublethal exposure to permethrin. *Physiological Entomology*, *15*(1), 55–60. https://doi.org/10.1111/j.1365-3032.1990.tb00492.x

Matsumoto, Y., Menzel, R., Sandoz, J.-C., & Giurfa, M. (2012). Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: A step toward standardized procedures. *Journal of Neuroscience Methods*, *211*(1), 159–167. https://doi.org/10.1016/j.jneumeth.2012.08.018

Mayack, C., Macherone, A., Zaki, A. G., Filiztekin, E., Özkazanç, B., Koperly, Y., Schick, S. J., Eppley, E. J., Deb, M., Ambiel, N., Schafsnitz, A. M., & Broadrup, R. L. (2022). Environmental exposures associated with honey bee health. *Chemosphere*, *286*, 131948. https://doi.org/10.1016/j.chemosphere.2021.131948

Mengoni Goñalons, C., & Farina, W. M. (2015). Effects of Sublethal Doses of Imidacloprid on Young Adult Honeybee Behaviour. *PLOS ONE*, *10*(10), e0140814. https://doi.org/10.1371/journal.pone.0140814

Mengoni Goñalons, C., & Farina, W. M. (2018). Impaired associative learning after chronic exposure to pesticides in young adult honey bees. *Journal of Experimental Biology*, *221*(7). https://doi.org/10.1242/jeb.176644

Milone, J. P., & Tarpy, D. R. (2021). Effects of developmental exposure to pesticides in wax and pollen on honey bee (*Apis mellifera*) queen reproductive phenotypes. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-020-80446-3

Monchanin, C., Blanc-Brude, A., Drujont, E., Negahi, M. M., Pasquaretta, C., Silvestre, J., Baqué, D., Elger, A., Barron, A. B., Devaud, J.-M., & Lihoreau, M. (2021a). Chronic exposure to trace lead impairs honey bee learning. *Ecotoxicology and Environmental Safety*, *212*, 112008. https://doi.org/10.1016/j.ecoenv.2021.112008

Monchanin, C., Drujont, E., Devaud, J.-M., Lihoreau, M., & Barron, A. B. (2021b). Metal pollutants have additive negative effects on honey bee cognition. *Journal of Experimental Biology*, *jeb.241869*. https://doi.org/10.1242/jeb.241869

Mullin, C. A., Chen, J., Fine, J. D., Frazier, M. T., & Frazier, J. L. (2015). The formulation makes the honey bee poison. *Pesticide Biochemistry and Physiology*, *120*, 27–35. https://doi.org/10.1016/j.pestbp.2014.12.026

Mullin, C. A., Fine, J. D., Reynolds, R. D., & Frazier, M. T. (2016). Toxicological Risks of Agrochemical Spray Adjuvants: Organosilicone Surfactants May Not Be Safe. *Frontiers in Public Health*, *4*. https://doi.org/10.3389/fpubh.2016.00092

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., & Pettis, J. S. (2010). High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLOS ONE*, *5*(3), e9754. https://doi.org/10.1371/journal.pone.0009754

Mustard, J. A., Gott, A., Scott, J., Chavarria, N. L., & Wright, G. A. (2020). Honeybees fail to discriminate floral scents in a complex learning task after consuming a neonicotinoid pesticide. *Journal of Experimental Biology*, *223*(5). https://doi.org/10.1242/jeb.217174

Nakasu, E. Y. T., Williamson, S. M., Edwards, M. G., Fitches, E. C., Gatehouse, J. A., Wright, G. A., & Gatehouse, A. M. R. (2014). Novel biopesticide based on a spider venom peptide shows no adverse effects on honeybees. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1787), 20140619. https://doi.org/10.1098/rspb.2014.0619

Ohlinger, B. D., Schürch, R., Durzi, S., Kietzman, P. M., Silliman, M. R., & Couvillon, M. J. (2022). Honey Bees (Hymenoptera: Apidae) Decrease Foraging But Not Recruitment After Neonicotinoid Exposure. *Journal of Insect Science*, *22*(1), 16. https://doi.org/10.1093/jisesa/ieab095

Ostiguy, N., Drummond, F. A., Aronstein, K., Eitzer, B., Ellis, J. D., Spivak, M., & Sheppard, W. S. (2019). Honey Bee Exposure to Pesticides: A Four-Year Nationwide Study. *Insects*, *10*(1), Article 1. https://doi.org/10.3390/insects10010013

Pahl, M., Zhu, H., Tautz, J., & Zhang, S. (2011). Large Scale Homing in Honeybees. *PLOS ONE*, *6*(5), e19669. https://doi.org/10.1371/journal.pone.0019669

Papach, A., Fortini, D., Grateau, S., Aupinel, P., & Richard, F.-J. (2017). Larval exposure to thiamethoxam and American foulbrood: Effects on mortality and cognition in the honey bee *Apis mellifera*. *Journal of Apicultural Research*, *56*(4), 475–486. https://doi.org/10.1080/00218839.2017.1332541

Pham-Delègue, M.-H., Girard, C., Métayer, M. L., Picard-Nizou, A.-L., Hennequet, C., Pons, O., & Jouanin, L. (2000). Long-term effects of soybean protease inhibitors on digestive enzymes, survival and learning abilities of honeybees. *Entomologia Experimentalis et Applicata*, *95*(1), 21–29. https://doi.org/10.1046/j.1570-7458.2000.00637.x

Picard-Nizou, A. L., Grison, R., Olsen, L., Pioche, C., Arnold, G., & Pham-Delegue, M. H. (1997). Impact of Proteins Used in Plant Genetic Engineering: Toxicity and Behavioral Study in the Honeybee. *Journal of Economic Entomology*, *90*(6), 1710–1716. https://doi.org/10.1093/jee/90.6.1710

Piiroinen, S., & Goulson, D. (2016). Chronic neonicotinoid pesticide exposure and parasite stress differentially affects learning in honeybees and bumblebees. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1828), 20160246. https://doi.org/10.1098/rspb.2016.0246

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology & Evolution*, *25*(6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007

Ramirez-Romero, R., Chaufaux, J., & Pham-Delègue, M.-H. (2005). Effects of Cry1Ab protoxin, deltamethrin and imidacloprid on the foraging activity and the learning performances of the honeybee Apis mellifera, a comparative approach. *Apidologie*, *36*(4), 601–611. https://doi.org/10.1051/apido:2005039

Ramirez-Romero, R., Desneux, N., Decourtye, A., Chaffiol, A., & Pham-Delègue, M. H. (2008). Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L. (Hymenoptera, Apidae)? *Ecotoxicology and Environmental Safety*, *70*(2), 327–333. https://doi.org/10.1016/j.ecoenv.2007.12.002

Reilly, J. R., Artz, D. R., Biddinger, D., Bobiwash, K., Boyle, N. K., Brittain, C., Brokaw, J., Campbell, J. W., Daniels, J., Elle, E., Ellis, J. D., Fleischer, S. J., Gibbs, J., Gillespie, R. L., Gundersen, K. B., Gut, L., Hoffman, G., Joshi, N., Lundin, O., … Winfree, R. (2020). Crop production in the USA is frequently limited by a lack of pollinators. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1931), 20200922. https://doi.org/10.1098/rspb.2020.0922

Reitmayer, C. M., Ryalls, J. M. W., Farthing, E., Jackson, C. W., Girling, R. D., & Newman, T. A. (2019). Acute exposure to diesel exhaust induces central nervous system stress and altered learning and memory in honey bees. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-41876-w

Rescorla, R. A. (1988). Behavioral Studies of Pavlovian Conditioning. *Annual Review of Neuroscience*, *11*(1), 329–352. https://doi.org/10.1146/annurev.ne.11.030188.001553

Ricke, D. F., Lin, C.-H., & Johnson, R. M. (2021). Pollen Treated with a Combination of Agrochemicals Commonly Applied During Almond Bloom Reduces the Emergence Rate and Longevity of Honey Bee (Hymenoptera: Apidae) Queens. *Journal of Insect Science*, *21*(6). https://doi.org/10.1093/jisesa/ieab074

Rix, R. R., & Cutler, G. C. (2017). Acute Exposure to Worst-Case Concentrations of Amitraz Does Not Affect Honey Bee Learning, Short-Term Memory, or Hemolymph Octopamine Levels. *Journal of Economic Entomology*, *110*(1), 127–132. https://doi.org/10.1093/jee/tow250

Scheiner, R., Erber, J., & Page Jr., R. E. (1999). Tactile learning and the individual evaluation of the reward in honey bees (Apis mellifera L.). *Journal of Comparative Physiology A*, *185*(1), 1–10. https://doi.org/10.1007/s003590050360

Schneider, C. W., Tautz, J., Grünewald, B., & Fuchs, S. (2012). RFID Tracking of Sublethal Effects of Two Neonicotinoid Insecticides on the Foraging Behavior of *Apis mellifera*. *PLOS ONE*, *7*(1), e30023. https://doi.org/10.1371/journal.pone.0030023

Schneider, S., Eisenhardt, D., & Rademacher, E. (2012). Sublethal effects of oxalic acid on *Apis mellifera* (Hymenoptera: Apidae): changes in behaviour and longevity. *Apidologie*, *43*(2), 218–225. https://doi.org/10.1007/s13592-011-0102-0

Schwartz, K. R., Minor, H., Magro, C., McConnell, J., Capani, J., Griffin, J., & Doebel, H. (2021). The neonicotinoid imidacloprid alone alters the cognitive behavior in *Apis mellifera L.* and the combined exposure of imidacloprid and *Varroa destructor* mites synergistically contributes to trial attrition. *Journal of Apicultural Research*, *0*(0), 1–8. https://doi.org/10.1080/00218839.2020.1866233

Sgolastra, F., Medrzycki, P., Bortolotti, L., Maini, S., Porrini, C., Simon-Delso, N., & Bosch, J. (2020). Bees and pesticide regulation: Lessons from the neonicotinoid experience. *Biological Conservation*, *241*, 108356. https://doi.org/10.1016/j.biocon.2019.108356

Shapira, M., Thompson, C. K., Soreq, H., & Robinson, G. E. (2001). Changes in neuronal acetylcholinesterase gene expression and division of labor in honey bee colonies. *Journal of Molecular Neuroscience*, *17*(1), 1–12. https://doi.org/10.1385/JMN:17:1:1

Siviter, H., Koricheva, J., Brown, M. J. F., & Leadbeater, E. (2018). Quantifying the impact of pesticides on learning and memory in bees. *Journal of Applied Ecology*, *55*(6), 2812–2821. https://doi.org/10.1111/1365-2664.13193

Siviter, H., Scott, A., Pasquier, G., Pull, C. D., Brown, M. J. F., & Leadbeater, E. (2019). No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. *PeerJ*, *7*, e7208. https://doi.org/10.7717/peerj.7208

Smith, B. H., & Burden, C. M. (2014). A Proboscis Extension Response Protocol for Investigating Behavioral Plasticity in Insects: Application to Basic, Biomedical, and Agricultural Research. *JoVE (Journal of Visualized Experiments)*, *91*, e51057. https://doi.org/10.3791/51057

Sol Balbuena, M., Tison, L., Hahn, M.-L., Greggers, U., Menzel, R., & Farina, W. M. (2015). Effects of sublethal doses of glyphosate on honeybee navigation. *Journal of Experimental Biology*, *218*(17), 2799–2805. https://doi.org/10.1242/jeb.117291

Stone, J. C., Abramson, C. I., & Price, J. M. (1997). Task-Dependent Effects of Dicofol (Kelthane) on Learning in the Honey Bee (*Apis mellifera*). *Bulletin of Environmental Contamination and Toxicology*, *58*(2), 177–183. https://doi.org/10.1007/s001289900317

Takeda, K. (1961). Classical conditioned response in the honey bee. *Journal of Insect Physiology*, *6*(3), 168–179. https://doi.org/10.1016/0022-1910(61)90060-9

Taylor, K. S., Waller, G. D., & Crowder, L. A. (1987). IMPAIRMENT OF A CLASSICAL CONDITIONED RESPONSE OF THE HONEY BEE (*APIS MELLIFERA* L.) BY SUBLETHAL DOSES OF SYNTHETIC PYRETHROID INSECTICIDES. *Apidologie*, *18*(3), 243–252. https://doi.org/10.1051/apido:19870304

Thany, S. H., Bourdin, C. M., Graton, J., Laurent, A. D., Mathé-Allainmat, M., Lebreton, J., & Le Questel, J.-Y. (2015). Similar Comparative Low and High Doses of Deltamethrin and Acetamiprid Differently Impair the Retrieval of the Proboscis Extension Reflex in the Forager Honey Bee (*Apis mellifera*). *Insects*, *6*(4), 805–814. https://doi.org/10.3390/insects6040805

Tison, L., Duer, A., Púčiková, V., Greggers, U., & Menzel, R. (2020). Detrimental effects of clothianidin on foraging and dance communication in honey bees. *PLOS ONE*, *15*(10), e0241134. https://doi.org/10.1371/journal.pone.0241134

Tison, L., Holtz, S., Adeoye, A., Kalkan, Ö., Irmisch, N. S., Lehmann, N., & Menzel, R. (2017). Effects of sublethal doses of thiacloprid and its formulation Calypso® on the learning and memory performance of honey bees. *Journal of Experimental Biology*, *220*(20), 3695–3705. https://doi.org/10.1242/jeb.154518

Tison, L., Rößner, A., Gerschewski, S., & Menzel, R. (2019). The neonicotinoid clothianidin impairs memory processing in honey bees. *Ecotoxicology and Environmental Safety*, *180*, 139–145. https://doi.org/10.1016/j.ecoenv.2019.05.007

Traynor, K. S., Pettis, J. S., Tarpy, D. R., Mullin, C. A., Frazier, J. L., Frazier, M., & vanEngelsdorp, D. (2016). In-hive Pesticide Exposome: Assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. *Scientific Reports*, *6*(1), 1–16. https://doi.org/10.1038/srep33207

Traynor, K. S., Tosi, S., Rennich, K., Steinhauer, N., Forsgren, E., Rose, R., Kunkel, G., Madella, S., Lopez, D., Eversole, H., Fahey, R., Pettis, J., Evans, J. D., & vanEngelsdorp, D. (2021). Pesticides in honey bee colonies: Establishing a baseline for real world exposure over seven years in the USA. *Environmental Pollution*, *279*, 116566. https://doi.org/10.1016/j.envpol.2021.116566

Urlacher, E., Monchanin, C., Rivière, C., Richard, F.-J., Lombardi, C., Michelsen-Heath, S., Hageman, K. J., & Mercer, A. R. (2016). Measurements of Chlorpyrifos Levels in Forager Bees and Comparison with Levels that Disrupt Honey Bee Odor-Mediated Learning Under Laboratory Conditions. *Journal of Chemical Ecology*, *42*(2), 127–138. https://doi.org/10.1007/s10886-016-0672-4

Van Dame, R., Meled, M., Colin, M.-E., & Belzunces, L. P. (1995). Alteration of the homing-flight in the honey bee *Apis mellifera* L. Exposed to sublethal dose of deltamethrin. *Environmental Toxicology and Chemistry*, *14*(5), 855–860. https://doi.org/10.1002/etc.5620140517

Weick, J., & Thorn, R. S. (2002). Effects of Acute Sublethal Exposure to Coumaphos or Diazinon on Acquisition and Discrimination of Odor Stimuli in the Honey Bee (Hymenoptera: Apidae). *Journal of Economic Entomology*, *95*(2), 227–236. https://doi.org/10.1603/0022-0493-95.2.227

Williamson, S. M., Baker, D. D., & Wright, G. A. (2013). Acute exposure to a sublethal dose of imidacloprid and coumaphos enhances olfactory learning and memory in the honeybee *Apis mellifera*. *Invertebrate Neuroscience*, *13*(1), 63–70. https://doi.org/10.1007/s10158-012-0144-7

Williamson, S. M., & Wright, G. A. (2013). Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. *Journal of Experimental Biology*, *216*(10), 1799–1807. https://doi.org/10.1242/jeb.083931

Wright, G. A., Softley, S., & Earnshaw, H. (2015). Low doses of neonicotinoid pesticides in food rewards impair short-term olfactory memory in foraging-age honeybees. *Scientific Reports*, *5*(1), 1–7. https://doi.org/10.1038/srep15322

Yang, E. C., Chuang, Y. C., Chen, Y. L., & Chang, L. H. (2008). Abnormal Foraging Behavior Induced by Sublethal Dosage of Imidacloprid in the Honey Bee (Hymenoptera: Apidae). *Journal of Economic Entomology*, *101*(6), 1743–1748. https://doi.org/10.1603/0022-0493-101.6.1743

Yang, E.-C., Chang, H.-C., Wu, W.-Y., & Chen, Y.-W. (2012). Impaired Olfactory Associative Behavior of Honeybee Workers Due to Contamination of Imidacloprid in the Larval Stage. *PLOS ONE*, *7*(11), e49472. https://doi.org/10.1371/journal.pone.0049472

Zaluski, R., Justulin, L. A., & Orsi, R. de O. (2017). Field-relevant doses of the systemic insecticide fipronil and fungicide pyraclostrobin impair mandibular and hypopharyngeal glands in nurse honeybees (*Apis mellifera*). *Scientific Reports*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-15581-5

Zhang, E., & Nieh, J. C. (2015). The neonicotinoid imidacloprid impairs honey bee aversive learning of simulated predation. *Journal of Experimental Biology*, *218*(20), 3199–3205. https://doi.org/10.1242/jeb.127472

CHAPTER 2

A COMMON FUNGICIDE, PRISTINE®, IMPAIRS OLFACTORY ASSOCIATIVE LEARNING PERFORMANCE IN HONEY BEES (*APIS MELLIFERA*)

ABSTRACT

Although fungicides were previously considered to be safe for important agricultural pollinators such as honey bees, recent evidence has shown that they can cause a number of behavioral and physiological sublethal effects. Here, I focus on the fungicide Pristine® (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin), which is sprayed during the blooming period on a variety of crops and is known to affect honey bee mitochondria at field-relevant levels. To date, no study has tested the effects of a field-relevant concentration of a fungicide on associative learning ability in honey bees. I tested whether chronic, colony-level exposure at field-relevant and higher concentrations of Pristine® impairs performance on the proboscis extension reflex (PER) paradigm, an associative learning task. Learning performance was reduced at higher field-relevant concentrations of Pristine®. The reductions in learning performance could not be explained by effects on hunger or motivation, as sucrose responsiveness was not affected by Pristine® exposure. To determine whether Pristine®'s negative effects on learning performance were mediated at a specific life stage, I conducted a cross-fostering experiment that exposed bees to the fungicide either only as larvae, only as adults, or during both stages. I found that exposure across the entire life was necessary to significantly reduce learning performance, although non-significant reductions occurred when bees were exposed during just one stage. My study provides strong evidence that Pristine® has significant sublethal effects on learning performance. As associative

learning is a necessary ability for foraging, my results raise concerns that Pristine® could impair foraging abilities and substantially weaken colony health.

INTRODUCTION

Insect pollinator populations are declining globally (Sánchez-Bayo & Wyckhuys, 2019), threatening long-term food security. Thirty-five percent of global food production comes from pollinator-dependent crops (Klein et al., 2007), and animal pollination is valued at $14.2–23.8 billion in the United States (Chopra et al., 2015). There has been particular concern regarding the supply of domesticated honey bees, which is growing globally, but more slowly than needed for pollination (Aizen & Harder, 2009). Honey bee population declines in the United States and Europe have caused concern in recent years (Gray et al., 2020; Kulhanek et al., 2017). Pathogens and parasites (Genersch, 2010), poor nutrition (Naug, 2009), and agrochemical exposure (Mullin et al., 2010) have all been implicated as direct contributors to colony losses. It is likely that interacting stressors are to blame (Goulson et al., 2015; Potts et al., 2010). However, agrochemicals in particular have caused concern in the scientific community, as honey bees may be more sensitive than other insects due to a relative lack of detoxification enzymes (Claudianos et al., 2006).

The term “sublethal effects” describes harmful physiological and behavioral effects of agrochemicals that occur in individuals that have survived exposure (Desneux et al., 2006). These effects can weaken honey bee health and contribute to colony loss over time. Sublethal effects shown to be induced by agrochemicals include altered development, reduced fecundity, altered hygienic and foraging behaviors (Wu-Smart & Spivak, 2016), reduced immune function (Di Prisco et al., 2013), lowered fecundity (Wu-

Smart & Spivak, 2016), impaired mobility (Lambin et al., 2001), impaired navigation ability (Henry et al., 2012), and impaired communication (Eiri & Nieh, 2012).

Insecticides in particular can impair bees' performance in olfactory associative learning tasks, disrupting their ability or motivation to learn to associate a novel odor with a food reward (see Siviter et al. (2018) for a meta-analysis). Some insecticides can also alter bees' motivation or ability to extend their proboscis in response to sucrose (Eiri & Nieh, 2012), a main phagostimulatory component of floral nectar (Wykes, 1952). Most insecticides target various components of the insect nervous system, providing a potential mechanism for the effects on learning behavior and sucrose responsiveness (Belzunces et al., 2012). For example, the effects of neonicotinoids on learning performance have been particularly well-studied (e.g. Aliouane et al., 2009; Decourtye et al., 2004, 2005).

Bees are also exposed to other agrochemicals while foraging, including fungicides (Mullin et al., 2010). Short-term toxicity tests for fungicides have suggested that they are safe for bees (Ladurner et al., 2005). However, standard toxicity tests do not take sublethal effects into account (Desneux et al., 2006). Sublethal exposure to fungicides may cause significant stress to honey bees, as residues found in hives have been correlated with colony decline (Simon-Delso et al., 2014). Here, I focus on the fungicide Pristine®, which is licensed for use on a variety of crops pollinated by honey bees including bulb vegetables, berries, pome and stone fruits, and tree nuts. Of significant importance is that Pristine® is registered for use on almonds during the bloom period (BASF, 2019). More than 70 percent of all commercial honey bee hives in the US are brought to California for almond pollination during bloom each year (Lee et al., 2019). Residues of the active ingredients of Pristine®, boscalid and pyraclostrobin, have been found in bee hives in the US and Europe (Mullin et al., 2010; Simon-Delso et al., 2014). Boscalid and pyraclostrobin inhibit respiration in fungal cells (Earley et al., 2012), and Pristine® also directly inhibits honey bee mitochondrial function *in vitro* (Campbell et al., 2016). When consumed with pollen, Pristine® interferes with protein digestion and increases virus titers (DeGrandi-Hoffman et al., 2015). When consumed in pollen at field-realistic levels, it reduces thorax mass (Glass et al., 2021), colony population size and worker lifespan, and causes earlier foraging that is more focused on pollen-collection (Fisher et al., 2021a). The latter finding suggests that the toxic effects may be at least partially behaviorally-mediated.

Here, I evaluate how chronic exposure of honey bees to Pristine® affects learning performance and sucrose responsiveness. The proboscis extension reflex (PER) paradigm, which measures how well bees learn to associate a neutral odor with a sucrose reward (Bitterman et al., 1983; Smith & Burden, 2014), is well-suited to measure the learning abilities in bees exposed to agrochemicals (Pham-Delègue et al., 2002). Some non-insecticide agrochemicals, such as spray adjuvants (Ciarlo et al., 2012), herbicides (Farina et al., 2019), and one other fungicide (prochloraz) (Decourtye et al., 2005), as well as some heavy metals (Burden et al., 2016, 2019), have been shown to affect associative learning and sucrose responsiveness. I tested olfactory associative learning and sucrose responsiveness in individual bees from colonies that chronically consumed Pristine®-contaminated pollen, mimicking natural exposure in the field. I also investigated whether the behavioral effects of Pristine® on honey bees were mediated at the larval or adult stage, or both.

METHODS

Honey bee colonies and fungicide exposure. Initiation and fungicide treatment of the experimental colonies is described in detail in Fisher et al. (2021a); the basic protocols are presented here. In April 2018, forty 1.36 kg Italian honey bee (*Apis mellifera linguistica*) packages (Pendell Apiaries, Inc., Stonyford, CA) were used to initiate colonies in Apimaye insulated hives (Kaftan LLC, Tempe, AZ) at the Arizona State University Bee Lab in Mesa, AZ (33.293173, −111.684520). To prevent exposure to stored agrochemicals, each colony was initially stocked with five wooden frames with plastic cell foundation, which the workers used to construct new comb. Colonies were supplied with 30 percent sucrose solution *ad libitum* for the first three weeks after initiation to help them build combs. Pollen traps were placed internally over the hive entrance to restrict the amount of pollen that workers could bring inside. Pollen patties (50 g) containing a mixture of pollen (30.6%), sucrose (30.6%), fondant sugar (30.6%), and water (8.2%) were placed in a Petri dish inside each hive. A week before fungicide treatments began, the hives were equalized so they each had five drawn combs and approximately equal adult populations. As colonies continued to grow, additional frames with bare, plastic cell foundation were provided to minimize possible exposure to chemicals in old wax.

The fungicide treatments began in May 2018, one month after colony initiation. Pristine® (BASF Corporation, Research Triangle Park, NC) was dissolved in deionized water and mixed into pollen patties at the following concentrations: 0.23 ppm, 2.3 ppm, 23, ppm, and 230 ppm (this replaced the 8.2% water described above in pollen patty preparation). These concentrations were meant to bracket those measured in corbicular pollen collected from bees foraging in Pristine®-treated almond orchards (which ranged from 3.13 ppm to 24 ppm, as measured by Fisher et al., 2021a). The quantity of the pollen patty consumed was measured at least weekly (Fisher et al., 2021a). The per larva and per adult doses of Pristine®, boscalid, and pyraclostrobin were calculated by Fisher et al. (2021a) and are shown in Table 2.1. A fifth group, the control, received pollen patties with only deionized water. Each of the five treatment groups contained eight hives. Hives were continually monitored and by very briefly looking inside the hive every other day to check the amount of pollen left in the patty. Pollen patties were replaced as soon as they were consumed, or at least weekly to maintain freshness.

General olfactory associative learning. Preparation and conditioning methods were based on those described in Smith & Burden (2014). I sampled from 20 hives (out of the 40 total involved in the larger experiment). Among each treatment group, each hive was assigned a number from one through eight, and a pseudo-random number generator (random.org) was used to randomly select four numbers within that range, which corresponded to the hives used in the experiment. Of the 20 hives selected, three showed poor brood production and foraging, and because of this, they were not sampled from during the learning experiment. Thus, a total of 17 hives were used (3–4 per treatment group). Ten bees were trained at a time; they were collected at the hive entrance as they returned from foraging trips and immediately brought into the lab and anesthetized on ice. They were then harnessed in plastic drinking straws (3 cm tall, 0.9 cm inner diameter) and held in place by strips of duct tape (0.2 × 6 cm) so that only their antennae and mouthparts were moveable. Each individual was then fed 3–5 μL of 1.0 M sucrose one time and allowed to rest for 45 min at room temperature. This round of feeding served to keep them from depleting their energy reserves during the acclimation period (Smith & Burden, 2014). After the resting period, the bees' antennae were stimulated with a droplet of 1.5 M sucrose. Bees that did not extend their proboscis in response to antennal stimulation were considered not sufficiently motivated to learn and were removed from the experiment prior to its start.

I used a discrimination conditioning procedure to evaluate associative learning and reduce the possibility that changes in behavior were due to non-associative mechanisms (Benatar et al., 1995). Conditioned Stimuli (CS) were always odors. Thus, I included both a CS^+, which was followed by reinforcement with a sucrose droplet, and a CS^-, which was not. The chemicals 1-hexanol (Sigma-Aldrich, St. Louis, MO) and 2-octanone (Sigma-Aldrich, St. Louis, MO) were counterbalanced as the CS^+ and CS^- odors. Prior to training, strips of filter paper (Sigma-Aldrich, St. Louis, MO) containing 0.7 μL of the target odor were placed into glass cartridges (1 cc tuberculin syringe barrels, BD Medical, Franklin Lakes, NJ). Odor cartridges were changed after every fifth trial, according to standard protocols (Smith & Burden, 2014).

The plexiglass conditioning arena consisted of a circular stand on which a harness could be placed, a piece of modeling clay on which the odor tube could be mounted in front of the bee, and an exhaust system (hooked up to the laboratory vacuum system with dryer tubing) to prevent odors from lingering. The odor delivery system consisted of an air tube that could be hooked up to an odor cartridge and automated via a programmable logic controller (Automation-Direct, Cumming, GA) to deliver the odor at the correct times. While not being actively trained, bees were kept in a staging area approximately 30 cm from the training arena.

During acquisition trials with the CS^+ odor, a bee was placed into the arena and allowed to sit for 25 s. After that, an air pulse (flowing at 7 mL/s) was directed through the odor cartridge and toward the bee for 4 s. During the last second of odor delivery, the bee was manually fed 0.4 µL of 1.5 M sucrose using a 0.2 mL Gilmont syringe (Cole-Parmer, Vernon Hills, IL). If the bee extended her proboscis after odor delivery but before sucrose presentation, she had learned successfully (denoted by a '1' in my scoring system). If a bee extended her proboscis after sucrose presentation, she had responded to sucrose but not to the odor (denoted by a '0' in my scoring system). Bees that did not extend their proboscis to either odor or sucrose were scored with an 'NA'.

After sucrose presentation, the bee was left in the arena for another 30 s, allowing her to form initial memories before being moved back to the staging area. This 1-min process was repeated for each of the other nine bees before starting the second trial with the first bee, allowing for a 10-min inter-trial interval.

During unrewarded trials, the process was the same as above, except the bee was not fed sucrose. The Gilmont syringe was still held close to the bees' heads during the time when sucrose would normally be fed in order to reduce changes in stimulation as much as possible.

	Pristine	**Boscalid**	**Pyraclostrobin**
Pollen patty, ppm	**0.23**	**0.06**	**0.03**
Per larva dose, ng	0.086	0.022	0.011
Per adult dose, ng	6.9	1.73	0.88
Pollen patty, ppm	**2.3**	**0.6**	**0.3**
Per larva dose, ng	1.0	0.25	0.13
Per adult dose, ng	79.7	20.1	10.2
Pollen patty, ppm	**23**	**6**	**3**
Per larva dose, ng	8.3	2.09	1.06
Per adult dose, ng	663	167.1	84.9
Pollen patty, ppm	**230**	**60**	**30**
Per larva dose, ng	89.9	22.7	11.5
Per adult dose, ng	7,194	1813	921

Table 2.1. Concentrations of Pristine®, boscalid, and pyraclostrobin in the pollen patties for each treatment group, along with the calculated per larva and per adult dose (Fisher et al., 2021).

Sucrose responsiveness. Changes in learning performance due to fungicide treatment could be caused by changes in taste responsiveness rather than impaired associative learning *per se*. To test this, bees were sampled from the same 17 hives that were used in the first experiment. Bee collection and harnessing protocols were the same as those described above. After the 45-min resting period, bees were exposed via antennal stimulation to 1–2 µL of increasing concentrations of sucrose: 0.1, 0.3, 1, 3, 10, and 30 percent solutions (w/v in deionized water). They were not allowed to feed. Deionized water was presented after each sucrose concentration, with 8-min intervals between each trial. For each sucrose or water presentation, I recorded whether the bee extended its proboscis (denoted by a '1') or did not (denoted by a '0'). For data analysis, each bee was given a "sensitivity score" for each sucrose concentration: response to sucrose minus response to the following water presentation. This gave me a range of values of either '0' or '1' (A '-1' value was also theoretically possible; however, this was not actually observed). A '0' value indicated that a bee had responded to both the sucrose and water, or neither the sucrose nor the water. A '1' value indicated that an individual had responded to the particular concentration of sucrose but not the water.

Cross-fostering olfactory associative learning. For this experiment, bees were exposed to Pristine® either only as larvae, only as adults, during both developmental stages, or not at all, using a cross-fostering design (Fisher et al., 2021b). Because adults consume much more pollen than larvae and therefore receive a much greater dose (Table 1), I hypothesized that effects of Pristine® on learning would stem from exposure during the adult stage. To rigorously test this hypothesis, I used a supra-field concentration of Pristine®. Randomly-chosen capped brood frames from three control (0 ppm Pristine®) hives and three hives treated with 230 ppm Pristine® were brought into the lab and placed in a wire mesh frame cage (L x W x H: 53.3 × 5.1 × 27.9 cm) in an incubator (34 °C, 90% relative humidity) until ~600 adults had emerged from each frame. The adults were marked on the mesonotum with a paint color corresponding to their hive of origin.

The bees from the control and 230 ppm Pristine® colonies were each divided into four subsets of ~450 individuals. Two control colonies each received ~450 bees from control colonies and ~450 bees that had been reared as larvae in the 230 ppm Pristine® treatment group. Two colonies from the 230 ppm treatment group each received ~450 bees that had been reared as larvae in control colonies and ~450 bees that had been reared as larvae in colonies in the 230 ppm Pristine® treatment group. This resulted in four treatment groups that are named according to their larval and adult Pristine® exposure: larval control/adult control, larval control/adult Pristine®, larval Pristine®/adult Pristine®, and larval Pristine®/adult control. The learning abilities of bees from these four treatment groups were tested once they began foraging outside the hive, using the same protocol as described above.

Statistical analyses. All data were analyzed in R version 3.6.2 (R Core Team, 2019). Learning data were analyzed using generalized linear mixed effects models and the lme4 package (Bates et al., 2015). Trial, hive, and treatment group were fixed effects and individual was a random effect. For the five-concentration learning experiment, another fixed effect was added—season—in order to determine whether there were any differences between the bees tested in December and March. Season and hive did not have significant effects, so these variables were pooled in the model. For post-hoc pairwise comparisons, the estimated marginal means were calculated using the emmeans package (Lenth, 2019). P-values were adjusted using the Tukey method for multiple comparisons.

For the sucrose response data, I considered that a bee had responded to a particular sucrose concentration if she extended her proboscis in response to that concentration, but not to the water presented immediately afterward. Thus, I subtracted water responses from the corresponding sucrose responses and used this “sensitivity score” to run a generalized linear mixed effects model. Sucrose concentration, treatment group, and season were fixed effects, and individual was a random effect. The season effect revealed a significant difference between the January–February and March–May experiments, so two models were constructed to analyze each experiment separately.

RESULTS

General olfactory associative learning. Colony-level Pristine® consumption affected performance (proportion of bees that successfully learned the odor-reward association across eight acquisition trials) on my PER associative learning assay (χ^2=7.9, p=0.0050). By the eighth acquisition trial, control bees performed, on average, 20% better than bees consuming pollen containing the 23 ppm and 230 ppm Pristine® concentrations. (Figure 2.1A). Hive and season did not significantly affect results. Bees rarely responded to the CS^- odor, and there were no trends across trial or treatment group. Pristine®-exposed bees also responded to the odor less often than control bees throughout the unrewarded test trials (χ^2=17, p<0.001, Figure 2.1B). Post-hoc pairwise comparisons showed that the 23 ppm and 230 ppm treatment groups performed significantly worse than the control treatments during the test phase.

Sucrose responsiveness. Here, I measured the proportion of bees that responded to ascending sucrose concentrations, corrected for their responses to water. An initial model revealed significant differences (χ^2=5.9, p=0.015) between results obtained in January/February versus May/June, corroborating earlier results that show seasonal variation in forager sucrose responsiveness (Scheiner et al., 2003). Because of this, the data from the two time periods were analyzed separately. Pristine® exposure did not have an effect in either January/February (χ^2=1.7, p=0.19, Figure 2.2A) or May/June (χ^2=0.62, p=0.43, Figure 2.2B). Thus, regardless of season, Pristine® consumption did not affect sucrose responsiveness, suggesting that the differences in associative learning ability between treatment groups were not caused by effects on sucrose responsiveness.

Cross-fostering olfactory associative learning. Performance (proportion of bees that successfully learned the odor-reward association) in the PER olfactory learning assay differed significantly among treatment groups during both the acquisition (χ^2=8.95, p=0.030, Figure 2.3A) and test phases (χ^2=10.2, p=0.017, Figure 2.3B). Hive did not significantly affect the results. Post-hoc pairwise comparisons revealed that the control group (larval control/adult control) performed better than the bees exposed to Pristine® at both larval and adult stages (larval 230 ppm/adult 230 ppm). Bees exposed during just the larval or adult stage performed intermediate to the two constant treatment groups, but these groups did not differ significantly from either treatment group.

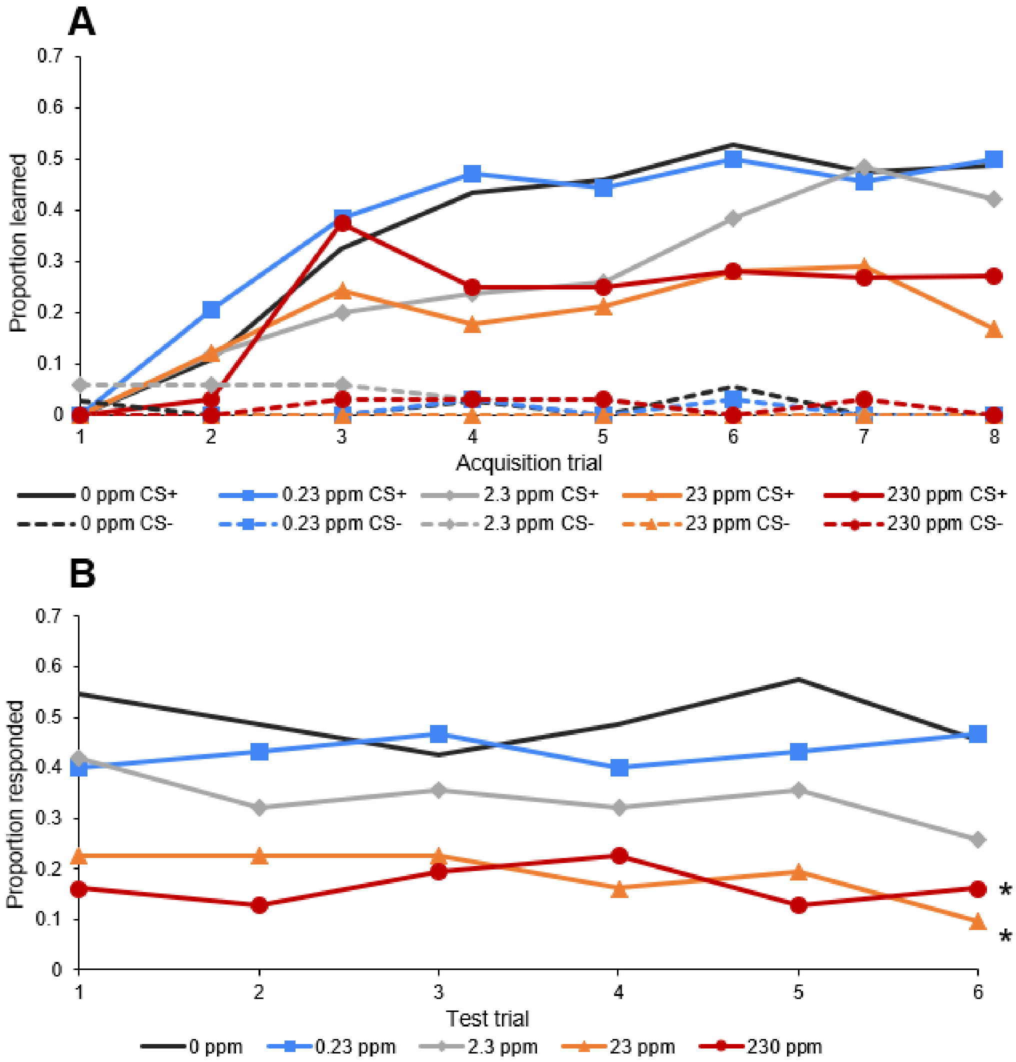
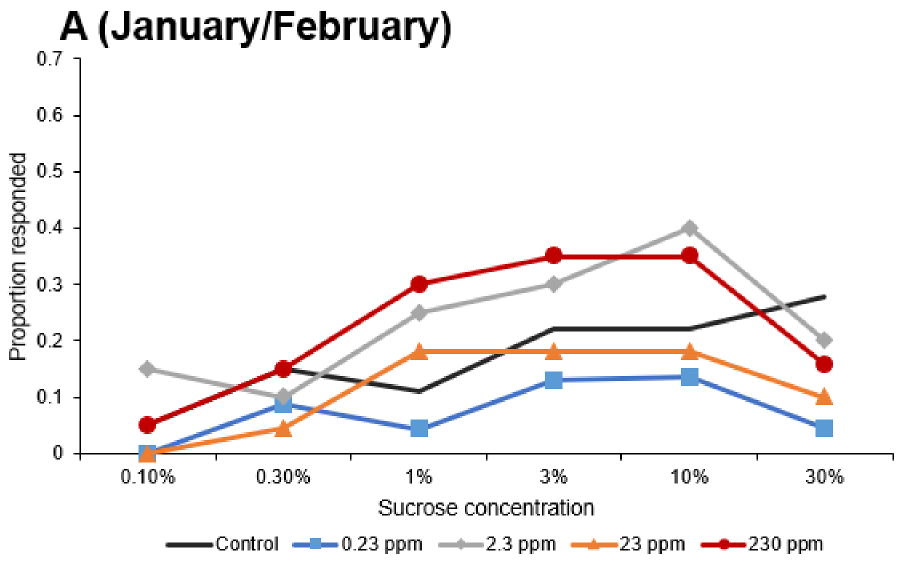


Figure 2.1. Proportion of bees chronically exposed to Pristine$^{®}$ fungicide showing learning in a PER olfactory learning assay, during (***A***) the acquisition phase and (***B***) the test phase. N=33-37 individuals per treatment group. In (***A***), solid lines indicate responses to the CS^{+}, while dashed lines represent responses to the CS^{-}. Asterisks indicate that a treatment group is significantly different from the control.



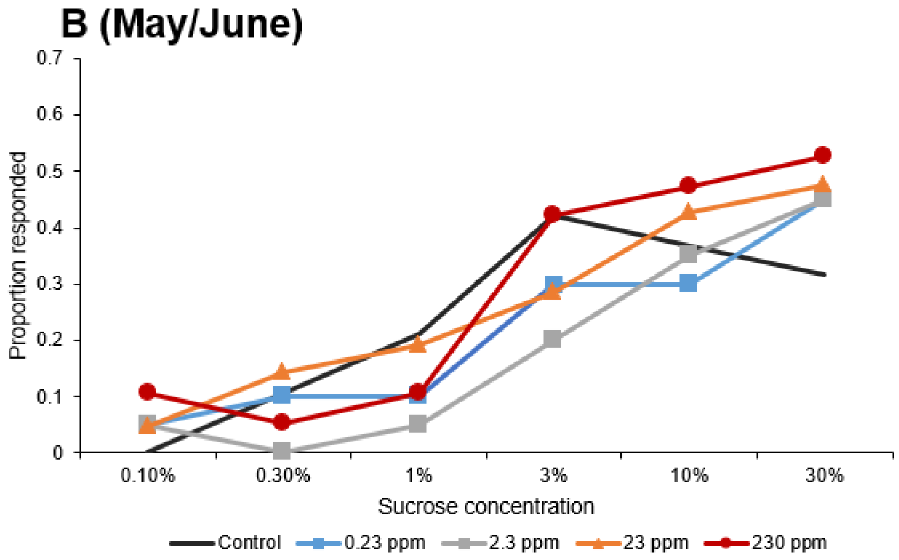


Figure 2.2. Sensitivity to different sucrose concentrations for bees chronically exposed to five concentrations of Pristine® fungicide. Bees were tested in either January-February (***A***) or May-June (***B***). Graphs represent sensitivity score—proportion of bees responding to sucrose minus the proportion responding to water presented immediately afterward. For both graphs, N=19-23 individuals per treatment group.

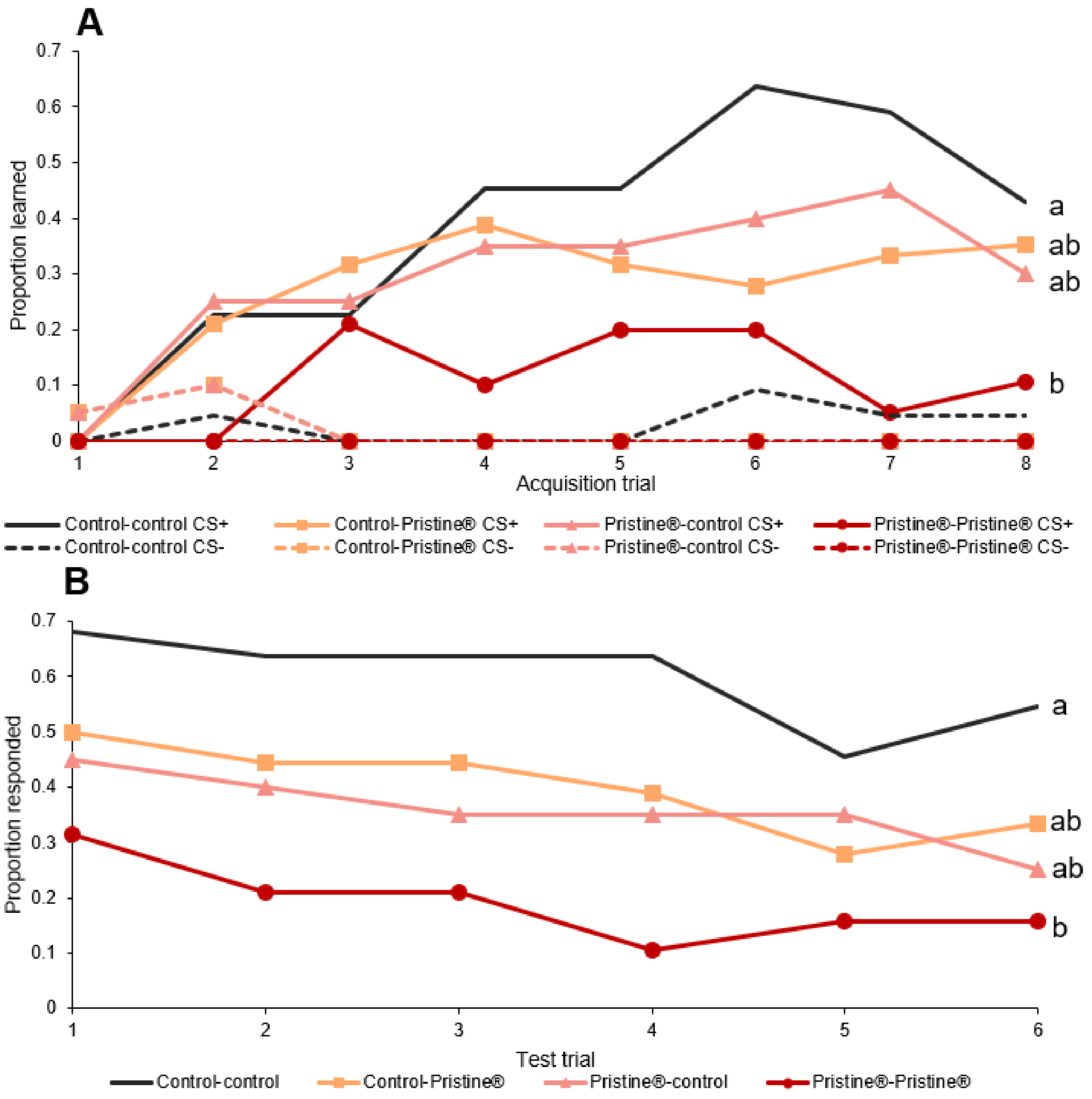


Figure 2.3. Proportion of bees exposed to Pristine® fungicide either as larvae, adults, neither, or both showing learned responses in a PER olfactory learning assay, during (***A***) the acquisition phase and (***B***) the test phase. In the legend, each treatment group is indicated in the following manner: larval treatment-adult treatment. N=19-21 individuals per treatment group. In (***A***), solid lines indicate responses to the CS^+, while dashed lines represent responses to the CS^-. Lowercase letters on the right indicate statistically significant differences between treatment groups.

DISCUSSION

The issue of sublethal effects of agrochemicals, including fungicides, on pollinators is very concerning, with recent studies demonstrating that field application levels previously thought of as non-toxic may have significant longer-term impact on honey bee health (Fisher et al., 2021a). My study demonstrates an important impact of field-relevant doses of the fungicide Pristine® on olfactory associative learning. I found that consumption of pollen containing 23 and 230 ppm of the fungicide in field colonies reduced olfactory associative learning performance with no concurrent effect on sucrose responsiveness. The impaired associative learning caused by Pristine® consumption was thus not due to a simple suppression of the ability to taste sucrose or reduced motivation to feed.

Reduced learning ability, as demonstrated here, can negatively impact colony functioning through impairing foraging or navigation ability in exposed bees. It is well-documented that olfactory associative learning is necessary to remember the locations of food sources (Gerber et al., 1996; Kirchner & Grasser, 1998; von Frisch, 1993). Some agrochemicals that can impair learning can also negatively impact navigation or foraging ability (Fischer et al., 2014; Henry et al., 2012; Schneider et al., 2012; Sol Balbuena et al., 2015), further suggesting that impaired associative learning performance could damage proper colony functioning by impairing the bees' ability to find and communicate the locations of food resources.

Although the effects of neuroactive insecticides on bee associative learning performance are well-documented, only one other study showed impaired learning as the result of exposure to a fungicide, prochloraz (Decourtye et al., 2005). Prochloraz acts by a completely different mechanism than boscalid and pyraclostrobin, as it is an inhibitor of cytochrome P450 enzymes and is considered an endocrine disruptor (Vinggaard et al., 2006).

A variety of other agrochemicals beyond fungicides impair PER learning in honey bees (Siviter et al., 2018). Many of these are insecticides that are designed to be neuroactive, specifically to affect cholinergic transmission, which provides a logical underlying mechanism for their effects on learning, as acetylcholine is the main neurotransmitter involved in learning and memory in the honey bee (Lozano et al., 2001). Ingestion of a few other agrochemicals that are not designed to be neuroactive has been shown to affect learning, including organosilicone agricultural spray adjuvants (Ciarlo et al., 2012), the fungicide prochloraz (Decourtye et al., 2005), and the herbicide glyphosate (Farina et al., 2019). The heavy metal selenium also negatively affects learning (Burden et al., 2016). The mechanisms underlying these effects remain mostly unknown. Regardless, these studies reveal that a wide variety of chemicals, including those that are not meant to target the insect nervous system, can cause negative sublethal effects on learning in honey bees.

A key question is whether the observed toxic and behavioral effects of Pristine® on honey bees were mediated at the larval or adult stage (or both). Young adults eat a substantial amount of pollen, while larvae consume only a few mg late in development (Babendreier et al., 2004; Crailsheim et al., 1992), suggesting that adult exposure is the most critical, at least for Pristine® in pollen. Young adults feed larvae by producing glandular secretions that could contain consumed fungicides. However, concentrations of boscalid and pyraclostrobin were undetectable in royal jelly produced by nurse bees fed

Pristine®, suggesting that the active ingredients in Pristine® are not passed on to larvae in the brood food (DeGrandi-Hoffman et al., 2013). Also, Fisher et al. (2021a) reported no effects of consumption of Pristine® on colony egg and brood levels. Therefore, I hypothesized that Pristine® exposure exerts its negative effects on honey bee colonies only at the adult stage. To rigorously test this hypothesis, I reared bees in colonies provided Pristine® only in the larval, only in the adult, or in both stages, testing effects on learning capabilities of adult foraging-age workers. I used a supra-field concentration (230 ppm) of Pristine®, as peak exposures are likely higher than average concentrations, and my goal was to exclude the possibility that larvae are negatively impacted by colonial Pristine® consumption in pollen. I found that consumption of pollen containing 230 ppm Pristine® during both the larval and adult life stages significantly reduced learning ability, while non-significant reductions occurred when bees were exposed during only one life stage. These results suggest that the sublethal behavioral effects of Pristine® are mediated at both the larval and adult life stages. At present it is unclear whether such effects are due to the consumption of Pristine® by the larvae, or to the effects of Pristine® consumption on the feeding and care provided to the larvae by the adults.

The findings of this study lead naturally to the question of mechanisms for impaired learning. Other agrochemicals that can impair associative learning, such as imidacloprid (Eiri & Nieh, 2012; Lambin et al., 2001), thiamethoxam (Aliouane et al., 2009; Démares et al., 2016), flupyradifurone (Hesselbach & Scheiner, 2018), and fluvalinate (Frost et al., 2013) have been shown simultaneously to affect sucrose responsiveness in honey bees. Sucrose is the unconditioned stimulus in the PER paradigm, and individual differences in sucrose responsiveness correlate with performances during the assay (Scheiner et al., 2004). However, as Pristine® does not appear to affect sucrose responsiveness, there is likely some other mechanism behind its effects on learning.

Pristine® may impair learning indirectly through effects on nutrient absorption via the gut. Ultimately, impaired nutrient absorption could impact learning either by interfering with post-ingestive feedback mechanisms necessary for learning or with proper brain development. Pristine® may interfere with nutrient transport in honey bees, as it impairs protein digestion (DeGrandi-Hoffman et al., 2015), and one of its active ingredients (pyraclostrobin) damages the midgut epithelium (Tadei et al., 2020). If bees are stimulated with but not fed sugar during PER training, their memory consolidation is worse overall than when allowed to feed during training (Wright et al., 2007). Furthermore, feeding with a tasteless but nutritional sugar produces robust learning (Mustard et al., 2018). These results suggest that the rise in hemolymph glucose levels following sugar ingestion may provide a post-ingestive signal and help the bee form a robust memory of the association (Simcock et al., 2018). The exact physiological mechanisms underlying this post-ingestive feedback remain unknown; however, it is possible that the brain is able to sense either changes in hemolymph glucose levels or a rise in intracellular ATP levels (Simcock et al., 2018). If Pristine® interferes with nutrient absorption, it may block this post-ingestive feedback mechanism from promoting memory consolidation. Pristine® could also affect nutrient absorption during larval and pupal development, possibly impairing proper brain development. It has been well-established that malnutrition during development can alter cognitive performance later in life in other animals, especially in rodents (Halas et al., 1979; Morgane et al., 1993;

Tonkiss & Galler, 1990). Relatively few studies have examined this phenomenon in honey bees, although two studies failed to find a link between nutrient deprivation at either the individual (Steijven et al., 2017) or colony (Mattila & Smith, 2008) level and impaired learning. Regardless, it is possible that Pristine® affects nutrient uptake and development in larvae, as both larval and adult exposure was necessary to cause significantly reduced learning in adults.

Alternatively, Pristine® could affect learning by directly poisoning mitochondria in the brain (Campbell et al., 2016). Although the degree to which Pristine® can pass through the gut and into the hemolymph remains unclear, it is plausible that some amount could reach the brain. Previous studies have used cytochrome oxidase activity as a proxy for cellular respiration in the bee brain; increased activity in the mushroom body calyces correlates with acquisition in the PER learning assay (Déglise et al., 2003). When the neonicotinoid insecticide imidacloprid is fed to bees, they show altered cytochrome oxidase activity in the mushroom body calyces, which correlates with impaired PER learning (Decourtye et al., 2004). Future studies are necessary to determine whether any amount of consumed Pristine® enters the bee brain. It will remain unclear whether Pristine® directly poisons mitochondria in the bee brain or whether its effects on associative learning result from more indirect causes, such as impaired nutrient absorption.

CONCLUSION

My study shows that the fungicide Pristine® may not be entirely safe for pollinators. Along with its effects on learning, a variety of other negative effects have been reported, including disrupted nestmate recognition in solitary bees (Artz & Pitts-

Singer, 2015), reduced queen emergence (DeGrandi-Hoffman et al., 2013), impaired protein digestion and increased virus titers (DeGrandi-Hoffman et al., 2015), precocious foraging, and reduced adult worker population (Fisher et al., 2021a). Future research should seek to determine whether additional behaviors are impaired, such as navigation and foraging ability. Further work is also necessary to determine the mechanisms underlying impaired learning, including whether Pristine® is directly affecting mitochondrial function in the brain or whether it is acting through more indirect mechanisms, such as impaired nutrient absorption. Additionally, due to the multitude of sublethal effects that have been reported across a variety of agrochemical classes, standard toxicity tests may need to be redesigned by stakeholders such as the United States EPA and USDA to take these into account (Barascou et al., 2021; Fisher, 2021). These results suggest that this commonly-used agrochemical may not be safe for honey bees, an important agricultural pollinator.

REFERENCES

Aizen, M. A., & Harder, L. D. (2009). The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. *Current Biology*, *19*(11), 915–918. https://doi.org/10.1016/j.cub.2009.03.071

Aliouane, Y., El Hassani, A. K., Gary, V., Armengaud, C., Lambin, M., & Gauthier, M. (2009). Subchronic exposure of honeybees to sublethal doses of pesticides: Effects on behavior. *Environmental Toxicology and Chemistry*, *28*(1), 113–122. https://doi.org/10.1897/08-110.1

Artz, D. R., & Pitts-Singer, T. L. (2015). Effects of Fungicide and Adjuvant Sprays on Nesting Behavior in Two Managed Solitary Bees, *Osmia lignaria* and *Megachile rotundata*. *PLOS ONE*, *10*(8), e0135688. https://doi.org/10.1371/journal.pone.0135688

Babendreier, D., Kalberer, N., Romeis, J., Fluri, P., & Bigler, F. (2004). Pollen consumption in honey bee larvae: A step forward in the risk assessment of transgenic plants. *Apidologie*, *35*(3), 293–300. https://doi.org/10.1051/apido:2004016

Barascou, L., Brunet, J.-L., Belzunces, L., Decourtye, A., Henry, M., Fourrier, J., Le Conte, Y., & Alaux, C. (2021). Pesticide risk assessment in honeybees: Toward the use of behavioral and reproductive performances as assessment endpoints. *Chemosphere*, *276*, 130134. https://doi.org/10.1016/j.chemosphere.2021.130134

BASF. (2019). *Pristine*®*: Specimen label*. http://www.cdms.net/ldat/ld6DD016.pdf

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Belzunces, L. P., Tchamitchian, S., & Brunet, J.-L. (2012). Neural effects of insecticides in the honey bee. *Apidologie*, *43*(3), 348–370. https://doi.org/10.1007/s13592-012-0134-0

Benatar, S. T., Cobey, S., & Smith, B. H. (1995). Selection on a haploid genotype for discrimination learning performance: Correlation between drone honey bees (*Apis mellifera*) and their worker progeny (Hymenoptera: Apidae). *Journal of Insect Behavior*, *8*(5), 637–652. https://doi.org/10.1007/BF01997235

Bitterman, M. E., Menzel, R., Fietz, A., & Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Psychology*, *97*(2), 107–119. https://doi.org/10.1037/0735-7036.97.2.107

Burden, C. M., Elmore, C., Hladun, K. R., Trumble, J. T., & Smith, B. H. (2016). Acute exposure to selenium disrupts associative conditioning and long-term memory recall in honey bees (*Apis mellifera*). *Ecotoxicology and Environmental Safety*, *127*, 71–79. https://doi.org/10.1016/j.ecoenv.2015.12.034

Burden, C. M., Morgan, M. O., Hladun, K. R., Amdam, G. V., Trumble, J. J., & Smith, B. H. (2019). Acute sublethal exposure to toxic heavy metals alters honey bee (*Apis mellifera*) feeding behavior. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-019-40396-x

Campbell, J. B., Nath, R., Gadau, J., Fox, T., DeGrandi-Hoffman, G., & Harrison, J. F. (2016). The fungicide Pristine® inhibits mitochondrial function *in vitro* but not flight metabolic rates in honey bees. *Journal of Insect Physiology*, *86*, 11–16. https://doi.org/10.1016/j.jinsphys.2015.12.003

Chopra, S. S., Bakshi, B. R., & Khanna, V. (2015). Economic Dependence of U.S. Industrial Sectors on Animal-Mediated Pollination Service. *Environmental Science & Technology*, *49*(24), 14441–14451. https://doi.org/10.1021/acs.est.5b03788

Ciarlo, T. J., Mullin, C. A., Frazier, J. L., & Schmehl, D. R. (2012). Learning Impairment in Honey Bees Caused by Agricultural Spray Adjuvants. *PLOS ONE*, *7*(7), e40848. https://doi.org/10.1371/journal.pone.0040848

Claudianos, C., Ranson, H., Johnson, R. M., Biswas, S., Schuler, M. A., Berenbaum, M. R., Feyereisen, R., & Oakeshott, J. G. (2006). A deficit of detoxification enzymes: Pesticide sensitivity and environmental response in the honeybee. *Insect Molecular Biology*, *15*(5), 615–636. https://doi.org/10.1111/j.1365-2583.2006.00672.x

Crailsheim, K., Schneider, L. H. W., Hrassnigg, N., Bühlmann, G., Brosch, U., Gmeinbauer, R., & Schöffmann, B. (1992). Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): Dependence on individual age and function. *Journal of Insect Physiology*, *38*(6), 409–419. https://doi.org/10.1016/0022-1910(92)90117-V

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M., & Pham-Delègue, M.-H. (2004). Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). *Pesticide Biochemistry and Physiology*, *78*(2), 83–92. https://doi.org/10.1016/j.pestbp.2003.10.001

Decourtye, A., Devillers, J., Genecque, E., Menach, K. L., Budzinski, H., Cluzeau, S., & Pham-Delègue, M. H. (2005). Comparative Sublethal Toxicity of Nine Pesticides on Olfactory Learning Performances of the Honeybee *Apis mellifera*. *Archives of Environmental Contamination and Toxicology*, *48*(2), 242–250. https://doi.org/10.1007/s00244-003-0262-7

Déglise, P., Dacher, M., Dion, E., Gauthier, M., & Armengaud, C. (2003). Regional brain variations of cytochrome oxidase staining during olfactory learning in the honeybee (*Apis mellifera*). *Behavioral Neuroscience*, *117*(3), 540–547. https://doi.org/10.1037/0735-7044.117.3.540

DeGrandi-Hoffman, G., Chen, Y., & Simonds, R. (2013). The Effects of Pesticides on Queen Rearing and Virus Titers in Honey Bees (*Apis mellifera* L.). *Insects*, *4*(1), 71–89. https://doi.org/10.3390/insects4010071

DeGrandi-Hoffman, G., Chen, Y., Watkins Dejong, E., Chambers, M. L., & Hidalgo, G. (2015). Effects of Oral Exposure to Fungicides on Honey Bee Nutrition and Virus Levels. *Journal of Economic Entomology*, *108*(6), 2518–2528. https://doi.org/10.1093/jee/tov251

Démares, F. J., Crous, K. L., Pirk, C. W. W., Nicolson, S. W., & Human, H. (2016). Sucrose Sensitivity of Honey Bees Is Differently Affected by Dietary Protein and a Neonicotinoid Pesticide. *PLOS ONE*, *11*(6), e0156584. https://doi.org/10.1371/journal.pone.0156584

Desneux, N., Decourtye, A., & Delpuech, J.-M. (2006). The Sublethal Effects of Pesticides on Beneficial Arthropods. *Annual Review of Entomology*, *52*(1), 81–106. https://doi.org/10.1146/annurev.ento.52.110405.091440

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G., & Pennacchio, F. (2013). Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences*, *110*(46), 18466–18471. https://doi.org/10.1073/pnas.1314923110

Earley, F., Sauter, H., Rheinheimer, J., Rieck, H., Coqueron, P.-Y., Whittingham, W. G., & Walter, H. (2012). Fungicides Acting on Oxidative Phosphorylation. In Krämer, W., Schirmer, U., Jeschke, P., & Witschel, M. (Eds.), *Modern Crop Protection Compounds* (pp. 559–691). John Wiley & Sons, Ltd. https://doi.org/10.1002/9783527644179.ch15

Eiri, D. M., & Nieh, J. C. (2012). A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. *Journal of Experimental Biology*, *215*(12), 2022–2029. https://doi.org/10.1242/jeb.068718

Farina, W. M., Sol Balbuena, M., Herbert, L. T., Mengoni Goñalons, C., & Vázquez, D. E. (2019). Effects of the Herbicide Glyphosate on Honey Bee Sensory and Cognitive Abilities: Individual Impairments with Implications for the Hive. *Insects*, *10*(10), 354. https://doi.org/10.3390/insects10100354

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B., & Menzel, R. (2014). Neonicotinoids Interfere with Specific Components of Navigation in Honeybees. *PLOS ONE*, *9*(3), e91364. https://doi.org/10.1371/journal.pone.0091364

Fisher, A. (2021). Protect pollinators—Reform pesticide regulations. *Nature*, *595*(7866), 172–172. https://doi.org/10.1038/d41586-021-01818-x

Fisher, A., DeGrandi-Hoffman, G., Smith, B. H., Johnson, M., Kaftanoglu, O., Cogley, T., Fewell, J. H., & Harrison, J. F. (2021a). Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*). *Environmental Pollution*, *269*, 115964. https://doi.org/10.1016/j.envpol.2020.115964

Fisher, A., DeGrandi-Hoffman, G., Smith, B. H., Ozturk, C., Kaftanoglu, O., Fewell, J. H., & Harrison, J. F. (2021b). Field cross-fostering and *in vitro* rearing demonstrate negative effects of both larval and adult exposure to a widely used fungicide in honey bees (*Apis mellifera*). *Ecotoxicology and Environmental Safety*, *217*, 112251. https://doi.org/10.1016/j.ecoenv.2021.112251

Frost, E. H., Shutler, D., & Hillier, N. K. (2013). Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival. *Journal of Experimental Biology*, *216*(15), 2931–2938. https://doi.org/10.1242/jeb.086538

Genersch, E. (2010). Honey bee pathology: Current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*, *87*(1), 87–97. https://doi.org/10.1007/s00253-010-2573-8

Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalksy, O., Wüstenberg, D., & Menzel, R. (1996). Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Animal Behaviour*, *52*(6), 1079–1085. https://doi.org/10.1006/anbe.1996.0255

Glass, J. R., Fisher, A., Fewell, J. H., DeGrandi-Hoffman, G., Ozturk, C., & Harrison, J. F. (2021). Consumption of field-realistic doses of a widely used mito-toxic fungicide reduces thorax mass but does not negatively impact flight capacities of the honey bee (*Apis mellifera*). *Environmental Pollution*, *274*, 116533. https://doi.org/10.1016/j.envpol.2021.116533

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957. https://doi.org/10.1126/science.1255957

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Charrière, J.-D., Chlebo, R., Coffey, M. F., Cornelissen, B., da Costa, C. A., Dahle, B., Danihlík, J., Dražić, M. M., Evans, G., Fedoriak, M., Forsythe, I., Gajda, A., de Graaf, D. C., Gregorc, A., … Brodschneider, R. (2020). Honey bee colony winter loss rates for 35 countries participating in the COLOSS survey for winter 2018–2019, and the effects of a new queen on the risk of colony winter loss. *Journal of Apicultural Research*, *59*(5), 744–751. https://doi.org/10.1080/00218839.2020.1797272

Halas, E. S., Heinrich, M. D., & Sandstead, H. H. (1979). Long term memory deficits in adult rats due to postnatal malnutrition. *Physiology & Behavior*, *22*(5), 991–997. https://doi.org/10.1016/0031-9384(79)90345-7

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., & Decourtye, A. (2012). A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. *Science*, *336*(6079), 348–350. https://doi.org/10.1126/science.1215039

Hesselbach, H., & Scheiner, R. (2018). Effects of the novel pesticide flupyradifurone (Sivanto) on honeybee taste and cognition. *Scientific Reports*, *8*(1), 1–8. https://doi.org/10.1038/s41598-018-23200-0

Kirchner, W. H., & Grasser, A. (1998). The Significance of Odor Cues and Dance Language Information for the Food Search Behavior of Honeybees (Hymenoptera: Apidae). *Journal of Insect Behavior*, *11*(2), 169–178. https://doi.org/10.1023/A:1021098405564

Klein, A.-M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 303–313. https://doi.org/10.1098/rspb.2006.3721

Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D. M., Sagili, R. R., Pettis, J. S., Ellis, J. D., Wilson, M. E., Wilkes, J. T., Tarpy, D. R., Rose, R., Lee, K., Rangel, J., & vanEngelsdorp, D. (2017). A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *Journal of Apicultural Research*, *56*(4), 328–340. https://doi.org/10.1080/00218839.2017.1344496

Ladurner, E., Bosch, J., Kemp, W. P., & Maini, S. (2005). Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* Say and *Apis mellifera*. *Apidologie*, *36*(3), 449–460. https://doi.org/10.1051/apido:2005032

Lambin, M., Armengaud, C., Raymond, S., & Gauthier, M. (2001). Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. *Archives of Insect Biochemistry and Physiology*, *48*(3), 129–134. https://doi.org/10.1002/arch.1065

Lee, H., Sumner, D. A., & Champetier, A. (2019). Pollination Markets and the Coupled Futures of Almonds and Honey Bees: Simulating Impacts of Shifts in Demands and Costs. *American Journal of Agricultural Economics*, *101*(1), 230–249. https://doi.org/10.1093/ajae/aay063

Lenth, R. (2019). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.4.3.01, https://CRAN.R-project.org/package=emmeans

Lozano, V., Armengaud, C., & Gauthier, M. (2001). Memory impairment induced by cholinergic antagonists injected into the mushroom bodies of the honeybee. *Journal of Comparative Physiology A*, *187*(4), 249–254. https://doi.org/10.1007/s003590100196

Mattila, H. R., & Smith, B. H. (2008). Learning and memory in workers reared by nutritionally stressed honey bee (*Apis mellifera* L.) colonies. *Physiology & Behavior*, *95*(5), 609–616. https://doi.org/10.1016/j.physbeh.2008.08.003

Morgane, P. J., Austin-LaFrance, A., Bronzino, J., Tonkiss, J., Diaz-Cintra, S., Cintra, L., Kemper, T., & Galler, J. R. (1993). Prenatal malnutrition and development of the brain. *Neuroscience & Biobehavioral Reviews*, *17*(1), 91–128. https://doi.org/10.1016/S0149-7634(05)80234-9

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., & Pettis, J. S. (2010). High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLOS ONE*, *5*(3), e9754. https://doi.org/10.1371/journal.pone.0009754

Mustard, J. A., Alvarez, V., Barocio, S., Mathews, J., Stoker, A., & Malik, K. (2018). Nutritional value and taste play different roles in learning and memory in the honey bee (*Apis mellifera*). *Journal of Insect Physiology*, *107*, 250–256. https://doi.org/10.1016/j.jinsphys.2018.04.014

Naug, D. (2009). Nutritional stress due to habitat loss may explain recent honeybee colony collapses. *Biological Conservation*, *142*(10), 2369–2372. https://doi.org/10.1016/j.biocon.2009.04.007

Pham-Delègue, M.-H., Decourtye, A., Kaiser, L., & Devillers, J. (2002). Behavioural methods to assess the effects of pesticides on honey bees. *Apidologie*, *33*(5), 425–432. https://doi.org/10.1051/apido:2002033

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology & Evolution*, *25*(6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Sánchez-Bayo, F., & Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, *232*, 8–27. https://doi.org/10.1016/j.biocon.2019.01.020

Scheiner, R., Barnert, M., & Erber, J. (2003). Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. *Apidologie*, *34*(1), 67–72. https://doi.org/10.1051/apido:2002050

Scheiner, R., Page, R. E., & Erber, J. (2004). Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). *Apidologie*, *35*(2), 133–142. https://doi.org/10.1051/apido:2004001

Schneider, C. W., Tautz, J., Grünewald, B., & Fuchs, S. (2012). RFID Tracking of Sublethal Effects of Two Neonicotinoid Insecticides on the Foraging Behavior of *Apis mellifera*. *PLOS ONE*, *7*(1), e30023. https://doi.org/10.1371/journal.pone.0030023

Simcock, N. K., Gray, H., Bouchebti, S., & Wright, G. A. (2018). Appetitive olfactory learning and memory in the honeybee depend on sugar reward identity. *Journal of Insect Physiology*, *106*, 71–77. https://doi.org/10.1016/j.jinsphys.2017.08.009

Simon-Delso, N., San Martin, G., Bruneau, E., Minsart, L.-A., Mouret, C., & Hautier, L. (2014). Honeybee Colony Disorder in Crop Areas: The Role of Pesticides and Viruses. *PLOS ONE*, *9*(7), e103073. https://doi.org/10.1371/journal.pone.0103073

Siviter, H., Koricheva, J., Brown, M. J. F., & Leadbeater, E. (2018). Quantifying the impact of pesticides on learning and memory in bees. *Journal of Applied Ecology*, *55*(6), 2812–2821. https://doi.org/10.1111/1365-2664.13193

Smith, B. H., & Burden, C. M. (2014). A Proboscis Extension Response Protocol for Investigating Behavioral Plasticity in Insects: Application to Basic, Biomedical, and Agricultural Research. *JoVE (Journal of Visualized Experiments)*, *91*, e51057. https://doi.org/10.3791/51057

Sol Balbuena, M., Tison, L., Hahn, M.-L., Greggers, U., Menzel, R., & Farina, W. M. (2015). Effects of sublethal doses of glyphosate on honeybee navigation. *Journal of Experimental Biology*, *218*(17), 2799–2805. https://doi.org/10.1242/jeb.117291

Steijven, K., Spaethe, J., Steffan-Dewenter, I., & Härtel, S. (2017). Learning performance and brain structure of artificially-reared honey bees fed with different quantities of food. *PeerJ*, *5*, e3858. https://doi.org/10.7717/peerj.3858

Tadei, R., Menezes-Oliveira, V. B., & Silva-Zacarin, E. C. M. (2020). Silent effect of the fungicide pyraclostrobin on the larval exposure of the non-target organism Africanized *Apis mellifera* and its interaction with the pathogen *Nosema ceranae* in adulthood. *Environmental Pollution*, *267*, 115622. https://doi.org/10.1016/j.envpol.2020.115622

Tonkiss, J., & Galler, J. R. (1990). Prenatal protein malnutrition and working memory performance in adult rats. *Behavioural Brain Research*, *40*(2), 95–107. https://doi.org/10.1016/0166-4328(90)90002-V

Vinggaard, A. M., Hass, U., Dalgaard, M., Andersen, H. R., Bonefeld-Jørgensen, E., Christiansen, S., Laier, P., & Poulsen, M. E. (2006). Prochloraz: An imidazole fungicide with multiple mechanisms of action. *International Journal of Andrology*, *29*(1), 186–192. https://doi.org/10.1111/j.1365-2605.2005.00604.x

von Frisch, K. (1993). *The Dance Language and Orientation of Bees*. Harvard University Press. https://doi.org/10.4159/harvard.9780674418776

Wright, G. A., Mustard, J. A., Kottcamp, S. M., & Smith, B. H. (2007). Olfactory memory formation and the influence of reward pathway during appetitive learning by honey bees. *Journal of Experimental Biology*, *210*(22), 4024–4033. https://doi.org/10.1242/jeb.006585

Wu-Smart, J., & Spivak, M. (2016). Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development. *Scientific Reports*, *6*, 32108. https://doi.org/10.1038/srep32108

Wykes, G. R. (1952). The Preferences of Honeybees for Solutions of Various Sugars Which Occur in Nectar. *Journal of Experimental Biology*, *29*(4), 511–519. https://doi.org/10.1242/jeb.29.4.511

CHAPTER 3

A MITOTOXIC FUNGICIDE ALTERS POST-INGESTIVE GLUCOSE SIGNALS NECESSARY FOR ASSOCIATIVE LEARNING IN HONEY BEES

ABSTRACT

The Proboscis Extension Reflex (PER) paradigm trains honey bees to associate an odor with a sugar reward and is commonly used to assess impacts on associative learning after exposure to pesticides or other stressors. A rise in hemolymph glucose levels during PER training serves as a post-ingestive signal and is essential for the bee to form a robust memory of the association. Foraging honey bees are exposed to a wide variety of toxins in agricultural ecosystems. While the effects of some types of pesticides have been well-investigated, relatively little attention has been focused on fungicides that are applied to flowering crops to control fungal infections. I have previously shown that the fungicide Pristine® (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin) impairs honey bee performance in an associative learning assay. Pristine® disrupts protein digestion, but the mechanism of its action has not been investigated. Here, I investigate Pristine®'s impacts on carbohydrate absorption by measuring hemolymph sugar levels post-feeding. Pristine®-exposed bees had elevated baseline glucose concentrations in the hemolymph relative to control bees. Hemolymph glucose levels rose significantly within five minutes of feeding in control bees, but not in Pristine®-fed bees. These data suggest that the post-ingestive feedback mechanisms necessary for robust learning are disrupted in bees that have consumed this fungicide, providing a plausible mechanistic explanation for its effects on learning performance in the PER assay. Pristine®-exposed bees may have elevated hemolymph glucose levels because the fungicide damages the midgut, or

because it elicits an inflammatory response. These results are an important step forward in understanding the physiological effects of mitotoxic fungicides on this important pollinator.

INTRODUCTION

Classical conditioning is the process by which an animal learns to associate an environmental stimulus with either a good (appetitive) or bad (aversive) biological outcome (Rescorla, 1988). Post-ingestive feedback is a mechanism underlying associative learning and, in a general sense, dictates how an animal's body reacts to and learns about a particular food. Post-ingestive feedback mechanisms may be either appetitive or aversive, teaching an animal to either seek out or avoid the food in the future.

Honey bees can learn to associate the odors and tastes of toxins with the post-ingestive consequences (malaise) of consuming them (Wright et al., 2010). On the appetitive side, some compounds naturally present in nectar can enhance a bee's memory of the food source, making it more likely that the bee will seek out that source again later. These compounds include certain amino acids (Carlesso et al., 2021; Kim & Smith, 2000; Marchi et al., 2021), flavonoids (Gong et al., 2021; Hernández et al., 2019), and caffeine (Gong et al., 2021; Marchi et al., 2021; Wright et al., 2013). While substances like these are most well-known as deterrents to herbivory and are undoubtably toxic at high doses, the small concentrations found in nectar can actually enhance pollinators' memory of the flower by making them feel good—likened to a "buzz" similar to how a human might feel after caffeine consumption (Vignieri, 2013).

Appetitive post-ingestive feedback can be studied in the proboscis extension reflex (PER) conditioning paradigm. PER, a method of classical conditioning for honey bees and other insects, conditions bees to associate a neutral stimulus (usually an odor) with a sugar reward (Bitterman et al., 1983; Smith & Burden, 2014; Takeda, 1961). During the PER assay, bees are fed a small amount of a sucrose solution following each acquisition trial. A few such trials is usually sufficient for the bees to show good recall of that association 24 or more hours later. Bees that are allowed to taste but not feed on sucrose learn in the short term but do not remember the association between odor and reward 24 hours later (Wright et al., 2007), and conversely, feeding bees tasteless but metabolizable sugars mitigates this effect (Mustard et al., 2018). Sucrose is quickly digested into glucose and fructose and absorbed by honey bees, causing hemolymph glucose levels to rise. Rising hemolymph glucose levels within 5-10 minutes of feeding serve as a cue for the brain to reinforce the association between odor and reward (Simcock et al., 2018).

I have previously shown that a common agricultural fungicide, Pristine® (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin), impairs honey bee olfactory associative learning performance in the PER paradigm (DesJardins et al., 2021). These two active ingredients interfere with components of respiration in fungal cells (boscalid targets succinate dehydrogenase and pyraclostrobin targets cytochrome bc1 at Qo site) (Fungicide Resistance Action Committee [FRAC], 2022), and inhibit mitochondrial activity in honey bees (Campbell et al., 2016). Additionally, pyraclostrobin damages the honey bee midgut (da Costa Domingues et al., 2020; Tadei et al., 2020) and the Pristine® formulation impairs protein absorption (DeGrandi-Hoffman et al., 2015). It is unknown whether Pristine® also impacts carbohydrate absorption, but if it does, the post-feeding rise in hemolymph glucose levels necessary for robust memory formation in the PER paradigm may not be sufficient to support the formation of an associative memory. Here, I test this idea by measuring carbohydrate absorption in honey bees exposed to Pristine® by feeding them a set amount of sucrose and measuring their hemolymph sugar levels at known time points afterward. By doing so, I seek to determine the mechanism underlying this fungicide's effects on associative learning and take another step towards understanding its impacts on honey bee health.

METHODS

Honey bee colonies and fungicide exposure. Colony initiation and exposure protocols were the same as described in previous studies (DesJardins et al., 2021; Fisher et al., 2021). For these experiments, I obtained six 3 lb. Italian honey bee (*Apis mellifera linguistica*) packages from Pendell Apiaries in Stonyford, California in April 2021. The packages were used to initiate new colonies in Apimaye plastic hive boxes (Kaftan LLC, Tempe, AZ) at the Arizona State University Bee Lab in Mesa, AZ (33.293173, −111.684520).

In September 2021, pollen traps were placed on hive entrances to limit the amount of outside pollen that foragers were able to bring in. A random number generator (random.org) was used to assign three hives to the fungicide treatment group and three hives to the control group. Pollen patties containing either plain deionized water (control) or deionized water mixed with 23 ppm Pristine® (treatment group) (BASF Corporation, Research Triangle Park, NC) were placed inside the hive *ad libitum* starting at the beginning of September 2021 and ending in December 2021, after bee hemolymph collection was finished. I chose 23 ppm Pristine® as my focal concentration because bees could realistically be exposed to that amount while foraging in a treated almond orchard (Fisher et al., 2021), and this concentration also impairs associative learning performance in the laboratory (DesJardins et al., 2021).

Bee feeding and hemolymph collection. In November and December 2021, bees were captured from hives entrances, either Pristine®-fed or controls. They were taken inside, anesthetized on ice, and then harnessed so that only their antennae and mouthparts were moveable, just as they would be for the PER assay (DesJardins et al., 2021; Smith & Burden, 2014). To control for any effect of time since last feeding, bees were fed and then subjected to an overnight starvation procedure. Each bee was fed 30 µL of a 1.0 M table sugar and water solution using a 0.2 mL Gilmont syringe (Cole-Parmer, Vernon Hills, IL). I chose table sugar and this concentration because it matches standard PER procedures (DesJardins et al., 2021; Smith & Burden, 2014). Most bees consumed the full 30 µL, but if a bee consumed less than that, a note of the amount consumed was recorded. After feeding, bees were placed in a plastic box with damp paper towels at the bottom to provide humidity, which was then placed in a dark cabinet overnight.

The following day, bees were randomly assigned to one of six time points: 0, 0.5, 1, 3, 5, or 10 minutes post-feeding, chosen to mimic the procedures of Simcock et al. (2018). Bees in the “zero minutes” treatment group were not fed; their hemolymph was collected immediately. Bees in the other five treatment groups were fed 30 µL of the sugar water solution, and their hemolymph was collected at the time post-feeding corresponding with their treatment group.

To collect hemolymph, as in Simcock et al. (2018), a small incision was made above the median ocellus. A 1 µL microcapillary tube (Drummond Scientific, Broomall, PA) was inserted into the incision, and was left there until it was completely full. Its contents were then diluted in 300 µL deionized water and emptied into a microcentrifuge tube, which was immediately placed in a -80°C freezer. If the bee refused the sugar water or 1 µL hemolymph could not be obtained, it was excluded.

Glucose assays. I used a glucose oxidase colorimetric assay kit (GAGO20, Sigma-Aldrich, St. Louis, MO) to determine the amount of glucose in the hemolymph samples. The kit was prepared according to its instructions, but the amounts of reagents added during each step of the assay were modified to accommodate the small volumes of my hemolymph samples. I created a glucose standard curve with standards containing 0, 2, 4, 6, 8, and 10 percent glucose, which was run with each plate. R^2 values for the standard curves ranged from 0.990 to 0.999. I added 33 µL of each diluted hemolymph sample (using 99 µL total) in triplicate to a 96-well plate. I added 66 µL of the glucose oxidase mix to each well, incubated for a half hour at 37°C, and then added 66 µL of a 12 N sulfuric acid solution to each well to stop the reaction. The plate was run through a microplate spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories, Hercules, CA) at 540 nm, and then glucose amounts for each sample (averaged across the three wells) were calculated using the standard curve and adjusted based on the prior hemolymph dilution.

Trehalose assays. Trehalose was measured by adding another 33 µL of each diluted hemolymph sample in triplicate to a 96-well plate. One µL of the enzyme trehalase (T8778, Sigma-Aldrich, St. Louis, MO) was added to each well, and then the plate was incubated overnight at 37°C (Wang et al., 2016). Preliminary experiments confirmed that this procedure broke down all the trehalose present in the sample into its component glucose, and that the trehalase enzyme did not lyse other disaccharides such as sucrose. Each sample was then run through another round of the glucose assay, and the difference in glucose between the first and second runs was used to determine the amount of trehalose in the sample.

Statistical analyses. Data were analyzed in R version 4.2.2 (R Core Team, 2022). Linear models were created to analyze the results for glucose and trehalose separately, looking for effects of time post-feeding, treatment group, and the interaction between the two on hemolymph sugar concentrations. For the glucose assay, post hoc pairwise comparisons were conducted using t-tests with Bonferroni-corrected p-values to determine which time points were significantly different from time point zero within each treatment group. Because five t-tests were performed for each treatment group, α was set to 0.01.

RESULTS

Hemolymph glucose concentrations. In the control group, average hemolymph glucose concentrations started relatively low (2.50 µg/µL) and rose steadily, reaching 8.13 µg/µL three minutes post-feeding. In the Pristine® group, average hemolymph glucose concentrations were higher than controls at time point zero (7.14 µg/µL), and there were apparently larger peaks at both 30 seconds (14.4 µg/µL) and five minutes (15.6 µg/µL) post-feeding, but there was no systematic pattern in hemolymph glucose concentrations over time.

Exposure to 23 ppm Pristine® significantly increased the concentration of glucose present in honey bee hemolymph on average across all time points relative to controls (F=12.8, p<0.001, Figure 3.1). In all but two intermediate time points, bees from the Pristine® treatment group had markedly higher concentrations of hemolymph glucose than controls. Hemolymph glucose levels also increased across post-feeding time points in control bees (F=4.523, p=0.0354). Based on post hoc comparisons, hemolymph concentrations were significantly different from time point zero at one (t=2.8353, p= 0.008079), five (t=3.6095, p=0.001644), and ten (t=4.0174, p=0.0003823) minutes post-feeding. However, hemolymph glucose levels did not change linearly with time in Pristine®-fed bees (F=2.53, p=0.114), and glucose levels did not differ significantly from those measured prior to feeding at any time after feeding according to post hoc tests.

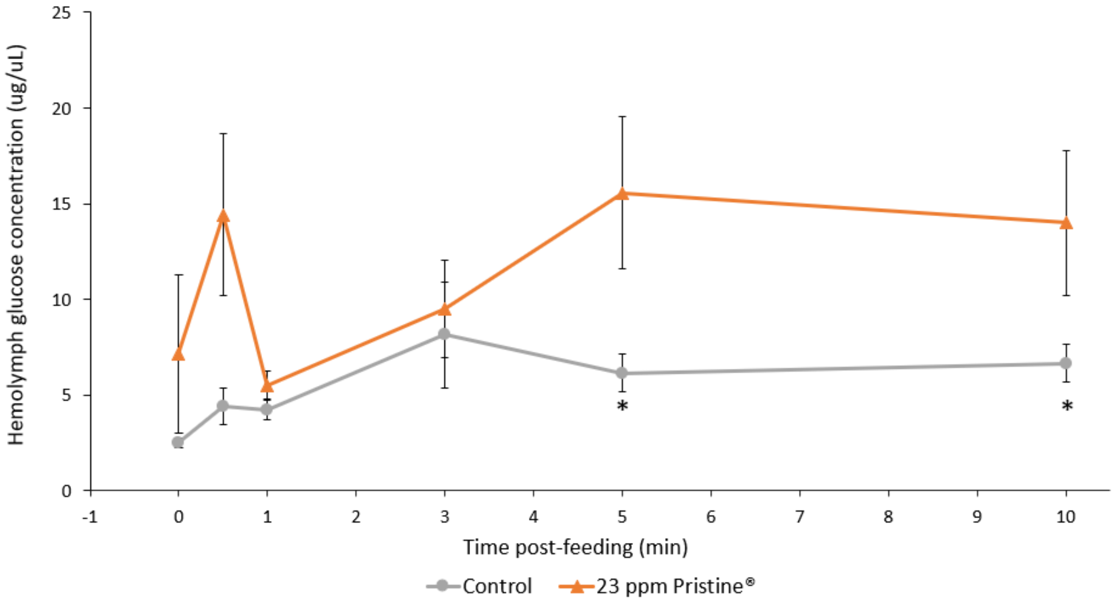


Figure 3.1. Average hemolymph glucose concentrations for bees exposed to Pristine® fungicide (orange) or for unexposed control bees (gray) at set times after consuming 30 µL of a 1.0 M sucrose-water solution. Asterisks indicate time points that were significantly different from time point zero for that treatment group. Error bars indicate standard error. N=17-26 individuals per time point/treatment group.

Hemolymph trehalose concentrations. Pristine® consumption had no statistically significant effect on honey bee hemolymph trehalose concentrations (F=0.350, p=0.852, Figure 3.2). Trehalose concentrations did not vary across post-feeding time points (F=0.0199, p=0.888), staying within the range of 10-17 µg/µL, even in bees that had not been fed (time point zero). The interaction between time post-feeding and treatment group was also nonsignificant (F=0.0579, p=0.810).

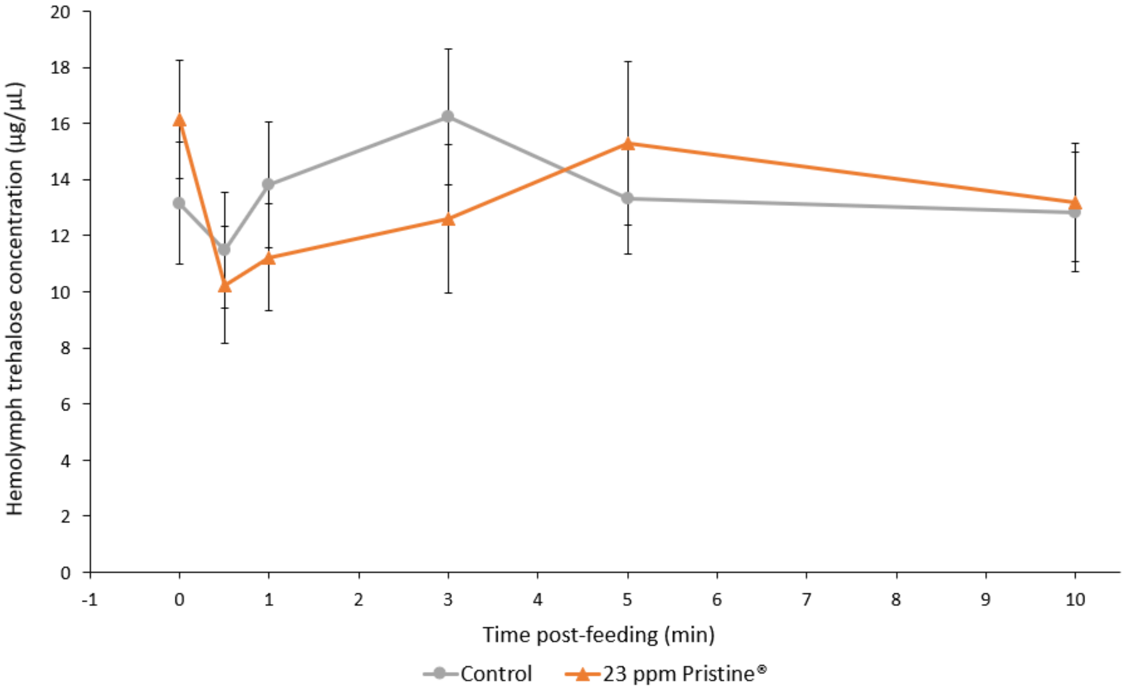


Figure 3.2. Average hemolymph trehalose concentrations for bees exposed to Pristine® fungicide (orange) or for unexposed control bees (gray) at set times after consuming 30 µL of a 1.0 M sucrose-water solution. Error bars indicate standard error. N=14-23 individuals per time point/treatment group.

DISCUSSION

My results support the hypothesis that disrupted glucose absorption could account for why Pristine® reduces the learning abilities of honey bees. Pristine® consumption through larval and adult development reduces associative learning in a dose-dependent manner, with significant effects observed at field-relevant concentrations (DesJardins et al., 2021). Because the results of PER assays can be predictive of effects on real-world foraging behaviors (Cabirol et al., 2018; Gerber et al., 1996), it is important to understand the mechanisms through which agrochemicals impact the critical abilities of bees to learn about food sources in their environments. Here, I present data showing that Pristine® impacts carbohydrate absorption, possibly interfering with the post-ingestive feedback mechanisms necessary for learning. Simcock et al. (2018) demonstrated that a rise in hemolymph glucose levels within five minutes of feeding was necessary for bees to form robust memories of the association between odor and sugar reward. I found that this rise happened in control but not Pristine® bees, likely because Pristine® bees had exceptionally high hemolymph glucose levels across most time points post-feeding, including at time point zero (which represented bees that had not been fed).

I also observed that average hemolymph trehalose concentrations haphazardly ranged from 10-17 µg/µL across all time points and treatment groups, with no significant differences over time or between treatments. Other studies have reported similar concentrations (Blatt & Roces, 2001; Woodring et al., 1993) and have confirmed that these concentrations remain relatively consistent within the first 20 minutes after feeding (Simcock et al., 2018). I conclude that the alterations in hemolymph glucose concentrations do not translate to differences in trehalose, suggesting that Pristine®-exposed bees are still able to regulate storage of hemolymph sugars over time.

The PER paradigm mimics the process through which bees learn about which flowers provide the best sources of nutrients. To understand the post-ingestive feedback mechanisms at play during PER learning, it is important to understand how nectar is digested and absorbed. Nectar consists of mostly sucrose, with smaller concentrations of glucose and fructose (Wykes, 1952). As sucrose is consumed, it is rapidly converted to its constituent monosaccharides, glucose and fructose, in the crop (Oertel et al., 1951). Although some studies report that sucrose can be present in the hemolymph after bees have fed on large amounts of sucrose (Bounias & Morgan, 1984; Simcock et al., 2018), it is generally accepted that sucrose is mainly broken down entirely within the gut, and that glucose, fructose, and trehalose are the main sugars found in the hemolymph (Arslan et al., 1986; Woodring et al., 1993). Sugar absorption from the midgut into the hemolymph is entirely passive, and glucose levels in the hemolymph rise within five minutes of feeding (Crailsheim, 1988). Within another five minutes, the fat body converts most of the glucose into trehalose, which is the dominant hemolymph sugar in honey bees (Blatt & Roces, 2001; Gmeinbauer & Crailsheim, 1993).

During the PER assay, bees fed with sucrose or glucose form robust long-term memories of the association between odor and reward, while bees fed with fructose do not. This suggests that a rise in hemolymph glucose levels is the driving force behind post-ingestive feedback (Simcock et al., 2018). The change in glucose levels in the brain could be detected by gustatory receptors expressed in neurons or glia that interact with the neurons that encode memories, reinforcing the association. Alternatively, the circuit involved in memory could sense rising intracellular ATP levels that are brought about by rising glucose levels (Simcock et al., 2018). If the rise in hemolymph glucose levels does not happen, perhaps because a fungicide is interfering with a bee’s ability to absorb nutrients, it follows that these post-ingestive processes would be disrupted and the bee would not learn as well.

I observed that average hemolymph glucose concentrations rose to 8.13 µg/µL in control bees three minutes after feeding on 30 µL of a 1.0 M sucrose solution. This is consistent with most reports of hemolymph glucose concentrations soon after feeding (which usually peak somewhere between 5-10 µg/µL within five minutes) (Blatt & Roces, 2001; Crailsheim, 1988; Woodring et al., 1993), but falls short of the 150 mM (~27 µg/µL) peak reported by Simcock et al. (2018). The group of bees that consumed Pristine® had higher average hemolymph glucose concentrations than controls at every time point measured, with exceptionally large peaks at both 30 seconds (14.4 µg/µL) and five minutes (15.6 µg/µL) after feeding. However, these rises were non-significant when compared to hemolymph glucose concentrations in unfed (time point zero) Pristine® bees, which were also relatively high (7.14 µg/µL).

What could cause elevated hemolymph glucose levels in Pristine®-fed bees? In one study, pyraclostrobin, one of the active ingredients, caused a variety of changes to the morphology of honey bee midgut cells. These included increased epithelial cytoplasmic vacuolization, elimination of cells to the lumen, morphological alterations in regenerative cells, and reduced staining for neutral polysaccharides and glycoconjugates (da Costa Domingues et al., 2020). This damage to the midgut epithelia suggests that Pristine® (or pyraclostrobin, at least) compromises the midgut's ability to properly absorb carbohydrates, may create a "leaky gut" where midgut contents are prematurely leaked into the hemolymph, potentially explaining elevated baseline glucose levels. Also, glucose transport by the midgut is likely to be at least partially paracellular, in conjunction with fluid transport driven by active ion reabsorption by the midgut epithelia, which could be inhibited by boscalid and pyraclostrobin (Campbell et al., 2016).

Another potential explanation for increased hemolymph glucose levels Pristine® bees is that they could have greater immune system activity, either from the inflammation caused by gut damage or from the increase in virus titers associated with exposure (DeGrandi-Hoffman et al., 2015). Inflammation in insects infected with parasites has been linked to increased hemolymph sugar levels (Schilder & Marden, 2006; Xu et al., 2015), including in *Nosema*-infected honey bees (Aliferis et al., 2012). Hemolymph sugar levels are not as responsive to insulin in infected insects compared to healthy controls. This response has been compared to mammalian metabolic syndrome (Schilder & Marden, 2006), which is also linked to increased inflammation.

CONCLUSION

My study is the first to report that carbohydrate absorption in honey bees is impacted by the fungicide Pristine®, adding to reports that it damages the midgut (da Costa Domingues et al., 2020; Tadei et al., 2020), and interferes with protein digestion (DeGrandi-Hoffman et al., 2015). This fungicide's effects on altered nutrient absorption are worth further study, and may ultimately relate to other observed effects such as reduced individual longevity, increased pollen collection, and reduced population sizes of adult workers in colonies (Fisher et al., 2021). Although glucose levels in Pristine®-fed bees were higher than in control bees, which intuitively should support better memory, it may be the relative rise that triggers memory rather than the absolute levels. I found that hemolymph glucose levels rose after feeding in control but not Pristine® bees, which is consistent with the idea that these effects on nutrient absorption interfere with the post-ingestive feedback mechanisms necessary for robust learning (Simcock et al., 2018). Associative learning is critical for successful foraging in honey bees; therefore, my results take an important step forward in understanding how Pristine® impacts the health and behavior of this important pollinator.

REFERENCES

Aliferis, K. A., Copley, T., & Jabaji, S. (2012). Gas chromatography–mass spectrometry metabolite profiling of worker honey bee (*Apis mellifera* L.) hemolymph for the study of *Nosema ceranae* infection. *Journal of Insect Physiology*, *58*(10), 1349–1359. https://doi.org/10.1016/j.jinsphys.2012.07.010

Arslan, A., Standifer, L. N., & Don, H. (1986). Carbohydrates in honey bee hemolymph. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *84*(3), 363–367. https://doi.org/10.1016/0305-0491(86)90090-8

Bitterman, M. E., Menzel, R., Fietz, A., & Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Psychology*, *97*(2), 107–119. https://doi.org/10.1037/0735-7036.97.2.107

Blatt, J., & Roces, F. (2001). Haemolymph sugar levels in foraging honeybees (*Apis mellifera carnica*): Dependence on metabolic rate and *in vivo* measurement of maximal rates of trehalose synthesis. *Journal of Experimental Biology*, *204*(15), 2709–2716. https://doi.org/10.1242/jeb.204.15.2709

Bounias, M., & Morgan, M. R. J. (1984). Induction of honeybee haemolymph sucrase activity by high levels of dietary sucrose. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *79*(1), 75–80. https://doi.org/10.1016/0305-0491(84)90080-4

Cabirol, A., Cope, A. J., Barron, A. B., & Devaud, J.-M. (2018). Relationship between brain plasticity, learning and foraging performance in honey bees. *PLOS ONE*, *13*(4), e0196749. https://doi.org/10.1371/journal.pone.0196749

Campbell, J. B., Nath, R., Gadau, J., Fox, T., DeGrandi-Hoffman, G., & Harrison, J. F. (2016). The fungicide Pristine® inhibits mitochondrial function *in vitro* but not flight metabolic rates in honey bees. *Journal of Insect Physiology*, *86*, 11–16. https://doi.org/10.1016/j.jinsphys.2015.12.003

Carlesso, D., Smargiassi, S., Pasquini, E., Bertelli, G., & Baracchi, D. (2021). Nectar non-protein amino acids (NPAAs) do not change nectar palatability but enhance learning and memory in honey bees. *Scientific Reports*, *11*(1), 11721. https://doi.org/10.1038/s41598-021-90895-z

Crailsheim, K. (1988). Intestinal transport of sugars in the honeybee (*Apis mellifera* L.). *Journal of Insect Physiology*, *34*(9), 839–845. https://doi.org/10.1016/0022-1910(88)90117-5 da Costa Domingues, C. E., Bello Inoue, L. V., Mathias da Silva-Zacarin, E. C., & Malaspina, O. (2020). Foragers of Africanized honeybee are more sensitive to fungicide pyraclostrobin than newly emerged bees. *Environmental Pollution*, *266*, 115267. https://doi.org/10.1016/j.envpol.2020.115267

DeGrandi-Hoffman, G., Chen, Y., Watkins Dejong, E., Chambers, M. L., & Hidalgo, G. (2015). Effects of Oral Exposure to Fungicides on Honey Bee Nutrition and Virus Levels. *Journal of Economic Entomology*, *108*(6), 2518–2528. https://doi.org/10.1093/jee/tov251

DesJardins, N. S., Fisher, A., Ozturk, C., Fewell, J. H., DeGrandi-Hoffman, G., Harrison, J. F., & Smith, B. H. (2021). A common fungicide, Pristine®, impairs olfactory associative learning performance in honey bees (*Apis mellifera*). *Environmental Pollution*, 117720. https://doi.org/10.1016/j.envpol.2021.117720

Fisher, A., DeGrandi-Hoffman, G., Smith, B. H., Johnson, M., Kaftanoglu, O., Cogley, T., Fewell, J. H., & Harrison, J. F. (2021). Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*). *Environmental Pollution*, *269*, 115964. https://doi.org/10.1016/j.envpol.2020.115964

Fungicide Resistance Action Committee. (2022, March). *FRAC Code List©*2022: Fungal control agents sorted by cross-resistance pattern and mode of action (including coding for FRAC Groups on product labels).* https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2022--final.pdf?sfvrsn=b6024e9a_2

Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalksy, O., Wüstenberg, D., & Menzel, R. (1996). Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Animal Behaviour*, *52*(6), 1079–1085. https://doi.org/10.1006/anbe.1996.0255

Gmeinbauer, R., & Crailsheim, K. (1993). Glucose utilization during flight of honeybee (*Apis mellifera*) workers, drones and queens. *Journal of Insect Physiology*, *39*(11), 959–967. https://doi.org/10.1016/0022-1910(93)90005-C

Gong, Z., Gu, G., Wang, Y., Dong, S., Tan, K., & Nieh, J. C. (2021). Floral tea polyphenols can improve honey bee memory retention and olfactory sensitivity. *Journal of Insect Physiology*, *128*, 104177. https://doi.org/10.1016/j.jinsphys.2020.104177

Hernández, I. G., Palottini, F., Macri, I., Galmarini, C. R., & Farina, W. M. (2019). Appetitive behavior of the honey bee *Apis mellifera* in response to phenolic compounds naturally found in nectars. *Journal of Experimental Biology*, *222*(2). https://doi.org/10.1242/jeb.189910

Kim, Y. S., & Smith, B. H. (2000). Effect of an amino acid on feeding preferences and learning behavior in the honey bee, *Apis mellifera*. *Journal of Insect Physiology*, *46*(5), 793–801. https://doi.org/10.1016/S0022-1910(99)00168-7

Marchi, I. L., Palottini, F., & Farina, W. M. (2021). Combined secondary compounds naturally found in nectars enhance honeybee cognition and survival. *Journal of Experimental Biology*, *224*(6). https://doi.org/10.1242/jeb.239616

Mustard, J. A., Alvarez, V., Barocio, S., Mathews, J., Stoker, A., & Malik, K. (2018). Nutritional value and taste play different roles in learning and memory in the honey bee (*Apis mellifera*). *Journal of Insect Physiology*, *107*, 250–256. https://doi.org/10.1016/j.jinsphys.2018.04.014

Oertel, E., Fieger, E. A., Williams, V. R., & Andrews, E. A. (1951). Inversion of Cane Sugar in the Honey Stomach of the Bee. *Journal of Economic Entomology*, *44*(4), 487–492. https://doi.org/10.1093/jee/44.4.487

R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rescorla, R. A. (1988). Behavioral Studies of Pavlovian Conditioning. *Annual Review of Neuroscience*, *11*(1), 329–352. https://doi.org/10.1146/annurev.ne.11.030188.001553

Schilder, R. J., & Marden, J. H. (2006). Metabolic syndrome and obesity in an insect. *Proceedings of the National Academy of Sciences*, *103*(49), 18805–18809. https://doi.org/10.1073/pnas.0603156103

Simcock, N. K., Gray, H., Bouchebti, S., & Wright, G. A. (2018). Appetitive olfactory learning and memory in the honeybee depend on sugar reward identity. *Journal of Insect Physiology*, *106*, 71–77. https://doi.org/10.1016/j.jinsphys.2017.08.009

Smith, B. H., & Burden, C. M. (2014). A Proboscis Extension Response Protocol for Investigating Behavioral Plasticity in Insects: Application to Basic, Biomedical, and Agricultural Research. *JoVE (Journal of Visualized Experiments)*, *91*, e51057. https://doi.org/10.3791/51057

Tadei, R., Menezes-Oliveira, V. B., & Silva-Zacarin, E. C. M. (2020). Silent effect of the fungicide pyraclostrobin on the larval exposure of the non-target organism Africanized *Apis mellifera* and its interaction with the pathogen *Nosema ceranae* in adulthood. *Environmental Pollution*, *267*, 115622. https://doi.org/10.1016/j.envpol.2020.115622

Takeda, K. (1961). Classical conditioned response in the honey bee. *Journal of Insect Physiology*, *6*(3), 168–179. https://doi.org/10.1016/0022-1910(61)90060-9

Vignieri, S. (2013). Bees Get That Caffeine "Buzz." *Science Signaling*, *6*(266), ec64–ec64. https://doi.org/10.1126/scisignal.2004130

Wang, Y., Campbell, J. B., Kaftanoglu, O., Page, R. E., Amdam, G. V., & Harrison, J. F. (2016). Larval starvation improves metabolic response to adult starvation in honey bees (*Apis mellifera* L.). *Journal of Experimental Biology*, *219*(7), 960–968. https://doi.org/10.1242/jeb.136374

Woodring, J., Boulden, M., Das, S., & Gäde, G. (1993). Studies on blood sugar homeostasis in the honeybee (*Apis mellifera*, L.). *Journal of Insect Physiology*, *39*(1), 89–97. https://doi.org/10.1016/0022-1910(93)90022-J

Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. A., Power, E. F., Borland, A. M., & Stevenson, P. C. (2013). Caffeine in Floral Nectar Enhances a Pollinator's Memory of Reward. *Science*, *339*(6124), 1202–1204. https://doi.org/10.1126/science.1228806

Wright, G. A., Mustard, J. A., Kottcamp, S. M., & Smith, B. H. (2007). Olfactory memory formation and the influence of reward pathway during appetitive learning by honey bees. *Journal of Experimental Biology*, *210*(22), 4024–4033. https://doi.org/10.1242/jeb.006585

Wright, G. A., Mustard, J. A., Simcock, N. K., Ross-Taylor, A. A. R., McNicholas, L. D., Popescu, A., & Marion-Poll, F. (2010). Parallel Reinforcement Pathways for Conditioned Food Aversions in the Honeybee. *Current Biology*, *20*(24), 2234–2240. https://doi.org/10.1016/j.cub.2010.11.040

Wykes, G. R. (1952). The Preferences of Honeybees for Solutions of Various Sugars Which Occur in Nectar. *Journal of Experimental Biology*, *29*(4), 511–519. https://doi.org/10.1242/jeb.29.4.511

Xu, Y.-J., Luo, F., Gao, Q., Shang, Y., & Wang, C. (2015). Metabolomics reveals insect metabolic responses associated with fungal infection. *Analytical and Bioanalytical Chemistry*, *407*(16), 4815–4821. https://doi.org/10.1007/s00216-015-8648-8

CHAPTER 4

SYNERGISTIC EFFECTS BETWEEN A FUNGICIDE AND HIGH TEMPERATURES ON FORAGING AND NAVIGATION BEHAVIORS IN HONEY BEES

ABSTRACT

Interactions between environmental stressors may contribute significantly to ongoing pollinator declines. Here, I examined the interaction between the agricultural fungicide Pristine® (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin) and high temperatures on navigation behaviors in managed honey bees. I have previously shown that this fungicide impairs associative learning performance in a laboratory-based assay. Because of that, I hypothesized that it would impair navigation and foraging behaviors in the field as well. By timing the return of foragers released from a novel site one kilometer from their colony, I show that exposure to Pristine® at field-relevant levels reduced the probability of successful return to the colony, and this effect was exacerbated when paired with the high temperatures typical of an Arizona summer. Pristine® did not affect the masses of corbicular pollen or volumes of nectar or water brought back to the hive by returning foragers, and it did not affect the total ratio of nectar:pollen:water foragers in a colony. However, Pristine®-fed bees brought more concentrated nectar back to the hive, suggesting that the fungicide may raise sucrose response thresholds in foraging honey bees. My results show that this commonly-used fungicide can affect important foraging behaviors in honey bees under natural conditions, especially when paired with extreme temperatures. As agrochemical usage continues to increase, and particularly as heat waves become more common under climate change, it will become more important to manage the effects of interacting stressors to ensure pollinator health and food security.

INTRODUCTION

Global pollinator populations have been declining in recent years (Brown et al., 2016; Goulson et al., 2015; Potts et al., 2010). In parallel, managed honey bee populations in the US and Europe are experiencing high rates of annual colony loss (Bruckner et al., 2023; Gray et al., 2022). These losses are important because honey bees and other animal pollinators contribute substantially to agricultural production. Bee-pollinated crops constitute one third of the global human food supply (Khalifa et al., 2021). In the US, animal pollinators are worth $14.2–23.8 billion annually (Chopra et al., 2015). There is also evidence that certain US crops, including apples, cherries and blueberries, are already limited by lack of pollinator visitation (Reilly et al., 2020). Given the economic and societal importance of pollinators, it is essential to understand the causes of their population declines.

Among the likely causes of pollinator population declines are habitat loss and fragmentation, agrochemicals, pathogens, non-native species, and climate change (Potts et al., 2010). While environmental stressors are often studied in isolation, interactions between these stressors are likely. For example, poor nutrition can make bees more vulnerable to viruses (Dolezal et al., 2019), as can pesticide exposure (Mayack et al., 2022). Of particular interest are interactions between pesticides and extreme temperatures, such as the heat waves that are expected to occur as a result of climate change. Neonicotinoids reduce bee ability to survive exceptionally warm or cold temperatures (Alburaki et al., 2023; Bester et al., 2023), with greater differences in gene expression found in bees exposed to both stressors as opposed to one or the other (Alburaki et al., 2023; Kim et al., 2022; Manzi et al., 2020). A recent study also found that the neonicotinoid imidacloprid interacted with high temperatures to reduce the distances that bumble bees were able to fly in a tethered flight mill (Kenna et al., 2023).

Navigation and foraging are complex tasks for honey bees that require associative learning (Menzel et al., 2006). Honey bees are central place foragers, which means they need to navigate to floral resources outside the colony and bring nectar and pollen home (Bell, 1990). Successful navigation requires bees to learn about their surroundings using multiple sensory modalities (Buehlmann et al., 2020), integrating visual landmarks (Pahl et al., 2011), polarized light (Kobayashi et al., 2020), and optic flow (Si et al., 2003) cues to understand the direction and distance needed to travel in order to return to the hive. Odor learning also plays a role in navigation and foraging, as workers learn floral odors from their nestmates in the hive, which helps them locate and exploit the source later (Farina et al., 2007). Because navigation and foraging are complex tasks that involve associative learning, stressors such as high temperatures (Gérard et al., 2022a) and pesticides (Siviter et al., 2018) that impair associative learning may impact these critical behaviors as well.

Indeed, a number of studies have found that such stressors negatively affect navigation and foraging behaviors in honey bees. Neonicotinoids and other insecticides can reduce the frequency and increase the average duration of foraging trips (Barascou et al., 2022; Colin et al., 2021; Guez et al., 2005; Schneider et al., 2012), reduce the average age of first foraging (Colin et al., 2019; Shi et al., 2020), and alter the amount of pollen and nectar resources brought back to the hive (Prado et al., 2019). Neonicotinoids (Christen et al., 2021; Fischer et al., 2014; Henry et al., 2012; Tison et al., 2016), pyrethroids (Van Dame et al., 1995), and the herbicide glyphosate (Sol Balbuena et al.,

2015) can also negatively impact the ability of bees to successfully return to the hive after foraging, and these homing failures can contribute to colony collapse over time (Henry et al., 2012). High temperatures can lead to reduced foraging activity in bumble bees (Gérard et al., 2022b) and can induce precocious foraging and reduce longevity in honey bees (Medina et al., 2018). No studies have experimentally investigated the synergistic effects of high temperature and pesticides on navigation and foraging in bees, although some have found that exposure to neonicotinoids during cold weather can reduce homing success (Henry et al., 2014; Monchanin et al., 2019).

I have previously shown that the fungicide Pristine® impairs olfactory associative learning performance in honey bees (DesJardins et al., 2021). I therefore hypothesized that it would negatively impact navigation and foraging behaviors, which depend on the bee's learning ability. Pristine®'s active ingredients are boscalid (25.2%) and pyraclostrobin (12.8%), both of which interfere with the election transport chain in fungal cellular respiration (Fungicide Resistance Action Committee [FRAC], 2022). I used a combination of manual methods and RFID tracking technology to test whether field-relevant Pristine® exposure reduced homing success and the amounts of various types of resources collected by foragers. The homing experiments were conducted across a wide range of air temperatures, from approximately 20-40°C, enabling me to determine whether air temperature influenced any potential negative effect of Pristine® on the ability of honey bees to return to their hives.

METHODS

Honey bee colonies and fungicide exposure. Colony initiation and exposure protocols were similar to those described in previous studies (DesJardins et al., 2021; Fisher et al., 2021a). For the 2021 homing experiment, I obtained six 3 lb. Italian honey bee (*Apis mellifera linguistica*) packages from Pendell Apiaries in Stonyford, California in April. The packages were used to initiate new colonies in Apimaye plastic hive boxes (Kaftan LLC, Tempe, AZ) at the Arizona State University Bee Lab in Mesa, AZ (33.293173, −111.684520).

For the 2022 homing and foraging experiments, I took approximately 3 lb. of adult bees from the 2021 hives and paired them with new queen bees purchased from Pendell Apiaries. In April, the bees and new queen bees were used to initiate new colonies (with new frames and hive boxes). Since this procedure kept only adult bees from the initial 2021 colonies (which were also quickly replaced by emerging brood), it prevented the 2022 colonies from being influenced by any potentially leftover fungicide residues from the 2021 experiments.

For the 2021 experiments, fungicide exposure began in September. Pollen traps were placed on hive entrances to limit the amount of outside pollen that foragers were able to bring in. A random number generator (random.org) was used to assign three hives each to the fungicide treatment and control groups. Pollen patties containing either plain deionized water (control) or deionized water mixed with 23 ppm Pristine® (treatment group) (BASF Corporation, Research Triangle Park, NC) were placed inside the hive *ad libitum*. I chose 23 ppm Pristine® as the focal concentration because bees could realistically be exposed to that amount while foraging in a treated almond orchard (Fisher et al., 2021a), and this concentration also impairs associative learning performance in the laboratory (DesJardins et al., 2021).

In 2022, fungicide exposure began in July. Hives were assigned to treatment groups and fed in the same manner as in 2021. Fungicide exposure continued until experiments concluded in October.

To ensure that all focal bees in my experiment would be of similar age, I completed an age-marking process similar to the one described in Fisher et al. (2021b), once in 2021 (late September) and twice in 2022 (early August and late September). Capped brood frames were removed from each hive and placed in separate wire cages in an incubator (34 °C, 90% relative humidity) overnight. The following day, newly-emerged adults were marked on the mesonotum with a paint color corresponding to their date of emergence, and then they were returned to their hive of origin. This process was repeated until there were at least 550 marked bees in each hive. The homing and foraging experiments began after marked bees from all hives had begun foraging (approximately two weeks later).

2021 homing success. Marked foragers were collected in glass vials as they exited the hive. They were brought inside, anesthetized on ice, and then wood glue (Gorilla Glue, Cincinnati, OH) was used to attach a queen tag (Mann Lake Bee & Ag Supply, Hackensack, MN) with a unique color and number to their mesonotum. Workers were then placed back in their glass vials along with a piece of cotton soaked in a 1.0 M sucrose solution. They were allowed to feed *ad libitum* on the sucrose during the time between marking and their release (approximately 20 minutes).

Bees were released from a location 1 km east of the ASU Bee Lab, as determined using the measurement tool in Google Maps. This distance was chosen because it was comparable to the distance honey bees would usually cover when foraging (Steffan-Dewenter & Kuhn, 2003), and it has been used in other similar homing studies (Henry et al., 2012). The release site was located along Old Pecos Road, which runs through vacant ASU-owned property and is closed to vehicle traffic. This direction was chosen because, compared to other locations around the Bee Lab, it contained relatively few landmarks, which can influence results (Henry et al., 2014). Weather data (cloud cover, air temperature, wind speed, and wind direction) for the time of release were taken from a weather station at the Phoenix-Mesa Gateway Airport (https://www.wunderground.com/weather/KIWA), which was located approximately 2 km from the release site. Bees were released by removing the caps from the glass vials, placing them on the ground, and allowing individuals to exit on their own. The vials were left open for five minutes, after which any remaining bees were considered unmotivated to fly and removed from the experiment.

The colony entrances were partially covered with mesh wire at all times, which slowed down returning foragers and made it easier for the observers to see their tag numbers. Observers watched the colony entrances starting when the bees were released and ending one hour later. The length of the observation period was chosen because previous studies suggested that the majority of bees to return would do so within the first hour (Henry et al., 2012). Observers noted the time that each marked bee landed at the colony entrance.

2022 homing success. The capture and marking process was the same as in 2021, except bees were marked with RFID tags (BEE-TAG mic3®Q1.6, microsensys GmbH, Erfurt, Germany) instead of queen tags. Each tag was assigned to a treatment group (fungicide or control) and hive using a specialized RFID device (iID®PENsolid UHFcc, microsensys GmbH). The release site and methods were the same as in 2021. Before each bout of the experiment, RFID readers (iID®science reader device AEB-03.C2D, microsensys GmbH) were installed at hive entrances. They remained installed for three hours following the release time. Whenever the readers were not installed on the hives, 3D printed "sham" readers were present instead so that the entrance would always look the same to the bees. As bees returned, their tag IDs were recorded along with timestamps on a specialized system controller (iID®BEEcontroller, microsensys GmbH).

2022 forager resource collection. Forager sample collection occurred at the same time as both of the 2022 homing runs, but was not conducted in 2021. Weather was recorded using the same method as in the homing experiment. Marked foragers were captured in glass vials as they returned to the hive. They were brought inside and immediately euthanized in a mixture of ethanol and dry ice.

Bees were classified as pollen, nectar, or water foragers following a similar methodology to the one outlined in Prado et al. (2019). If the bee was carrying pollen, the pollen was scraped off using forceps and weighed on an analytical balance. The bee's abdomen was cut off with dissecting scissors and its crop contents were collected using 2, 5, and 10 µL microcapillary tubes (Drummond Scientific, Broomall, PA) to measure crop volume. The crop contents were then transferred to a BRIX refractometer (VLT032, V-RESOURCING) in order to measure sugar concentration. If the sugar concentration was

10 percent Brix or higher, the bee was counted as a nectar forager, otherwise, it was classified as a water forager (Prado et al., 2019).

Statistical analysis—navigation. Data were analyzed in R version 4.2.2 (R Core Team, 2022). I analyzed two dependent variables: whether the bee returned to the hive after it was released (which I refer to as probability of return) and the time it took successful bees to return. For both variables, I considered the effect of treatment group (Pristine$^{®}$ versus control), air temperature at the time of release, and hive. I analyzed the two years separately as well as combined (with year as a fixed effect in the combined models); I report both results here.

To evaluate the probability that bees successfully returned to the hive, I ran generalized linear mixed models (logit link function, binomial family) using the lme4 package (Bates et al., 2015). For the model that combined the two years, I first tested for a three-way interaction between treatment, temperature, and year, as well as for effects of all those variables separately. Hive was included as a random effect. I then dropped non-significant interactions and re-ran the model. For the models that analyzed the years separately, I tested for an interaction between treatment and temperature, as well as effects of each variable separately.

To evaluate the time it took bees to return to the hive, I ran mixed effects Cox models using the coxme package (Therneau, 2022). The procedure was the same as described above for the probability of return GLMMs. I included hive as a random effect and tested for three-way interactions between treatment, temperature, and year in the combined model. In the models for each separate year, I tested for interactions between treatment and temperature.

Statistical analysis—foraging. Data were analyzed in R version 4.2.2 (R Core Team, 2022). T-tests were performed using the t.test() function to analyze the differences in corbicular pollen mass, nectar forager crop volume, water forager crop volume, and nectar forager crop sugar concentration between treatment groups and runs. Pearson's chi-squared tests were performed using the chisq.test() function to analyze the difference in proportions of foragers carrying different resource types (water, nectar, pollen, or nothing) between treatment groups and runs.

RESULTS

Navigation—probability of return. The model that combined years produced statistically significant effects of treatment (χ^2=5.82, p=0.0158), temperature (χ^2=17.0, p<0.001), and the interaction between year and temperature (χ^2=6.87, p=0.00878). In 2021, neither treatment, temperature, nor the interaction between them produced a significant effect (Figure 1B). In 2022, treatment alone did not produce a significant effect, but temperature (χ^2=19.6, p<0.001) and the interaction between treatment and temperature (χ^2=4.62, p=0.0316, Figure 1E) did.

Navigation—time to return. The model that combined the years produced statistically significant effects of year (χ^2=13.0, p<0.001) and the interaction between treatment and temperature (χ^2=8.42, p=0.00372). In 2021, neither treatment, temperature, nor the interaction between them was significant, although the interaction produced a p-value very close to the significance threshold (χ^2=3.64, p=0.0565, Figure 1C). In 2022, neither treatment nor temperature produced a statistically significant effect on their own, but the interaction between the two was significant (χ^2=4.63, p=0.0314).

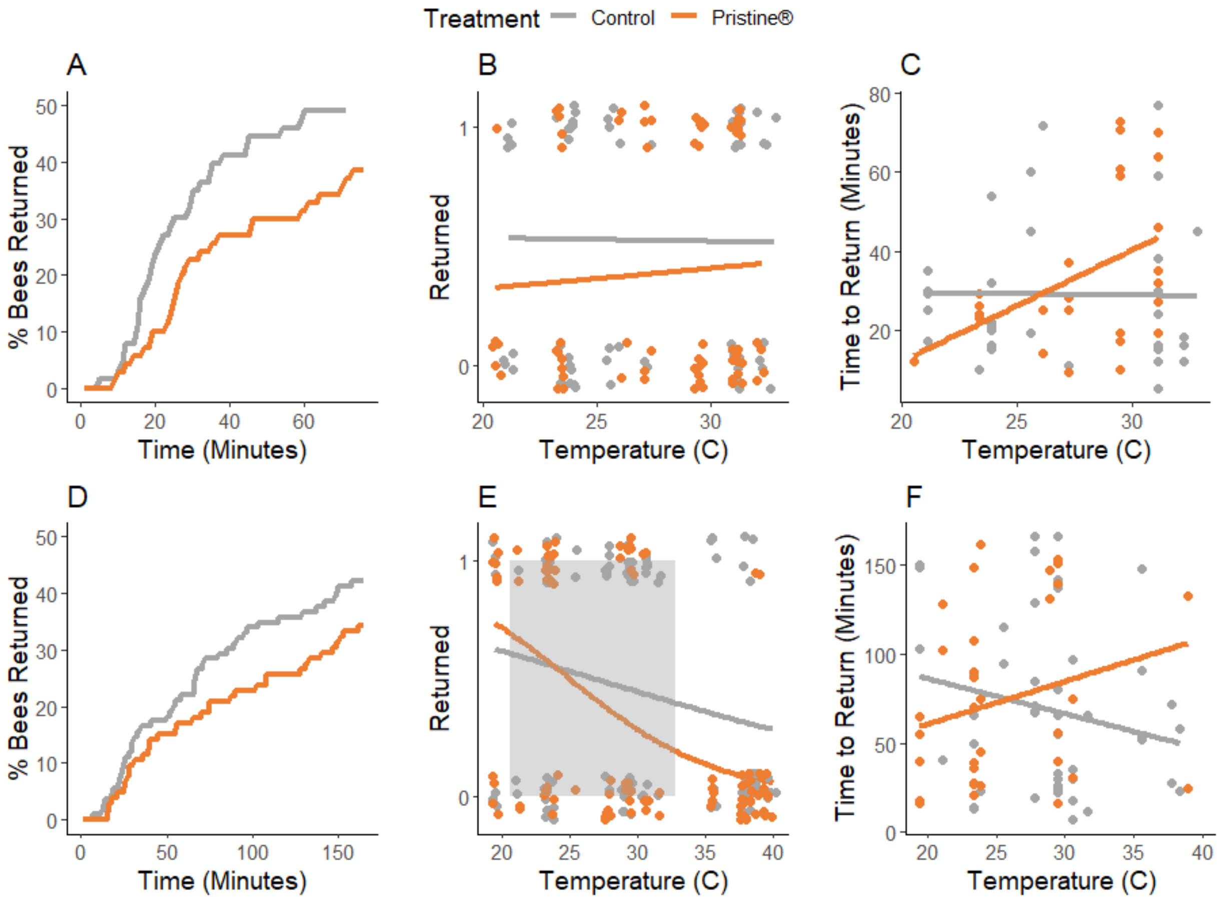


Figure 4.1. Effects of Pristine® and air temperature on homing performance during experiments in 2021 (top row) and 2022 (bottom row). ***A.*** Cumulative percentage of bees that returned to the hive over time during 2021 experiments. ***B.*** Plot showing the potential interaction between air temperature and bee probability of return in 2021. In this case, the lines do not cross, indicating that there was no interaction between temperature and treatment group. ***C.*** Plot showing the potential interaction between temperature and the time it took bees to return in 2021. In this case, the lines do cross, indicating a likely interaction (Pristine® bees took longer to return to the hive at higher temperatures). ***D.*** Cumulative percentage of bees that returned to the hive over time during 2022 experiments. E. Plot showing the interaction between temperature and probability of return in 2022. Lines cross, indicating an interaction (Pristine® bees were less likely to return to the hive at higher temperatures). The gray box represents the range of temperatures measured in 2021. ***F.*** Plot showing the interaction between temperature and time to return in 2022. Lines cross, indicating an interaction (Pristine® bees took longer to return to the hive at higher temperatures).

Foraging—pollen, water, and nectar loads. Preliminary tests confirmed that hive had no significant effects on any of the parameters measured. Run (August versus October) had no significant effects on anything except for the sugar concentration of nectar forager crop contents (t=2.25, p=0.0287), with higher concentrations recorded in August (mean=44.5 percent, 95% CI=5.86 percent) compared to October (mean=36.8 percent, 95% CI=3.76 percent).

Fungicide treatment had no significant effect on corbicular pollen mass (t=0.640, p=0.525) (Figure 2A), volume of nectar forager crop contents, (t=-0.474, p=0.637) (Figure 2B), or volume of water forager crop contents (t=-1.81, p=0.0792) (Figure 2C). Treatment did produce a significant effect on the sugar concentration of nectar forger crop contents (t=-2.31, p=0.0238) (Figure 2D), with higher concentrations recorded in the Pristine® group (mean=43.6 percent, 95% CI=4.80 percent) compared to the control group (mean=36.3 percent, 95% CI=4.35 percent).

Foraging—proportion of foragers collecting each resource type. Run (August versus October) had a significant effect on the proportion of foragers carrying nectar, water, pollen, or nothing (χ^2=22.9, p<0.001), with more pollen foragers collected in October. Treatment group (Pristine® versus control) had no significant effect on the proportion of foragers carrying each resource type (χ^2=0.420, p=0.936) (Figure 3).

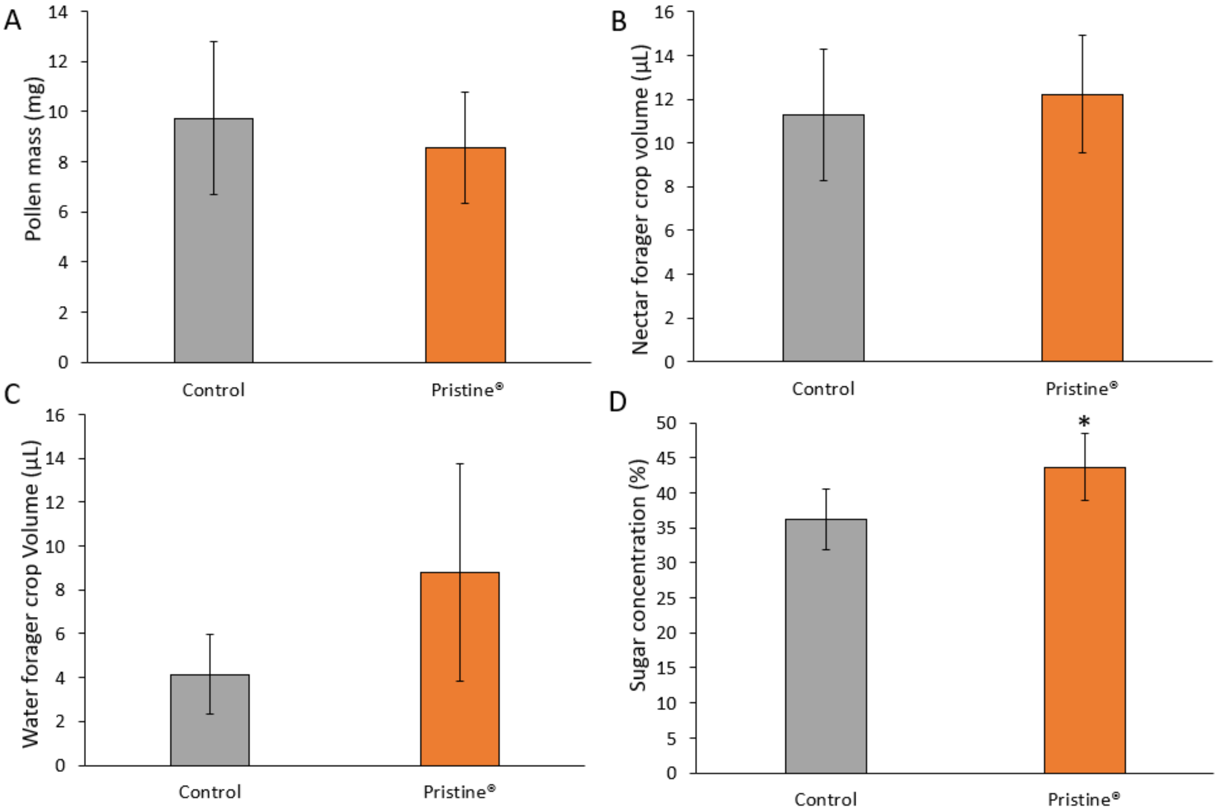


Figure 4.2. Comparisons of nectar, pollen, and water amounts brought back to the hive between control and Pristine® treatment groups. Bars indicate means, while error bars indicate 95 percent confidence intervals. Asterisks denote significant differences between groups. ***A.*** Comparison of corbicular pollen masses between groups. ***B.*** Comparison of the volume of nectar forager crop contents between groups. ***C.*** Comparison of the volume of water forager crop contents between groups. ***D.*** Comparison of the sugar concentration of nectar forager crop contents between groups.

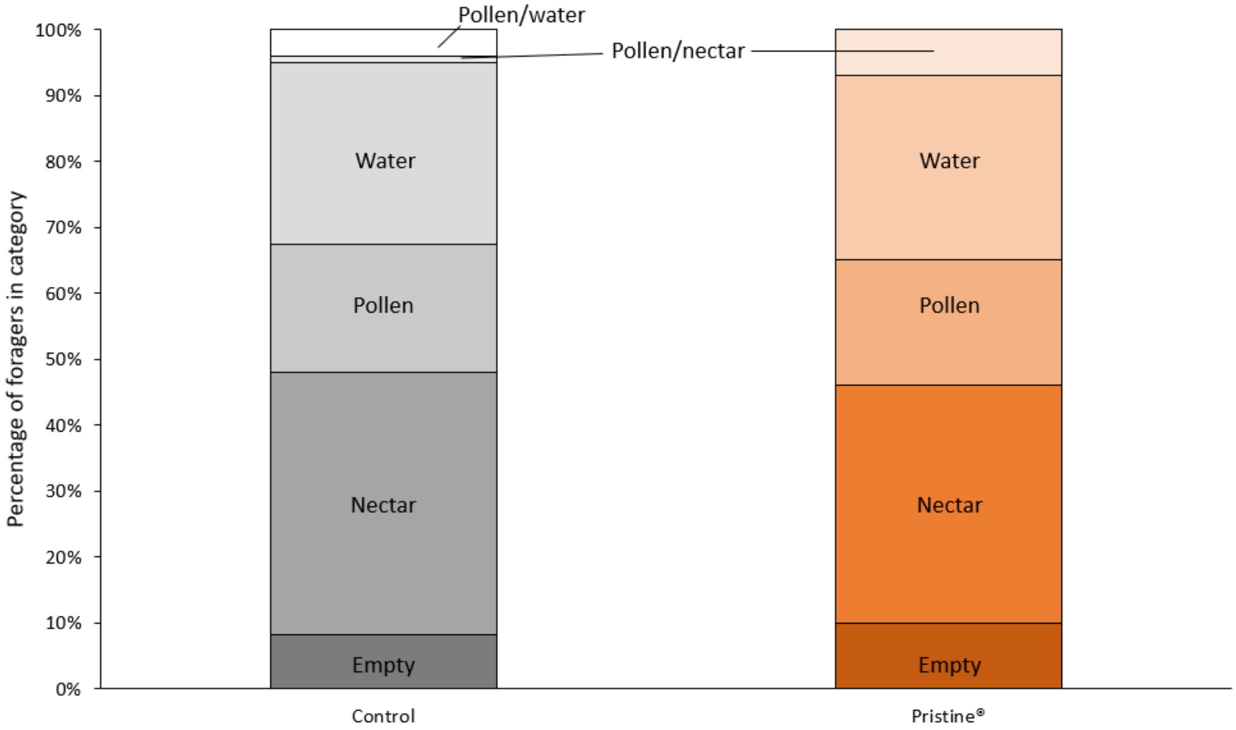


Figure 4.3. Proportions of foragers in both treatment groups devoted to each type of resource.

DISCUSSION

In the navigation experiments, I found that Pristine® exposure reduced the probability that a bee would successfully return to the colony. Although fungicide treatment alone had no significant effect when the two years were analyzed separately (Figure 1A for 2021 and 1D for 2022), the model with combined years (with a larger sample size and more power) did show a significant effect. To my knowledge, this is the first study to show a direct effect of a fungicide on homing, a critical bee behavior. Aside from the herbicide glyphosate (Sol Balbuena et al., 2015), most of the compounds found to affect honey bee homing have been neuroactive insecticides (Christen et al., 2021; Fischer et al., 2014; Henry et al., 2012; Tison et al., 2016; Van Dame et al., 1995). My study adds to a growing body of literature suggesting that Pristine® is not safe for honey bees (DeGrandi-Hoffman et al., 2015; DesJardins et al., 2021; Fisher et al., 2021a, b; Glass et al., 2021).

I also found that these homing failures were exacerbated when paired with the high temperatures typical of an Arizona summer. The model that measured probability of return with the two years combined showed a significant effect of temperature (suggesting that all bees, regardless of treatment group, were less likely to successfully return to the hive at higher temperatures), but did not show a significant interaction between treatment and temperature. There was a significant interaction between year and temperature, conveying the wider range of temperatures sampled in 2022 compared to 2021. When the two years were analyzed separately, 2022 showed a significant interaction between fungicide treatment and temperature (Figure 1E), while 2021 did not (Figure 1B). Therefore, I conclude that this effect only becomes prevalent at temperatures above 32°C, which were sampled in 2022 but not 2021.

I also found that Pristine® interacted with high temperatures to increase the amount of time it took the bees to return. This effect was significant in the model with combined years and in the 2022 model (Figure 1F). The p-value for the interaction also closely approached significance in the 2021 model (Figure 1C). I conclude that the interactive effect between fungicide treatment and temperature on time to return was present in both years; therefore, this effect is prevalent even at lower temperatures.

The negative effects of this fungicide on homing abilities at high air temperatures are worrisome given current trends of climactic warming. Pristine® has been used extensively on almond trees in California. Although temperatures are unlikely to climb as high as 32°C during the February almond bloom in California's Central Valley in the near term (with average temperatures ranging from approximately 5-15°C and all-time highs at approximately 25°C) (National Weather Service [NWS], 2023), Pristine® is used on a variety of other honey bee-pollinated crops, some of which bloom in the summer. Normal summer temperatures usually peak around 30°C in the Central Valley, but heat waves can bring temperatures in excess of 40°C (NWS, 2023). The interaction between the two stressors is interesting and signifies that this fungicide is not completely innocuous. Especially as heat waves become more frequent in California and elsewhere as a result of climate change (Pathak et al., 2018), growers and regulators should exercise caution when deciding which agrochemicals are safe to use on blooming crops.

A handful of other studies have shown interactions between pesticides and temperature extremes. Neonicotinoids reduce bee ability to survive exceptionally warm or cold temperatures (Alburaki et al., 2023; Bester et al., 2023). A recent study also found that the neonicotinoid imidacloprid interacted with high temperatures to reduce the distances that bumble bees were able to fly in a tethered flight mill (Kenna et al., 2023). My study focused on homing success, but also required bees to fly one kilometer, a distance which may have been challenging for Pristine®-exposed bees, particularly at higher temperatures. No study to date has shown an interactive effect between high temperatures and pesticides on homing success specifically, although the neonicotinoid thiamethoxam can interact with low temperatures to exacerbate honey bee homing failure (Henry et al., 2014; Monchanin et al., 2019).

One possible mechanism underlying this interaction could involve an effect of the fungicide on energetic metabolism, causing exposed bees to tire more quickly than controls, especially at high temperatures. Pristine® directly inhibits cellular respiration in isolated honey bee mitochondria (Campbell et al., 2016), and it also lowers ATP levels in honey bee flight muscles (DeGrandi-Hoffman et al., 2015). However, supporting evidence for this hypothesis is mixed, as some studies have reported no effects of Pristine® on carbon dioxide production and thorax temperatures during flight (Campbell et al., 2016). Glass et al. (2021) reported a negative effect of Pristine® on flight performance, but only when fed at 230 ppm, which is 10 times higher than the concentration used in my experiments.

Another possible mechanistic explanation for these homing results involves Pristine®'s effects on nutrient absorption. The active ingredient pyraclostrobin damages the honey bee midgut (da Costa Domingues et al., 2020; Tadei et al., 2020) and the formulation interferes with protein absorption (DeGrandi-Hoffman et al., 2015). These changes could potentially result in Pristine®-fed bees lacking the fuels necessary to sustain flight. Glass et al. (2021) also found that Pristine® bees had smaller thoraxes than controls, suggesting that flight muscles were not as well-developed in these bees.

A third possible explanation for the increase in homing failures is that bees could have reduced cognitive abilities as a result of exposure to Pristine®, high temperatures, or both. It is unknown whether Pristine® can pass through the gut and into the hemolymph, but it is plausible that it could poison mitochondria in the brain. This explanation goes along with my previous finding that Pristine® impairs olfactory associative learning performance (DesJardins et al., 2021), which provides a basic mechanism for measuring a bee's ability to learn about relevant environmental stimuli, a necessary ability for successful homing. Likewise, short-term exposure to high temperatures can impair both learning (Gérard et al., 2022a) and foraging (Gérard et al., 2022b) behaviors in bumble bees, although the underlying mechanisms remain unknown.

In the foraging experiments, foragers brought back similar masses of corbicular pollen and similar volumes of nectar and water regardless of Pristine® exposure, but foragers from the fungicide treatment group returned with more concentrated nectar than controls. Nectar was also more concentrated in August compared to October. I also observed different proportions of forager types in August compared to October, with more pollen foragers observed in October. However, I observed no differences between treatment groups. Interestingly, this differs from a previous study involving Pristine®, which found that the fungicide increased colony pollen collection and consumption (Fisher et al., 2021a). Overall, my results suggest that Pristine® does not significantly affect foraging efficiency, as Pristine® bees returned to their colonies with comparable amounts of nectar, pollen, and water and even greater concentrations of nectar when compared to controls.

The difference in sugar content of nectar is an interesting result, and is possibly suggestive of possible broader sensory effects of the fungicide. Sucrose response threshold is the lowest concentration of sucrose that will elicit proboscis extension (Page et al., 1998; Pankiw & Page, 1999, 2000). There is some precedence for fungicides affecting sucrose responsiveness in honey bees (Jiang et al., 2018). Bees with higher sucrose response thresholds may be more likely to return to the colony after foraging with higher concentrations of nectar (Page et al., 1998; Pankiw & Page, 1999), so it follows that perhaps Pristine®-exposed bees have higher sucrose response thresholds compared to controls. Despite this, a previous study found that sucrose responsiveness in a laboratory assay was not affected by Pristine® consumption (DesJardins et al., 2021). However, sucrose responsiveness in laboratory assays does not always correlate with sucrose responsiveness in the field (Mujagic & Erber, 2009), so it is still possible that the fungicide could be affecting this critical sensory trait.

Another possible explanation for the difference in nectar concentrations between the two treatment groups could be that the Pristine® bees required more concentrated nectar to successfully return to the hive, and that the Pristine® bees carrying more dilute nectar concentrations died before returning. As discussed above, Pristine® has a variety of effects on nutrient absorption (da Costa Domingues et al., 2020; DeGrandi-Hoffman et al., 2015; Tadei et al., 2020), which may result in them lacking the necessary fuels to sustain flight. Bees carrying more concentrated nectar may have used it to fuel their own return flight to the colony.

CONCLUSION

I found that the fungicide Pristine® reduced the proportion of bees that successfully returned to the colony during a homing test, and that this effect was exacerbated when paired with temperatures above 32°C. I found limited effects of the fungicide on foraging efficiency overall, although Pristine®-exposed bees returned with more concentrated nectar in their crops than controls. My results reinforce the idea that fungicides are not safe for pollinating insects (Cullen et al., 2019), even if they act via non-neurotoxic mechanisms. They also highlight the importance of testing for sublethal effects of agrochemicals in the field (Barascou et al., 2021), in part because important interactive effects become apparent, such as the interaction between Pristine® and high temperatures. Future studies should further examine the effects of Pristine® on behaviors in the field; for example, Pristine® may still have effects on the frequency and/or duration of foraging trips performed by bees that consumed the fungicide.

REFERENCES

Alburaki, M., Madella, S., & Cook, S. C. (2023). Non-optimal ambient temperatures aggravate insecticide toxicity and affect honey bees *Apis mellifera* L. gene regulation. *Scientific Reports*, *13*(1), Article 1. https://doi.org/10.1038/s41598-023-30264-0

Barascou, L., Brunet, J.-L., Belzunces, L., Decourtye, A., Henry, M., Fourrier, J., Le Conte, Y., & Alaux, C. (2021). Pesticide risk assessment in honeybees: Toward the use of behavioral and reproductive performances as assessment endpoints. *Chemosphere*, *276*, 130134. https://doi.org/10.1016/j.chemosphere.2021.130134

Barascou, L., Requier, F., Sené, D., Crauser, D., Le Conte, Y., & Alaux, C. (2022). Delayed effects of a single dose of a neurotoxic pesticide (sulfoxaflor) on honeybee foraging activity. *Science of The Total Environment*, *805*, 150351. https://doi.org/10.1016/j.scitotenv.2021.150351

Bell, W. J. (1990). Central place foraging. In W. J. Bell (Ed.), *Searching Behaviour: The behavioural ecology of finding resources* (pp. 171–187). Springer Netherlands. https://doi.org/10.1007/978-94-011-3098-1_12

Bester, L. C., Wege, M., & Pirk, C. W. W. (2023). The influence of sublethal neonicotinoid doses on individual *Apis mellifera scutellata* thermotolerance. *Apidologie*, *54*(1), 7. https://doi.org/10.1007/s13592-023-00989-y

Brown, M. J. F., Dicks, L. V., Paxton, R. J., Baldock, K. C. R., Barron, A. B., Chauzat, M.-P., Freitas, B. M., Goulson, D., Jepsen, S., Kremen, C., Li, J., Neumann, P., Pattemore, D. E., Potts, S. G., Schweiger, O., Seymour, C. L., & Stout, J. C. (2016). A horizon scan of future threats and opportunities for pollinators and pollination. *PeerJ*, *4*, e2249. https://doi.org/10.7717/peerj.2249

Bruckner, S., Wilson, M., Aurell, D., Rennich, K., vanEngelsdorp, D., Steinhauer, N., & Williams, G. R. (2023). A national survey of managed honey bee colony losses in the USA: Results from the Bee Informed Partnership for 2017–18, 2018–19, and 2019–20. *Journal of Apicultural Research*, 1–15. https://doi.org/10.1080/00218839.2022.2158586

Buehlmann, C., Mangan, M., & Graham, P. (2020). Multimodal interactions in insect navigation. *Animal Cognition*. https://doi.org/10.1007/s10071-020-01383-2

Campbell, J. B., Nath, R., Gadau, J., Fox, T., DeGrandi-Hoffman, G., & Harrison, J. F. (2016). The fungicide Pristine® inhibits mitochondrial function *in vitro* but not flight metabolic rates in honey bees. *Journal of Insect Physiology*, *86*, 11–16. https://doi.org/10.1016/j.jinsphys.2015.12.003

Chopra, S. S., Bakshi, B. R., & Khanna, V. (2015). Economic Dependence of U.S. Industrial Sectors on Animal-Mediated Pollination Service. *Environmental Science & Technology*, *49*(24), 14441–14451. https://doi.org/10.1021/acs.est.5b03788

Christen, V., Grossar, D., Charrière, J.-D., Eyer, M., & Jeker, L. (2021). Correlation Between Increased Homing Flight Duration and Altered Gene Expression in the Brain of Honey Bee Foragers After Acute Oral Exposure to Thiacloprid and Thiamethoxam. *Frontiers in Insect Science*, *0*. https://doi.org/10.3389/finsc.2021.765570

Colin, T., Forster, C. C., Westacott, J., Wu, X., Meikle, W. G., & Barron, A. B. (2021). Effects of late miticide treatments on foraging and colony productivity of European honey bees (*Apis mellifera*). *Apidologie*. https://doi.org/10.1007/s13592-020-00837-3

Colin, T., Meikle, W. G., Wu, X., & Barron, A. B. (2019). Traces of a Neonicotinoid Induce Precocious Foraging and Reduce Foraging Performance in Honey Bees. *Environmental Science & Technology*, *53*(14), 8252–8261. https://doi.org/10.1021/acs.est.9b02452

Cullen, M. G., Thompson, L. J., Carolan, J. C., Stout, J. C., & Stanley, D. A. (2019). Fungicides, herbicides and bees: A systematic review of existing research and methods. *PLOS ONE*, *14*(12), e0225743. https://doi.org/10.1371/journal.pone.0225743

da Costa Domingues, C. E., Bello Inoue, L. V., Mathias da Silva-Zacarin, E. C., & Malaspina, O. (2020). Foragers of Africanized honeybee are more sensitive to fungicide pyraclostrobin than newly emerged bees. *Environmental Pollution*, *266*, 115267. https://doi.org/10.1016/j.envpol.2020.115267

DeGrandi-Hoffman, G., Chen, Y., Watkins Dejong, E., Chambers, M. L., & Hidalgo, G. (2015). Effects of Oral Exposure to Fungicides on Honey Bee Nutrition and Virus Levels. *Journal of Economic Entomology*, *108*(6), 2518–2528. https://doi.org/10.1093/jee/tov251

DesJardins, N. S., Fisher, A., Ozturk, C., Fewell, J. H., DeGrandi-Hoffman, G., Harrison, J. F., & Smith, B. H. (2021). A common fungicide, Pristine®, impairs olfactory associative learning performance in honey bees (*Apis mellifera*). *Environmental Pollution*, 117720. https://doi.org/10.1016/j.envpol.2021.117720

Dolezal, A. G., Carrillo-Tripp, J., Judd, T. M., Allen Miller, W., Bonning, B. C., & Toth, A. L. (2019). Interacting stressors matter: Diet quality and virus infection in honeybee health. *Royal Society Open Science*, *6*(2), 181803. https://doi.org/10.1098/rsos.181803

Farina, W. M., Grüter, C., Acosta, L., & Mc Cabe, S. (2007). Honeybees learn floral odors while receiving nectar from foragers within the hive. *Naturwissenschaften*, *94*(1), 55–60. https://doi.org/10.1007/s00114-006-0157-3

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B., & Menzel, R. (2014). Neonicotinoids Interfere with Specific Components of Navigation in Honeybees. *PLOS ONE*, *9*(3), e91364. https://doi.org/10.1371/journal.pone.0091364

Fisher, A., DeGrandi-Hoffman, G., Smith, B. H., Johnson, M., Kaftanoglu, O., Cogley, T., Fewell, J. H., & Harrison, J. F. (2021a). Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*). *Environmental Pollution*, *269*, 115964. https://doi.org/10.1016/j.envpol.2020.115964

Fisher, A., DeGrandi-Hoffman, G., Smith, B. H., Ozturk, C., Kaftanoglu, O., Fewell, J. H., & Harrison, J. F. (2021b). Field cross-fostering and *in vitro* rearing demonstrate negative effects of both larval and adult exposure to a widely used fungicide in honey bees (*Apis mellifera*). *Ecotoxicology and Environmental Safety*, *217*, 112251. https://doi.org/10.1016/j.ecoenv.2021.112251

Fungicide Resistance Action Committee. (2022, March). *FRAC Code List©*2022: Fungal control agents sorted by cross-resistance pattern and mode of action (including coding for FRAC Groups on product labels).* https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2022--final.pdf?sfvrsn=b6024e9a_2

Gérard, M., Amiri, A., Cariou, B., & Baird, E. (2022a). Short-term exposure to heatwave-like temperatures affects learning and memory in bumblebees. *Global Change Biology*, *28*(14), 4251–4259. https://doi.org/10.1111/gcb.16196

Gérard, M., Cariou, B., Henrion, M., Descamps, C., & Baird, E. (2022b). Exposure to elevated temperature during development affects bumblebee foraging behavior. *Behavioral Ecology*, *33*(4), 816–824. https://doi.org/10.1093/beheco/arac045

Glass, J. R., Fisher, A., Fewell, J. H., DeGrandi-Hoffman, G., Ozturk, C., & Harrison, J. F. (2021). Consumption of field-realistic doses of a widely used mito-toxic fungicide reduces thorax mass but does not negatively impact flight capacities of the honey bee (*Apis mellifera*). *Environmental Pollution*, *274*, 116533. https://doi.org/10.1016/j.envpol.2021.116533

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957. https://doi.org/10.1126/science.1255957

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Bugeja Douglas, A., Cadahía, L., Charrière, J.-D., Chlebo, R., Coffey, M. F., Cornelissen, B., Costa, C. A. da, Danneels, E., Danihlík, J., Dobrescu, C., Evans, G., Fedoriak, M., Forsythe, I., Gregorc, A., … Brodschneider, R. (2022). Honey bee colony loss rates in 37 countries using the COLOSS survey for winter 2019–2020: The combined effects of operation size, migration and queen replacement. *Journal of Apicultural Research*, 1–7. https://doi.org/10.1080/00218839.2022.2113329

Guez, D., Zhang, S.-W., & Srinivasan, M. V. (2005). Methyl Parathion Modifies Foraging Behaviour in Honeybees (*Apis mellifera*). *Ecotoxicology*, *14*(4), 431–437. https://doi.org/10.1007/s10646-004-1348-3

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., & Decourtye, A. (2012). A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. *Science*, *336*(6079), 348–350. https://doi.org/10.1126/science.1215039

Henry, M., Bertrand, C., Le Féon, V., Requier, F., Odoux, J.-F., Aupinel, P., Bretagnolle, V., & Decourtye, A. (2014). Pesticide risk assessment in free-ranging bees is weather and landscape dependent. *Nature Communications*, *5*(1), 4359. https://doi.org/10.1038/ncomms5359

Kenna, D., Graystock, P., & Gill, R. J. (2023). Toxic temperatures: Bee behaviours exhibit divergent pesticide toxicity relationships with warming. *Global Change Biology*. https://doi.org/10.1111/gcb.16671

Khalifa, S. A. M., Elshafiey, E. H., Shetaia, A. A., El-Wahed, A. A. A., Algethami, A. F., Musharraf, S. G., AlAjmi, M. F., Zhao, C., Masry, S. H. D., Abdel-Daim, M. M., Halabi, M. F., Kai, G., Al Naggar, Y., Bishr, M., Diab, M. A. M., & El-Seedi, H. R. (2021). Overview of Bee Pollination and Its Economic Value for Crop Production. *Insects*, *12*(8), Article 8. https://doi.org/10.3390/insects12080688

Kim, S., Cho, S., & Lee, S. H. (2022). Synergistic effects of imidacloprid and high temperature on honey bee colonies. *Apidologie*, *53*(6), 67. https://doi.org/10.1007/s13592-022-00980-z

Kobayashi, N., Okada, R., & Sakura, M. (2020). Orientation to polarized light in tethered flying honeybees. *Journal of Experimental Biology*. https://doi.org/10.1242/jeb.228254

Manzi, C., Vergara-Amado, J., Franco, L. M., & Silva, A. X. (2020). The effect of temperature on candidate gene expression in the brain of honey bee *Apis mellifera* (Hymenoptera: Apidae) workers exposed to neonicotinoid imidacloprid. *Journal of Thermal Biology*, *93*, 102696. https://doi.org/10.1016/j.jtherbio.2020.102696

Mayack, C., Macherone, A., Zaki, A. G., Filiztekin, E., Özkazanç, B., Koperly, Y., Schick, S. J., Eppley, E. J., Deb, M., Ambiel, N., Schafsnitz, A. M., & Broadrup, R. L. (2022). Environmental exposures associated with honey bee health. *Chemosphere*, *286*, 131948. https://doi.org/10.1016/j.chemosphere.2021.131948

Medina, R. G., Paxton, R. J., De Luna, E., Fleites-Ayil, F. A., Medina Medina, L. A., & Quezada-Euán, J. J. G. (2018). Developmental stability, age at onset of foraging and longevity of Africanized honey bees (*Apis mellifera* L.) under heat stress (Hymenoptera: Apidae). *Journal of Thermal Biology*, *74*, 214–225. https://doi.org/10.1016/j.jtherbio.2018.04.003

Menzel, R., De Marco, R. J., & Greggers, U. (2006). Spatial memory, navigation and dance behaviour in *Apis mellifera*. *Journal of Comparative Physiology A*, *192*(9), 889–903. https://doi.org/10.1007/s00359-006-0136-3

Monchanin, C., Henry, M., Decourtye, A., Dalmon, A., Fortini, D., Bœuf, E., Dubuisson, L., Aupinel, P., Chevallereau, C., Petit, J., & Fourrier, J. (2019). Hazard of a neonicotinoid insecticide on the homing flight of the honeybee depends on climatic conditions and *Varroa* infestation. *Chemosphere*, *224*, 360–368. https://doi.org/10.1016/j.chemosphere.2019.02.129

National Weather Service (2023). *Central and Southern San Joaquin Valley Climate Graphs*. NOAA’s National Weather Service. Retrieved April 5, 2023, from https://www.weather.gov/hnx/Cliplot

Page, R. E., Erber, J., & Fondrk, M. K. (1998). The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (Apis mellifera L.). *Journal of Comparative Physiology A*, *182*(4), 489–500. https://doi.org/10.1007/s003590050196

Pahl, M., Zhu, H., Tautz, J., & Zhang, S. (2011). Large Scale Homing in Honeybees. *PLOS ONE*, *6*(5), e19669. https://doi.org/10.1371/journal.pone.0019669

Pankiw, T., & Page Jr., R. E. (1999). The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (Apis mellifera L.). *Journal of Comparative Physiology A*, *185*(2), 207–213. https://doi.org/10.1007/s003590050379

Pankiw, T., & Page Jr, R. E. (2000). Response thresholds to sucrose predict foraging division of labor in honeybees. *Behavioral Ecology and Sociobiology*, *47*(4), 265–267. https://doi.org/10.1007/s002650050664

Pathak, T. B., Maskey, M. L., Dahlberg, J. A., Kearns, F., Bali, K. M., & Zaccaria, D. (2018). Climate Change Trends and Impacts on California Agriculture: A Detailed Review. *Agronomy*, *8*(3), Article 3. https://doi.org/10.3390/agronomy8030025

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology & Evolution*, *25*(6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007

Prado, A., Pioz, M., Vidau, C., Requier, F., Jury, M., Crauser, D., Brunet, J.-L., Le Conte, Y., & Alaux, C. (2019). Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees. *Science of The Total Environment*, *650*, 1250–1260. https://doi.org/10.1016/j.scitotenv.2018.09.102

Reilly, J. R., Artz, D. R., Biddinger, D., Bobiwash, K., Boyle, N. K., Brittain, C., Brokaw, J., Campbell, J. W., Daniels, J., Elle, E., Ellis, J. D., Fleischer, S. J., Gibbs, J., Gillespie, R. L., Gundersen, K. B., Gut, L., Hoffman, G., Joshi, N., Lundin, O., … Winfree, R. (2020). Crop production in the USA is frequently limited by a lack of pollinators. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1931), 20200922. https://doi.org/10.1098/rspb.2020.0922

Schneider, C. W., Tautz, J., Grünewald, B., & Fuchs, S. (2012). RFID Tracking of Sublethal Effects of Two Neonicotinoid Insecticides on the Foraging Behavior of *Apis mellifera*. *PLOS ONE*, *7*(1), e30023. https://doi.org/10.1371/journal.pone.0030023

Shi, J., Yang, H., Yu, L., Liao, C., Liu, Y., Jin, M., Yan, W., & Wu, X. B. (2020). Sublethal acetamiprid doses negatively affect the lifespans and foraging behaviors of honey bee (*Apis mellifera* L.) workers. *Science of The Total Environment*, *738*, 139924. https://doi.org/10.1016/j.scitotenv.2020.139924

Si, A., Srinivasan, M. V., & Zhang, S. (2003). Honeybee navigation: Properties of the visually driven `odometer’. *Journal of Experimental Biology*, *206*(8), 1265–1273. https://doi.org/10.1242/jeb.00236

Siviter, H., Koricheva, J., Brown, M. J. F., & Leadbeater, E. (2018). Quantifying the impact of pesticides on learning and memory in bees. *Journal of Applied Ecology*, *55*(6), 2812–2821. https://doi.org/10.1111/1365-2664.13193

Sol Balbuena, M., Tison, L., Hahn, M.-L., Greggers, U., Menzel, R., & Farina, W. M. (2015). Effects of sublethal doses of glyphosate on honeybee navigation. *Journal of Experimental Biology*, *218*(17), 2799–2805. https://doi.org/10.1242/jeb.117291

Steffan-Dewenter, I., & Kuhn, A. (2003). Honeybee foraging in differentially structured landscapes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1515), 569–575. https://doi.org/10.1098/rspb.2002.2292

Tadei, R., Menezes-Oliveira, V. B., & Silva-Zacarin, E. C. M. (2020). Silent effect of the fungicide pyraclostrobin on the larval exposure of the non-target organism Africanized *Apis mellifera* and its interaction with the pathogen *Nosema ceranae* in adulthood. *Environmental Pollution*, *267*, 115622. https://doi.org/10.1016/j.envpol.2020.115622

Tison, L., Hahn, M.-L., Holtz, S., Rößner, A., Greggers, U., Bischoff, G., & Menzel, R. (2016). Honey Bees' Behavior Is Impaired by Chronic Exposure to the Neonicotinoid Thiacloprid in the Field. *Environmental Science & Technology*, *50*(13), 7218–7227. https://doi.org/10.1021/acs.est.6b02658

Therneau, T.M. (2022). coxme: Mixed Effects Cox Models. R package version 2.2-18.1. https://CRAN.R-project.org/package=coxme

Van Dame, R., Meled, M., Colin, M.-E., & Belzunces, L. P. (1995). Alteration of the homing-flight in the honey bee *Apis mellifera* L. Exposed to sublethal dose of deltamethrin. *Environmental Toxicology and Chemistry*, *14*(5), 855–860. https://doi.org/10.1002/etc.5620140517

CHAPTER 5

CONCLUSIONS

My dissertation work investigated the effects of the fungicide formulation Pristine® (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin) on the behavior and physiology of honey bees. First, I found that Pristine® impaired associative learning performance in the Proboscis Extension Reflex (PER) assay (Chapter 2; DesJardins et al., 2021). I found altered carbohydrate absorption in bees that had consumed Pristine®, suggesting that the fungicide's negative effects on learning performance could be related to disrupted post-ingestive feedback mechanisms (Chapter 3). I found that Pristine® interacted with hot summer temperatures to reduce homing performance, as exposed bees were less likely to return to the hive than controls. I also found that Pristine® bees returned to the hive with more concentrated nectar than controls, again, possibly due to the fungicide's effects on nutrient absorption (Chapter 4).

Via a literature review, I concluded that learning studies are useful proxies for assessing whether foraging and navigation behaviors in the field may be impaired in honey bees that have been exposed to toxins. However, lab learning studies that are paired with data from field foraging and navigation studies are more likely to influence policymakers and lead to changes in pesticide regulations (Chapter 1). I therefore paired my lab-based learning study with field-based foraging and navigation experiments, which demonstrated that Pristine® also produces negative effects on honey bee behaviors under natural field conditions.

My experiments contribute to a substantial body of literature that shows a variety of lethal and sublethal effects of Pristine® and its active ingredients on honey bees.

Pristine® inhibits honey bee mitochondrial function *in vitro* (Campbell et al., 2016). It reduces pollen consumption, protein absorption, and flight muscle ATP concentrations and increases virus titers (DeGrandi-Hoffman et al., 2015). In field colonies, it induces precocious foraging and reduces worker longevity, which leads to reduced adult worker populations and a lower probability of overwintering survival (Fisher et al., 2021). Pristine® also reduces thorax mass at field-relevant concentrations and flight performance at higher-than-field-relevant concentrations (Glass et al., 2021). Boscalid reduces wingbeat frequency during flight (Liao et al., 2019), and pyraclostrobin produces changes in midgut morphophysiology (da Costa Domingues et al., 2020; Tadei et al., 2020). Based on this relatively large body of evidence, I conclude that Pristine® degrades a number of critical bee behaviors, causing decreased colony fitness.

These results are significant, as Pristine® is registered for use on a variety of bee-pollinated fruit, vegetable, and nut crops (BASF, 2022). Pristine® has been the focus of so much honey bee toxicology research because it has been used during bloom on almonds (Fisher et al., 2021), which depend very heavily on honey bees for pollination (Lee et al., 2019; Reilly et al., 2020). Pristine® is also used on blueberries during bloom. A study conducted during blueberry pollination reported the active ingredients of Pristine in large proportions in corbicular pollen, wax, bee body, and flower samples (Graham et al., 2022). The active ingredients of Pristine® have also been found in bee bread, honey, and wax samples in a number of other studies (Mullin et al., 2010; Ostiguy et al., 2019; Rondeau & Raine, 2022; Traynor et al., 2016, 2021).

Given that Pristine® can significantly affect the health and functioning of honey bee colonies, and that bees are likely to be exposed as they forage on treated crops, should this fungicide be banned? There would be both benefits and drawbacks to this approach. If Pristine® continues to be used and colony losses increase as a result, beekeepers will face rising operational costs. This will drive up pollination fees (Baylis et al., 2021; Lee et al., 2019), therefore leading to increased costs for growers. This has already been happening to some degree; pollination fees have risen since Colony Collapse Disorder was first reported in 2006 (Baylis et al., 2021). However, growers would likely also face decreased yields (and revenue) if Pristine® was banned, as their crops would be more vulnerable to fungal diseases. Severe yield losses would lead to crop shortages, which would have negative implications for human nutrition and well-being (Cooper & Dobson, 2007). Because of this, I do not recommend banning Pristine® outright; rather, I recommend that various stakeholders work together to ameliorate the negative effects and find alternatives.

Beekeepers and growers can both implement strategies to mitigate the effects of pesticides on pollinators. Growers can avoid spraying during bloom whenever possible, use more modern and efficient spraying technologies (allowing for less drift and more targeted applications), use lower doses (since many of the reported negative effects of Pristine® are dose-dependent), and implement Integrated Pest Management (IPM) strategies (Zhang et al., 2023). IPM strategies are especially promising—for example, one study focused on watermelon IPM resulted in dramatically decreased pesticide use and increased yields, which were attributed to increased pollinator visitation (Pecenka et al., 2021). Beekeepers can temporarily move hives away from areas that are being sprayed or block off entrances to stop foraging (Zhang et al., 2023). There are also promising dietary supplements that may be able to aid bees in detoxification—for example, quercetin can mitigate some of boscalid’s negative effects (Liao et al., 2019). Beekeepers can also work to keep their hives as healthy as possible (well-nourished and disease-free), as other stressors can interact with agrochemicals to increase the chances of negative outcomes (Mayack et al., 2022).

Indeed, widespread agrochemical use is only one potential cause of pollinator decline, and it should not be considered in isolation. Other likely causes include agricultural intensification and habitat loss causing nutritional stress, parasites and pathogens, invasive species, and climate change (Brown et al., 2016; Dicks et al., 2021; Goulson et al., 2015; Insolia et al., 2022; Potts et al., 2010; Wagner, 2020). The world’s economic dependence on pollinators is significant (Chopra et al., 2015; Jordan et al., 2021; Lippert et al., 2021), and although rising managed honey bee stocks have been able to keep up with demand so far (Baylis et al., 2021; Calderone, 2012), our global dependence on pollinators is increasing and may become unsustainable in the future (Aizen et al., 2019; Phiri et al., 2022). The impacts of pesticides and other stressors should also be more closely examined in wild bees, which contribute more than honey bees to pollination of certain crops (such as apples and cherries in the US) (Reilly et al., 2020). Scientists should work more closely with growers and beekeepers to consider how all of these stressors interact in order to avoid further pollinator losses which could have catastrophic effects on food security and human health (Gallai et al., 2009; López-Uribe et al., 2020; Smith et al., 2022).

REFERENCES

Aizen, M. A., Aguiar, S., Biesmeijer, J. C., Garibaldi, L. A., Inouye, D. W., Jung, C., Martins, D. J., Medel, R., Morales, C. L., Ngo, H., Pauw, A., Paxton, R. J., Sáez, A., & Seymour, C. L. (2019). Global agricultural productivity is threatened by increasing pollinator dependence without a parallel increase in crop diversification. *Global Change Biology*, *25*(10), 3516–3527. https://doi.org/10.1111/gcb.14736

BASF. (2022). *Pristine*®*: Specimen label*. https://www.cdms.net/ldat/ld6DD004.pdf

Baylis, K., Lichtenberg, E. M., & Lichtenberg, E. (2021). Economics of Pollination. *Annual Review of Resource Economics*. https://doi.org/10.1146/annurev-resource-101420-110406

Brown, M. J. F., Dicks, L. V., Paxton, R. J., Baldock, K. C. R., Barron, A. B., Chauzat, M.-P., Freitas, B. M., Goulson, D., Jepsen, S., Kremen, C., Li, J., Neumann, P., Pattemore, D. E., Potts, S. G., Schweiger, O., Seymour, C. L., & Stout, J. C. (2016). A horizon scan of future threats and opportunities for pollinators and pollination. *PeerJ*, *4*, e2249. https://doi.org/10.7717/peerj.2249

Calderone, N. W. (2012). Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. *PLOS ONE*, *7*(5), e37235. https://doi.org/10.1371/journal.pone.0037235

Campbell, J. B., Nath, R., Gadau, J., Fox, T., DeGrandi-Hoffman, G., & Harrison, J. F. (2016). The fungicide Pristine® inhibits mitochondrial function *in vitro* but not flight metabolic rates in honey bees. *Journal of Insect Physiology*, *86*, 11–16. https://doi.org/10.1016/j.jinsphys.2015.12.003

Chopra, S. S., Bakshi, B. R., & Khanna, V. (2015). Economic Dependence of U.S. Industrial Sectors on Animal-Mediated Pollination Service. *Environmental Science & Technology*, *49*(24), 14441–14451. https://doi.org/10.1021/acs.est.5b03788

Cooper, J., & Dobson, H. (2007). The benefits of pesticides to mankind and the environment. *Crop Protection*, *26*(9), 1337–1348. https://doi.org/10.1016/j.cropro.2007.03.022

da Costa Domingues, C. E., Bello Inoue, L. V., Mathias da Silva-Zacarin, E. C., & Malaspina, O. (2020). Foragers of Africanized honeybee are more sensitive to fungicide pyraclostrobin than newly emerged bees. *Environmental Pollution*, *266*, 115267. https://doi.org/10.1016/j.envpol.2020.115267

DeGrandi-Hoffman, G., Chen, Y., Watkins Dejong, E., Chambers, M. L., & Hidalgo, G. (2015). Effects of Oral Exposure to Fungicides on Honey Bee Nutrition and Virus Levels. *Journal of Economic Entomology*, *108*(6), 2518–2528. https://doi.org/10.1093/jee/tov251

DesJardins, N. S., Fisher, A., Ozturk, C., Fewell, J. H., DeGrandi-Hoffman, G., Harrison, J. F., & Smith, B. H. (2021). A common fungicide, Pristine®, impairs olfactory associative learning performance in honey bees (*Apis mellifera*). *Environmental Pollution*, 117720. https://doi.org/10.1016/j.envpol.2021.117720

Dicks, L. V., Breeze, T. D., Ngo, H. T., Senapathi, D., An, J., Aizen, M. A., Basu, P., Buchori, D., Galetto, L., Garibaldi, L. A., Gemmill-Herren, B., Howlett, B. G., Imperatriz-Fonseca, V. L., Johnson, S. D., Kovács-Hostyánszki, A., Kwon, Y. J., Lattorff, H. M. G., Lungharwo, T., Seymour, C. L., … Potts, S. G. (2021). A global-scale expert assessment of drivers and risks associated with pollinator decline. *Nature Ecology & Evolution*, *5*(10), Article 10. https://doi.org/10.1038/s41559-021-01534-9

Fisher, A., DeGrandi-Hoffman, G., Smith, B. H., Johnson, M., Kaftanoglu, O., Cogley, T., Fewell, J. H., & Harrison, J. F. (2021). Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*). *Environmental Pollution*, *269*, 115964. https://doi.org/10.1016/j.envpol.2020.115964

Gallai, N., Salles, J.-M., Settele, J., & Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, *68*(3), 810–821. https://doi.org/10.1016/j.ecolecon.2008.06.014

Glass, J. R., Fisher, A., Fewell, J. H., DeGrandi-Hoffman, G., Ozturk, C., & Harrison, J. F. (2021). Consumption of field-realistic doses of a widely used mito-toxic fungicide reduces thorax mass but does not negatively impact flight capacities of the honey bee (*Apis mellifera*). *Environmental Pollution*, *274*, 116533. https://doi.org/10.1016/j.envpol.2021.116533

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957. https://doi.org/10.1126/science.1255957

Graham, K. K., Milbrath, M. O., Zhang, Y., Baert, N., McArt, S., & Isaacs, R. (2022). Pesticide risk to managed bees during blueberry pollination is primarily driven by off-farm exposures. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-11156-1

Insolia, L., Molinari, R., Rogers, S. R., Williams, G. R., Chiaromonte, F., & Calovi, M. (2022). Honey bee colony loss linked to parasites, pesticides and extreme weather across the United States. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-24946-4

Jordan, A., Patch, H. M., Grozinger, C. M., & Khanna, V. (2021). Economic Dependence and Vulnerability of United States Agricultural Sector on Insect-Mediated Pollination Service. *Environmental Science & Technology*, *55*(4), 2243–2253. https://doi.org/10.1021/acs.est.0c04786

Lee, H., Sumner, D. A., & Champetier, A. (2019). Pollination Markets and the Coupled Futures of Almonds and Honey Bees: Simulating Impacts of Shifts in Demands and Costs. *American Journal of Agricultural Economics*, *101*(1), 230–249. https://doi.org/10.1093/ajae/aay063

Liao, L.-H., Wu, W.-Y., Dad, A., & Berenbaum, M. R. (2019). Fungicide suppression of flight performance in the honeybee (*Apis mellifera*) and its amelioration by quercetin. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1917), 20192041. https://doi.org/10.1098/rspb.2019.2041

Lippert, C., Feuerbacher, A., & Narjes, M. (2021). Revisiting the economic valuation of agricultural losses due to large-scale changes in pollinator populations. *Ecological Economics*, *180*, 106860. https://doi.org/10.1016/j.ecolecon.2020.106860

López-Uribe, M. M., Ricigliano, V. A., & Simone-Finstrom, M. (2020). Defining Pollinator Health: A Holistic Approach Based on Ecological, Genetic, and Physiological Factors. *Annual Review of Animal Biosciences*, *8*(1), 269–294. https://doi.org/10.1146/annurev-animal-020518-115045

Mayack, C., Macherone, A., Zaki, A. G., Filiztekin, E., Özkazanç, B., Koperly, Y., Schick, S. J., Eppley, E. J., Deb, M., Ambiel, N., Schafsnitz, A. M., & Broadrup, R. L. (2022). Environmental exposures associated with honey bee health. *Chemosphere*, *286*, 131948. https://doi.org/10.1016/j.chemosphere.2021.131948

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., & Pettis, J. S. (2010). High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLOS ONE*, *5*(3), e9754. https://doi.org/10.1371/journal.pone.0009754

Ostiguy, N., Drummond, F. A., Aronstein, K., Eitzer, B., Ellis, J. D., Spivak, M., & Sheppard, W. S. (2019). Honey Bee Exposure to Pesticides: A Four-Year Nationwide Study. *Insects*, *10*(1), Article 1. https://doi.org/10.3390/insects10010013

Pecenka, J. R., Ingwell, L. L., Foster, R. E., Krupke, C. H., & Kaplan, I. (2021). IPM reduces insecticide applications by 95% while maintaining or enhancing crop yields through wild pollinator conservation. *Proceedings of the National Academy of Sciences, 118*(44). https://doi.org/10.1073/pnas.2108429118

Phiri, B. J., Fèvre, D., & Hidano, A. (2022). Uptrend in global managed honey bee colonies and production based on a six-decade viewpoint, 1961–2017. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-25290-3

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology & Evolution*, *25*(6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007

Reilly, J. R., Artz, D. R., Biddinger, D., Bobiwash, K., Boyle, N. K., Brittain, C., Brokaw, J., Campbell, J. W., Daniels, J., Elle, E., Ellis, J. D., Fleischer, S. J., Gibbs, J., Gillespie, R. L., Gundersen, K. B., Gut, L., Hoffman, G., Joshi, N., Lundin, O., … Winfree, R. (2020). Crop production in the USA is frequently limited by a lack of pollinators. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1931), 20200922. https://doi.org/10.1098/rspb.2020.0922

Rondeau, S., & Raine, N. E. (2022). Fungicides and bees: A review of exposure and risk. *Environment International*, *165*, 107311. https://doi.org/10.1016/j.envint.2022.107311

Smith, M. R., Mueller, N. D., Springmann, M., Sulser, T. B., Garibaldi, L. A., Gerber, J., Wiebe, K., & Myers, S. S. (2022). Pollinator Deficits, Food Consumption, and Consequences for Human Health: A Modeling Study. *Environmental Health Perspectives*, *130*(12), 127003. https://doi.org/10.1289/EHP10947

Tadei, R., Menezes-Oliveira, V. B., & Silva-Zacarin, E. C. M. (2020). Silent effect of the fungicide pyraclostrobin on the larval exposure of the non-target organism Africanized *Apis mellifera* and its interaction with the pathogen *Nosema ceranae* in adulthood. *Environmental Pollution*, *267*, 115622. https://doi.org/10.1016/j.envpol.2020.115622

Traynor, K. S., Pettis, J. S., Tarpy, D. R., Mullin, C. A., Frazier, J. L., Frazier, M., & vanEngelsdorp, D. (2016). In-hive Pesticide Exposome: Assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. *Scientific Reports*, *6*(1), 1–16. https://doi.org/10.1038/srep33207

Traynor, K. S., Tosi, S., Rennich, K., Steinhauer, N., Forsgren, E., Rose, R., Kunkel, G., Madella, S., Lopez, D., Eversole, H., Fahey, R., Pettis, J., Evans, J. D., & vanEngelsdorp, D. (2021). Pesticides in honey bee colonies: Establishing a baseline for real world exposure over seven years in the USA. *Environmental Pollution*, *279*, 116566. https://doi.org/10.1016/j.envpol.2021.116566

Wagner, D. L. (2020). Insect Declines in the Anthropocene. *Annual Review of Entomology*, *65*(1), 457–480. https://doi.org/10.1146/annurev-ento-011019-025151

Zhang, G., Olsson, R. L., & Hopkins, B. K. (2023). Strategies and techniques to mitigate the negative impacts of pesticide exposure to honey bees. *Environmental Pollution*, *318*, 120915. https://doi.org/10.1016/j.envpol.2022.120915

REFERENCES

Abramson, C. I., Aquino, I. S., Ramalho, F. S., & Price, J. M. (1999). The Effect of Insecticides on Learning in the Africanized Honey Bee (*Apis mellifera* L.). *Archives of Environmental Contamination and Toxicology*, *37*(4), 529–535. https://doi.org/10.1007/s002449900548

Abramson, C. I., Singleton, J. B., Wilson, M. K., Wanderley, P. A., Ramalho, F. S., & Michaluk, L. M. (2006). The Effect of an Organic Pesticide on Mortality and Learning in Africanized Honey Bees (*Apis mellifera* L.) in Brasil. *American Journal of Environmental Sciences*, *2*(1), 33–40. https://doi.org/10.3844/ajessp.2006.33.40

Abramson, C. I., Sokolowski, M. B. C., Brown, E. A., & Pilard, S. (2012). The effect of pymetrozine (Plenum WG-50®) on proboscis extension conditioning in honey bees (*Apis mellifera*: Hybrid var. Buckfast). *Ecotoxicology and Environmental Safety*, *78*, 287–295. https://doi.org/10.1016/j.ecoenv.2011.11.038

Abramson, C. I., Squire, J., Sheridan, A., & Mulder, P. G. (2004). The Effect of Insecticides Considered Harmless to Honey Bees (*Apis mellifera*): Proboscis Conditioning Studies by Using the Insect Growth Regulators Tebufenozide and Diflubenzuron. *Environmental Entomology*, *33*(2), 378–388. https://doi.org/10.1603/0046-225X-33.2.378

Aizen, M. A., Aguiar, S., Biesmeijer, J. C., Garibaldi, L. A., Inouye, D. W., Jung, C., Martins, D. J., Medel, R., Morales, C. L., Ngo, H., Pauw, A., Paxton, R. J., Sáez, A., & Seymour, C. L. (2019). Global agricultural productivity is threatened by increasing pollinator dependence without a parallel increase in crop diversification. *Global Change Biology*, *25*(10), 3516–3527. https://doi.org/10.1111/gcb.14736

Aizen, M. A., & Harder, L. D. (2009). The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. *Current Biology*, *19*(11), 915–918. https://doi.org/10.1016/j.cub.2009.03.071

Al Naggar, Y., Wiseman, S., Sun, J., Cutler, G. C., Aboul-Soud, M., Naiem, E., Mona, M., Seif, A., & Giesy, J. P. (2015). Effects of environmentally-relevant mixtures of four common organophosphorus insecticides on the honey bee (*Apis mellifera* L.). *Journal of Insect Physiology*, *82*, 85–91. https://doi.org/10.1016/j.jinsphys.2015.09.004

Alburaki, M., Madella, S., & Cook, S. C. (2023). Non-optimal ambient temperatures aggravate insecticide toxicity and affect honey bees *Apis mellifera* L. gene regulation. *Scientific Reports*, *13*(1), Article 1. https://doi.org/10.1038/s41598-023-30264-0

Aliferis, K. A., Copley, T., & Jabaji, S. (2012). Gas chromatography–mass spectrometry metabolite profiling of worker honey bee (*Apis mellifera* L.) hemolymph for the study of *Nosema ceranae* infection. *Journal of Insect Physiology*, *58*(10), 1349–1359. https://doi.org/10.1016/j.jinsphys.2012.07.010

Aliouane, Y., El Hassani, A. K., Gary, V., Armengaud, C., Lambin, M., & Gauthier, M. (2009). Subchronic exposure of honeybees to sublethal doses of pesticides: Effects on behavior. *Environmental Toxicology and Chemistry*, *28*(1), 113–122. https://doi.org/10.1897/08-110.1

Alkassab, A. T., & Kirchner, W. H. (2016). Impacts of chronic sublethal exposure to clothianidin on winter honeybees. *Ecotoxicology*, *25*(5), 1000–1010. https://doi.org/10.1007/s10646-016-1657-3

Alma, A. M., de Groot, G. S., & Buteler, M. (2023). Microplastics incorporated by honeybees from food are transferred to honey, wax and larvae. *Environmental Pollution*, *320*, 121078. https://doi.org/10.1016/j.envpol.2023.121078

Arenas, A., Fernández, V. M., & Farina, W. M. (2007). Floral odor learning within the hive affects honeybees’ foraging decisions. *Naturwissenschaften*, *94*(3), 218–222. https://doi.org/10.1007/s00114-006-0176-0

Arslan, A., Standifer, L. N., & Don, H. (1986). Carbohydrates in honey bee hemolymph. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *84*(3), 363–367. https://doi.org/10.1016/0305-0491(86)90090-8

Artz, D. R., & Pitts-Singer, T. L. (2015). Effects of Fungicide and Adjuvant Sprays on Nesting Behavior in Two Managed Solitary Bees, *Osmia lignaria* and *Megachile rotundata*. *PLOS ONE*, *10*(8), e0135688. https://doi.org/10.1371/journal.pone.0135688

Auteri, D., Arena, M., Barmaz, S., Ippolito, A., Linguadoca, A., Molnar, T., Sharp, R., Szentes, C., Vagenende, B., & Verani, A. (2017). Neonicotinoids and bees: The case of the European regulatory risk assessment. *Science of The Total Environment*, *579*, 966–971. https://doi.org/10.1016/j.scitotenv.2016.10.158

Babendreier, D., Kalberer, N., Romeis, J., Fluri, P., & Bigler, F. (2004). Pollen consumption in honey bee larvae: A step forward in the risk assessment of transgenic plants. *Apidologie*, *35*(3), 293–300. https://doi.org/10.1051/apido:2004016

Balzani, P., Galeotti, G., Scheggi, S., Masoni, A., Santini, G., & Baracchi, D. (2022). Acute and chronic ingestion of polyethylene (PE) microplastics has mild effects on honey bee health and cognition. *Environmental Pollution*, *305*, 119318. https://doi.org/10.1016/j.envpol.2022.119318

Barascou, L., Brunet, J.-L., Belzunces, L., Decourtye, A., Henry, M., Fourrier, J., Le Conte, Y., & Alaux, C. (2021). Pesticide risk assessment in honeybees: Toward the use of behavioral and reproductive performances as assessment endpoints. *Chemosphere*, *276*, 130134. https://doi.org/10.1016/j.chemosphere.2021.130134

Barascou, L., Requier, F., Sené, D., Crauser, D., Le Conte, Y., & Alaux, C. (2022). Delayed effects of a single dose of a neurotoxic pesticide (sulfoxaflor) on honeybee foraging activity. *Science of The Total Environment*, *805*, 150351. https://doi.org/10.1016/j.scitotenv.2021.150351

BASF. (2019). *Pristine®: Specimen label*. http://www.cdms.net/ldat/ld6DD016.pdf

BASF. (2022). *Pristine®: Specimen label*. https://www.cdms.net/ldat/ld6DD004.pdf

Baylis, K., Lichtenberg, E. M., & Lichtenberg, E. (2021). Economics of Pollination. *Annual Review of Resource Economics*. https://doi.org/10.1146/annurev-resource-101420-110406

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Begna, T., & Jung, C. (2021). Effects of sequential exposures of sub-lethal doses of amitraz and thiacloprid on learning and memory of honey bee foragers, *Apis mellifera*. *Journal of Asia-Pacific Entomology*, *24*(2), 77–83. https://doi.org/10.1016/j.aspen.2021.03.012

Bell, H. C., Montgomery, C. N., Benavides, J. E., & Nieh, J. C. (2020). Effects of *Nosema ceranae* (Dissociodihaplophasida: Nosematidae) and Flupyradifurone on Olfactory Learning in Honey Bees, *Apis mellifera* (Hymenoptera: Apidae). *Journal of Insect Science*, *20*(6), 29. https://doi.org/10.1093/jisesa/ieaa130

Bell, W. J. (1990). Central place foraging. In W. J. Bell (Ed.), *Searching Behaviour: The behavioural ecology of finding resources* (pp. 171–187). Springer Netherlands. https://doi.org/10.1007/978-94-011-3098-1_12

Belzunces, L. P., Tchamitchian, S., & Brunet, J.-L. (2012). Neural effects of insecticides in the honey bee. *Apidologie*, *43*(3), 348–370. https://doi.org/10.1007/s13592-012-0134-0

Benatar, S. T., Cobey, S., & Smith, B. H. (1995). Selection on a haploid genotype for discrimination learning performance: Correlation between drone honey bees (*Apis mellifera*) and their worker progeny (Hymenoptera: Apidae). *Journal of Insect Behavior*, *8*(5), 637–652. https://doi.org/10.1007/BF01997235

Bernadou, A., Démares, F., Couret-Fauvel, T., Sandoz, J. C., & Gauthier, M. (2009). Effect of fipronil on side-specific antennal tactile learning in the honeybee. *Journal of Insect Physiology*, *55*(12), 1099–1106. https://doi.org/10.1016/j.jinsphys.2009.08.019

Bester, L. C., Wege, M., & Pirk, C. W. W. (2023). The influence of sublethal neonicotinoid doses on individual *Apis mellifera scutellata* thermotolerance. *Apidologie*, *54*(1), 7. https://doi.org/10.1007/s13592-023-00989-y

Bitterman, M. E., Menzel, R., Fietz, A., & Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Psychology*, *97*(2), 107–119. https://doi.org/10.1037/0735-7036.97.2.107

Blatt, J., & Roces, F. (2001). Haemolymph sugar levels in foraging honeybees (*Apis mellifera carnica*): Dependence on metabolic rate and *in vivo* measurement of maximal rates of trehalose synthesis. *Journal of Experimental Biology*, *204*(15), 2709–2716. https://doi.org/10.1242/jeb.204.15.2709

Bonnafé, E., Alayrangues, J., Hotier, L., Massou, I., Renom, A., Souesme, G., Marty, P., Allaoua, M., Treilhou, M., & Armengaud, C. (2018). Monoterpenoid-based preparations in beehives affect learning, memory, and gene expression in the bee brain. *Environmental Toxicology and Chemistry*, 337–345. https://doi.org/10.1002/etc.3527

Bonnafé, E., Drouard, F., Hotier, L., Carayon, J.-L., Marty, P., Treilhou, M., & Armengaud, C. (2015). Effect of a thymol application on olfactory memory and gene expression levels in the brain of the honeybee *Apis mellifera*. *Environmental Science and Pollution Research*, *22*(11), 8022–8030. https://doi.org/10.1007/s11356-014-2616-2

Bounias, M., & Morgan, M. R. J. (1984). Induction of honeybee haemolymph sucrase activity by high levels of dietary sucrose. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *79*(1), 75–80. https://doi.org/10.1016/0305-0491(84)90080-4

Brown, M. J. F., Dicks, L. V., Paxton, R. J., Baldock, K. C. R., Barron, A. B., Chauzat, M.-P., Freitas, B. M., Goulson, D., Jepsen, S., Kremen, C., Li, J., Neumann, P., Pattemore, D. E., Potts, S. G., Schweiger, O., Seymour, C. L., & Stout, J. C. (2016). A horizon scan of future threats and opportunities for pollinators and pollination. *PeerJ*, *4*, e2249. https://doi.org/10.7717/peerj.2249

Braun, G., & Bicker, G. (1992). Habituation of an appetitive reflex in the honeybee. *Journal of Neurophysiology*, *67*(3), 588–598. https://doi.org/10.1152/jn.1992.67.3.588

Bruckner, S., Wilson, M., Aurell, D., Rennich, K., vanEngelsdorp, D., Steinhauer, N., & Williams, G. R. (2023). A national survey of managed honey bee colony losses in the USA: Results from the Bee Informed Partnership for 2017–18, 2018–19, and 2019–20. *Journal of Apicultural Research*, *0*(0), 1–15. https://doi.org/10.1080/00218839.2022.2158586

Buehlmann, C., Mangan, M., & Graham, P. (2020). Multimodal interactions in insect navigation. *Animal Cognition*. https://doi.org/10.1007/s10071-020-01383-2

Burden, C. M., Elmore, C., Hladun, K. R., Trumble, J. T., & Smith, B. H. (2016). Acute exposure to selenium disrupts associative conditioning and long-term memory recall in honey bees (*Apis mellifera*). *Ecotoxicology and Environmental Safety*, *127*, 71–79. https://doi.org/10.1016/j.ecoenv.2015.12.034

Burden, C. M., Morgan, M. O., Hladun, K. R., Amdam, G. V., Trumble, J. J., & Smith, B. H. (2019). Acute sublethal exposure to toxic heavy metals alters honey bee (*Apis mellifera*) feeding behavior. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-019-40396-x

Cabirol, A., Cope, A. J., Barron, A. B., & Devaud, J.-M. (2018). Relationship between brain plasticity, learning and foraging performance in honey bees. *PLOS ONE*, *13*(4), e0196749. https://doi.org/10.1371/journal.pone.0196749

Calderone, N. W. (2012). Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. *PLOS ONE*, *7*(5), e37235. https://doi.org/10.1371/journal.pone.0037235

Campbell, J. B., Nath, R., Gadau, J., Fox, T., DeGrandi-Hoffman, G., & Harrison, J. F. (2016). The fungicide Pristine® inhibits mitochondrial function *in vitro* but not flight metabolic rates in honey bees. *Journal of Insect Physiology*, *86*, 11–16. https://doi.org/10.1016/j.jinsphys.2015.12.003

Capaldi, E. A., & Dyer, F. C. (1999). The role of orientation flights on homing performance in honeybees. *Journal of Experimental Biology*, *202*(12), 1655–1666. https://doi.org/10.1242/jeb.202.12.1655

Cappa, F., Baracchi, D., & Cervo, R. (2022). Biopesticides and insect pollinators: Detrimental effects, outdated guidelines, and future directions. *Science of The Total Environment*, *837*, 155714. https://doi.org/10.1016/j.scitotenv.2022.155714

Cappa, F., Petrocelli, I., Dani, F. R., Dapporto, L., Giovannini, M., Silva-Castellari, J., Turillazzi, S., & Cervo, R. (2019). Natural biocide disrupts nestmate recognition in honeybees. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-019-38963-3

Carlesso, D., Smargiassi, S., Pasquini, E., Bertelli, G., & Baracchi, D. (2021). Nectar non-protein amino acids (NPAAs) do not change nectar palatability but enhance learning and memory in honey bees. *Scientific Reports*, *11*(1), 11721. https://doi.org/10.1038/s41598-021-90895-z

Carlesso, D., Smargiassi, S., Sassoli, L., Cappa, F., Cervo, R., & Baracchi, D. (2020). Exposure to a biopesticide interferes with sucrose responsiveness and learning in honey bees. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-020-76852-2

Cartereau, A., Pineau, X., Lebreton, J., Mathé-Allainmat, M., Taillebois, E., & Thany, S. H. (2022). Impairments in learning and memory performances associated with nicotinic receptor expression in the honeybee *Apis mellifera* after exposure to a sublethal dose of sulfoxaflor. *PLOS ONE*, *17*(8), e0272514. https://doi.org/10.1371/journal.pone.0272514

Catae, A. F., Roat, T. C., Oliveira, R. A. D., Nocelli, R. C. F., & Malaspina, O. (2014). Cytotoxic effects of thiamethoxam in the midgut and malpighian tubules of Africanized *Apis mellifera* (Hymenoptera: Apidae). *Microscopy Research and Technique*, *77*(4), 274–281. https://doi.org/10.1002/jemt.22339

Chaffiol, A., Laloi, D., & Pham-Delègue, M.-H. (2005). Prior classical olfactory conditioning improves odour-cued flight orientation of honey bees in a wind tunnel. *Journal of Experimental Biology*, *208*(19), 3731–3737. https://doi.org/10.1242/jeb.01796

Chapuy, C., Ribbens, L., Renou, M., Dacher, M., & Armengaud, C. (2019). Thymol Affects Congruency Between Olfactory and Gustatory Stimuli in Bees. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-43614-8

Chopra, S. S., Bakshi, B. R., & Khanna, V. (2015). Economic Dependence of U.S. Industrial Sectors on Animal-Mediated Pollination Service. *Environmental Science & Technology*, *49*(24), 14441–14451. https://doi.org/10.1021/acs.est.5b03788

Christen, V., Grossar, D., Charrière, J.-D., Eyer, M., & Jeker, L. (2021). Correlation Between Increased Homing Flight Duration and Altered Gene Expression in the Brain of Honey Bee Foragers After Acute Oral Exposure to Thiacloprid and Thiamethoxam. *Frontiers in Insect Science*, *0*. https://doi.org/10.3389/finsc.2021.765570

Ciarlo, T. J., Mullin, C. A., Frazier, J. L., & Schmehl, D. R. (2012). Learning Impairment in Honey Bees Caused by Agricultural Spray Adjuvants. *PLOS ONE*, *7*(7), e40848. https://doi.org/10.1371/journal.pone.0040848

Claudianos, C., Ranson, H., Johnson, R. M., Biswas, S., Schuler, M. A., Berenbaum, M. R., Feyereisen, R., & Oakeshott, J. G. (2006). A deficit of detoxification enzymes: Pesticide sensitivity and environmental response in the honeybee. *Insect Molecular Biology*, *15*(5), 615–636. https://doi.org/10.1111/j.1365-2583.2006.00672.x

Colin, T., Forster, C. C., Westacott, J., Wu, X., Meikle, W. G., & Barron, A. B. (2021). Effects of late miticide treatments on foraging and colony productivity of European honey bees (*Apis mellifera*). *Apidologie*. https://doi.org/10.1007/s13592-020-00837-3

Colin, T., Plath, J. A., Klein, S., Vine, P., Devaud, J.-M., Lihoreau, M., Meikle, W. G., & Barron, A. B. (2020). The miticide thymol in combination with trace levels of the neonicotinoid imidacloprid reduces visual learning performance in honey bees (*Apis mellifera*). *Apidologie*. https://doi.org/10.1007/s13592-020-00737-6

Cooper, J., & Dobson, H. (2007). The benefits of pesticides to mankind and the environment. *Crop Protection*, *26*(9), 1337–1348. https://doi.org/10.1016/j.cropro.2007.03.022

Crailsheim, K. (1988). Intestinal transport of sugars in the honeybee (*Apis mellifera* L.). *Journal of Insect Physiology*, *34*(9), 839–845. https://doi.org/10.1016/0022-1910(88)90117-5

Crailsheim, K., Schneider, L. H. W., Hrassnigg, N., Bühlmann, G., Brosch, U., Gmeinbauer, R., & Schöffmann, B. (1992). Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): Dependence on individual age and function. *Journal of Insect Physiology*, *38*(6), 409–419. https://doi.org/10.1016/0022-1910(92)90117-V

Cullen, M. G., Thompson, L. J., Carolan, J. C., Stout, J. C., & Stanley, D. A. (2019). Fungicides, herbicides and bees: A systematic review of existing research and methods. *PLOS ONE*, *14*(12), e0225743. https://doi.org/10.1371/journal.pone.0225743

da Costa Domingues, C. E., Bello Inoue, L. V., Mathias da Silva-Zacarin, E. C., & Malaspina, O. (2020). Foragers of Africanized honeybee are more sensitive to fungicide pyraclostrobin than newly emerged bees. *Environmental Pollution*, *266*, 115267. https://doi.org/10.1016/j.envpol.2020.115267

Dai, P.-L., Jia, H.-R., Geng, L.-L., & Diao, Q.-Y. (2016). Bt Toxin Cry1Ie Causes No Negative Effects on Survival, Pollen Consumption, or Olfactory Learning in Worker Honey Bees (Hymenoptera: Apidae). *Journal of Economic Entomology*, *109*(3), 1028–1033. https://doi.org/10.1093/jee/tow088

Dai, P.-L., Zhou, W., Zhang, J., Cui, H.-J., Wang, Q., Jiang, W.-Y., Sun, J.-H., Wu, Y.-Y., & Zhou, T. (2012). Field assessment of Bt *cry1Ah* corn pollen on the survival, development and behavior of *Apis mellifera ligustica*. *Ecotoxicology and Environmental Safety*, *79*, 232–237. https://doi.org/10.1016/j.ecoenv.2012.01.005

De Stefano, L. A., Stepanov, I. I., & Abramson, C. I. (2014). The First Order Transfer Function in the Analysis of Agrochemical Data in Honey Bees (*Apis Mellifera* L.): Proboscis Extension Reflex (PER) Studies. *Insects*, *5*(1), 167–198. https://doi.org/10.3390/insects5010167

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M., & Pham-Delègue, M.-H. (2004). Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). *Pesticide Biochemistry and Physiology*, *78*(2), 83–92. https://doi.org/10.1016/j.pestbp.2003.10.001

Decourtye, A., Devillers, J., Aupinel, P., Brun, F., Bagnis, C., Fourrier, J., & Gauthier, M. (2011). Honeybee tracking with microchips: A new methodology to measure the effects of pesticides. *Ecotoxicology*, *20*(2), 429–437. https://doi.org/10.1007/s10646-011-0594-4

Decourtye, A., Devillers, J., Cluzeau, S., Charreton, M., & Pham-Delègue, M.-H. (2004). Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotoxicology and Environmental Safety*, *57*(3), 410–419. https://doi.org/10.1016/j.ecoenv.2003.08.001

Decourtye, A., Devillers, J., Genecque, E., Menach, K. L., Budzinski, H., Cluzeau, S., & Pham-Delègue, M. H. (2005). Comparative Sublethal Toxicity of Nine Pesticides on Olfactory Learning Performances of the Honeybee *Apis mellifera*. *Archives of Environmental Contamination and Toxicology*, *48*(2), 242–250. https://doi.org/10.1007/s00244-003-0262-7

Decourtye, A., Henry, M., & Desneux, N. (2013). Overhaul pesticide testing on bees. *Nature*, *497*(7448), 188–188. https://doi.org/10.1038/497188a

Decourtye, A., Lacassie, E., & Pham-Delègue, M.-H. (2003). Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Management Science*, *59*(3), 269–278. https://doi.org/10.1002/ps.631

Déglise, P., Dacher, M., Dion, E., Gauthier, M., & Armengaud, C. (2003). Regional brain variations of cytochrome oxidase staining during olfactory learning in the honeybee (*Apis mellifera*). *Behavioral Neuroscience*, *117*(3), 540–547. https://doi.org/10.1037/0735-7044.117.3.540

DeGrandi-Hoffman, G., Chen, Y., & Simonds, R. (2013). The Effects of Pesticides on Queen Rearing and Virus Titers in Honey Bees (*Apis mellifera* L.). *Insects*, *4*(1), 71–89. https://doi.org/10.3390/insects4010071

DeGrandi-Hoffman, G., Chen, Y., Watkins Dejong, E., Chambers, M. L., & Hidalgo, G. (2015). Effects of Oral Exposure to Fungicides on Honey Bee Nutrition and Virus Levels. *Journal of Economic Entomology*, *108*(6), 2518–2528. https://doi.org/10.1093/jee/tov251

Delkash-Roudsari, S., Chicas-Mosier, A. M., Goldansaz, S. H., Talebi-Jahromi, K., Ashouri, A., & Abramson, C. I. (2020). Assessment of lethal and sublethal effects of imidacloprid, ethion, and glyphosate on aversive conditioning, motility, and lifespan in honey bees (*Apis mellifera* L.). *Ecotoxicology and Environmental Safety*, *204*, 111108. https://doi.org/10.1016/j.ecoenv.2020.111108

Démares, F. J., Crous, K. L., Pirk, C. W. W., Nicolson, S. W., & Human, H. (2016). Sucrose Sensitivity of Honey Bees Is Differently Affected by Dietary Protein and a Neonicotinoid Pesticide. *PLOS ONE*, *11*(6), e0156584. https://doi.org/10.1371/journal.pone.0156584

Démares, F., Gibert, L., Creusot, P., Lapeyre, B., & Proffit, M. (2022). Acute ozone exposure impairs detection of floral odor, learning, and memory of honey bees, through olfactory generalization. *Science of The Total Environment*, *827*, 154342. https://doi.org/10.1016/j.scitotenv.2022.154342

DesJardins, N. S., Fisher, A., Ozturk, C., Fewell, J. H., DeGrandi-Hoffman, G., Harrison, J. F., & Smith, B. H. (2021). A common fungicide, Pristine®, impairs olfactory associative learning performance in honey bees (*Apis mellifera*). *Environmental Pollution*, 117720. https://doi.org/10.1016/j.envpol.2021.117720

Desneux, N., Decourtye, A., & Delpuech, J.-M. (2006). The Sublethal Effects of Pesticides on Beneficial Arthropods. *Annual Review of Entomology*, *52*(1), 81–106. https://doi.org/10.1146/annurev.ento.52.110405.091440

Dicks, L. V., Breeze, T. D., Ngo, H. T., Senapathi, D., An, J., Aizen, M. A., Basu, P., Buchori, D., Galetto, L., Garibaldi, L. A., Gemmill-Herren, B., Howlett, B. G., Imperatriz-Fonseca, V. L., Johnson, S. D., Kovács-Hostyánszki, A., Kwon, Y. J., Lattorff, H. M. G., Lungharwo, T., Seymour, C. L., … Potts, S. G. (2021). A global-scale expert assessment of drivers and risks associated with pollinator decline. *Nature Ecology & Evolution*, *5*(10), Article 10. https://doi.org/10.1038/s41559-021-01534-9

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G., & Pennacchio, F. (2013). Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences*, *110*(46), 18466–18471. https://doi.org/10.1073/pnas.1314923110

Dolezal, A. G., Carrillo-Tripp, J., Judd, T. M., Allen Miller, W., Bonning, B. C., & Toth, A. L. (2019). Interacting stressors matter: Diet quality and virus infection in honeybee health. *Royal Society Open Science*, *6*(2), 181803. https://doi.org/10.1098/rsos.181803

Douglas, M. R., Sponsler, D. B., Lonsdorf, E. V., & Grozinger, C. M. (2020). County-level analysis reveals a rapidly shifting landscape of insecticide hazard to honey bees (*Apis mellifera*) on US farmland. *Scientific Reports*, *10*(1), 1–11. https://doi.org/10.1038/s41598-019-57225-w

Earley, F., Sauter, H., Rheinheimer, J., Rieck, H., Coqueron, P.-Y., Whittingham, W. G., & Walter, H. (2012). Fungicides Acting on Oxidative Phosphorylation. In Krämer, W., Schirmer, U., Jeschke, P., & Witschel, M. (Eds.), *Modern Crop Protection Compounds* (pp. 559–691). John Wiley & Sons, Ltd. https://doi.org/10.1002/9783527644179.ch15

Eiri, D. M., & Nieh, J. C. (2012). A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. *Journal of Experimental Biology*, *215*(12), 2022–2029. https://doi.org/10.1242/jeb.068718

El Hassani, A. K., Dacher, M., Gary, V., Lambin, M., Gauthier, M., & Armengaud, C. (2008). Effects of Sublethal Doses of Acetamiprid and Thiamethoxam on the Behavior of the Honeybee (*Apis mellifera*). *Archives of Environmental Contamination and Toxicology*, *54*(4), 653–661. https://doi.org/10.1007/s00244-007-9071-8

El Hassani, A. K., Dacher, M., Gauthier, M., & Armengaud, C. (2005). Effects of sublethal doses of fipronil on the behavior of the honeybee (*Apis mellifera*). *Pharmacology Biochemistry and Behavior*, *82*(1), 30–39. https://doi.org/10.1016/j.pbb.2005.07.008

El Hassani, A. K., Dupuis, J. P., Gauthier, M., & Armengaud, C. (2009). Glutamatergic and GABAergic effects of fipronil on olfactory learning and memory in the honeybee. *Invertebrate Neuroscience*, *9*(2), 91. https://doi.org/10.1007/s10158-009-0092-z

Faita, M. R., Oliveira, E. de M., Alves, V. V., Orth, A. I., & Nodari, R. O. (2018). Changes in hypopharyngeal glands of nurse bees (*Apis mellifera*) induced by pollen-containing sublethal doses of the herbicide Roundup®. *Chemosphere*, *211*, 566–572. https://doi.org/10.1016/j.chemosphere.2018.07.189

Farina, W. M., Grüter, C., Acosta, L., & Mc Cabe, S. (2007). Honeybees learn floral odors while receiving nectar from foragers within the hive. *Naturwissenschaften*, *94*(1), 55–60. https://doi.org/10.1007/s00114-006-0157-3

Farina, W. M., Sol Balbuena, M., Herbert, L. T., Mengoni Goñalons, C., & Vázquez, D. E. (2019). Effects of the Herbicide Glyphosate on Honey Bee Sensory and Cognitive Abilities: Individual Impairments with Implications for the Hive. *Insects*, *10*(10), 354. https://doi.org/10.3390/insects10100354

Fine, J. D. (2020). Evaluation and comparison of the effects of three insect growth regulators on honey bee queen oviposition and egg eclosion. *Ecotoxicology and Environmental Safety*, *205*, 111142. https://doi.org/10.1016/j.ecoenv.2020.111142

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B., & Menzel, R. (2014). Neonicotinoids Interfere with Specific Components of Navigation in Honeybees. *PLOS ONE*, *9*(3), e91364. https://doi.org/10.1371/journal.pone.0091364

Fisher, A. (2021). Protect pollinators—Reform pesticide regulations. *Nature*, *595*(7866), 172–172. https://doi.org/10.1038/d41586-021-01818-x

Fisher, A., DeGrandi-Hoffman, G., Smith, B. H., Johnson, M., Kaftanoglu, O., Cogley, T., Fewell, J. H., & Harrison, J. F. (2021). Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*). *Environmental Pollution*, *269*, 115964. https://doi.org/10.1016/j.envpol.2020.115964

Fisher, A., DeGrandi-Hoffman, G., Smith, B. H., Ozturk, C., Kaftanoglu, O., Fewell, J. H., & Harrison, J. F. (2021). Field cross-fostering and *in vitro* rearing demonstrate negative effects of both larval and adult exposure to a widely used fungicide in honey bees (*Apis mellifera*). *Ecotoxicology and Environmental Safety*, *217*, 112251. https://doi.org/10.1016/j.ecoenv.2021.112251

Frost, E. H., Shutler, D., & Hillier, N. K. (2012). The proboscis extension reflex to evaluate learning and memory in honeybees (*Apis mellifera*): Some caveats. *Naturwissenschaften*, *99*(9), 677–686. https://doi.org/10.1007/s00114-012-0955-8

Frost, E. H., Shutler, D., & Hillier, N. K. (2013). Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival. *Journal of Experimental Biology*, *216*(15), 2931–2938. https://doi.org/10.1242/jeb.086538

Fungicide Resistance Action Committee. (2022, March). *FRAC Code List©*2022: Fungal control agents sorted by cross-resistance pattern and mode of action (including coding for FRAC Groups on product labels).* https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2022--final.pdf?sfvrsn=b6024e9a_2

Gallai, N., Salles, J.-M., Settele, J., & Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, *68*(3), 810–821. https://doi.org/10.1016/j.ecolecon.2008.06.014

Gashout, H. A., Guzman-Novoa, E., Goodwin, P. H., & Correa-Benítez, A. (2020). Impact of sublethal exposure to synthetic and natural acaricides on honey bee (*Apis mellifera*) memory and expression of genes related to memory. *Journal of Insect Physiology*, *121*, 104014. https://doi.org/10.1016/j.jinsphys.2020.104014

Genersch, E. (2010). Honey bee pathology: Current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*, *87*(1), 87–97. https://doi.org/10.1007/s00253-010-2573-8

Gérard, M., Amiri, A., Cariou, B., & Baird, E. (2022). Short-term exposure to heatwave-like temperatures affects learning and memory in bumblebees. *Global Change Biology*, *28*(14), 4251–4259. https://doi.org/10.1111/gcb.16196

Gérard, M., Cariou, B., Henrion, M., Descamps, C., & Baird, E. (2022). Exposure to elevated temperature during development affects bumblebee foraging behavior. *Behavioral Ecology*, *33*(4), 816–824. https://doi.org/10.1093/beheco/arac045

Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalksy, O., Wüstenberg, D., & Menzel, R. (1996). Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Animal Behaviour*, *52*(6), 1079–1085. https://doi.org/10.1006/anbe.1996.0255

Giurfa, M., & Sandoz, J.-C. (2012). Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learning & Memory*, *19*(2), 54–66. https://doi.org/10.1101/lm.024711.111

Glass, J. R., Fisher, A., Fewell, J. H., DeGrandi-Hoffman, G., Ozturk, C., & Harrison, J. F. (2021). Consumption of field-realistic doses of a widely used mito-toxic fungicide reduces thorax mass but does not negatively impact flight capacities of the honey bee (*Apis mellifera*). *Environmental Pollution*, *274*, 116533. https://doi.org/10.1016/j.envpol.2021.116533

Glavinic, U., Tesovnik, T., Stevanovic, J., Zorc, M., Cizelj, I., Stanimirovic, Z., & Narat, M. (2019). Response of adult honey bees treated in larval stage with prochloraz to infection with *Nosema ceranae*. *PeerJ*, *7*, e6325. https://doi.org/10.7717/peerj.6325

Gmeinbauer, R., & Crailsheim, K. (1993). Glucose utilization during flight of honeybee (*Apis mellifera*) workers, drones and queens. *Journal of Insect Physiology*, *39*(11), 959–967. https://doi.org/10.1016/0022-1910(93)90005-C

Gong, Z., Gu, G., Wang, Y., Dong, S., Tan, K., & Nieh, J. C. (2021). Floral tea polyphenols can improve honey bee memory retention and olfactory sensitivity. *Journal of Insect Physiology*, *128*, 104177. https://doi.org/10.1016/j.jinsphys.2020.104177

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957. https://doi.org/10.1126/science.1255957

Graham, K. K., Milbrath, M. O., Zhang, Y., Baert, N., McArt, S., & Isaacs, R. (2022). Pesticide risk to managed bees during blueberry pollination is primarily driven by off-farm exposures. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-11156-1

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Bugeja Douglas, A., Cadahía, L., Charrière, J.-D., Chlebo, R., Coffey, M. F., Cornelissen, B., Costa, C. A. da, Danneels, E., Danihlík, J., Dobrescu, C., Evans, G., Fedoriak, M., Forsythe, I., Gregorc, A., … Brodschneider, R. (2022). Honey bee colony loss rates in 37 countries using the COLOSS survey for winter 2019–2020: The combined effects of operation size, migration and queen replacement. *Journal of Apicultural Research*, *0*(0), 1–7. https://doi.org/10.1080/00218839.2022.2113329

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Charrière, J.-D., Chlebo, R., Coffey, M. F., Cornelissen, B., da Costa, C. A., Dahle, B., Danihlík, J., Dražić, M. M., Evans, G., Fedoriak, M., Forsythe, I., Gajda, A., de Graaf, D. C., Gregorc, A., … Brodschneider, R. (2020). Honey bee colony winter loss rates for 35 countries participating in the COLOSS survey for winter 2018–2019, and the effects of a new queen on the risk of colony winter loss. *Journal of Apicultural Research*, *59*(5), 744–751. https://doi.org/10.1080/00218839.2020.1797272

Guez, D., Suchail, S., Gauthier, M., Maleszka, R., & Belzunces, L. P. (2001). Contrasting Effects of Imidacloprid on Habituation in 7- and 8-Day-Old Honeybees (*Apis mellifera*). *Neurobiology of Learning and Memory*, *76*(2), 183–191. https://doi.org/10.1006/nlme.2000.3995

Guez, D., Zhang, S.-W., & Srinivasan, M. V. (2005). Methyl Parathion Modifies Foraging Behaviour in Honeybees (*Apis mellifera*). *Ecotoxicology*, *14*(4), 431–437. https://doi.org/10.1007/s10646-004-1348-3

Halas, E. S., Heinrich, M. D., & Sandstead, H. H. (1979). Long term memory deficits in adult rats due to postnatal malnutrition. *Physiology & Behavior*, *22*(5), 991–997. https://doi.org/10.1016/0031-9384(79)90345-7

Han, P., Niu, C.-Y., Lei, C.-L., Cui, J.-J., & Desneux, N. (2010). Use of an innovative T-tube maze assay and the proboscis extension response assay to assess sublethal effects of GM products and pesticides on learning capacity of the honey bee *Apis mellifera* L. *Ecotoxicology*, *19*(8), 1612–1619. https://doi.org/10.1007/s10646-010-0546-4

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., & Decourtye, A. (2012). A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. *Science*, *336*(6079), 348–350. https://doi.org/10.1126/science.1215039

Henry, M., Bertrand, C., Le Féon, V., Requier, F., Odoux, J.-F., Aupinel, P., Bretagnolle, V., & Decourtye, A. (2014). Pesticide risk assessment in free-ranging bees is weather and landscape dependent. *Nature Communications*, *5*(1), 4359. https://doi.org/10.1038/ncomms5359

Henry, M., Cerrutti, N., Aupinel, P., Decourtye, A., Gayrard, M., Odoux, J.-F., Pissard, A., Rüger, C., & Bretagnolle, V. (2015). Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1819), 20152110. https://doi.org/10.1098/rspb.2015.2110

Herbert, L. T., Vázquez, D. E., Arenas, A., & Farina, W. M. (2014). Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour. *Journal of Experimental Biology*, *217*(19), 3457–3464. https://doi.org/10.1242/jeb.109520

Herbicide Resistance Action Committee. (2022, January 6). *HRAC Mode of Action Classification 2022.* https://hracglobal.com/files/HRAC_MOA_Poster_January_6_2022.pdf

Hernández, I. G., Palottini, F., Macri, I., Galmarini, C. R., & Farina, W. M. (2019). Appetitive behavior of the honey bee *Apis mellifera* in response to phenolic compounds naturally found in nectars. *Journal of Experimental Biology*, *222*(2). https://doi.org/10.1242/jeb.189910

Hernández, J., Riveros, A. J., & Amaya-Márquez, M. (2021). Sublethal doses of glyphosate impair olfactory memory retention, but not learning in the honey bee (*Apis mellifera scutellata*). *Journal of Insect Conservation*, *25*(4), 683–694. https://doi.org/10.1007/s10841-021-00335-6

Hesselbach, H., & Scheiner, R. (2018). Effects of the novel pesticide flupyradifurone (Sivanto) on honeybee taste and cognition. *Scientific Reports*, *8*(1), 1–8. https://doi.org/10.1038/s41598-018-23200-0

Hori, S., Takeuchi, H., Arikawa, K., Kinoshita, M., Ichikawa, N., Sasaki, M., & Kubo, T. (2006). Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L. *Journal of Comparative Physiology A*, *192*(7), 691–700. https://doi.org/10.1007/s00359-005-0091-4

Insecticide Resistance Action Committee. (2022, December). *Mode of Action Classification*. https://irac-online.org/documents/moa-brochure/

Insolia, L., Molinari, R., Rogers, S. R., Williams, G. R., Chiaromonte, F., & Calovi, M. (2022). Honey bee colony loss linked to parasites, pesticides and extreme weather across the United States. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-24946-4

Johnson, R. M. (2015). Honey Bee Toxicology. *Annual Review of Entomology*, *60*(1), 415–434. https://doi.org/10.1146/annurev-ento-011613-162005

Jordan, A., Patch, H. M., Grozinger, C. M., & Khanna, V. (2021). Economic Dependence and Vulnerability of United States Agricultural Sector on Insect-Mediated Pollination Service. *Environmental Science & Technology*, *55*(4), 2243–2253. https://doi.org/10.1021/acs.est.0c04786

Karahan, A., Çakmak, I., Hranitz, J. M., Karaca, I., & Wells, H. (2015). Sublethal imidacloprid effects on honey bee flower choices when foraging. *Ecotoxicology*, *24*(9), 2017–2025. https://doi.org/10.1007/s10646-015-1537-2

Kenna, D., Graystock, P., & Gill, R. J. (2023). Toxic temperatures: Bee behaviours exhibit divergent pesticide toxicity relationships with warming. *Global Change Biology*. https://doi.org/10.1111/gcb.16671

Khalifa, S. A. M., Elshafiey, E. H., Shetaia, A. A., El-Wahed, A. A. A., Algethami, A. F., Musharraf, S. G., AlAjmi, M. F., Zhao, C., Masry, S. H. D., Abdel-Daim, M. M., Halabi, M. F., Kai, G., Al Naggar, Y., Bishr, M., Diab, M. A. M., & El-Seedi, H. R. (2021). Overview of Bee Pollination and Its Economic Value for Crop Production. *Insects*, *12*(8), Article 8. https://doi.org/10.3390/insects12080688

Kim, S., Cho, S., & Lee, S. H. (2022). Synergistic effects of imidacloprid and high temperature on honey bee colonies. *Apidologie*, *53*(6), 67. https://doi.org/10.1007/s13592-022-00980-z

Kim, Y. S., & Smith, B. H. (2000). Effect of an amino acid on feeding preferences and learning behavior in the honey bee, *Apis mellifera*. *Journal of Insect Physiology*, *46*(5), 793–801. https://doi.org/10.1016/S0022-1910(99)00168-7

Kirchner, W. H., & Grasser, A. (1998). The Significance of Odor Cues and Dance Language Information for the Food Search Behavior of Honeybees (Hymenoptera: Apidae). *Journal of Insect Behavior*, *11*(2), 169–178. https://doi.org/10.1023/A:1021098405564

Klein, S., Cabirol, A., Devaud, J.-M., Barron, A. B., & Lihoreau, M. (2017). Why Bees Are So Vulnerable to Environmental Stressors. *Trends in Ecology & Evolution*, *32*(4), 268–278. https://doi.org/10.1016/j.tree.2016.12.009

Klein, A.-M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 303–313. https://doi.org/10.1098/rspb.2006.3721

Kobayashi, N., Okada, R., & Sakura, M. (2020). Orientation to polarized light in tethered flying honeybees. *Journal of Experimental Biology*. https://doi.org/10.1242/jeb.228254

Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D. M., Sagili, R. R., Pettis, J. S., Ellis, J. D., Wilson, M. E., Wilkes, J. T., Tarpy, D. R., Rose, R., Lee, K., Rangel, J., & vanEngelsdorp, D. (2017). A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *Journal of Apicultural Research*, *56*(4), 328–340. https://doi.org/10.1080/00218839.2017.1344496

Ladurner, E., Bosch, J., Kemp, W. P., & Maini, S. (2005). Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* Say and *Apis mellifera*. *Apidologie*, *36*(3), 449–460. https://doi.org/10.1051/apido:2005032

Lambin, M., Armengaud, C., Raymond, S., & Gauthier, M. (2001). Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. *Archives of Insect Biochemistry and Physiology*, *48*(3), 129–134. https://doi.org/10.1002/arch.1065

Lee, H., Sumner, D. A., & Champetier, A. (2019). Pollination Markets and the Coupled Futures of Almonds and Honey Bees: Simulating Impacts of Shifts in Demands and Costs. *American Journal of Agricultural Economics*, *101*(1), 230–249. https://doi.org/10.1093/ajae/aay063

Lenth, R. (2019). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.4.3.01, https://CRAN.R-project.org/package=emmeans

Leonard, R. J., Vergoz, V., Proschogo, N., McArthur, C., & Hochuli, D. F. (2019). Petrol exhaust pollution impairs honey bee learning and memory. *Oikos*, *128*(2), 264–273. https://doi.org/10.1111/oik.05405

Li, B., Ke, L., Li, A.-R., Diao, Q.-Y., Wang, Q., & Liu, Y.-J. (2022). Exposure of Larvae to Sublethal Thiacloprid Delays Bee Development and Affects Transcriptional Responses of Newly Emerged Honey Bees. *Frontiers in Insect Science*, *2*. https://www.frontiersin.org/article/10.3389/finsc.2022.844957

Li, Z., Li, M., Huang, J., Ma, C., Xiao, L., Huang, Q., Zhao, Y., Nie, H., & Su, S. (2017). Effects of Sublethal Concentrations of Chlorpyrifos on Olfactory Learning and Memory Performances in Two Bee Species, *Apis mellifera* and *Apis cerana*. *Sociobiology*, *64*(2), 174–181. https://doi.org/10.13102/sociobiology.v64i2.1385

Li, Z., Qiu, Y., Li, J., Wan, K., Nie, H., & Su, S. (2022). Chronic Cadmium Exposure Induces Impaired Olfactory Learning and Altered Brain Gene Expression in Honey Bees (*Apis mellifera). Insects*, *13*(11), Article 11. https://doi.org/10.3390/insects13110988

Li, Z., Yu, T., Chen, Y., Heerman, M., He, J., Huang, J., Nie, H., & Su, S. (2019). Brain transcriptome of honey bees (*Apis mellifera*) exhibiting impaired olfactory learning induced by a sublethal dose of imidacloprid. *Pesticide Biochemistry and Physiology*, *156*, 36–43. https://doi.org/10.1016/j.pestbp.2019.02.001

Liao, C., He, X., Wang, Z., Barron, A. B., Zhang, B., Zeng, Z., & Wu, X. (2018). Short-Term Exposure to Lambda-Cyhalothrin Negatively Affects the Survival and Memory-Related Characteristics of Worker Bees *Apis mellifera*. *Archives of Environmental Contamination and Toxicology*, *75*(1), 59–65. https://doi.org/10.1007/s00244-018-0514-1

Liao, L.-H., Wu, W.-Y., Dad, A., & Berenbaum, M. R. (2019). Fungicide suppression of flight performance in the honeybee (*Apis mellifera*) and its amelioration by quercetin. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1917), 20192041. https://doi.org/10.1098/rspb.2019.2041

Lippert, C., Feuerbacher, A., & Narjes, M. (2021). Revisiting the economic valuation of agricultural losses due to large-scale changes in pollinator populations. *Ecological Economics*, *180*, 106860. https://doi.org/10.1016/j.ecolecon.2020.106860

López-Uribe, M. M., Ricigliano, V. A., & Simone-Finstrom, M. (2020). Defining Pollinator Health: A Holistic Approach Based on Ecological, Genetic, and Physiological Factors. *Annual Review of Animal Biosciences*, *8*(1), 269–294. https://doi.org/10.1146/annurev-animal-020518-115045

Lozano, V., Armengaud, C., & Gauthier, M. (2001). Memory impairment induced by cholinergic antagonists injected into the mushroom bodies of the honeybee. *Journal of Comparative Physiology A*, *187*(4), 249–254. https://doi.org/10.1007/s003590100196

Ludicke, J. C., & Nieh, J. C. (2020). Thiamethoxam impairs honey bee visual learning, alters decision times, and increases abnormal behaviors. *Ecotoxicology and Environmental Safety*, *193*, 110367. https://doi.org/10.1016/j.ecoenv.2020.110367

Luo, Q.-H., Gao, J., Guo, Y., Liu, C., Ma, Y.-Z., Zhou, Z.-Y., Dai, P.-L., Hou, C.-S., Wu, Y.-Y., & Diao, Q.-Y. (2021). Effects of a commercially formulated glyphosate solutions at recommended concentrations on honeybee (*Apis mellifera* L.) behaviours. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-020-80445-4

Mamood, A. N., & Waller, G. D. (1990). Recovery of learning responses by honeybees following a sublethal exposure to permethrin. *Physiological Entomology*, *15*(1), 55–60. https://doi.org/10.1111/j.1365-3032.1990.tb00492.x

Manzi, C., Vergara-Amado, J., Franco, L. M., & Silva, A. X. (2020). The effect of temperature on candidate gene expression in the brain of honey bee *Apis mellifera* (Hymenoptera: Apidae) workers exposed to neonicotinoid imidacloprid. *Journal of Thermal Biology*, *93*, 102696. https://doi.org/10.1016/j.jtherbio.2020.102696

Marchi, I. L., Palottini, F., & Farina, W. M. (2021). Combined secondary compounds naturally found in nectars enhance honeybee cognition and survival. *Journal of Experimental Biology*, *224*(6). https://doi.org/10.1242/jeb.239616

Matsumoto, Y., Menzel, R., Sandoz, J.-C., & Giurfa, M. (2012). Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: A step toward standardized procedures. *Journal of Neuroscience Methods*, *211*(1), 159–167. https://doi.org/10.1016/j.jneumeth.2012.08.018

Mattila, H. R., & Smith, B. H. (2008). Learning and memory in workers reared by nutritionally stressed honey bee (*Apis mellifera* L.) colonies. *Physiology & Behavior*, *95*(5), 609–616. https://doi.org/10.1016/j.physbeh.2008.08.003

Mayack, C., Macherone, A., Zaki, A. G., Filiztekin, E., Özkazanç, B., Koperly, Y., Schick, S. J., Eppley, E. J., Deb, M., Ambiel, N., Schafsnitz, A. M., & Broadrup, R. L. (2022). Environmental exposures associated with honey bee health. *Chemosphere*, *286*, 131948. https://doi.org/10.1016/j.chemosphere.2021.131948

Medina, R. G., Paxton, R. J., De Luna, E., Fleites-Ayil, F. A., Medina Medina, L. A., & Quezada-Euán, J. J. G. (2018). Developmental stability, age at onset of foraging and longevity of Africanized honey bees (*Apis mellifera* L.) under heat stress (Hymenoptera: Apidae). *Journal of Thermal Biology*, *74*, 214–225. https://doi.org/10.1016/j.jtherbio.2018.04.003

Mengoni Goñalons, C., & Farina, W. M. (2015). Effects of Sublethal Doses of Imidacloprid on Young Adult Honeybee Behaviour. *PLOS ONE*, *10*(10), e0140814. https://doi.org/10.1371/journal.pone.0140814

Mengoni Goñalons, C., & Farina, W. M. (2018). Impaired associative learning after chronic exposure to pesticides in young adult honey bees. *Journal of Experimental Biology*, *221*(7). https://doi.org/10.1242/jeb.176644

Menzel, R., De Marco, R. J., & Greggers, U. (2006). Spatial memory, navigation and dance behaviour in *Apis mellifera*. *Journal of Comparative Physiology A*, *192*(9), 889–903. https://doi.org/10.1007/s00359-006-0136-3

Milone, J. P., & Tarpy, D. R. (2021). Effects of developmental exposure to pesticides in wax and pollen on honey bee (*Apis mellifera*) queen reproductive phenotypes. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-020-80446-3

Monchanin, C., Blanc-Brude, A., Drujont, E., Negahi, M. M., Pasquaretta, C., Silvestre, J., Baqué, D., Elger, A., Barron, A. B., Devaud, J.-M., & Lihoreau, M. (2021). Chronic exposure to trace lead impairs honey bee learning. *Ecotoxicology and Environmental Safety*, *212*, 112008. https://doi.org/10.1016/j.ecoenv.2021.112008

Monchanin, C., Drujont, E., Devaud, J.-M., Lihoreau, M., & Barron, A. B. (2021). Metal pollutants have additive negative effects on honey bee cognition. *Journal of Experimental Biology*, *jeb.241869*. https://doi.org/10.1242/jeb.241869

Monchanin, C., Henry, M., Decourtye, A., Dalmon, A., Fortini, D., Bœuf, E., Dubuisson, L., Aupinel, P., Chevallereau, C., Petit, J., & Fourrier, J. (2019). Hazard of a neonicotinoid insecticide on the homing flight of the honeybee depends on climatic conditions and *Varroa* infestation. *Chemosphere*, *224*, 360–368. https://doi.org/10.1016/j.chemosphere.2019.02.129

Morgane, P. J., Austin-LaFrance, A., Bronzino, J., Tonkiss, J., Diaz-Cintra, S., Cintra, L., Kemper, T., & Galler, J. R. (1993). Prenatal malnutrition and development of the brain. *Neuroscience & Biobehavioral Reviews*, *17*(1), 91–128. https://doi.org/10.1016/S0149-7634(05)80234-9

Mullin, C. A., Chen, J., Fine, J. D., Frazier, M. T., & Frazier, J. L. (2015). The formulation makes the honey bee poison. *Pesticide Biochemistry and Physiology*, *120*, 27–35. https://doi.org/10.1016/j.pestbp.2014.12.026

Mullin, C. A., Fine, J. D., Reynolds, R. D., & Frazier, M. T. (2016). Toxicological Risks of Agrochemical Spray Adjuvants: Organosilicone Surfactants May Not Be Safe. *Frontiers in Public Health*, *4*. https://doi.org/10.3389/fpubh.2016.00092

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., & Pettis, J. S. (2010). High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLOS ONE*, *5*(3), e9754. https://doi.org/10.1371/journal.pone.0009754

Mustard, J. A., Alvarez, V., Barocio, S., Mathews, J., Stoker, A., & Malik, K. (2018). Nutritional value and taste play different roles in learning and memory in the honey bee (*Apis mellifera*). *Journal of Insect Physiology*, *107*, 250–256. https://doi.org/10.1016/j.jinsphys.2018.04.014

Mustard, J. A., Gott, A., Scott, J., Chavarria, N. L., & Wright, G. A. (2020). Honeybees fail to discriminate floral scents in a complex learning task after consuming a neonicotinoid pesticide. *Journal of Experimental Biology*, *223*(5). https://doi.org/10.1242/jeb.217174

Nakasu, E. Y. T., Williamson, S. M., Edwards, M. G., Fitches, E. C., Gatehouse, J. A., Wright, G. A., & Gatehouse, A. M. R. (2014). Novel biopesticide based on a spider venom peptide shows no adverse effects on honeybees. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1787), 20140619. https://doi.org/10.1098/rspb.2014.0619

National Weather Service (2023). *Central and Southern San Joaquin Valley Climate Graphs*. NOAA's National Weather Service. Retrieved April 5, 2023, from https://www.weather.gov/hnx/Cliplot

Naug, D. (2009). Nutritional stress due to habitat loss may explain recent honeybee colony collapses. *Biological Conservation*, *142*(10), 2369–2372. https://doi.org/10.1016/j.biocon.2009.04.007

Oertel, E., Fieger, E. A., Williams, V. R., & Andrews, E. A. (1951). Inversion of Cane Sugar in the Honey Stomach of the Bee. *Journal of Economic Entomology*, *44*(4), 487–492. https://doi.org/10.1093/jee/44.4.487

Ohlinger, B. D., Schürch, R., Durzi, S., Kietzman, P. M., Silliman, M. R., & Couvillon, M. J. (2022). Honey Bees (Hymenoptera: Apidae) Decrease Foraging But Not Recruitment After Neonicotinoid Exposure. *Journal of Insect Science*, *22*(1), 16. https://doi.org/10.1093/jisesa/ieab095

Ostiguy, N., Drummond, F. A., Aronstein, K., Eitzer, B., Ellis, J. D., Spivak, M., & Sheppard, W. S. (2019). Honey Bee Exposure to Pesticides: A Four-Year Nationwide Study. *Insects*, *10*(1), Article 1. https://doi.org/10.3390/insects10010013

Page, R. E., Erber, J., & Fondrk, M. K. (1998). The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (Apis mellifera L.). *Journal of Comparative Physiology A*, *182*(4), 489–500. https://doi.org/10.1007/s003590050196

Pahl, M., Zhu, H., Tautz, J., & Zhang, S. (2011). Large Scale Homing in Honeybees. *PLOS ONE*, *6*(5), e19669. https://doi.org/10.1371/journal.pone.0019669

Pankiw, T., & Page Jr., R. E. (1999). The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (Apis mellifera L.). *Journal of Comparative Physiology A*, *185*(2), 207–213. https://doi.org/10.1007/s003590050379

Pankiw, T., & Page Jr, R. E. (2000). Response thresholds to sucrose predict foraging division of labor in honeybees. *Behavioral Ecology and Sociobiology*, *47*(4), 265–267. https://doi.org/10.1007/s002650050664

Papach, A., Fortini, D., Grateau, S., Aupinel, P., & Richard, F.-J. (2017). Larval exposure to thiamethoxam and American foulbrood: Effects on mortality and cognition in the honey bee *Apis mellifera*. *Journal of Apicultural Research*, *56*(4), 475–486. https://doi.org/10.1080/00218839.2017.1332541

Pathak, T. B., Maskey, M. L., Dahlberg, J. A., Kearns, F., Bali, K. M., & Zaccaria, D. (2018). Climate Change Trends and Impacts on California Agriculture: A Detailed Review. *Agronomy*, *8*(3), Article 3. https://doi.org/10.3390/agronomy8030025

Pecenka, J. R., Ingwell, L. L., Foster, R. E., Krupke, C. H., & Kaplan, I. (2021). IPM reduces insecticide applications by 95% while maintaining or enhancing crop yields through wild pollinator conservation. *Proceedings of the National Academy of Sciences*, *118*(44). https://doi.org/10.1073/pnas.2108429118

Pham-Delègue, M.-H., Decourtye, A., Kaiser, L., & Devillers, J. (2002). Behavioural methods to assess the effects of pesticides on honey bees. *Apidologie*, *33*(5), 425–432. https://doi.org/10.1051/apido:2002033

Pham-Delègue, M.-H., Girard, C., Métayer, M. L., Picard-Nizou, A.-L., Hennequet, C., Pons, O., & Jouanin, L. (2000). Long-term effects of soybean protease inhibitors on digestive enzymes, survival and learning abilities of honeybees. *Entomologia Experimentalis et Applicata*, *95*(1), 21–29. https://doi.org/10.1046/j.1570-7458.2000.00637.x

Phiri, B. J., Fèvre, D., & Hidano, A. (2022). Uptrend in global managed honey bee colonies and production based on a six-decade viewpoint, 1961–2017. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-25290-3

Picard-Nizou, A. L., Grison, R., Olsen, L., Pioche, C., Arnold, G., & Pham-Delegue, M. H. (1997). Impact of Proteins Used in Plant Genetic Engineering: Toxicity and Behavioral Study in the Honeybee. *Journal of Economic Entomology*, *90*(6), 1710–1716. https://doi.org/10.1093/jee/90.6.1710

Piiroinen, S., & Goulson, D. (2016). Chronic neonicotinoid pesticide exposure and parasite stress differentially affects learning in honeybees and bumblebees. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1828), 20160246. https://doi.org/10.1098/rspb.2016.0246

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology & Evolution*, *25*(6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007

Prado, A., Pioz, M., Vidau, C., Requier, F., Jury, M., Crauser, D., Brunet, J.-L., Le Conte, Y., & Alaux, C. (2019). Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees. *Science of The Total Environment*, *650*, 1250–1260. https://doi.org/10.1016/j.scitotenv.2018.09.102

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Ramirez-Romero, R., Chaufaux, J., & Pham-Delègue, M.-H. (2005). Effects of Cry1Ab protoxin, deltamethrin and imidacloprid on the foraging activity and the learning performances of the honeybee Apis mellifera, a comparative approach. *Apidologie*, *36*(4), 601–611. https://doi.org/10.1051/apido:2005039

Ramirez-Romero, R., Desneux, N., Decourtye, A., Chaffiol, A., & Pham-Delègue, M. H. (2008). Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L. (Hymenoptera, Apidae)? *Ecotoxicology and Environmental Safety*, *70*(2), 327–333. https://doi.org/10.1016/j.ecoenv.2007.12.002

Reilly, J. R., Artz, D. R., Biddinger, D., Bobiwash, K., Boyle, N. K., Brittain, C., Brokaw, J., Campbell, J. W., Daniels, J., Elle, E., Ellis, J. D., Fleischer, S. J., Gibbs, J., Gillespie, R. L., Gundersen, K. B., Gut, L., Hoffman, G., Joshi, N., Lundin, O., … Winfree, R. (2020). Crop production in the USA is frequently limited by a lack of pollinators. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1931), 20200922. https://doi.org/10.1098/rspb.2020.0922

Reitmayer, C. M., Ryalls, J. M. W., Farthing, E., Jackson, C. W., Girling, R. D., & Newman, T. A. (2019). Acute exposure to diesel exhaust induces central nervous system stress and altered learning and memory in honey bees. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-41876-w

Rescorla, R. A. (1988). Behavioral Studies of Pavlovian Conditioning. *Annual Review of Neuroscience*, *11*(1), 329–352. https://doi.org/10.1146/annurev.ne.11.030188.001553

Ricke, D. F., Lin, C.-H., & Johnson, R. M. (2021). Pollen Treated with a Combination of Agrochemicals Commonly Applied During Almond Bloom Reduces the Emergence Rate and Longevity of Honey Bee (Hymenoptera: Apidae) Queens. *Journal of Insect Science*, *21*(6). https://doi.org/10.1093/jisesa/ieab074

Rix, R. R., & Cutler, G. C. (2017). Acute Exposure to Worst-Case Concentrations of Amitraz Does Not Affect Honey Bee Learning, Short-Term Memory, or Hemolymph Octopamine Levels. *Journal of Economic Entomology*, *110*(1), 127–132. https://doi.org/10.1093/jee/tow250

Rondeau, S., & Raine, N. E. (2022). Fungicides and bees: A review of exposure and risk. *Environment International*, *165*, 107311. https://doi.org/10.1016/j.envint.2022.107311

Sánchez-Bayo, F., & Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, *232*, 8–27. https://doi.org/10.1016/j.biocon.2019.01.020

Scheiner, R., Barnert, M., & Erber, J. (2003). Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. *Apidologie*, *34*(1), 67–72. https://doi.org/10.1051/apido:2002050

Scheiner, R., Erber, J., & Page Jr., R. E. (1999). Tactile learning and the individual evaluation of the reward in honey bees (Apis mellifera L.). *Journal of Comparative Physiology A*, *185*(1), 1–10. https://doi.org/10.1007/s003590050360

Scheiner, R., Page, R. E., & Erber, J. (2004). Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). *Apidologie*, *35*(2), 133–142. https://doi.org/10.1051/apido:2004001

Schilder, R. J., & Marden, J. H. (2006). Metabolic syndrome and obesity in an insect. *Proceedings of the National Academy of Sciences*, *103*(49), 18805–18809. https://doi.org/10.1073/pnas.0603156103

Schneider, C. W., Tautz, J., Grünewald, B., & Fuchs, S. (2012). RFID Tracking of Sublethal Effects of Two Neonicotinoid Insecticides on the Foraging Behavior of *Apis mellifera*. *PLOS ONE*, *7*(1), e30023. https://doi.org/10.1371/journal.pone.0030023

Schneider, S., Eisenhardt, D., & Rademacher, E. (2012). Sublethal effects of oxalic acid on *Apis mellifera* (Hymenoptera: Apidae): changes in behaviour and longevity. *Apidologie*, *43*(2), 218–225. https://doi.org/10.1007/s13592-011-0102-0

Schwartz, K. R., Minor, H., Magro, C., McConnell, J., Capani, J., Griffin, J., & Doebel, H. (2021). The neonicotinoid imidacloprid alone alters the cognitive behavior in *Apis mellifera L.* and the combined exposure of imidacloprid and *Varroa destructor* mites synergistically contributes to trial attrition. *Journal of Apicultural Research*, *0*(0), 1–8. https://doi.org/10.1080/00218839.2020.1866233

Sgolastra, F., Medrzycki, P., Bortolotti, L., Maini, S., Porrini, C., Simon-Delso, N., & Bosch, J. (2020). Bees and pesticide regulation: Lessons from the neonicotinoid experience. *Biological Conservation*, *241*, 108356. https://doi.org/10.1016/j.biocon.2019.108356

Shapira, M., Thompson, C. K., Soreq, H., & Robinson, G. E. (2001). Changes in neuronal acetylcholinesterase gene expression and division of labor in honey bee colonies. *Journal of Molecular Neuroscience*, *17*(1), 1–12. https://doi.org/10.1385/JMN:17:1:1

Shi, J., Yang, H., Yu, L., Liao, C., Liu, Y., Jin, M., Yan, W., & Wu, X. B. (2020). Sublethal acetamiprid doses negatively affect the lifespans and foraging behaviors of honey bee (*Apis mellifera* L.) workers. *Science of The Total Environment*, *738*, 139924. https://doi.org/10.1016/j.scitotenv.2020.139924

Si, A., Srinivasan, M. V., & Zhang, S. (2003). Honeybee navigation: Properties of the visually driven `odometer’. *Journal of Experimental Biology*, *206*(8), 1265–1273. https://doi.org/10.1242/jeb.00236

Simcock, N. K., Gray, H., Bouchebti, S., & Wright, G. A. (2018). Appetitive olfactory learning and memory in the honeybee depend on sugar reward identity. *Journal of Insect Physiology*, *106*, 71–77. https://doi.org/10.1016/j.jinsphys.2017.08.009

Simon-Delso, N., San Martin, G., Bruneau, E., Minsart, L.-A., Mouret, C., & Hautier, L. (2014). Honeybee Colony Disorder in Crop Areas: The Role of Pesticides and Viruses. *PLOS ONE*, *9*(7), e103073. https://doi.org/10.1371/journal.pone.0103073

Siviter, H., Koricheva, J., Brown, M. J. F., & Leadbeater, E. (2018). Quantifying the impact of pesticides on learning and memory in bees. *Journal of Applied Ecology*, *55*(6), 2812–2821. https://doi.org/10.1111/1365-2664.13193

Siviter, H., Scott, A., Pasquier, G., Pull, C. D., Brown, M. J. F., & Leadbeater, E. (2019). No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. *PeerJ*, *7*, e7208. https://doi.org/10.7717/peerj.7208

Smith, B. H., & Burden, C. M. (2014). A Proboscis Extension Response Protocol for Investigating Behavioral Plasticity in Insects: Application to Basic, Biomedical, and Agricultural Research. *JoVE (Journal of Visualized Experiments)*, *91*, e51057. https://doi.org/10.3791/51057

Smith, M. R., Mueller, N. D., Springmann, M., Sulser, T. B., Garibaldi, L. A., Gerber, J., Wiebe, K., & Myers, S. S. (2022). Pollinator Deficits, Food Consumption, and Consequences for Human Health: A Modeling Study. *Environmental Health Perspectives*, *130*(12), 127003. https://doi.org/10.1289/EHP10947

Sol Balbuena, M., Tison, L., Hahn, M.-L., Greggers, U., Menzel, R., & Farina, W. M. (2015). Effects of sublethal doses of glyphosate on honeybee navigation. *Journal of Experimental Biology*, *218*(17), 2799–2805. https://doi.org/10.1242/jeb.117291

Steffan-Dewenter, I., & Kuhn, A. (2003). Honeybee foraging in differentially structured landscapes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1515), 569–575. https://doi.org/10.1098/rspb.2002.2292

Steijven, K., Spaethe, J., Steffan-Dewenter, I., & Härtel, S. (2017). Learning performance and brain structure of artificially-reared honey bees fed with different quantities of food. *PeerJ*, *5*, e3858. https://doi.org/10.7717/peerj.3858

Stone, J. C., Abramson, C. I., & Price, J. M. (1997). Task-Dependent Effects of Dicofol (Kelthane) on Learning in the Honey Bee (*Apis mellifera*). *Bulletin of Environmental Contamination and Toxicology*, *58*(2), 177–183. https://doi.org/10.1007/s001289900317

Tadei, R., Menezes-Oliveira, V. B., & Silva-Zacarin, E. C. M. (2020). Silent effect of the fungicide pyraclostrobin on the larval exposure of the non-target organism Africanized *Apis mellifera* and its interaction with the pathogen *Nosema ceranae* in adulthood. *Environmental Pollution*, *267*, 115622. https://doi.org/10.1016/j.envpol.2020.115622

Takeda, K. (1961). Classical conditioned response in the honey bee. *Journal of Insect Physiology*, *6*(3), 168–179. https://doi.org/10.1016/0022-1910(61)90060-9

Taylor, K. S., Waller, G. D., & Crowder, L. A. (1987). IMPAIRMENT OF A CLASSICAL CONDITIONED RESPONSE OF THE HONEY BEE (*APIS MELLIFERA* L.) BY SUBLETHAL DOSES OF SYNTHETIC PYRETHROID INSECTICIDES. *Apidologie*, *18*(3), 243–252. https://doi.org/10.1051/apido:19870304

Thany, S. H., Bourdin, C. M., Graton, J., Laurent, A. D., Mathé-Allainmat, M., Lebreton, J., & Le Questel, J.-Y. (2015). Similar Comparative Low and High Doses of Deltamethrin and Acetamiprid Differently Impair the Retrieval of the Proboscis Extension Reflex in the Forager Honey Bee (*Apis mellifera*). *Insects*, *6*(4), 805–814. https://doi.org/10.3390/insects6040805

Therneau, T.M. (2022). coxme: Mixed Effects Cox Models. R package version 2.2-18.1. https://CRAN.R-project.org/package=coxme

Tison, L., Duer, A., Púčiková, V., Greggers, U., & Menzel, R. (2020). Detrimental effects of clothianidin on foraging and dance communication in honey bees. *PLOS ONE*, *15*(10), e0241134. https://doi.org/10.1371/journal.pone.0241134

Tison, L., Hahn, M.-L., Holtz, S., Rößner, A., Greggers, U., Bischoff, G., & Menzel, R. (2016). Honey Bees' Behavior Is Impaired by Chronic Exposure to the Neonicotinoid Thiacloprid in the Field. *Environmental Science & Technology*, *50*(13), 7218–7227. https://doi.org/10.1021/acs.est.6b02658

Tison, L., Holtz, S., Adeoye, A., Kalkan, Ö., Irmisch, N. S., Lehmann, N., & Menzel, R. (2017). Effects of sublethal doses of thiacloprid and its formulation Calypso® on the learning and memory performance of honey bees. *Journal of Experimental Biology*, *220*(20), 3695–3705. https://doi.org/10.1242/jeb.154518

Tison, L., Rößner, A., Gerschewski, S., & Menzel, R. (2019). The neonicotinoid clothianidin impairs memory processing in honey bees. *Ecotoxicology and Environmental Safety*, *180*, 139–145. https://doi.org/10.1016/j.ecoenv.2019.05.007

Tonkiss, J., & Galler, J. R. (1990). Prenatal protein malnutrition and working memory performance in adult rats. *Behavioural Brain Research*, *40*(2), 95–107. https://doi.org/10.1016/0166-4328(90)90002-V

Traynor, K. S., Pettis, J. S., Tarpy, D. R., Mullin, C. A., Frazier, J. L., Frazier, M., & vanEngelsdorp, D. (2016). In-hive Pesticide Exposome: Assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. *Scientific Reports*, *6*(1), 1–16. https://doi.org/10.1038/srep33207

Traynor, K. S., Tosi, S., Rennich, K., Steinhauer, N., Forsgren, E., Rose, R., Kunkel, G., Madella, S., Lopez, D., Eversole, H., Fahey, R., Pettis, J., Evans, J. D., & vanEngelsdorp, D. (2021). Pesticides in honey bee colonies: Establishing a baseline for real world exposure over seven years in the USA. *Environmental Pollution*, *279*, 116566. https://doi.org/10.1016/j.envpol.2021.116566

Urlacher, E., Monchanin, C., Rivière, C., Richard, F.-J., Lombardi, C., Michelsen-Heath, S., Hageman, K. J., & Mercer, A. R. (2016). Measurements of Chlorpyrifos Levels in Forager Bees and Comparison with Levels that Disrupt Honey Bee Odor-Mediated Learning Under Laboratory Conditions. *Journal of Chemical Ecology*, *42*(2), 127–138. https://doi.org/10.1007/s10886-016-0672-4

Van Dame, R., Meled, M., Colin, M.-E., & Belzunces, L. P. (1995). Alteration of the homing-flight in the honey bee *Apis mellifera* L. Exposed to sublethal dose of deltamethrin. *Environmental Toxicology and Chemistry*, *14*(5), 855–860. https://doi.org/10.1002/etc.5620140517

Vignieri, S. (2013). Bees Get That Caffeine "Buzz." *Science Signaling*, *6*(266), ec64–ec64. https://doi.org/10.1126/scisignal.2004130

Vinggaard, A. M., Hass, U., Dalgaard, M., Andersen, H. R., Bonefeld-Jørgensen, E., Christiansen, S., Laier, P., & Poulsen, M. E. (2006). Prochloraz: An imidazole fungicide with multiple mechanisms of action. *International Journal of Andrology*, *29*(1), 186–192. https://doi.org/10.1111/j.1365-2605.2005.00604.x

von Frisch, K. (1993). *The Dance Language and Orientation of Bees*. Harvard University Press. https://doi.org/10.4159/harvard.9780674418776

Wagner, D. L. (2020). Insect Declines in the Anthropocene. *Annual Review of Entomology*, *65*(1), 457–480. https://doi.org/10.1146/annurev-ento-011019-025151

Wang, Y., Campbell, J. B., Kaftanoglu, O., Page, R. E., Amdam, G. V., & Harrison, J. F. (2016). Larval starvation improves metabolic response to adult starvation in honey bees (*Apis mellifera* L.). *Journal of Experimental Biology*, *219*(7), 960–968. https://doi.org/10.1242/jeb.136374

Weick, J., & Thorn, R. S. (2002). Effects of Acute Sublethal Exposure to Coumaphos or Diazinon on Acquisition and Discrimination of Odor Stimuli in the Honey Bee (Hymenoptera: Apidae). *Journal of Economic Entomology*, *95*(2), 227–236. https://doi.org/10.1603/0022-0493-95.2.227

Williamson, S. M., Baker, D. D., & Wright, G. A. (2013). Acute exposure to a sublethal dose of imidacloprid and coumaphos enhances olfactory learning and memory in the honeybee *Apis mellifera. Invertebrate Neuroscience*, *13*(1), 63–70. https://doi.org/10.1007/s10158-012-0144-7

Williamson, S. M., & Wright, G. A. (2013). Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. *Journal of Experimental Biology*, *216*(10), 1799–1807. https://doi.org/10.1242/jeb.083931

Woodring, J., Boulden, M., Das, S., & Gäde, G. (1993). Studies on blood sugar homeostasis in the honeybee (*Apis mellifera*, L.). *Journal of Insect Physiology*, *39*(1), 89–97. https://doi.org/10.1016/0022-1910(93)90022-J

Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. A., Power, E. F., Borland, A. M., & Stevenson, P. C. (2013). Caffeine in Floral Nectar Enhances a Pollinator's Memory of Reward. *Science*, *339*(6124), 1202–1204. https://doi.org/10.1126/science.1228806

Wright, G. A., Mustard, J. A., Kottcamp, S. M., & Smith, B. H. (2007). Olfactory memory formation and the influence of reward pathway during appetitive learning by honey bees. *Journal of Experimental Biology*, *210*(22), 4024–4033. https://doi.org/10.1242/jeb.006585

Wright, G. A., Mustard, J. A., Simcock, N. K., Ross-Taylor, A. A. R., McNicholas, L. D., Popescu, A., & Marion-Poll, F. (2010). Parallel Reinforcement Pathways for Conditioned Food Aversions in the Honeybee. *Current Biology*, *20*(24), 2234–2240. https://doi.org/10.1016/j.cub.2010.11.040

Wright, G. A., Softley, S., & Earnshaw, H. (2015). Low doses of neonicotinoid pesticides in food rewards impair short-term olfactory memory in foraging-age honeybees. *Scientific Reports*, *5*(1), 1–7. https://doi.org/10.1038/srep15322

Wu-Smart, J., & Spivak, M. (2016). Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development. *Scientific Reports*, *6*, 32108. https://doi.org/10.1038/srep32108

Wykes, G. R. (1952). The Preferences of Honeybees for Solutions of Various Sugars Which Occur in Nectar. *Journal of Experimental Biology*, *29*(4), 511–519. https://doi.org/10.1242/jeb.29.4.511

Xu, Y.-J., Luo, F., Gao, Q., Shang, Y., & Wang, C. (2015). Metabolomics reveals insect metabolic responses associated with fungal infection. *Analytical and Bioanalytical Chemistry*, *407*(16), 4815–4821. https://doi.org/10.1007/s00216-015-8648-8

Yang, E. C., Chuang, Y. C., Chen, Y. L., & Chang, L. H. (2008). Abnormal Foraging Behavior Induced by Sublethal Dosage of Imidacloprid in the Honey Bee (Hymenoptera: Apidae). *Journal of Economic Entomology*, *101*(6), 1743–1748. https://doi.org/10.1603/0022-0493-101.6.1743

Yang, E.-C., Chang, H.-C., Wu, W.-Y., & Chen, Y.-W. (2012). Impaired Olfactory Associative Behavior of Honeybee Workers Due to Contamination of Imidacloprid in the Larval Stage. *PLOS ONE*, *7*(11), e49472. https://doi.org/10.1371/journal.pone.0049472

Zaluski, R., Justulin, L. A., & Orsi, R. de O. (2017). Field-relevant doses of the systemic insecticide fipronil and fungicide pyraclostrobin impair mandibular and hypopharyngeal glands in nurse honeybees (*Apis mellifera*). *Scientific Reports*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-15581-5

Zhang, E., & Nieh, J. C. (2015). The neonicotinoid imidacloprid impairs honey bee aversive learning of simulated predation. *Journal of Experimental Biology*, *218*(20), 3199–3205. https://doi.org/10.1242/jeb.127472

Zhang, G., Olsson, R. L., & Hopkins, B. K. (2023). Strategies and techniques to mitigate the negative impacts of pesticide exposure to honey bees. *Environmental Pollution*, *318*, 120915. https://doi.org/10.1016/j.envpol.2022.120915

APPENDIX A

A COMMON FUNGICIDE, PRISTINE®, IMPAIRS OLFACTORY ASSOCIATIVE LEARNING PERFORMANCE IN HONEY BEES (*APIS MELLIFERA*), COAUTHOR PERMISSIONS

I, Nicole DesJardins, confirm that all co-authors granted permission to use the following, previously published work in this dissertation.

APPENDIX B

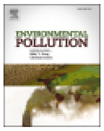
A COMMON FUNGICIDE, PRISTINE®, IMPAIRS OLFACTORY ASSOCIATIVE LEARNING PERFORMANCE IN HONEY BEES (*APIS MELLIFERA*), *ENVIRONMENTAL POLLUTION*

Environmental Pollution 288 (2021) 117720

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



A common fungicide, Pristine®, impairs olfactory associative learning performance in honey bees (*Apis mellifera*)[☆]

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ARTICLE INFO

Keywords:
Boscalid
Pyraclostrobin
Sublethal effects
Honey bee
Associative learning

ABSTRACT

Although fungicides were previously considered to be safe for important agricultural pollinators such as honey bees, recent evidence has shown that they can cause a number of behavioral and physiological sublethal effects. Here, we focus on the fungicide Pristine® (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin), which is sprayed during the blooming period on a variety of crops and is known to affect honey bee mitochondria at field-relevant levels. To date, no study has tested the effects of a field-relevant concentration of a fungicide on associative learning ability in honey bees. We tested whether chronic, colony-level exposure at field-relevant and higher concentrations of Pristine® impairs performance on the proboscis extension reflex (PER) paradigm, an associative learning task. Learning performance was reduced at higher field-relevant concentrations of Pristine®. The reductions in learning performance could not be explained by effects on hunger or motivation, as sucrose responsiveness was not affected by Pristine® exposure. To determine whether Pristine®'s negative effects on learning performance were mediated at a specific life stage, we conducted a cross-fostering experiment that exposed bees to the fungicide either only as larvae, only as adults, or during both stages. We found that exposure across the entire life was necessary to significantly reduce learning performance, although non-significant reductions occurred when bees were exposed during just one stage. Our study provides strong evidence that Pristine® has significant sublethal effects on learning performance. As associative learning is a necessary ability for foraging, our results raise concerns that Pristine® could impair foraging abilities and substantially weaken colony health.

1. Introduction

Insect pollinator populations are declining globally (Sánchez-Bayo and Wyckhuys, 2019), threatening long-term food security. Thirty-five percent of global food production comes from pollinator-dependent crops (Klein et al., 2007), and animal pollination is valued at $14.2–23.8 billion in the United States (Chopra et al., 2015). There has been particular concern regarding the supply of domesticated honey bees, which is growing globally, but more slowly than needed for pollination (Aizen and Harder, 2009). Honey bee population declines in the United States and Europe have caused concern in recent years (Gray et al., 2020; Kulhanek et al., 2017). Pathogens and parasites (Genersch, 2010), poor nutrition (Naug, 2009), and agrochemical exposure (Mullin et al., 2010) have all been implicated as direct contributors to colony losses. It is likely that interacting stressors are to blame (Goulson et al., 2015; Potts et al., 2010). However, agrochemicals in particular have caused concern in the scientific community, as honey bees may be more sensitive than other insects due to a relative lack of detoxification enzymes (Claudianos et al., 2006).

The term "sublethal effects" describes harmful physiological and behavioral effects of agrochemicals that occur in individuals that have survived exposure (Desneux et al., 2006). These effects can weaken honey bee health and contribute to colony loss over time. Sublethal effects shown to be induced by agrochemicals include altered development, reduced fecundity, altered hygienic and foraging behaviors (Wu-Smart and Spivak, 2016), reduced immune function (Di Prisco et al., 2013), lowered fecundity (Wu-Smart and Spivak, 2016), impaired mobility (Lambin et al., 2001), impaired navigation ability (Henry et al.,

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https://doi.org/10.1016/j.envpol.2021.117720
Received 21 April 2021; Received in revised form 23 June 2021; Accepted 3 July 2021

2012), and impaired communication (Eiri and Nieh, 2012).

Insecticides in particular can impair bees' performance in olfactory associative learning tasks, disrupting their ability or motivation to learn to associate a novel odor with a food reward (see Siviter et al., 2018 for a meta-analysis). Some insecticides can also alter bees' motivation or ability to extend their proboscis in response to sucrose (Eiri and Nieh, 2012), a main phagostimulatory component of floral nectar (Wykes, 1952). Most insecticides target various components of the insect nervous system, providing a potential mechanism for the effects on learning behavior and sucrose responsiveness (Belzunces et al., 2012). For example, the effects of neonicotinoids on learning performance have been particularly well-studied (e.g. Aliouane et al., 2009; Decourtye et al., 2005; Decourtye et al., 2004).

Bees are also exposed to other agrochemicals while foraging, including fungicides (Mullin et al., 2010). Short-term toxicity tests for fungicides have suggested that they are safe for bees (Ladurner et al., 2005). However, standard toxicity tests do not take sublethal effects into account (Desneux et al., 2006). Sublethal exposure to fungicides may cause significant stress to honey bees, as residues found in hives have been correlated with colony decline (Simon-Delso et al., 2014). Here, we focus on the fungicide Pristine®, which is licensed for use on a variety of crops pollinated by honey bees including bulb vegetables, berries, pome and stone fruits, and tree nuts. Of significant importance is that Pristine® is registered for use on almonds during the bloom period (BASF, 2019). More than 70 percent of all commercial honey bee hives in the US are brought to California for almond pollination during bloom each year (Lee et al., 2019). Residues of the active ingredients of Pristine®, boscalid and pyraclostrobin, have been found in bee hives in the US and Europe (Mullin et al., 2010; Simon-Delso et al., 2014). Boscalid and pyraclostrobin inhibit respiration in fungal cells (Earley et al., 2012), and Pristine® also directly inhibits honey bee mitochondrial function *in vitro* (Campbell et al., 2016). When consumed with pollen, Pristine® interferes with protein digestion and increases virus titers (DeGrandi-Hoffman et al., 2015). When consumed in pollen at field-realistic levels, it reduces thorax mass (Glass et al., 2021), colony population size and worker lifespan, and causes earlier foraging that is more focused on pollen-collection (Fisher et al., 2021a). The latter finding suggests that the toxic effects may be at least partially behaviorally-mediated.

Here, we evaluate how chronic exposure of honey bees to Pristine® affects learning performance and sucrose responsiveness. The proboscis extension reflex (PER) paradigm, which measures how well bees learn to associate a neutral odor with a sucrose reward (Bitterman et al., 1983; Smith and Burden, 2014), is well-suited to measure the learning abilities in bees exposed to agrochemicals (Pham-Delègue et al., 2002). Some non-insecticide agrochemicals, such as spray adjuvants (Ciarlo et al., 2012), herbicides (Farina et al., 2019), and one other fungicide (prochloraz) (Decourtye et al., 2005), as well as some heavy metals (Burden et al., 2016; Burden et al., 2019), have been shown to affect associative learning and sucrose responsiveness. We tested olfactory associative learning and sucrose responsiveness in individual bees from colonies that chronically consumed Pristine®-contaminated pollen, mimicking natural exposure in the field. We also investigated whether the behavioral effects of Pristine® on honey bees were mediated at the larval or adult stage, or both.

2. Materials and methods

2.1. Honey bee colonies and fungicide exposure

Initiation and fungicide treatment of the experimental colonies is described in detail in Fisher et al. (2021a); the basic protocols are presented here. In April 2018, forty 1.36 kg Italian honey bee (*Apis mellifera linguistica*) packages (Pendell Apiaries, Inc., Stonyford, CA) were used to initiate colonies in Apimaye insulated hives (Kaftan LLC, Tempe, AZ) at the Arizona State University Bee Lab in Mesa, AZ (33.293173, −111.684520). To prevent exposure to stored agrochemicals, each colony was initially stocked with five wooden frames with plastic cell foundation, which the workers used to construct new comb. Colonies were supplied with 30 percent sucrose solution *ad libitum* for the first three weeks after initiation to help them build combs. Pollen traps were placed internally over the hive entrance to restrict the amount of pollen that workers could bring inside. Pollen patties (50 g) containing a mixture of pollen (30.6%), sucrose (30.6%), fondant sugar (30.6%), and water (8.2%) were placed in a Petri dish inside each hive. A week before fungicide treatments began, the hives were equalized so they each had five drawn combs and approximately equal adult populations. As colonies continued to grow, additional frames with bare, plastic cell foundation were provided to minimize possible exposure to chemicals in old wax.

The fungicide treatments began in May 2018, one month after colony initiation. Pristine® (BASF Corporation, Research Triangle Park, NC) was dissolved in deionized water and mixed into pollen patties at the following concentrations: 0.23 ppm, 2.3 ppm, 23, ppm, and 230 ppm (this replaced the 8.2% water described above in pollen patty preparation). These concentrations were meant to bracket those measured in corbicular pollen collected from bees foraging in Pristine®-treated almond orchards (which ranged from 3.13 ppm to 24 ppm, as measured by Fisher et al., 2021a). The quantity of the pollen patty consumed was measured at least weekly (Fisher et al., 2021a). The per larva and per adult doses of Pristine®, boscalid, and pyraclostrobin were calculated by Fisher et al. (2021a) and are shown in Table 1. A fifth group, the control, received pollen patties with only deionized water. Each of the five treatment groups contained eight hives. Hives were continually monitored and by very briefly looking inside the hive every other day to check the amount of pollen left in the patty. Pollen patties were replaced as soon as they were consumed, or at least weekly to maintain freshness.

2.2. General olfactory associative learning

Preparation and conditioning methods were based on those described in Smith and Burden (2014). We sampled from 20 hives (out of the 40 total involved in the larger experiment). Among each treatment group, each hive was assigned a number from one through eight, and a pseudo-random number generator (random.org) was used to randomly select four numbers within that range, which corresponded to the hives used in the experiment. Of the 20 hives selected, three showed poor brood production and foraging, and because of this, they were not sampled from during the learning experiment. Thus, a total of 17 hives were used (3–4 per treatment group). Ten bees were trained at a time; they were collected at the hive entrance as they returned from foraging trips and immediately brought into the lab and anesthetized on ice. They were then harnessed in plastic drinking straws (3 cm tall, 0.9 cm inner diameter) and held in place by strips of duct tape (0.2 × 6 cm) so that

Table 1
Concentrations of Pristine®, boscalid, and pyraclostrobin in the pollen patties for each treatment group, along with the calculated per larva and per adult dose (Fisher et al., 2021).

	Pristine	Boscalid	Pyraclostrobin
Pollen patty, ppm	**0.23**	**0.06**	**0.03**
Per larva dose, ng	0.086	0.022	0.011
Per adult dose, ng	6.9	1.73	0.88
Pollen patty, ppm	**2.3**	**0.6**	**0.3**
Per larva dose, ng	1.0	0.25	0.13
Per adult dose, ng	79.7	20.1	10.2
Pollen patty, ppm	**23**	**6**	**3**
Per larva dose, ng	8.3	2.09	1.06
Per adult dose, ng	663	167.1	84.9
Pollen patty, ppm	**230**	**60**	**30**
Per larva dose, ng	89.9	22.7	11.5
Per adult dose, ng	7194	1813	921

only their antennae and mouthparts were moveable. Each individual was then fed 3–5 μL of 1.0 M sucrose one time and allowed to rest for 45 min at room temperature. This round of feeding served to keep them from depleting their energy reserves during the acclimation period (Smith and Burden, 2014). After the resting period, the bees' antennae were stimulated with a droplet of 1.5 M sucrose. Bees that did not extend their proboscis in response to antennal stimulation were considered not sufficiently motivated to learn and were removed from the experiment prior to its start.

We used a discrimination conditioning procedure to evaluate associative learning and reduce the possibility that changes in behavior were due to non-associative mechanisms (Benatar et al., 1995). Conditioned Stimuli (CS) were always odors. Thus, we included both a CS^+, which was followed by reinforcement with a sucrose droplet, and a CS^-, which was not. The chemicals 1-hexanol (Sigma-Aldrich, St. Louis, MO) and 2-octanone (Sigma-Aldrich, St. Louis, MO) were counterbalanced as the CS^+ and CS^- odors. Prior to training, strips of filter paper (Sigma-Aldrich, St. Louis, MO) containing 0.7 μL of the target odor were placed into glass cartridges (1 cc tuberculin syringe barrels, BD Medical, Franklin Lakes, NJ). Odor cartridges were changed after every fifth trial, according to standard protocols (Smith and Burden, 2014).

The acquisition phase consisted of eight rewarded trials, during which the CS^+ odor was delivered, and eight unrewarded trials, during which the CS^- odor was delivered. The types of trials were intermingled in a pseudorandomized order (+ − − + − + + − + − − + − + + −). After the acquisition phase, bees rested for 45 min at room temperature before beginning the test phase, which consisted of six unrewarded trials of the CS^+ odor only.

The plexiglass conditioning arena consisted of a circular stand on which a harness could be placed, a piece of modeling clay on which the odor tube could be mounted in front of the bee, and an exhaust system (hooked up to our laboratory vacuum system with dryer tubing) to prevent odors from lingering. The odor delivery system consisted of an air tube that could be hooked up to an odor cartridge and automated via a programmable logic controller (Automation-Direct, Cumming, GA) to deliver the odor at the correct times. While not being actively trained, bees were kept in a staging area approximately 30 cm from the training arena.

During acquisition trials with the CS^+ odor, a bee was placed into the arena and allowed to sit for 25 s. After that, an air pulse (flowing at 7 mL/s) was directed through the odor cartridge and toward the bee for 4 s. During the last second of odor delivery, the bee was manually fed 0.4 μL of 1.5 M sucrose using a 0.2 mL Gilmont syringe (Cole-Parmer, Vernon Hills, IL). If the bee extended her proboscis after odor delivery but before sucrose presentation, she had learned successfully (denoted by a '1' in our scoring system). If a bee extended her proboscis after sucrose presentation, she had responded to sucrose but not to the odor (denoted by a '0' in our scoring system). Bees that did not extend their proboscis to either odor or sucrose were scored with an 'NA'.

After sucrose presentation, the bee was left in the arena for another 30 s, allowing her to form initial memories before being moved back to the staging area. This 1-min process was repeated for each of the other nine bees before starting the second trial with the first bee, allowing for a 10-min inter-trial interval.

During unrewarded trials, the process was the same as above, except the bee was not fed sucrose. The Gilmont syringe was still held close to the bees' heads during the time when sucrose would normally be fed in order to reduce changes in stimulation as much as possible.

2.3. *Sucrose responsiveness*

Changes in learning performance due to fungicide treatment could be caused by changes in taste responsiveness rather than impaired associative learning *per se*. To test this, bees were sampled from the same 17 hives that were used in the first experiment. Bee collection and harnessing protocols were the same as those described above. After the 45-min resting period, bees were exposed via antennal stimulation to 1–2 μL of increasing concentrations of sucrose: 0.1, 0.3, 1, 3, 10, and 30 percent solutions (w/v in deionized water). They were not allowed to feed. Deionized water was presented after each sucrose concentration, with 8-min intervals between each trial. For each sucrose or water presentation, we recorded whether the bee extended its proboscis (denoted by a '1') or did not (denoted by a '0'). For data analysis, each bee was given a "sensitivity score" for each sucrose concentration: response to sucrose minus response to the following water presentation. This gave us a range of values of either '0' or '1' (A '-1' value was also theoretically possible; however, this was not actually observed). A '0' value indicated that a bee had responded to both the sucrose and water, or neither the sucrose nor the water. A '1' value indicated that an individual had responded to the particular concentration of sucrose but not the water.

2.4. *Cross-fostering olfactory associative learning*

For this experiment, bees were exposed to Pristine® either only as larvae, only as adults, during both developmental stages, or not at all, using a cross-fostering design (Fisher et al., 2021b). Because adults consume much more pollen than larvae and therefore receive a much greater dose (Table 1), we hypothesized that effects of Pristine® on learning would stem from exposure during the adult stage. To rigorously test this hypothesis, we used a supra-field concentration of Pristine®. Randomly-chosen capped brood frames from three control (0 ppm Pristine®) hives and three hives treated with 230 ppm Pristine® were brought into the lab and placed in a wire mesh frame cage (L x W x H: 53.3 × 5.1 × 27.9 cm) in an incubator (34 °C, 90% relative humidity) until ∼600 adults had emerged from each frame. The adults were marked on the mesonotum with a paint color corresponding to their hive of origin.

The bees from the control and 230 ppm Pristine® colonies were each divided into four subsets of ∼450 individuals. Two control colonies each received ∼450 bees from control colonies and ∼450 bees that had been reared as larvae in the 230 ppm Pristine® treatment group. Two colonies from the 230 ppm treatment group each received ∼450 bees that had been reared as larvae in control colonies and ∼450 bees that had been reared as larvae in colonies in the 230 ppm Pristine® treatment group. This resulted in four treatment groups that are named according to their larval and adult Pristine® exposure: larval control/adult control, larval control/adult Pristine®, larval Pristine®/adult Pristine®, and larval Pristine®/adult control. The learning abilities of bees from these four treatment groups were tested once they began foraging outside the hive, using the same protocol as described above.

2.5. *Statistical analyses*

All data were analyzed in R version 3.6.2 (R Core Team, 2019). Learning data were analyzed using generalized linear mixed effects models and the lme4 package (Bates et al., 2015). Trial, hive, and treatment group were fixed effects and individual was a random effect. For the five-concentration learning experiment, another fixed effect was added—season—in order to determine whether there were any differences between the bees tested in December and March. Season and hive did not have significant effects, so these variables were pooled in the model. For post-hoc pairwise comparisons, the estimated marginal means were calculated using the emmeans package (Lenth, 2019). P-values were adjusted using the Tukey method for multiple comparisons.

For the sucrose response data, we considered that a bee had responded to a particular sucrose concentration if she extended her proboscis in response to that concentration, but not to the water presented immediately afterward. Thus, we subtracted water responses from the corresponding sucrose responses and used this "sensitivity score" to run a generalized linear mixed effects model. Sucrose concentration, treatment group, and season were fixed effects, and individual was a random effect. The season effect revealed a significant difference between the January–February and March–May experiments, so two models were constructed to analyze each experiment separately.

3. Results

3.1. *General olfactory associative learning*

Colony-level Pristine® consumption affected performance (proportion of bees that successfully learned the odor-reward association across eight acquisition trials) on our PER associative learning assay ($X^2 = 7.9$, $p = 0.0050$). By the eighth acquisition trial, control bees performed, on average, 20% better than bees consuming pollen containing the 23 ppm and 230 ppm Pristine® concentrations. (Fig. 1A). Hive and season did not significantly affect results. Bees rarely responded to the CS^- odor, and there were no trends across trial or treatment group. Pristine®-exposed bees also responded to the odor less often than control bees throughout the unrewarded test trials ($X^2 = 17$, $p < 0.001$, Fig. 1B). Post-hoc pairwise comparisons showed that the 23 ppm and 230 ppm treatment groups performed significantly worse than the control treatments during the test phase.

3.2. *Sucrose responsiveness*

Here, we measured the proportion of bees that responded to ascending sucrose concentrations, corrected for their responses to water. An initial model revealed significant differences ($X^2 = 5.9$, $p = 0.015$) between results obtained in January/February versus May/June, corroborating earlier results that show seasonal variation in forager sucrose responsiveness (Scheiner et al., 2003). Because of this, the data from the two time periods were analyzed separately. Pristine® exposure did not have an effect in either January/February ($X^2 = 1.7$, $p = 0.19$, Fig. 2A) or May/June ($X^2 = 0.62$, $p = 0.43$, Fig. 2B). Thus, regardless of season, Pristine® consumption did not affect sucrose responsiveness, suggesting that the differences in associative learning ability between treatment groups were not caused by effects on sucrose responsiveness.

3.3. *Cross-fostering olfactory associative learning*

Performance (proportion of bees that successfully learned the odor-reward association) in the PER olfactory learning assay differed significantly among treatment groups during both the acquisition ($X^2 = 8.95$, $p = 0.030$, Fig. 3A) and test phases ($X^2 = 10.2$, $p = 0.017$, Fig. 3B). Hive did not significantly affect the results. Post-hoc pairwise comparisons revealed that the control group (larval control/adult control) performed better than the bees exposed to Pristine® at both larval and adult stages (larval 230 ppm/adult 230 ppm). Bees exposed during just the larval or adult stage performed intermediate to the two constant treatment groups, but these groups did not differ significantly from either treatment group.

4. Discussion

The issue of sublethal effects of agrochemicals, including fungicides, on pollinators is very concerning, with recent studies demonstrating that field application levels previously thought of as non-toxic may have significant longer-term impact on honey bee health (Fisher et al., 2021a). Our study demonstrates an important impact of field-relevant doses of the fungicide Pristine® on olfactory associative learning. We found that consumption of pollen containing 23 and 230 ppm of the fungicide in field colonies reduced olfactory associative learning performance with no concurrent effect on sucrose responsiveness. The impaired associative learning caused by Pristine® consumption was thus not due to a simple suppression of the ability to taste sucrose or reduced motivation to feed.

Reduced learning ability, as demonstrated here, can negatively impact colony functioning through impairing foraging or navigation ability in exposed bees. It is well-documented that olfactory associative learning is necessary to remember the locations of food sources (Gerber et al., 1996; Kirchner and Grasser, 1998; von Frisch, 1967). Some agrochemicals that can impair learning can also negatively impact navigation or foraging ability (Sol Balbuena et al., 2015; Fischer et al., 2014; Henry et al., 2012; Schneider et al., 2012), further suggesting that impaired associative learning performance could damage proper colony functioning by impairing the bees' ability to find and communicate the locations of food resources.

Although the effects of neuroactive insecticides on bee associative learning performance are well-documented, only one other study showed impaired learning as the result of exposure to a fungicide, prochloraz (Decourtye et al., 2005). Prochloraz acts by a completely different mechanism than boscalid and pyraclostrobin, as it is an inhibitor of cytochrome P450 enzymes and is considered an endocrine disruptor (Vinggaard et al., 2006).

A variety of other agrochemicals beyond fungicides impair PER learning in honey bees (Siviter et al., 2018). Many of these are insecticides that are designed to be neuroactive, specifically to affect cholinergic transmission, which provides a logical underlying mechanism for their effects on learning, as acetylcholine is the main neurotransmitter involved in learning and memory in the honey bee (Lozano et al., 2001). Ingestion of a few other agrochemicals that are not designed to be neuroactive has been shown to affect learning, including organosilicone agricultural spray adjuvants (Ciarlo et al., 2012), the fungicide prochloraz (Decourtye et al., 2005), and the herbicide glyphosate (Farina et al., 2019). The heavy metal selenium also negatively affects learning (Burden et al., 2016). The mechanisms underlying

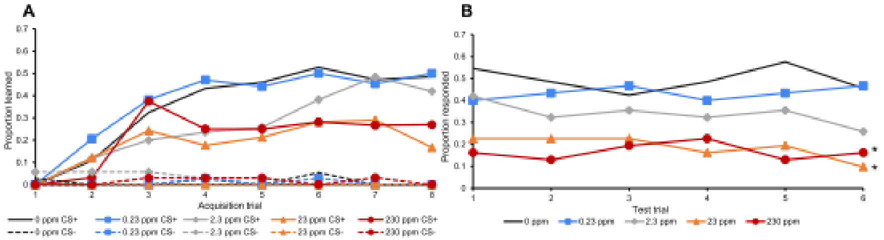

Fig. 1. Proportion of bees chronically exposed to Pristine® fungicide showing learning in a PER olfactory learning assay, during (A) the acquisition phase and (B) the test phase. N = 33–37 individuals per treatment group. In (A), solid lines indicate responses to the CS+, while dashed lines represent responses to the CS-. Asterisks indicate that a treatment group is significantly different from the control.

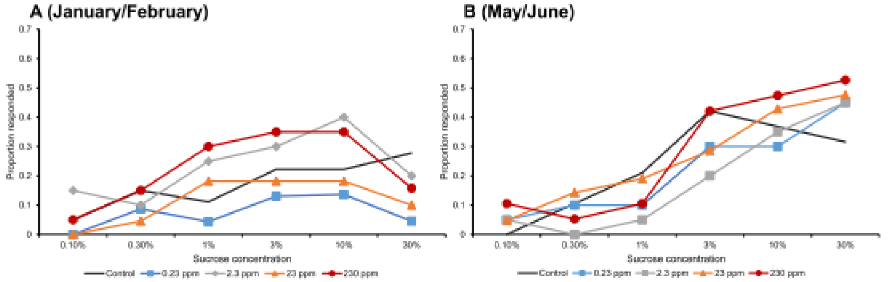

Fig. 2. Sensitivity to different sucrose concentrations for bees chronically exposed to five concentrations of Pristine® fungicide. Bees were tested in either January–February (A) or May–June (B). Graphs represent sensitivity score—proportion of bees responding to sucrose minus the proportion responding to water presented immediately afterward. For both graphs, N = 19–23 individuals per treatment group.

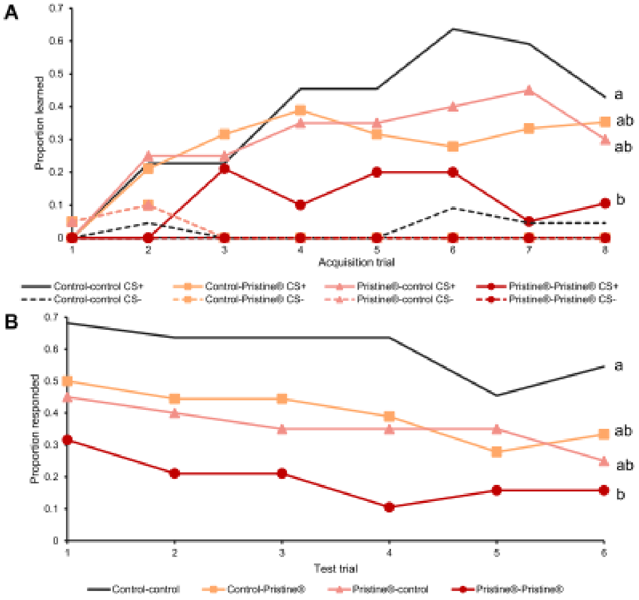

Fig. 3. Proportion of bees exposed to Pristine® fungicide either as larvae, adults, neither, or both showing learned responses in a PER olfactory learning assay, during (A) the acquisition phase and (B) the test phase. In the legend, each treatment group is indicated in the following manner: larval treatment-adult treatment. N = 19–21 individuals per treatment group. In (A), solid lines indicate responses to the CS+, while dashed lines represent responses to the CS-. Lowercase letters on the right indicate statistically significant differences between treatment groups.

these effects remain mostly unknown. Regardless, these studies reveal that a wide variety of chemicals, including those that are not meant to target the insect nervous system, can cause negative sublethal effects on learning in honey bees.

A key question is whether the observed toxic and behavioral effects of Pristine® on honey bees were mediated at the larval or adult stage (or both). Young adults eat a substantial amount of pollen, while larvae consume only a few mg late in development (Crailsheim et al., 1992; Babendreier et al., 2004), suggesting that adult exposure is the most critical, at least for Pristine® in pollen. Young adults feed larvae by producing glandular secretions that could contain consumed fungicides. However, concentrations of boscalid and pyraclostrobin were undetectable in royal jelly produced by nurse bees fed Pristine®, suggesting that the active ingredients in Pristine® are not passed on to larvae in the brood food (DeGrandi-Hoffman et al., 2013). Also, Fisher et al. (2021a) reported no effects of consumption of Pristine® on colony egg and brood levels. Therefore, we hypothesized that Pristine® exposure exerts its negative effects on honey bee colonies only at the adult stage. To rigorously test this hypothesis, we reared bees in colonies provided Pristine® only in the larval, only in the adult, or in both stages, testing effects on learning capabilities of adult foraging-age workers. We used a supra-field concentration (230 ppm) of Pristine®, as peak exposures are likely higher than average concentrations, and our goal was to exclude the possibility that larvae are negatively impacted by colonial Pristine® consumption in pollen. We found that consumption of pollen containing 230 ppm Pristine® during both the larval and adult life stages significantly reduced learning ability, while non-significant reductions occurred when bees were exposed during only one life stage. These results suggest that the sublethal behavioral effects of Pristine® are mediated at both the larval and adult life stages. At present it is unclear whether such effects are due to the consumption of Pristine® by the larvae, or to the effects of Pristine® consumption on the feeding and care provided to the larvae by the adults.

The findings of this study lead naturally to the question of mechanisms for impaired learning. Other agrochemicals that can impair associative learning, such as imidacloprid (Eiri and Nieh, 2012; Lambin et al., 2001), thiamethoxam (Aliouane et al., 2009; Démares et al., 2016), flupyradifurone (Hesselbach and Scheiner, 2018), and fluvalinate (Frost et al., 2013) have been shown simultaneously to affect sucrose responsiveness in honey bees. Sucrose is the unconditioned stimulus in the PER paradigm, and individual differences in sucrose responsiveness correlate with performances during the assay (Scheiner et al., 2004). However, as Pristine® does not appear to affect sucrose responsiveness, there is likely some other mechanism behind its effects on learning.

Pristine® may impair learning indirectly through effects on nutrient absorption via the gut. Ultimately, impaired nutrient absorption could impact learning either by interfering with post-ingestive feedback mechanisms necessary for learning or with proper brain development. Pristine® may interfere with nutrient transport in honey bees, as it impairs protein digestion (DeGrandi-Hoffman et al., 2015), and one of its active ingredients (pyraclostrobin) damages the midgut epithelium (Tadei et al., 2020). If bees are stimulated with but not fed sugar during PER training, their memory consolidation is worse overall than when allowed to feed during training (Wright et al., 2007). Furthermore, feeding with a tasteless but nutritional sugar produces robust learning (Mustard et al., 2018). These results suggest that the rise in hemolymph glucose levels following sugar ingestion may provide a post-ingestive signal and help the bee form a robust memory of the association (Simcock et al., 2018). The exact physiological mechanisms underlying this post-ingestive feedback remain unknown; however, it is possible that the brain is able to sense either changes in hemolymph glucose levels or a rise in intracellular ATP levels (Simcock et al., 2018). If Pristine® interferes with nutrient absorption, it may block this post-ingestive feedback mechanism from promoting memory consolidation. Pristine® could also affect nutrient absorption during larval and pupal development, possibly impairing proper brain development. It has been well-established that malnutrition during development can alter cognitive performance later in life in other animals, especially in rodents (Halas et al., 1979; Morgane et al., 1993; Tonkiss and Galler, 1990). Relatively few studies have examined this phenomenon in honey bees, although two studies failed to find a link between nutrient deprivation at either the individual (Steijven et al., 2017) or colony (Mattila and Smith, 2008) level and impaired learning. Regardless, it is possible that Pristine® affects nutrient uptake and development in larvae, as both larval and adult exposure was necessary to cause significantly reduced learning in adults.

Alternatively, Pristine® could affect learning by directly poisoning mitochondria in the brain (Campbell et al., 2016). Although the degree to which Pristine® can pass through the gut and into the hemolymph remains unclear, it is plausible that some amount could reach the brain. Previous studies have used cytochrome oxidase activity as a proxy for cellular respiration in the bee brain; increased activity in the mushroom body calyces correlates with acquisition in the PER learning assay (Déglise et al., 2003). When the neonicotinoid insecticide imidacloprid is fed to bees, they show altered cytochrome oxidase activity in the mushroom body calyces, which correlates with impaired PER learning (Decourtye et al., 2004). Future studies are necessary to determine whether any amount of consumed Pristine® enters the bee brain. It will remain unclear whether Pristine® directly poisons mitochondria in the bee brain or whether its effects on associative learning result from more indirect causes, such as impaired nutrient absorption.

5. Conclusion

Our study shows that the fungicide Pristine® may not be entirely safe for pollinators. Along with its effects on learning, a variety of other negative effects have been reported, including disrupted nestmate recognition in solitary bees (Artz and Pitts-Singer, 2015), reduced queen emergence (DeGrandi-Hoffman et al., 2013), impaired protein digestion and increased virus titers (DeGrandi-Hoffman et al., 2015), precocious foraging, and reduced adult worker population (Fisher et al., 2021a). Future research should seek to determine whether additional behaviors are impaired, such as navigation and foraging ability. Further work is also necessary to determine the mechanisms underlying impaired learning, including whether Pristine® is directly affecting mitochondrial function in the brain or whether it is acting through more indirect mechanisms, such as impaired nutrient absorption. Additionally, due to the multitude of sublethal effects that have been reported across a variety of agrochemical classes, standard toxicity tests may need to be redesigned by stakeholders such as the United States EPA and USDA to take these into account (Barascou et al., 2021; Fisher, 2021). These results suggest that this commonly-used agrochemical may not be safe for honey bees, an important agricultural pollinator.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Meredith Johnson and Shreya Nandy for assistance with data collection, Chelsea Cook for assistance with data analysis, and Trevor Fox and Meredith Johnson for feedback on the manuscript. This work was supported by the United States Department of Agriculture grant 2017-68004-26322.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117720.

Author contributions

ND: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. AF: Investigation, writing – reviewing and editing. CO: Conceptualization, Resources, writing – reviewing and editing. JF: Conceptualization, Funding acquisition, writing – reviewing and editing. GDH: Conceptualization, Funding acquisition, writing – reviewing and editing. JH: Conceptualization, writing – reviewing and editing, Supervision, Funding acquisition. BS: Conceptualization, Methodology, writing – reviewing and editing, Supervision, Funding acquisition.

References

Aizen, M.A., Harder, L.D., 2009. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr. Biol. 19 (11), 915–918. https://doi.org/10.1016/j.cub.2009.03.071.

Aliouane, Y., El Hassani, A.K., Gary, V., Armengaud, C., Lambin, M., Gauthier, M., 2009. Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. Environ. Toxicol. Chem. 28 (1), 113–122. https://doi.org/10.1897/08-110.1.

Artz, D.R., Pitts-Singer, T.L., 2015. Effects of fungicide and adjuvant sprays on nesting behavior in two managed solitary bees, *Osmia lignaria* and *Megachile rotundata*. PloS One 10 (8), e0135688. https://doi.org/10.1371/journal.pone.0135688.

Babendreier, D., Kalberer, N., Romeis, J., Fluri, P., Bigler, F., 2004. Pollen consumption in honey bee larvae: a step forward in the risk assessment of transgenic plants. Apidologie 35 (3), 293–300. https://doi.org/10.1051/apido:2004016.

Barascou, L., Brunet, J.-L., Belzunces, L., Decourtye, A., Henry, M., Fourrier, J., Le Conte, Y., Alaux, C., 2021. Pesticide risk assessment in honeybees: toward the use of behavioral and reproductive performances as assessment endpoints. Chemosphere 276, 130134. https://doi.org/10.1016/j.chemosphere.2021.130134.

BASF, 2019. *Pristine®: Specimen Label*. Retrieved from. http://www.cdms.net/ldat/ld6DD016.pdf.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Software 67 (1), 1–48. https://doi.org/10.18637/jss.v067.i01.

Belzunces, L.P., Tchamitchian, S., Brunet, J.-L., 2012. Neural effects of insecticides in the honey bee. Apidologie 43 (3), 348–370. https://doi.org/10.1007/s13592-012-0134-0.

Benatar, S.T., Cobey, S., Smith, B.H., 1995. Selection on a haploid genotype for discrimination learning performance: correlation between drone honey bees (*Apis mellifera*) and their worker progeny (Hymenoptera: apidae). J. Insect Behav. 8 (5), 637–652. https://doi.org/10.1007/BF01997235.

Bitterman, M.E., Menzel, R., Fietz, A., Schäfer, S., 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J. Comp. Psychol. 97 (2), 107–119. https://doi.org/10.1037/0735-7036.97.2.107.

Burden, C.M., Elmore, C., Hladun, K.R., Trumble, J.T., Smith, B.H., 2016. Acute exposure to selenium disrupts associative conditioning and long-term memory recall in honey bees (*Apis mellifera*). Ecotoxicol. Environ. Saf. 127, 71–79. https://doi.org/10.1016/j.ecoenv.2015.12.034.

Burden, C.M., Morgan, M.O., Hladun, K.R., Amdam, G.V., Trumble, J.J., Smith, B.H., 2019. Acute sublethal exposure to toxic heavy metals alters honey bee (*Apis mellifera*) feeding behavior. Sci. Rep. 9 (1), 1–10. https://doi.org/10.1038/s41598-019-40396-x.

Campbell, J.B., Nath, R., Gadau, J., Fox, T., DeGrandi-Hoffman, G., Harrison, J.F., 2016. The fungicide Pristine® inhibits mitochondrial function in vitro but not flight metabolic rates in honey bees. J. Insect Physiol. 86, 11–16. https://doi.org/10.1016/j.jinsphys.2015.12.003.

Chopra, S.S., Bakshi, B.R., Khanna, V., 2015. Economic dependence of U.S. Industrial sectors on animal-mediated pollination service. Environ. Sci. Technol. 49 (24), 14441–14451. https://doi.org/10.1021/acs.est.5b03788.

Ciarlo, T.J., Mullin, C.A., Frazier, J.L., Schmehl, D.R., 2012. Learning impairment in honey bees caused by agricultural spray adjuvants. PloS One 7 (7), e40848. https://doi.org/10.1371/journal.pone.0040848.

Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 15 (5), 615–636. https://doi.org/10.1111/j.1365-2583.2006.00672.x.

Crailsheim, K., Schneider, L.H.W., Hrassnigg, N., Bühlmann, G., Brosch, U., Gmeinbauer, R., Schöffmann, B., 1992. Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): dependence on individual age and function. J. Insect Physiol. 38 (6), 409–419. https://doi.org/10.1016/0022-1910(92)90117-V.

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M., Pham-Delègue, M.-H., 2004. Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). Pestic. Biochem. Physiol. 78 (2), 83–92. https://doi.org/10.1016/j.pestbp.2003.10.001.

Decourtye, A., Devillers, J., Genecque, E., Menach, K.L., Budzinski, H., Cluzeau, S., Pham-Delègue, M.H., 2005. Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee Apis mellifera. Arch. Environ. Contam. Toxicol. 48 (2), 242–250. https://doi.org/10.1007/s00244-003-0262-7.

Déglise, P., Dacher, M., Dion, E., Gauthier, M., Armengaud, C., 2003. Regional brain variations of cytochrome oxidase staining during olfactory learning in the honeybee (*Apis mellifera*). Behav. Neurosci. 117 (3), 540–547. https://doi.org/10.1037/0735-7044.117.3.540.

DeGrandi-Hoffman, G., Chen, Y., Simonds, R., 2013. The effects of pesticides on queen rearing and virus titers in honey bees (*Apis mellifera* L.). Insects 4 (1), 71–89. https://doi.org/10.3390/insects4010071.

DeGrandi-Hoffman, G., Chen, Y., Watkins Dejong, E., Chambers, M.L., Hidalgo, G., 2015. Effects of oral exposure to fungicides on honey bee nutrition and virus levels. J. Econ. Entomol. 108 (6), 2518–2528. https://doi.org/10.1093/jee/tov251.

Démares, F.J., Crous, K.L., Pirk, C.W.W., Nicolson, S.W., Human, H., 2016. Sucrose sensitivity of honey bees is differently affected by dietary protein and a neonicotinoid pesticide. PloS One 11 (6), e0156584. https://doi.org/10.1371/journal.pone.0156584.

Desneux, N., Decourtye, A., Delpuech, J.-M., 2006. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52 (1), 81–106. https://doi.org/10.1146/annurev.ento.52.110405.091440.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G., Pennacchio, F., 2013. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proc. Natl. Acad. Sci. Unit. States Am. 110 (46), 18466–18471. https://doi.org/10.1073/pnas.1314923110.

Earley, F., Sauter, H., Rheinheimer, J., Rieck, H., Coqueron, P.-Y., Whittingham, W.G., Walter, H., 2012. Fungicides acting on oxidative phosphorylation. In: Modern Crop Protection Compounds. John Wiley & Sons, Ltd, pp. 559–691. https://doi.org/10.1002/9783527644179.ch15.

Eiri, D.M., Nieh, J.C., 2012. A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. J. Exp. Biol. 215 (12), 2022–2029. https://doi.org/10.1242/jeb.068718.

Farina, W.M., Sol Balbuena, M., Herbert, L.T., Mengoni Goñalons, C., Vázquez, D.E., 2019. Effects of the herbicide glyphosate on honey bee sensory and cognitive abilities: individual impairments with implications for the hive. Insects 10 (10), 354. https://doi.org/10.3390/insects10100354.

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B., Menzel, R., 2014. Neonicotinoids interfere with specific components of navigation in honeybees. PloS One 9 (3), e91364. https://doi.org/10.1371/journal.pone.0091364.

Fisher, A., 2021. Protect pollinators—Reform pesticide regulations. Nature 595 (7866), 172. https://doi.org/10.1038/d41586-021-01818-x.

Fisher, A., DeGrandi-Hoffman, G., Smith, B.H., Johnson, M., Kaftanoglu, O., Cogley, T., Fewell, J.H., Harrison, J.F., 2021a. Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*). Environ. Pollut. 269, 115964. https://doi.org/10.1016/j.envpol.2020.115964.

Fisher, A., DeGrandi-Hoffman, G., Smith, B.H., Ozturk, C., Kaftanoglu, O., Fewell, J.H., Harrison, J.F., 2021b. Field cross-fostering and in vitro rearing demonstrate negative effects of both larval and adult exposure to a widely used fungicide in honey bees (*Apis mellifera*). Ecotoxicol. Environ. Saf. 217, 112251. https://doi.org/10.1016/j.ecoenv.2021.112251.

Frost, E.H., Shutler, D., Hillier, N.K., 2013. Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival. J. Exp. Biol. 216 (15), 2931–2938. https://doi.org/10.1242/jeb.086538.

Genersch, E., 2010. Honey bee pathology: current threats to honey bees and beekeeping. Appl. Microbiol. Biotechnol. 87 (1), 87–97. https://doi.org/10.1007/s00253-010-2573-8.

Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalksy, O., Wüstenberg, D., Menzel, R., 1996. Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. Anim. Behav. 52 (6), 1079–1085. https://doi.org/10.1006/anbe.1996.0255.

Glass, J.R., Fisher, A., Fewell, J.H., DeGrandi-Hoffman, G., Ozturk, C., Harrison, J.F., 2021. Consumption of field-realistic doses of a widely used mito-toxic fungicide reduces thorax mass but does not negatively impact flight capacities of the honey bee (*Apis mellifera*). Environ. Pollut. 274, 116533. https://doi.org/10.1016/j.envpol.2021.116533.

Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347 (6229), 1255957. https://doi.org/10.1126/science.1255957.

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Charrière, J.-D., Chlebo, R., Coffey, M.F., Cornelissen, B., da Costa, C.A., Dahle, B., Danihlík, J., Dražić, M.M., Evans, G., Fedoriak, M., Forsythe, I., Gajda, A., de Graaf, D.C., Gregorc, A., Brodschneider, R., 2020. Honey bee colony winter loss rates for 35 countries participating in the COLOSS survey for winter 2018–2019, and the effects of a new queen on the risk of colony winter loss. J. Apicult. Res. 59 (5), 744–751. https://doi.org/10.1080/00218839.2020.1797272.

Halas, E.S., Heinrich, M.D., Sandstead, H.H., 1979. Long term memory deficits in adult rats due to postnatal malnutrition. Physiol. Behav. 22 (5), 991–997. https://doi.org/10.1016/0031-9384(79)90345-7.

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., Decourtye, A., 2012. A common pesticide decreases foraging success and survival in honey bees. Science 336 (6079), 348–350. https://doi.org/10.1126/science.1215039.

Hesselbach, H., Scheiner, R., 2018. Effects of the novel pesticide flupyradifurone (Sivanto) on honeybee taste and cognition. Sci. Rep. 8 (1), 1–8. https://doi.org/10.1038/s41598-018-23200-0.

Kirchner, W.H., Grasser, A., 1998. The significance of odor cues and dance language information for the food search behavior of honeybees (hymenoptera: apidae). J. Insect Behav. 11 (2), 169–178. https://doi.org/10.1023/A:1021098405564.

Klein, A.-M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc. Biol. Sci. 274 (1608), 303–313. https://doi.org/10.1098/rspb.2006.3721.

Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D.M., Sagili, R.R., Pettis, J.S., Ellis, J.D., Wilson, M.E., Wilkes, J.T., Tarpy, D.R., Rose, R., Lee, K., Rangel, J., vanEngelsdorp, D., 2017. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. J. Apicult. Res. 56 (4), 328–340. https://doi.org/10.1080/00218839.2017.1344496.

Ladurner, E., Bosch, J., Kemp, W.P., Maini, S., 2005. Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* Say and *Apis mellifera*. Apidologie 36 (3), 449–460. https://doi.org/10.1051/apido:2005032.

Lambin, M., Armengaud, C., Raymond, S., Gauthier, M., 2001. Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. Arch. Insect Biochem. Physiol. 48 (3), 129–134. https://doi.org/10.1002/arch.1065.

Lee, H., Sumner, D.A., Champetier, A., 2019. Pollination markets and the coupled futures of almonds and honey bees: simulating impacts of shifts in demands and costs. Am. J. Agric. Econ. 101 (1), 230–249. https://doi.org/10.1093/ajae/aay063.

Lenth, R., 2019. Emmeans: Estimated Marginal Means, Aka Least-Squares Means. R Package Version 1.4.3.01. https://CRAN.R-project.org/package=emmeans.

Lozano, V., Armengaud, C., Gauthier, M., 2001. Memory impairment induced by cholinergic antagonists injected into the mushroom bodies of the honeybee. J. Comp. Physiol. 187 (4), 249–254. https://doi.org/10.1007/s003590100196.

Mattila, H.R., Smith, B.H., 2008. Learning and memory in workers reared by nutritionally stressed honey bee (*Apis mellifera* L.) colonies. Physiol. Behav. 95 (5), 609–616. https://doi.org/10.1016/j.physbeh.2008.08.003.

Morgane, P.J., Austin-LaFrance, A., Bronzino, J., Tonkiss, J., Diaz-Cintra, S., Cintra, L., Kemper, T., Galler, J.R., 1993. Prenatal malnutrition and development of the brain. Neurosci. Biobehav. Rev. 17 (1), 91–128. https://doi.org/10.1016/S0149-7634(05)80234-9.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., Pettis, J.S., 2010. High levels of miticides and agrochemicals in north American Apiaries: implications for honey bee health. PloS One 5 (3), e9754. https://doi.org/10.1371/journal.pone.0009754.

Mustard, J.A., Alvarez, V., Barocio, S., Mathews, J., Stoker, A., Malik, K., 2018. Nutritional value and taste play different roles in learning and memory in the honey bee (*Apis mellifera*). J. Insect Physiol. 107, 250–256. https://doi.org/10.1016/j.jinsphys.2018.04.014.

Naug, D., 2009. Nutritional stress due to habitat loss may explain recent honeybee colony collapses. Biol. Conserv. 142 (10), 2369–2372. https://doi.org/10.1016/j.biocon.2009.04.007.

Pham-Delègue, M.-H., Decourtye, A., Kaiser, L., Devillers, J., 2002. Behavioural methods to assess the effects of pesticides on honey bees. Apidologie 33 (5), 425–432. https://doi.org/10.1051/apido:2002033.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25 (6), 345–353. https://doi.org/10.1016/j.tree.2010.01.007.

R Core Team, 2019. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Sánchez-Bayo, F., Wyckhuys, K.A.G., 2019. Worldwide decline of the entomofauna: a review of its drivers. Biol. Conserv. 232, 8–27. https://doi.org/10.1016/j.biocon.2019.01.020.

Scheiner, R., Barnert, M., Erber, J., 2003. Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. Apidologie 34 (1), 67–72. https://doi.org/10.1051/apido:2002050.

Scheiner, R., Page, R.E., Erber, J., 2004. Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). Apidologie 35 (2), 133–142. https://doi.org/10.1051/apido:2004001.

Schneider, C.W., Tautz, J., Grünewald, B., Fuchs, S., 2012. RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of *Apis mellifera*. PloS One 7 (1), e30023. https://doi.org/10.1371/journal.pone.0030023.

Simcock, N.K., Gray, H., Bouchebti, S., Wright, G.A., 2018. Appetitive olfactory learning and memory in the honeybee depend on sugar reward identity. J. Insect Physiol. 106, 71–77. https://doi.org/10.1016/j.jinsphys.2017.08.009.

Simon-Delso, N., San Martin, G., Bruneau, E., Minsart, L.-A., Mouret, C., Hautier, L., 2014. Honeybee colony disorder in crop areas: the role of pesticides and viruses. PloS One 9 (7), e103073. https://doi.org/10.1371/journal.pone.0103073.

Siviter, H., Koricheva, J., Brown, M.J.F., Leadbeater, E., 2018. Quantifying the impact of pesticides on learning and memory in bees. J. Appl. Ecol. 55 (6), 2812–2821. https://doi.org/10.1111/1365-2664.13193.

Smith, B.H., Burden, C.M., 2014. A proboscis extension response protocol for investigating behavioral plasticity in insects: application to basic, biomedical, and agricultural research. JoVE 91, e51057. https://doi.org/10.3791/51057.

Sol Balbuena, M., Tison, L., Hahn, M.-L., Greggers, U., Menzel, R., Farina, W.M., 2015. Effects of sublethal doses of glyphosate on honeybee navigation. J. Exp. Biol. 218 (17), 2799–2805. https://doi.org/10.1242/jeb.117291.

Steijven, K., Spaethe, J., Steffan-Dewenter, I., Härtel, S., 2017. Learning performance and brain structure of artificially-reared honey bees fed with different quantities of food. PeerJ 5, e3858. https://doi.org/10.7717/peerj.3858.

Tadei, R., Menezes-Oliveira, V.B., Silva-Zacarin, E.C.M., 2020. Silent effect of the fungicide pyraclostrobin on the larval exposure of the non-target organism Africanized *Apis mellifera* and its interaction with the pathogen *Nosema ceranae* in adulthood. Environ. Pollut. 267, 115622. https://doi.org/10.1016/j.envpol.2020.115622.

Tonkiss, J., Galler, J.R., 1990. Prenatal protein malnutrition and working memory performance in adult rats. Behav. Brain Res. 40 (2), 95–107. https://doi.org/10.1016/0166-4328(90)90002-V.

Vinggaard, A.M., Hass, U., Dalgaard, M., Andersen, H.R., Bonefeld-Jørgensen, E., Christiansen, S., Laier, P., Poulsen, M.E., 2006. Prochloraz: an imidazole fungicide with multiple mechanisms of action. Int. J. Androl. 29 (1), 186–192. https://doi.org/10.1111/j.1365-2605.2005.00604.x.

von Frisch, K., 1967. The Dance Language and Orientation of Bees. Harvard University Press.

Wright, G.A., Mustard, J.A., Kottcamp, S.M., Smith, B.H., 2007. Olfactory memory formation and the influence of reward pathway during appetitive learning by honey bees. J. Exp. Biol. 210 (22), 4024–4033. https://doi.org/10.1242/jeb.006585.

Wu-Smart, J., Spivak, M., 2016. Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development. Sci. Rep. 6, 32108. https://doi.org/10.1038/srep32108.

Wykes, G.R., 1952. The preferences of honeybees for solutions of various sugars which occur in nectar. J. Exp. Biol. 29 (4), 511–519.