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LEARNING IMPAIRMENT IN HONEY BEES CAUSED BY AGRICULTURAL

SPRAY ADJUVANTS

A Thesis in

Entomology

by

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ABSTRACT

Spray adjuvants are often applied to crops in conjunction with agricultural pesticides in order to boost the efficacy of the active ingredient(s). The adjuvants themselves are largely assumed to be biologically inert and are therefore subject to minimal scrutiny and toxicological testing by regulatory agencies. Honey bees are exposed to a wide array of pesticides as they conduct normal foraging operations, meaning that they are likely exposed to spray adjuvants as well. It was previously unknown whether these agrochemicals have any deleterious effects on honey bee behavior. The proboscis extension reflex (PER) assay was used to measure the olfactory learning ability of honey bees treated orally with sublethal doses of the most widely used spray adjuvants on almonds in the Central Valley of California. Olfactory learning is important for foraging honey bees because it allows them to exploit the most productive floral resources in an area at any given time. Any impairment of this learning ability may have serious implications for foraging efficiency at the colony level. Several of the most widely used adjuvants from each of three different adjuvant classes (nonionic surfactants, crop oil concentrates, and organosilicone surfactants) were investigated in this study.

Learning was impaired after ingestion of 20 µg organosilicone surfactant, indicating harmful effects on honey bees caused by agrochemicals previously believed to be innocuous. Organosilicones were more active than the nonionic adjuvants, while the crop oil concentrates were inactive. Ingestion was required for the tested adjuvant to have an effect on learning, as exposure via antennal contact only induced no level of impairment. A decrease in percent conditioned response after ingestion of organosilicone surfactants, which

has been demonstrated here for the first time, may be an indication of severe, colony-level

impacts. Organosilicone spray adjuvants may therefore play a vital role in the ongoing losses

that characterize Colony Collapse Disorder (CCD).

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List of Abbreviations

AT	Acquisition Trial
CCD	Colony Collapse Disorder
CS	Conditioned stimulus
PER	Proboscis extension reflex
RT	Retention trial
US	Unconditioned stimulus
'A'	Antennal contact only treatment protocol
'A+O'	Antennal contact plus oral exposure treatment protocol
'O'	Oral exposure only treatment protocol

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Chapter I

The rationale for the investigation of learning impairment in honey bees caused by supposedly 'inert' agricultural spray adjuvants

Honey bees as pollinators in agricultural systems

Many of our most important agricultural crops depend on honey bees (*Apis mellifera* L.) for pollination. Indeed, 35% of the human diet benefits from animal pollinators, with 87 of the leading global food crops relying on animal pollination to some extent (Klein et al. 2007). Pollinators are essential for 13 of these crops (≥90% reduction in yield if absent), highly important for 30 crops (40-90% reduction in yield if absent), and moderately important for 27 crops (10-40% reduction in yield if absent) (Klein et al. 2007). Honey bees are well-suited to large-scale migratory beekeeping operations because they are generalists that can pollinate vast monoculture expanses in a relatively short period of time. Each hive generally contains between 10,000 and 40,000 individuals, one third of which are foragers (Daberkow et al. 2009). These hives can be transported in large numbers across the U.S. according to the progression of blooming periods of various crops. In the U.S. alone, pollination services from honey bees have been valued at $15 billion per year, making them by far the most economically important agricultural pollinator (Morse and Calderone 2000). Many of our grains and cereal crops such as wheat, corn, and rye are wind-pollinated, and are thus unaffected by animal pollinators (Gallai et al. 2009; vanEngelsdorp and Meixner 2010). While these crops do make up the majority of human caloric intake, there has been a shift in recent decades in terms of agricultural acreage toward higher value, pollinator-dependent crops (Aizen et al. 2008). This trend highlights the ever-increasing importance of insect pollinators, and honey bees in particular, to modern agriculture.

Colony Collapse Disorder

Beginning in the fall of 2006, beekeepers in North America and Europe began to notice a sudden and dramatic decline in their managed honey bee populations. The decline was characterized by three symptoms: 1) a rapid loss of adult bees coupled with an absence of dead bees in or around the hive, 2) the presence of both the queen and brood (indicting a sudden collapse of the adult population), and 3) reduced or delayed invasion of the collapsed hive by honey-robbing bees and hive pests (Cox-Foster et al. 2007; vanEngelsdorp et al.

2009). These scavenging behaviors occur much sooner in hives that have died off as a result of known causes, such as queenlessness or acute pesticide poisoning (vanEngelsdorp et al. 2009). This new phenomenon was subsequently termed Colony Collapse Disorder (CCD), and continues to be a major threat to honey bees worldwide. Colony losses have averaged 30%, 34%, 29%, 36%, and 32% in the winters of 2010-2011, 2009-2010, 2008-2009, 2007-2008, and 2006-2007, respectively (vanEngelsdorp et al. 2011). These figures do not include non-winter colony losses. At present, it is thought that multiple factors such as pathogens, parasites, malnutrition, and pesticide exposure are responsible for CCD (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009).

Role of pesticides in Colony Collapse Disorder

The pesticide hypothesis has received considerable attention since the emergence of CCD in 2006. Foraging worker bees are exposed to pesticides in agroecosystems as they gather nectar and pollen from flowers, but only recently has the extent of this pesticide exposure been investigated. A comparative study of CCD-affected hives and healthy hives revealed the presence of 121 different pesticides and metabolites in 887 wax, pollen, and bee samples taken from managed hives across the U.S., with an average of 6 detections per sample (Mullin et al. 2010). However, no correlation was found between any one pesticide and CCD (vanEngelsdorp et al. 2009; Mullin et al. 2010; Frazier et al. 2011). Managed honey bee colonies are also intentionally exposed to miticides in an effort by beekeepers to control *Varroa destructor*, an ectoparasite of honey bees and important vector of honey bee pathogens (Johnson et al. 2010). Not surprisingly, coumaphos and fluvalinate (two widely used in-hive miticides) were the two most frequently detected pesticide residues in managed hives (Mullin et al. 2010).

Typical ecotoxicological testing for pesticides focuses on short-term assays designed to determine the LD-50 or LC-50 of a particular chemical in a population of test organisms. Consequently, many of the effects from chronic or sublethal exposure to pesticides are largely unexplored, in part because of the difficulty of conducting these tests (Pham-Delegue et al. 2002). Given the complex foraging, communicative, and navigational tasks honey bees

must perform, sublethal effects of pesticides are especially important when compared to other, less sophisticated nontarget species (Devillers and Pham-Delegue 2002).

Pesticide adjuvants

While considerable progress has been made with regard to surveying the prevalence of pesticide active ingredients within hives, virtually no work has been done to examine the safety of pesticide adjuvants that are either included in pesticide formulations (formulation adjuvants) or tank-mixed and sprayed along with the formulated product (spray adjuvants). Adjuvants are designed to boost the efficacy of sprayed fungicides, herbicides, and insecticides by improving spreading, wetting, penetration, reducing UV degradation, and/or reducing foaming and evaporation (Stevens 1993; Hess 1999; Hartzler 2001; Stark and Walthall 2003). The adjuvants themselves are largely assumed to be biologically inert and are usually not included in risk assessment trials required to register a pesticide. Of the 20 toxicological tests required to register a new pesticide in the U.S., 13 are conducted with only the active ingredient(s); only 7 short-term acute mammalian and avian toxicity tests use the entire formulation (U.S. EPA 2005; Cox and Surgan 2006). Medium- and long-term toxicity tests only examine the active ingredient(s). Moreover, the specific ingredients that make up spray adjuvants are considered trade secrets of the chemical companies that manufacture them and are therefore usually not disclosed (Green 2000; Cox and Surgan 2006). Given the fact that migratory honey bees are exposed to so many pesticides, and the fact that these adjuvants are sprayed in conjunction with pesticide formulations, it follows that foragers are exposed to adjuvants as well. The role of these agrochemicals in the ongoing investigation of CCD and their effects on the physiology/behavior of honey bees have therefore been overlooked up to this point.

Despite the widespread assumption that formulation ingredients and adjuvants are biologically inert, substantial evidence suggests that this is often not the case. Numerous studies have found that pesticide active ingredients elicit very different physiological effects on nontarget organisms when combined with their formulation ingredients. Formulations containing glyphosate, the most widely used herbicide world-wide, have been shown to

reduce the activity of rat liver mitochondrial respiratory complexes (Peixoto 2005), inhibit progesterone production in mouse Leydig cells (Walsh et al. 2000), and interfere with the development of *Rana pipiens* tadpoles (Howe et al. 2004). In each of these studies, glyphosate alone did not induce these phenomena. Everett and Dickerson (2003) demonstrated that a glyphosate formulation was 100 times more toxic to ciliated protozoans than glyphosate alone. Formulations containing organophosphate insecticides, but not the organophosphates alone, caused mitochondrial damage and neurotoxicity in frogs (Swann et al. 1996) and caused 100 times greater toxicity to microbial flora in estuarine and river sediments (Garcia-Ortega et al. 2006). Tran et al. (2006) observed reduced viability and neurite outgrowth of PC12 neuronal cells treated with formulations containing bifenthrin but not in cells treated with bifenthrin alone. Lin and Garry (2000) described increased proliferation of MCF-7 breast cancer cells treated with formulations containing 2,4-D but not in cells treated with 2,4-D alone. Formulations containing glufosinate caused elevated acute mammalian toxicity (Watanabe and Sano 1998) and a decrease in blood pressure coupled with an increased heart rate in rats (Koyama et al. 1997), while glufosinate alone does not produce these effects. Formulations containing vinclozolin, but not vinclozolin alone, inhibit spermatogenesis in male Japanese medaka (Kiparissis et al. 2003).

Of equal or perhaps greater concern is the fact that adjuvants and inert ingredients themselves have been shown to cause harm to nontarget organisms. 4-nonylphenol, a degradate of a supposedly inert surfactant, has been shown to reduce the growth of Atlantic salmon smolts (Arsenault et al. 2004). R-11, one of the nonionic surfactants investigated in this thesis, reduced the growth rate of *Daphnia pulex* at concentrations that would be expected after application near aquatic systems at recommended field-use rates (Stark and Walthall 2003). Aqueous solutions of Silwet L-77, also investigated here, were toxic to two-spotted spider mites (Cowles et al. 2000), Pacific spider mites, cotton aphids, western flower thrips, and grape mealybugs (Tipping et al. 2003), and fruit flies (Purcell and Schroeder 1996) at concentrations within the range of field application rates. The researchers in these studies even suggested that they might be valuable tools for control of these pests as they act in much the same manner as insecticidal soaps. Honey bees can also be affected by surfactants. A simple detergent solution has been shown to kill swarms of Africanized honey bees (Sames et al. 1990). Goodwin and McBrydie (2000) found that 4 out of 11

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commercially available spray adjuvants (none of which were included in this thesis) were toxic to honey bees after topical application. Two of those 4 were toxic after oral administration. While very few studies have examined the toxicity of adjuvants to honey bees, virtually none have been conducted to determine their potential sublethal effects.

Associative learning in honey bees

Upon visiting a flower, a foraging honey bee reflexively extends her proboscis when gustatory receptors on her tarsi or antennae contact nectar in the flower (Takeda 1961; Bitterman et al. 1983). This reflex leads to the uptake of nectar via the extended proboscis. During nectar uptake, olfactory receptors on the antennae are stimulated by floral odors emanating from the nectar-donating flower (Menzel et al. 1993). The forager memorizes this particular odor-nectar association and will utilize it on subsequent foraging trips as a way of optimizing foraging efficiency.

Honey bees are prototypical generalist pollinators (Winston 1987; Gould and Gould 1988), making them ideal candidates for migratory beekeeping operations. While they will readily collect floral resources from a wide variety of plant species, foraging honey bees tend to exhibit flower constancy – repeatedly choosing to visit flowers from a single species after an initial floral relationship is established, even when profitable flowers from other species are present (Free 1963; Waser 1986; Greggers and Menzel 1993; Menzel et al. 1993). This behavior understandably benefits the plant's reproductive success by increasing the likelihood of conspecific pollen transfer (Free 1963; Waser 1986), but its advantages to the pollinator are not as readily apparent. One explanation for its adoption is that once a relationship with the flowers of a particular species is learned, subsequent visits to flowers of that species become more efficient. Laverty (1994) and Gegear and Laverty (1995) found that bumble bees that practice flower constancy on a species with complex flowers take 1-2 seconds longer to complete floral visits when they switch to another species with complex flowers. This increase in foraging time per visit was not seen with simple flowers. When multiplied by thousands of foragers visiting multiple flowers on multiple foraging trips, it

seems likely that a 1-2 second increase to collect the same amount of pollen and/or nectar could have substantial impacts on colony-level foraging efficiency.

The ability to learn in this system of foraging is therefore paramount. Specialist pollinators of a single plant species are generally more efficient than generalists that visit said plant species (Menzel and Müller 1996). However, generalist pollinators like honey bees that are able to learn new flower handling techniques via flower constancy can improve their performance on subsequent visitations to a level comparable with specialists (Laverty and Plowright 1988). In this way, generalists are able to collect floral resources nearly as efficiently as a specialist, but are still able to exploit a wide range of plant species.

Flower constancy in honey bees is driven by the recruitment of new foragers by foragers that have discovered a highly profitable food source. Upon returning to the hive, foragers are able to communicate the distance, direction, and quality of floral resources to recruits by utilizing the dance language described by von Frisch (1967). If the food source is particularly high in quality, recruits that attend to the dance performed by a given forager will preferentially visit the flowers discovered by said forager when they make their foraging trips (von Frisch 1967; Winston 1987; Gould and Gould 1988). Learning is important in this transfer of information. Arenas et al. (2007) have shown that after feeding on a scented in-hive feeder for 3 days, foragers will preferentially visit food sources outside the hive whose odor matches that of the in-hive feeder. This preference is maintained 24 hours after removal of the in-hive feeder and replacement of all hive comb, indicating the acquisition of medium-term learning. This methodology presumably simulates the nectar given to recruits via trophallaxis from incoming foragers. Learning is thus not only important for foragers in the field, but also for future foragers within the hive such as the bees used in the experiments that make up this thesis.

Proboscis extension reflex (PER) assay

The classical conditioning paradigm employed here was first described by Pavlov (1927) in dogs, although its principles are applicable to invertebrate systems as well. The proboscis extension reflex (PER) assay is a well-established associative learning assay that

effectively simulates the feeding events that occur at a flower as described above in a controlled laboratory setting (Takeda 1961; Bitterman et al. 1983). In the PER assay, a conditioned stimulus (CS) is presented to a harnessed honey bee immediately prior to an unconditioned stimulus (US). The US, a sucrose/water solution, reflexively elicits extension of the proboscis just as nectar would. The CS is generally an odor that is puffed over the bee's antennae for several seconds. After several paired trials (CS + US), the individual bee will extend her proboscis in subsequent trials in which the US is not administered (CS only). This is seen as a positive learning response (Takeda 1961; Bitterman et al. 1983).

Sublethal doses of pesticide active ingredients have been shown to impair this learning pathway in foraging honey bees. Decourtye et al. (2005) treated honey bees orally with one of nine pesticide active ingredients and found that learning performances were reduced in four of the nine treatment groups. One of three doses of each active ingredient was administered, the highest of which was only 1/20th of the 48-hour oral LD-50. Abramson et al. (2004) found that tebufenozide and diflubenzuron, two insect growth regulator pesticides believed to be harmless to honey bees, both reduce learning performance in honey bees at sublethal levels. The newly introduced neonicotinoid insecticides, in addition to being highly toxic to honey bees, also impair learning and memory at sublethal levels (Pham-Delegue et al. 2002; Decourtye 2004; Decourtye et al. 2004; Aliouane et al. 2009). This is particularly true for imidacloprid. These studies used the PER assay to examine only the effects of pesticide active ingredients, however. It is currently unknown what sublethal effects agricultural spray adjuvants have on foraging honey bees, specifically with respect to their learning abilities.

Thesis objectives

There are hundreds of agricultural spray adjuvants currently in use in the U.S., and the list of registered products is ever-expanding (Young 2010). In the interest of practicality, it was necessary to limit experimental investigation to the adjuvants most frequently encountered by honey bees in commercial beekeeping operations. I chose to search for the

most commonly used spray adjuvants applied to almonds in the Central Valley of California for two main reasons:

1) The almond pollination in the Central Valley of California is the single largest pollination event in the world. In February/March of 2011, approximately 1.3 million commercial honey bee hives were transported to California to pollinate ~740,000 acres of almond trees (Pollination Best Management Practices 2010). This figure represents roughly 50% of all honey bee hives in the U.S. (Johnson 2010). Agrochemicals applied to these almond trees are therefore likely to have the greatest impact on honey bee health relative to other cropping systems. Furthermore, some pesticides – especially fungicides – are applied to almonds while the flowers are in bloom (Mayer and Lunden 1986). Given that foragers visit open flowers to collect pollen and nectar, this scenario represents the greatest potential hazard to foraging honey bees in terms of exposure to agrochemicals.

2) The state of California is unique among all other U.S. states in that it requires growers of all important food crops to report their pesticide use. Spray adjuvants are considered pesticides and must therefore be reported in the same way pesticide active ingredients are. This usage information is contained in the California Pesticide Information Portal (CalPIP), a database maintained by the California Department of Pesticide Regulations (CDPR). One can search this database according to crop, product(s) applied, county, date of application, or any combination of these criteria. Pesticide usage records at this level of detail cannot be found in any other state.

The CalPIP database was searched for usage information regarding three major classes of spray adjuvants (organosilicone adjuvants, nonionic surfactants, and crop oil concentrates) on almonds in the top almond producing counties in California in 2009 (Almond Board of California 2010). The data gathered from these searches are shown in Figures 1, 2, and 3, respectively. Among the organosilicones, Dyne-Amic was the most widely used adjuvant, followed by Syl-Tac and Sylgard 309. Activator 90, R-11, and Britz B-85 were the most heavily used nonionic surfactants, while Penetrator, Crop Oil Concentrate, and Agri-Dex represented the most widely used crop oil concentrates. These top adjuvants were included in this study. Britz B-85 could not be obtained, so Induce was used instead.

The PER assay was used to measure the learning abilities of individual honey bees that were exposed to the most important spray adjuvants in each class (see above). While previous studies have shown that pesticide active ingredients can reduce the learning performances of honey bees at sublethal levels (Pham-Delegue et al. 2002; Abramson et al. 2004; Decourtye 2004; Decourtye et al. 2004, 2005; Aliouane et al. 2009), there are no reports on the effects of adjuvants on learning in honey bees. Foraging honey bees are exposed to spray adjuvants, and it is inappropriate to consider them harmless to honey bees until toxicological testing has been conducted, especially considering that their use is expanding (CDPR 2009) (Fig. 4). While the results of these laboratory assays may not necessarily extend directly to the colony level, they do indicate an inherent toxic action of certain adjuvants and provide a vital foundation for future field studies to determine actual colony-level impacts.

I hypothesize that the most commonly used agricultural spray adjuvants on almonds in the Central Valley of CA impair the learning performances of foraging honey bees.
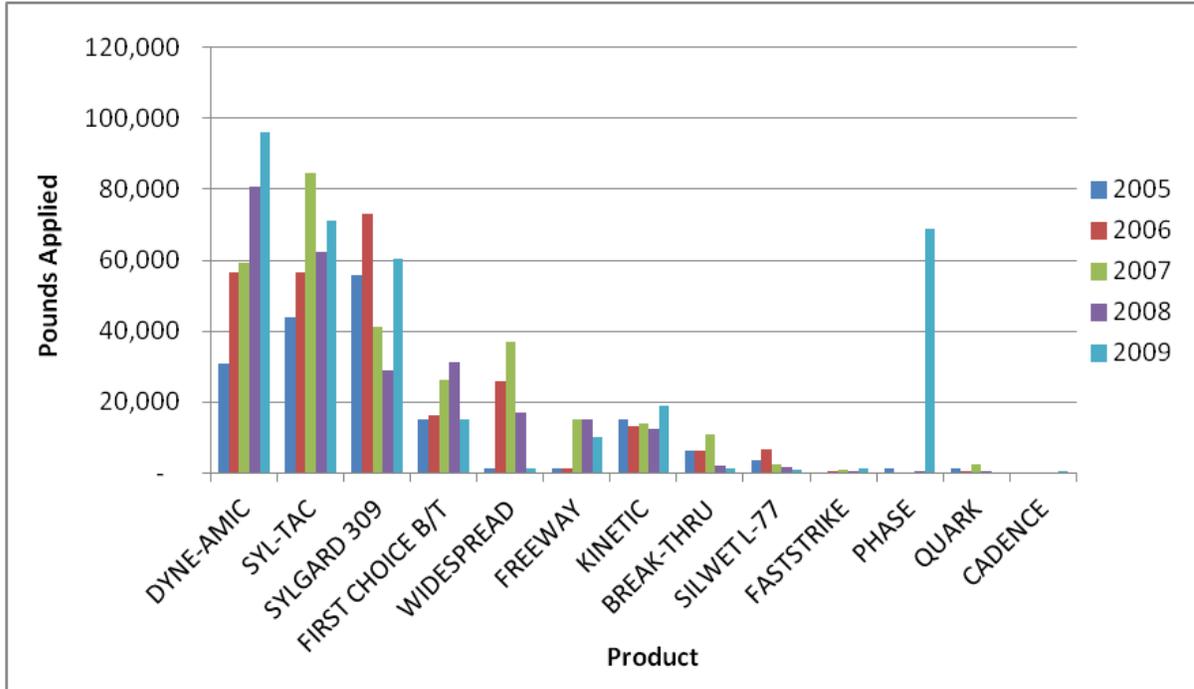
Figure 1. Organosilicone adjuvants applied to almonds in the Central Valley of CA from 2005-2009. Data compiled from CDPR CalPIP database.
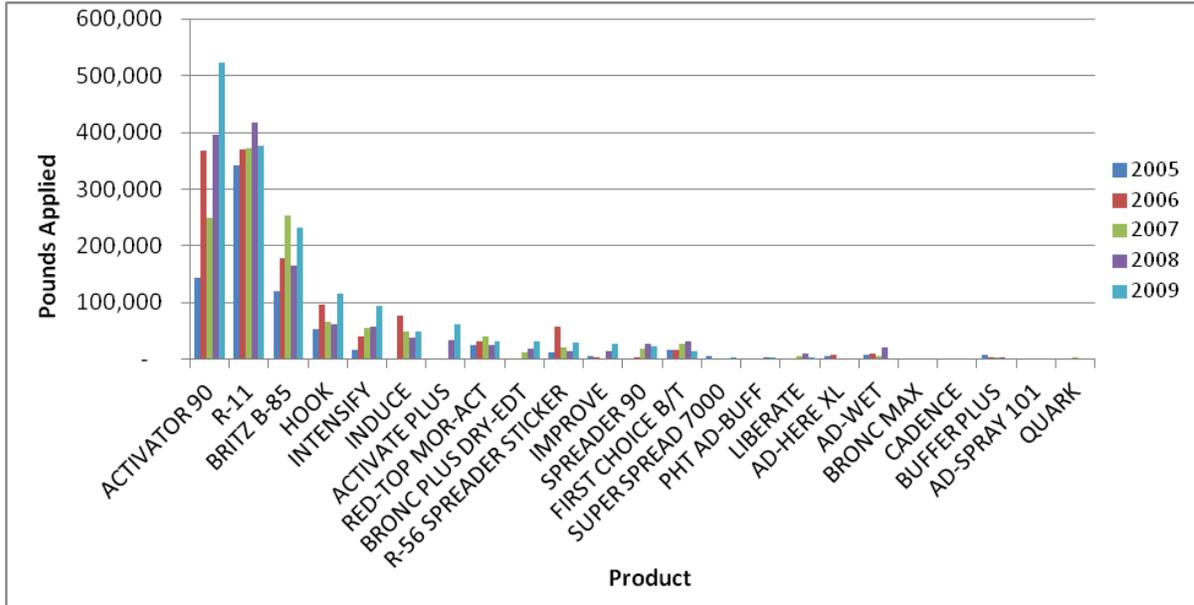
Figure 2. Nonionic surfactants applied to almonds in the Central Valley of CA from 2005-2009. Data compiled from CDPR CalPIP database.
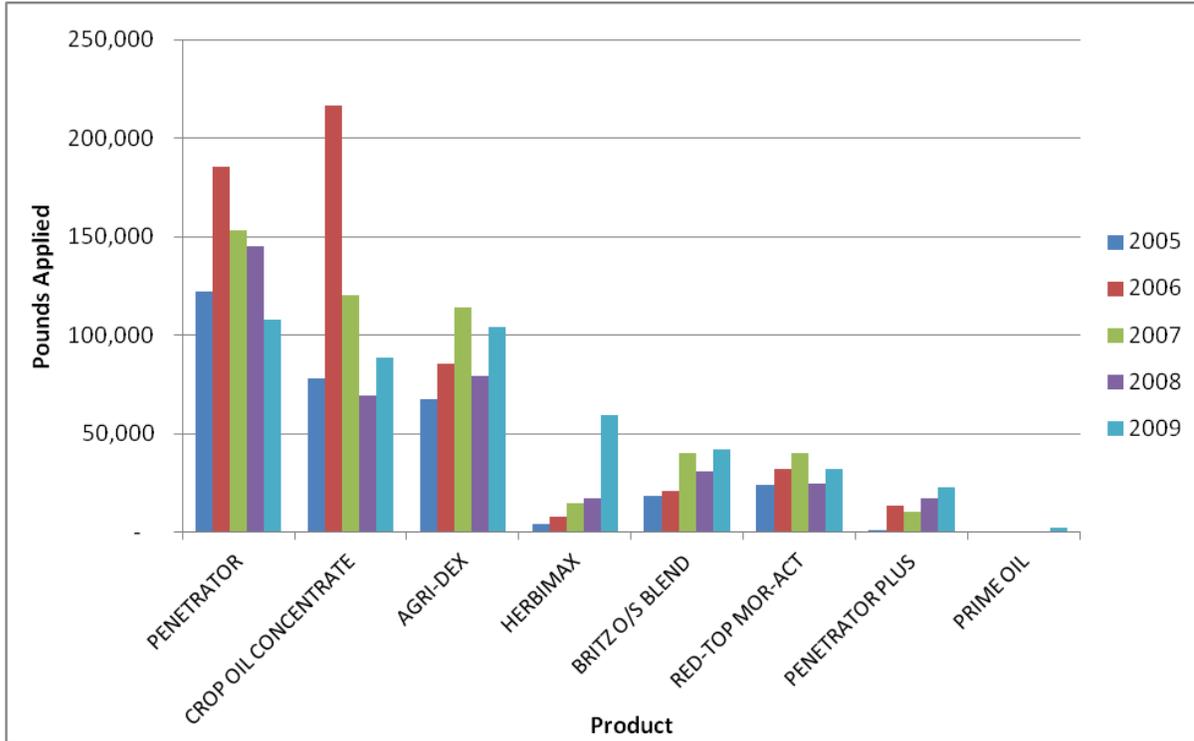
Figure 3. Crop oil concentrates applied to almonds in the Central Valley of CA from 2005-2009. Data compiled from CDPR CalPIP database.
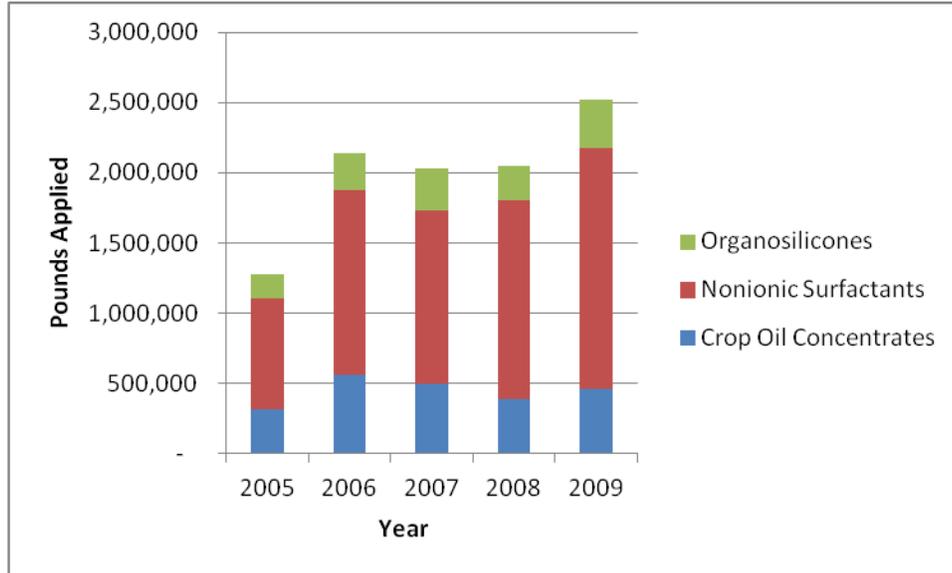
Figure 4. Agricultural spray adjuvants applied to almonds in the Central Valley of CA from 2005-2009 by class. Data compiled from CDPR CalPIP database.

Chapter II

Use of the proboscis extension reflex (PER) assay to assess learning impairment in honey bees caused by agricultural spray adjuvants

Materials and Methods

Adjuvants evaluated

The agricultural adjuvants investigated in this study are among the most widely used spray adjuvants on almonds in the Central Valley of California (CA DPR; see Chapter 1). As such, they have the greatest potential impact of all the adjuvants currently available on the market with respect to the health of honey bees in commercial beekeeping operations. Spray adjuvants from three classes were evaluated. Penetrator®, Agri-Dex®, and Crop Oil Concentrate® are crop oil concentrates. Activator 90®, R-11®, and Induce® are nonionic surfactants, and Dyne-Amic®, Sylgard 309®, Syl-Tac®, and Silwet L-77® are organosilicone surfactants. Imidacloprid is an active ingredient that reduces the learning ability of honey bees at sublethal doses (Decourtye et al. 2004) and was included in this study as a validation of the methods used. Syl-Tac and R-11 were provided by Wilbur-Ellis (San Francisco, CA), and Sylgard 309 was provided by Dow-Corning (Midland, MI). Dyne-Amic, Silwet L-77, Induce, Penetrator, Agri-Dex, and Crop Oil Concentrate were provided by Helena (Collierville, TN), and Activator 90 was provided by Loveland (Greeley, CO). Technical grade imidacloprid (99.5% purity) was purchased from Chem Service (West Chester, PA).

Animals

Worker honey bees (*Apis mellifera* L.) were collected from one of two hives on the campus of Penn State University (University Park, PA) during the months of July to September of 2011. To ensure a homogenous age distribution among the test organisms, only 'house' bees from the uppermost box of each hive were selected. Once collected, the bees were cold-anaesthetized for 3-4 minutes and individually harnessed in 2 cm lengths of 0.25" x 0.17" (OD x ID) polyethylene tubing. Each tube was cut lengthwise so that it could be opened, and a semicircular piece of the tubing was cut from one end with a cork borer to allow the bee's head and forelegs free range of motion (Fig. 5). Each bee's wings extended out of the tube via the lengthwise slit and were wrapped with a small piece of parafilm to

secure the bee in place. The harnessed bees were fed until satiated with a sucrose solution (50% w/v) and then starved for an 18-hour period at 25˚C prior to the learning assays. This established a uniform hunger level among the bees.

Dyne-Amic dose response

A dose-response study was undertaken to determine the lowest adjuvant concentration that leads to learning impairment. This concentration would then be used as a standard for all the adjuvants included in the three experimental protocols (A+O, A, and O). Dyne-Amic was selected because it was the most widely used organosilicone spray adjuvant on almonds in the Central Valley of California from 2005-2009 (CDPR). Dyne-Amic was added to sucrose solution (50% w/v), which was then administered to each bee in the treatment group according to the A+O exposure protocol. 0.1%, 0.5%, 1.0%, and 5.0% were the four concentrations of Dyne-Amic tested.

Exposure protocols

After the 18-hour starvation period, honey bees were treated with one of the aforementioned adjuvants in one of three ways: oral exposure only (O), antennal contact only (A), and oral exposure plus antennal contact (A+O). For all treatment modalities, the adjuvant being tested was added to sucrose solution (50% w/v) to achieve an adjuvant concentration of 1% (v/v). In the 'A+O' experiments, this 1% adjuvant/sucrose solution was presented to the antennae of each bee in the treatment group via cotton swab. Upon contacting the saturated cotton swab, proboscis extension reflexively occurred, and each bee was allowed to feed on the adjuvant/sucrose solution for 2 seconds to simulate a nectar-feeding event at an adjuvant-sprayed flower (Fig. 6). In the 'O' experiments, a drop of sucrose solution (50% w/v) was presented to the antennae via 16 gauge hypodermic needle to elicit proboscis extension such that the proboscis could not contact the drop. Instead, the proboscis (not antennae) was allowed to contact a cotton swab saturated with 1% adjuvant/sucrose solution for 2 seconds. In the 'A' experiments, the treatment method was

simply reversed so that only the antennae contacted the adjuvant/sucrose solution. An equivalent number of control bees was used in each experiment. They were subjected to the same treatment method, but sucrose-only solution (50% w/v) was used in place of 1% adjuvant/sucrose solution. Treatment was administered 5 minutes prior to PER testing. In all cases, bees that did not extend their proboscis after antennal contact with either the 1% adjuvant/sucrose solution or sucrose-only solution (50% w/v) (depending on the experiment) were removed from the study and replaced. 'A' and 'O' treatment protocols included only the most widely used adjuvant from each class (Dyne-Amic, Activator 90, and Penetrator) on almonds in the Central Valley of CA from 2005-2009.

Technical grade imidacloprid was first dissolved in acetone and then diluted in sucrose solution (50% w/v) to achieve a concentration of 6.25 mg L^{-1}. A 2 second feeding of this solution delivered an effective dose of 12 ng imidacloprid per bee, which has been shown to impair olfactory learning in honey bees (Decourtye et al. 2004). The final concentration of acetone in the treatment solution was 1%. Control bees received sucrose solution (50% w/v, 1% acetone v/v).

Learning assays

The PER assays described below were adapted from Takeda (1961) and Bitterman et al. (1983). The harnessed and treated honey bees were subjected to 5 acquisition trials followed by 6 retention trials. Acquisition trials consisted of presentation of the conditioned stimulus (CS) plus the unconditioned stimulus (US), while retention trials consisted of presentation of the CS only. Sucrose solution (50% w/v) was used as the US, and the odor of a 1% solution of pure cinnamon oil (Now Foods, Bloomingdale, IL) in mineral oil was used as the CS. 500 μL of this cinnamon oil solution was placed into a vial with one air inlet tube and one outlet tube (Fig. 7). During each trial, a computer-controlled solenoid valve (3-Way MIV, The Lee Co., Essex, CT) operated by LabView software (v. 8.6, National Instruments, Austin, TX) directed a 5 second pulse of air into the bottom of the vial to produce a bubbling effect. The outlet tube then carried the injected air from the headspace of the vial and delivered it to the bee's antennae. The bubbling ensured a consistent concentration of

cinnamon odor in the 5 second pulse. A glass Pasteur pipette glued to the odor delivery assembly housed the outlet tube as well as a 'non-pulse' tube. This non-pulse tube carried a constant flow of non-odorous humidified Ultra Zero air (Fairless Hills, PA) from a gas cylinder to the bee whenever the 5 second CS pulse was not in effect. The solenoid valve redirected this airflow into the vial when the odor pulse command was given to produce the CS. Thus, a constant airflow was achieved (250 mL min^{-1} was used in this study) throughout each experiment, even at the beginning of the CS pulse. This constancy ensured that any positive response observed was due to the olfactory cue and not mechanosensory stimulation from a change in airflow. A Pyrex 60° angle long-stem filtering funnel (VWR, Radnor, PA) with attached copper rings (Fig. 6) held a squad of 8 harnessed bees and was positioned in the arena with the aid of a manual micromanipulator (World Precision Instruments, Sarasota, FL). After each trial, the funnel remained in place but was rotated 45° to position the next bee in the squad in line with the air stream. After all 8 bees completed a single trial, the funnel was removed from the micromanipulator and another funnel introduced. The number of funnels used was dependent on the number of bees being tested. A separate glass funnel connected to a vacuum line, positioned directly behind the bee receiving the CS captured the exhaust once the CS odor passed over the antennae and removed it from the test arena. The automated odor delivery system described here is an improvement over previously described 'manual' methodologies (Takeda 1961; Bitterman et al. 1983; Abramson 1997; Abramson et al. 2004) that rely on a human operator to deliver a consistent pulse of odor in terms of duration and directional accuracy.

During an acquisition trial, a 5 second CS pulse was delivered to the bee's antennae. 3 seconds into this pulse, the US was presented via cotton swab first to the antennae to elicit proboscis extension, and then the proboscis itself (Fig. 6). The bee was permitted to feed on the US for 1 second. Presentation of the US was therefore completed before the CS pulse was finished. Proboscis extension in the 3 second window before US presentation was seen as a positive response, and was recorded as a 'yes' or 'no' event (Fig. 5). A 10 minute intertrial interval was used in all experiments for both acquisition and retention trials.

During a retention trial, only the 5 second CS pulse was presented to the bee. Proboscis extension in this 5 second window was seen as a positive response. A 1 to 1.5 hour break occurred between the last acquisition trial and the first retention trial.

Statistical analysis

Comparisons of positive learning responses at each trial between control and treated groups were performed with the Fisher's exact test. Student's t-test was used to compare the mean consumption amounts of treatment solution per bee according to adjuvant class. A P value of less than 0.05 was considered significant. Data analyses were performed using Minitab statistical software (v. 14, State College, PA).

Results

Control groups in each of the three treatment modalities, including the acetone control group in the imidacloprid experiment, were not statistically different from each other and were pooled into an aggregate control group for each treatment modality. All comparisons between treated and control bees were made using these aggregate controls.

Dyne-Amic dose response

The olfactory learning performances of bees treated with the 4 concentrations of Dyne-Amic according to the 'A+O' protocol are shown in Table 1. Dyne-Amic did not cause a significant reduction in learning at concentrations of 0.1% and 0.5%. Significant learning impairment occurred in the 1.0% Dyne-Amic treated bees, beginning with AT3 (Fig. 8) ($P < 0.05$, Fisher's exact test). An even greater reduction in learning ability was seen in bees treated with 5.0% Dyne-Amic. Since 1.0% was the lowest concentration that significantly reduced learning, it was chosen as the standard concentration for the other adjuvants investigated here.

Oral exposure + antennal contact

The olfactory learning performances of bees treated with adjuvants according to the 'A+O' protocol are shown in Table 2. The organosilicone adjuvants (Dyne-Amic, Syl-Tac, and Silwet L-77) induced learning impairment beginning with the third acquisition trial (AT3) (Fig. 9) ($P < 0.05$, Fisher's exact test). The only other organosilicone tested, Sylgard 309, induced learning impairment beginning with AT5 (Fig. 9C) ($P < 0.05$, Fisher's exact test). Honey bees treated with the nonionic surfactant Activator 90 experienced a similar reduction in learning ability beginning at AT5 (Fig. 10A) ($P < 0.05$, Fisher's exact test). The other nonionic surfactants tested, R-11 and Induce, did not significantly impair learning (Fig. 10), although a difference was seen at the fourth retention trial (RT4) for R-11 (Fig. 10B) ($P < 0.05$, Fisher's exact test). No learning impairment was seen in bees treated with the three crop oil concentrates (Penetrator, Agri-Dex, and Crop Oil Concentrate) (Fig. 11).

Imidacloprid caused the most dramatic reduction in learning seen in this study, beginning with AT2 (Fig. 12) ($P < 0.05$, Fisher's exact test). No more than 40% of imidacloprid-treated bees gave positive responses at any one trial during PER testing.

Oral exposure only

The olfactory learning performances of bees treated with the top adjuvant from each class according to the 'O' protocol are shown in Table 3. Bees treated with Dyne-Amic experienced a reduction in learning ability in line with those in the 'A+O' experiment, beginning with AT3 (Fig. 13A) ($P < 0.05$, Fisher's exact test). However, no statistical difference was seen at AT5, RT2, RT5, and RT6. Activator 90 did not significantly reduce learning, but there appears to be some degree of impairment (Fig. 13B). Penetrator did not cause any impact on learning ability (Fig. 13C). These results are similar to those observed in the 'A+O' experiments.

Antennal contact only

The olfactory learning performances of bees treated with the top adjuvant from each class according to the 'A' protocol are shown in Table 4. When bees were treated with the top three adjuvants in each class by antennal contact only, no difference in learning ability was seen (Fig. 14). This holds true for Dyne-Amic, which induced a significant reduction in learning in both the 'A+O' and 'O' experiments. Thus, ingestion is required for the tested adjuvant to have an effect on learning. Direct action on the olfactory chemosensory cells to modify olfactory input to the learning association seems highly unlikely.

Dose consumed during treatemnts

Amounts of treatment solutions consumed per bee are displayed in Figs. 15-19. These values were obtained by determining the mass of the treatment solution consumed by each treatment group and then dividing that value by the number of bees in each treatment group. The density of 50% sucrose solution (w/v) ($\rho = 1.1505$ g mL^{-1}) was used to convert mass to volume. 2 µL is the amount consumed per bee during a 2 second feeding. Since each test was not replicated, statistical comparisons between individual treatment groups were not possible. However, each adjuvant class was compared to the aggregate mean by treating each adjuvant within each class as a replicate. Mean consumption data according to class are shown in Fig. 20. These values were compared to the aggregate control (n=10) and were not significantly different ($P < 0.05$, Student's t-test). Given that the density of the nonionic and organosilicone surfactants is 1 g mL^{-1} or slightly greater than 1 g mL^{-1}, a 2 second feeding of this solution delivered an effective dose of 20 µg adjuvant per bee. The average density of the crop oil concentrates was 0.875 g mL^{-1}, meaning that a 2 second feeding delivered 17.5 µg per bee for these experiments.

Discussion

Oral ingestion of 20 µg of the organosilicone adjuvants tested here (Dyne-Amic, Syl-Tac, Sylgard 309, and Silwet L-77) significantly reduces honey bees' learning ability in the classical conditioning PER paradigm (Table 2, Fig. 9). This is the first investigation into adverse effects on honey bees caused by supposedly inert agricultural spray adjuvants. Previous studies have found that sublethal doses of neurotoxic insecticides such as deltamethrin (Decourtye et al. 2004; 2005), flucythrinate and cyfluthrin (Taylor et al. 1987), thiamethoxam (Aliouane et al. 2009), and imidacloprid (Decourtye et al. 2003; 2004; Decourtye 2004) impair learning ability in honey bees in a similar manner. Given that the neural connections in the honey bee brain, particularly those found in the mushroom bodies, play a large role in mediating associative learning and the formation of memory (Hammer 1993; Heisenberg 1998), these results are not entirely surprising. Abramson et al. (2004) showed that insecticides which are *not* neurotoxic, namely the insect growth regulators tebufenozide and diflubenzuron, can also interfere with associative learning in honey bees. Tebufenozide is an agonist of the molting hormone 20-hydroxyecdysone, causing development of a malformed cuticle when ingested by targeted insect larvae (Retnakaran et al. 2001). Diflubenzuron is a chitin inhibitor that disrupts synthesis of a new larval cuticle during molting (van Eck 1978). The mechanism of action of organosilicone adjuvants with respect to learning impairment in honey bees remains unknown, but a precedent has been set demonstrating that chemicals do not necessarily need to be classified as neurotoxic to have effects on learning and memory.

A series of initial experiments revealed 1% Dyne-Amic to be the lowest concentration that led to a significant decrease in learning ability following a 2 second feeding event (Table 1, Fig. 8). Therefore, 1% was chosen as the standard concentration to be used for the other adjuvants included in the main part of this study. The fact that a clear dose-response was seen with Dyne-Amic further substantiates the evidence of learning impairment caused by organosilicone surfactants.

A comparable reduction in learning was not seen in bees treated with nonionic surfactants (Table 2, Fig. 10), although percent conditioned responses were generally lower than those observed in the control bees. Activator 90 was the only nonionic surfactant to

induce significantly lower positive learning responses at more than one trial. None of the crop oil concentrates tested caused significant reductions in learning (Table 2, Fig. 11). The reasons as to why such a strong class effect was observed are unclear, but it may be due to the surfactant activity of each class. Organosilicones (Fig. 21) represent the newest class of agricultural surfactants and are known for their extreme spreading characteristics when added to aqueous solutions at very low concentrations (Stevens 1993; Stevens et al. 1993). Most agrochemicals are sprayed onto leaf surfaces as aqueous solutions, and would ordinarily either bead up or be repelled outright by the waxy cuticle of a leaf. In either case, the total leaf area covered by the sprayed material is reduced, which in turn reduces the efficacy of the agrochemical (Hess 1999). Surfactants, when added at to aqueous solutions, reduce the surface tension of the solution, thereby allowing it to spread more readily on a nonpolar surface. Like all surfactants, each organosilicone molecule is composed of a hydrophilic group and a hydrophobic moiety that allow it to readily interact with both polar and nonpolar compounds. The 'super-spreading' ability of organsilicones is thought to be due to the siloxane backbone of the hydrophobic group, which allows the hydrophobe to be far more compact than that of conventional, carbon-based surfactants (Stevens, 1993; Stevens et al. 1993). The methyl (CH_3) groups attached to each silicon atom are also more hydrophobic than the methylene (CH_2) groups that comprise the hydrophobe portion of more conventional hydrocarbon surfactants (Stevens 1993; Hess 1999). The end result is that organosilicones cause a greater reduction in surface tension than both nonionic surfactants and crop oil concentrates, making them the most potent surfactants available to growers even at lower concentrations. Thus, the mechanism of action within a honey bee that leads to learning impairment may be due to this extreme surfactant activity.

Organosilicone surfactants are also noted for their stomatal infiltration and penetrating characteristics. Most conventional herbicide adjuvants are not able to lower the surface tension of the sprayed solution to a point where stomatal infiltration is possible. Organosilicones can accomplish this in large part due to the compact nature of the siloxane hydrophobe, thereby increasing the efficacy of the herbicide with which they are sprayed (Stevens 1993; Hess 1999). Perhaps of greater concern with respect to honey bee health is the super-penetrating aspect of organosilicones. Knight and Kirkwood (1991) found that cuticular penetration of diflufenican in dicotyledonous weeds of winter cereals was enhanced

when mixed with Silwet L-77 (1.0 g L^{-1}). Again, this mechanism would increase the efficacy of herbicides by facilitating transport to their sites of action within the plant. Organosilicone adjuvants thus mediate both the mixing of hydrophobic pesticides with water to form solutions and the dissolution of hydrophobic cuticles and membranes to allow active ingredients to penetrate. It is unknown whether a similar phenomenon is taking place within the crop or midgut of honey bees.

In addition to being dependent on adjuvant class, learning impairment was also dependent on the route of exposure. No learning impairment was observed in the 'A' experiments (Table 4, Fig. 14). In contrast, bees in 'O' experiments receiving the same adjuvants exhibited reduced learning ability in line with those in the 'A+O' experiments. This would suggest that the organosilicones (Dyne-Amic, at least) are not acting directly on the chemosensory neurons in the antennae, but rather at a systemic level after entering the crop. It is possible that they are acting on the gustatory receptors located on the proboscis itself, but this seems unlikely due to the fact that the gustatory receptors of the antennae were unaffected by any adjuvant.

Learning impairment is characterized by significantly lower percent conditioned responses in treated bees relative to control bees at any given trial during PER testing (Takeda 1961; Bitterman et al. 1983; Taylor et al. 1987; Pham-Delegue et al. 2002; Decourtye 2004). Each PER experiment consists of 5 acquisition trials followed by 6 retention trials. The acquisition trials begin 5 minutes after treatment with the adjuvant being tested and are designed to measure how well bees can form the association between CS and US. Retention trials begin 1 to 1.5 hours after the fifth acquisition trial and are designed to measure medium-term memory, or how well bees can recall a learned association. They are governed by different parameters and give different insights into the temporal mechanics of learning. Lower percent conditioned responses during acquisition trials imply that the treatment has an immediate physiological effect on the bee, presumably in the central nervous system. It is important to note that a reduction in percent conditioned responses in the acquisition trials will have a concomitant effect on the retention trials for a given group of bees in a PER experiment. For instance the retention trials for a particular treatment group may be significantly lower than the respective retention trials of the control group, but only because the acquisition of learning was already impaired. In other words, if there was no

impairment during the acquisition trials, no significance would be seen in the retention trials. Indeed, this occurrence was seen in cases where organosilicones caused significant reductions in learning acquisition (Table 2, Fig. 9). Bees in the organosilicone groups that were able to learn appeared to retain the memory of the association to the same extent as the control bees (i.e. the slope between RT1 and RT6 appears to be the same). It is therefore difficult to draw conclusions with regard to information obtained from the retention trials in these experiments. A reduction in percent conditioned responses during the retention trials, but not in the acquisition trials, would suggest a delayed effect of the treatment, as retention trials begin 2-3 hours after treatment. This scenario was not observed in any experiment conducted here, however.

A slight decrease in percent conditioned responses over the course of the retention trials is expected regardless of whether or not an adjuvant was administered. This decrease is likely due to habituation – a diminution of positive responses caused by repeated over-excitation of sensory neurons. Habituation rates between organosilicone-treated bees and control bees do not appear to be different based on the slope between RT1 and RT2.

The findings of this thesis have potentially serious implications for the future use and safety of spray adjuvants in agricultural systems. Foraging honey bees are exposed to numerous visual and olfactory cues each time they gather floral resources from a flower, and quickly learn to associate these stimuli with a reward of nectar or pollen (von Frisch 1967). This helps the colony as a unit rapidly switch from a less profitable nectar/pollen source to a more profitable one (Winston, 1987; Gould and Gould 1988). The PER assay is a reliable and relatively easy way to simulate these events in the laboratory. A decrease in percent conditioned responses, which has been demonstrated here for the first time after ingestion of organosilicone surfactants, can be an indication of severe, colony-level impacts. The floral landscape is a dynamic one. The most profitable flowers in a given area change from day to day and can vary according to time of day (Gould and Gould 1988; Seeley et al. 1991). Weather and spatial distribution can also have huge impacts on floral resources. Optimal exploitation of the most profitable floral resources is vital to the success of a honey bee colony, and is dependent to a large extent on learning.

One of the hallmark symptoms of CCD is the rapid disappearance of adult bees away from the hive. This would seem to indicate that the causative agent is affecting the behavior

of honey bees, not merely causing them to die. If that were the case, we would expect to see piles of dead bees around collapsed hives much like we see in cases of acute pesticide poisoning (vanEngelsdorp et al. 2009). One hypothesis for the disappearance is that foragers are becoming disoriented while away on foraging trips and are unable to return to the hive. Chaffiol et al. (2005) demonstrated that short-distance orientation performance of honey bees toward a floral compound is increased by prior conditioning of that floral compound in the PER paradigm. This study suggests that learning impairment detected in PER assays could be an indication of orientation impairment as well. This would need to be verified by field or semi-field studies.

Given that the time between ingestion of the test adjuvant and AT1 is only 5 minutes, it is clear that effects on acquisition of learning are immediate. Thus, a forager ingesting nectar from an adjuvant-sprayed flower would be affected while it is still away from the hive. Disorientation at this time might prevent it from returning to the hive. The navigation system in honeybees relies on several mechanisms for orientation with respect to the hive and flowers. The sun's azimuth as well as polarized light are the main cues foraging honey bees utilize to find previously learned foraging locations (von Frisch 1967; Winston 1987; Gould and Gould 1988; Sakura et al. 2011), but these cues are not available during fully overcast weather conditions. Foraging, however, continues seemingly unhindered when the sky is overcast. Detection of the Earth's magnetic field is one strategy for overcoming this problem (Winston 1987; Gould and Gould 1988). Honey bees are also able to learn the spatial arrangement of landmarks to orient themselves with respect to the hive. The acquisition of this spatial awareness is based on memory (von Frisch and Lindauer 1954; Dyer and Gould 1981). It would be inappropriate to suggest that the PER assay is a valid measure of a honey bee's navigational ability since it relies mostly on visual cues, but memory is important in both cases. It is not inconceivable that learning impairment as indicated by the PER assay could also be associated with an impairment of the backup navigation system of honey bees.

Without sampling for these adjuvants in the field, it is inappropriate to make conclusions about the concentration of these materials in the nectar of sprayed flowers (if they are present at all). One percent represents an appropriate starting point for the investigation of sublethal effects caused by spray adjuvants. Generally, spray adjuvants are added to tank-mixes at concentrations of less than 1%, but they may accumulate in nectar to

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concentrations higher than 1%, especially if multiple applications take place over a relatively short period of time. Moreover, a forager visiting multiple flowers that have been sprayed with an adjuvant/pesticide will receive a much larger overall dose than the dose investigated in this thesis, which was designed to simulate a single feeding event at a contaminated flower. Nectar loads of returning foragers typically weigh 25-40 mg (Winston 1987). A 2 second feeding of 50% sucrose solution (w/v) using the methods described here corresponds to roughly 2 mg, or 5-8% of an average nectar load. Additionally, the chemicals that make up spray adjuvants are often included in pesticide formulations as formulation adjuvants. These factors suggest that 1% is a conservative estimate of actual exposure. A detection protocol for spray adjuvants using LC-MS would also need to be developed, as these compounds – most notably the organosilicones – are notoriously difficult to detect using standard analytical methods.

The experiments described here attempt to address the problem of disappearing honey bees that characterizes CCD from a behavioral standpoint. Traditional toxicological approaches that measure factors such as short-, or even long-term, mortality may fall short of accurately describing the effects of agrochemicals on the complex superorganism that is a honey bee colony. The PER assay is a well-established bioassay that measures the learning ability of honey bees, which is a vital component of effective foraging behavior. I have demonstrated here, for the first time, that agricultural spray adjuvants – and organosilicone surfactants in particular – do indeed cause significant learning impairment when ingested by honey bees. Their perceived status as 'inert' materials that can do no harm to biological organisms should be reconsidered. Field tests will need to be conducted to confirm these results on a colony-level, as events in the laboratory do not always translate to an organism's natural setting.

Figure 5. Administration of CS (odor of 1% cinnamon oil) to harnessed bee showing proboscis extension. This is recorded as a positive response and indicates that the bee has learned the association between CS and US.
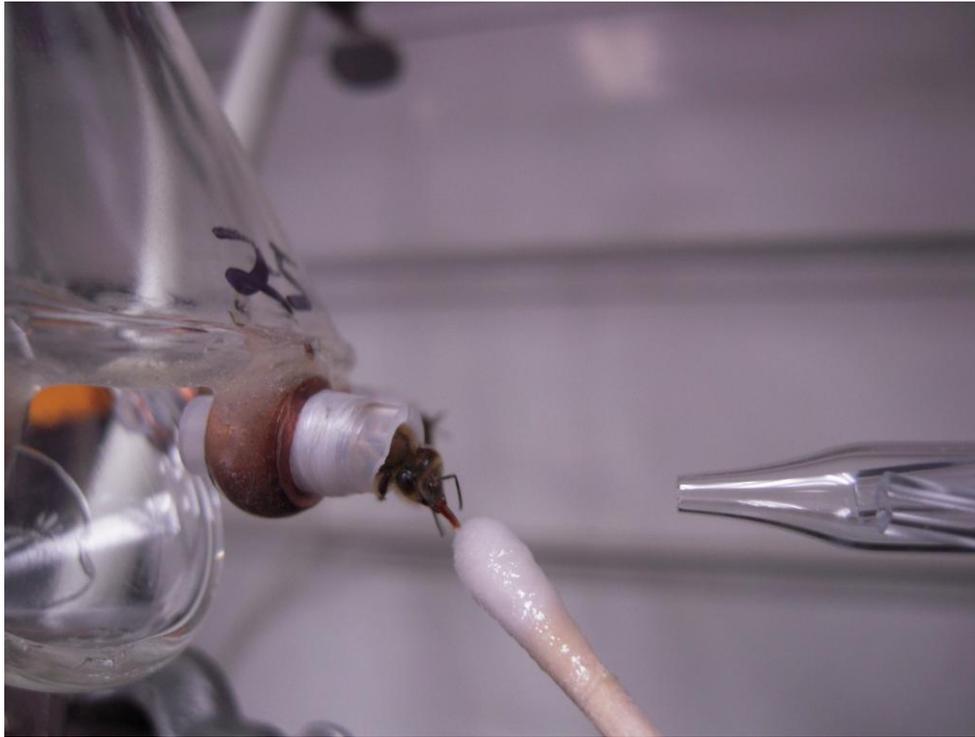
Figure 6. Administration of US (50% sucrose w/v). US is touched to the antennae first and then fed to bee for 2 seconds once the proboscis extends. Exhaust funnel to remove CS odor from the test area can be seen directly behind bee receiving stimuli.

Figure 7. Automated odor delivery apparatus showing 3-Way MIV solenoid valve. Vial contains 500 µL of 1% cinnamon oil/mineral oil solution (v/v). A 5 second pulse of Ultra Zero air is directed into the vial to produce the CS.
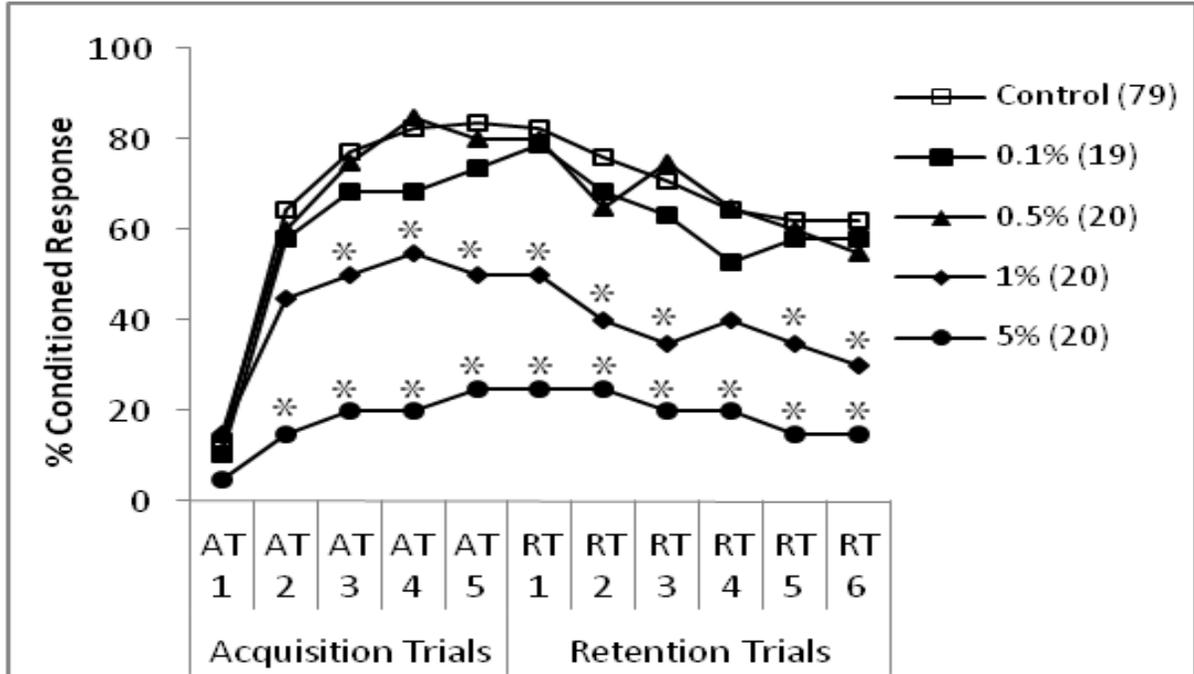
Figure 8. Learning performances of honey bees 5 minutes after antennal contact plus oral ingestion of four concentrations of Dyne-Amic. Percent conditioned response refers to the percentage of bees in each group that gave positive responses at each trial. Number of subjects in each group is indicated in parentheses. * $P < 0.05$ (Fisher's exact test) relative to the control.
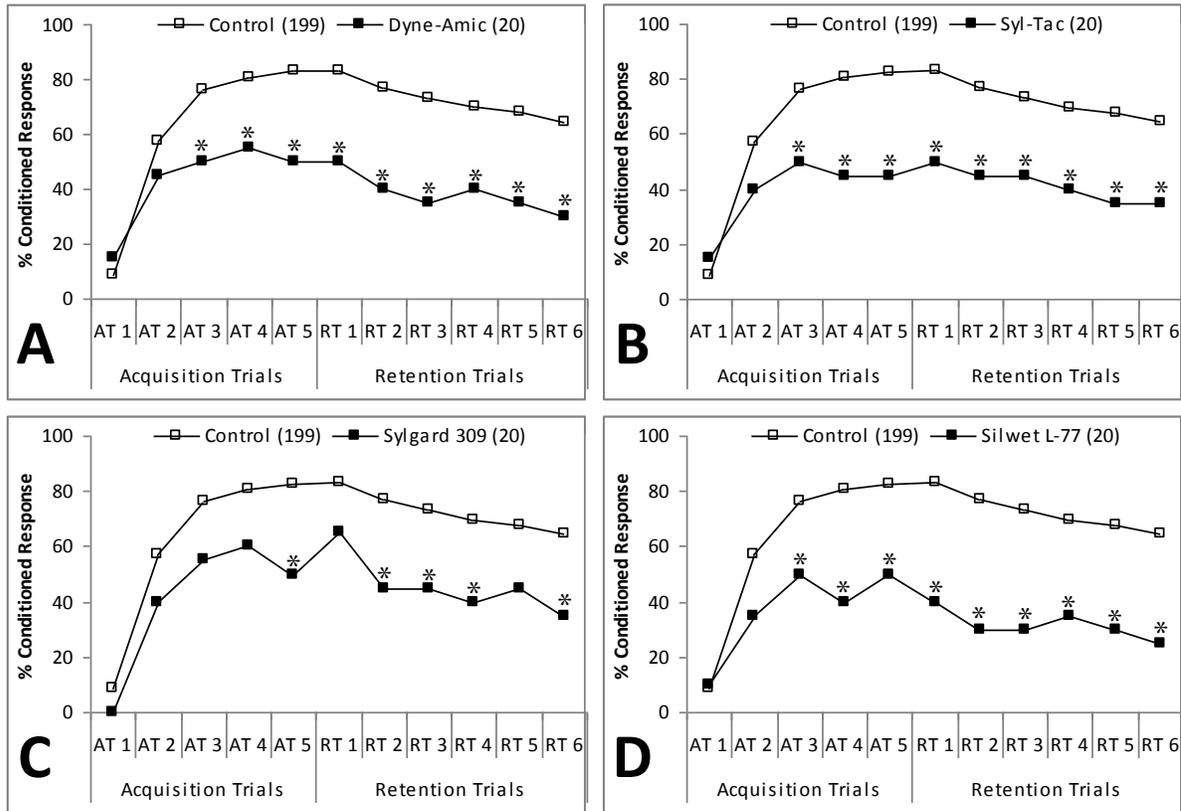
Figure 9. Learning performances of honey bees 5 minutes after antennal contact plus oral ingestion of four different organosilicone adjuvants (1% v/v). Percent conditioned response refers to the percentage of bees in each group that gave positive responses at each trial. Number of subjects in each group is indicated in parentheses. * $P < 0.05$ (Fisher's exact test).
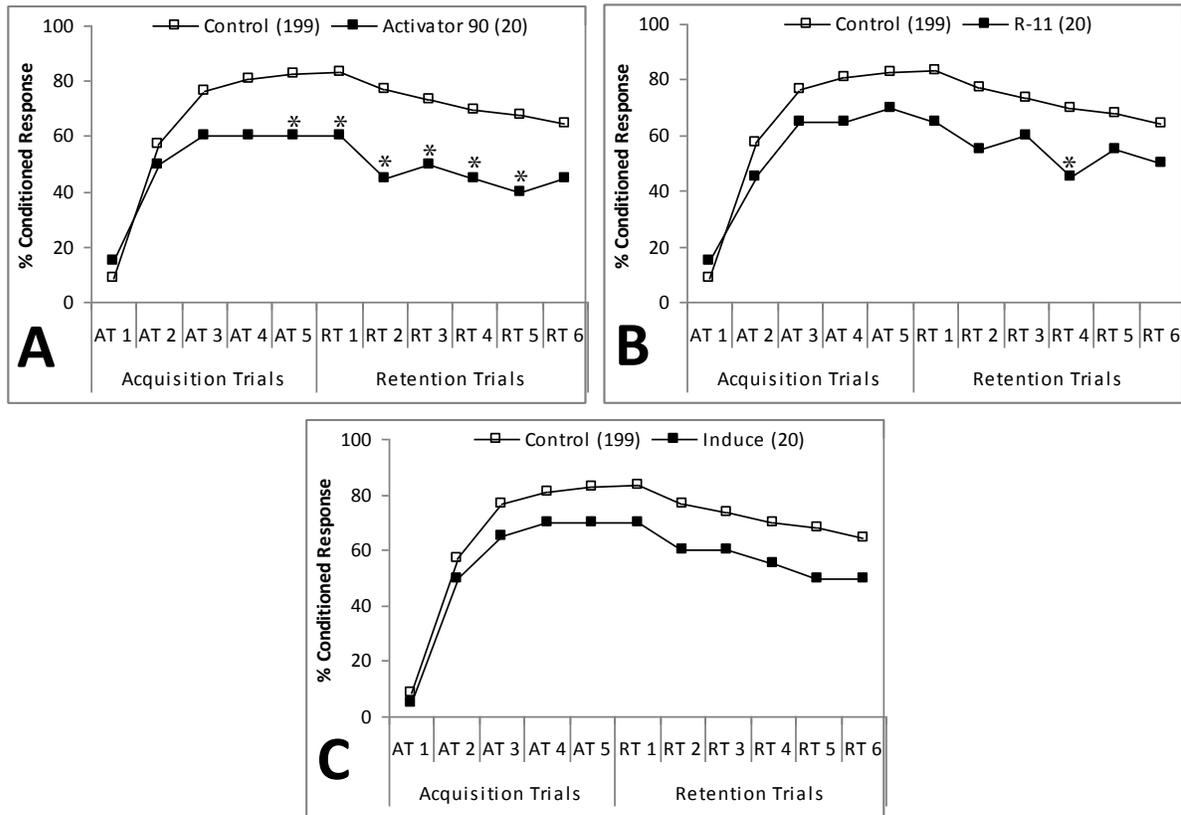
Figure 10. Learning performances of honey bees 5 minutes after antennal contact plus oral ingestion of three different nonionic surfactants (1% v/v). Percent conditioned response refers to the percentage of bees in each group that gave positive responses at each trial. Number of subjects in each group is indicated in parentheses. $* P < 0.05$ (Fisher's exact test).
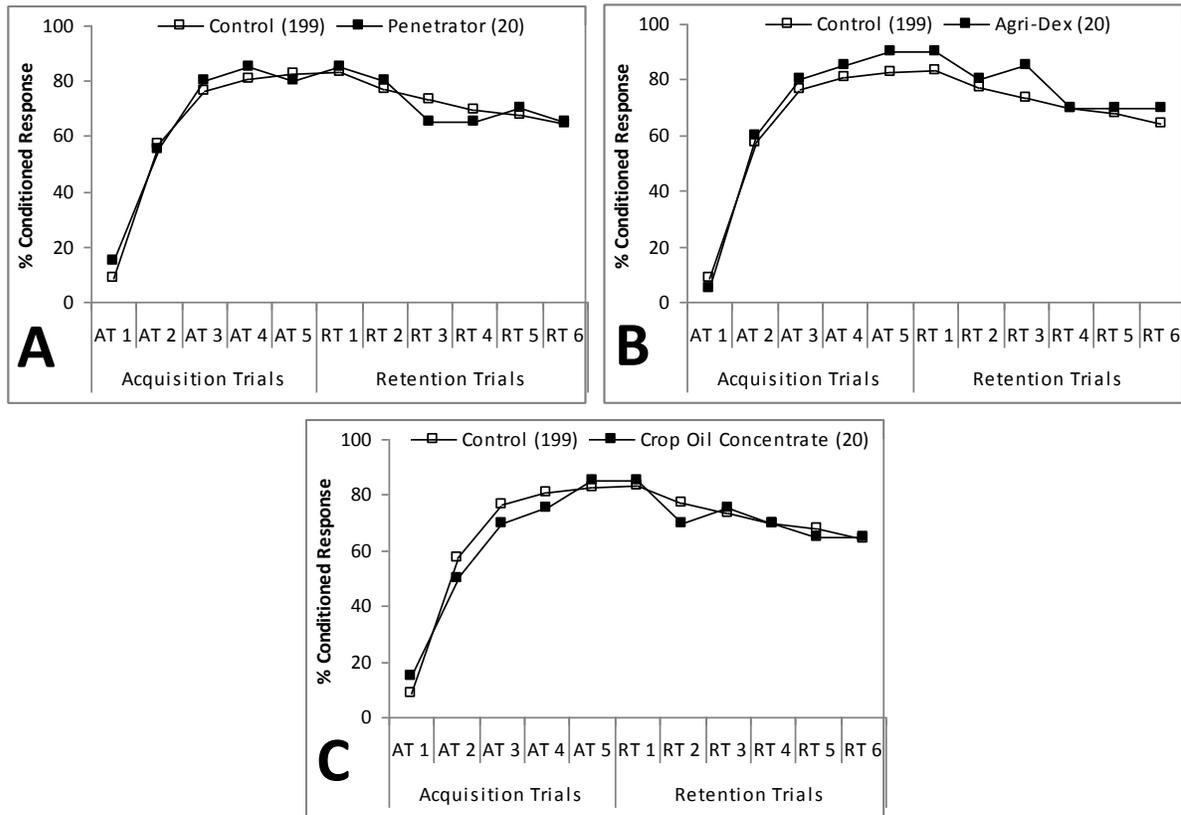
Figure 11. Learning performances of honey bees 5 minutes after antennal contact plus oral ingestion of three different crop oil concentrates (1% v/v). Percent conditioned response refers to the percentage of bees in each group that gave positive responses at each trial. Number of subjects in each group is indicated in parentheses. * $P < 0.05$ (Fisher's exact test).
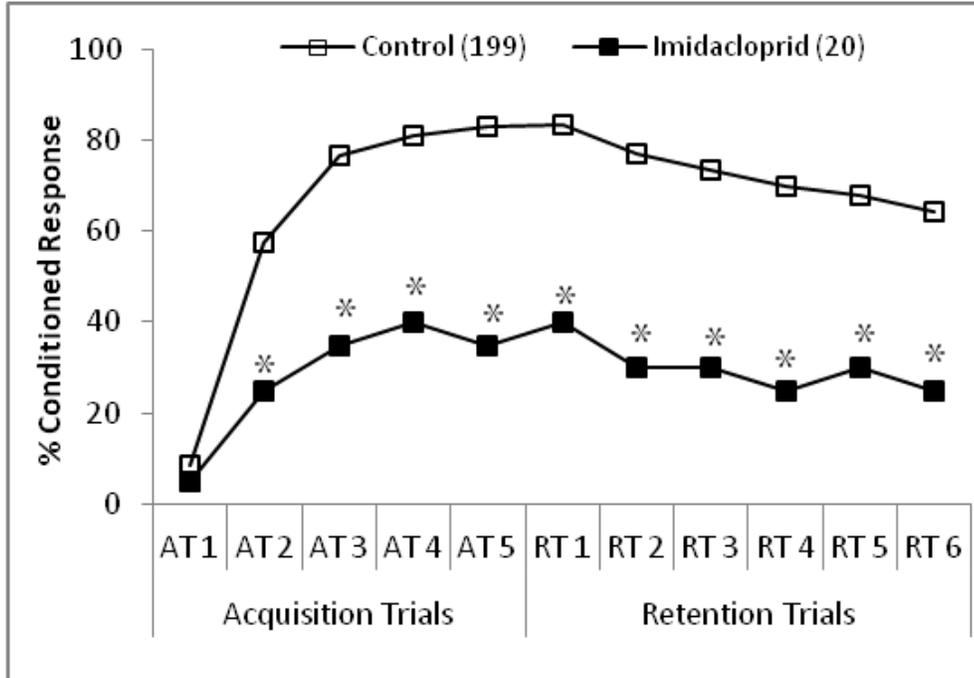
Figure 12. Learning performances of honey bees 5 minutes after antennal contact plus oral ingestion of 12 ng imidacloprid. Percent conditioned response refers to the percentage of bees in each group that gave positive responses at each trial. Number of subjects in each group is indicated in parentheses. * $P < 0.05$ (Fisher's exact test).
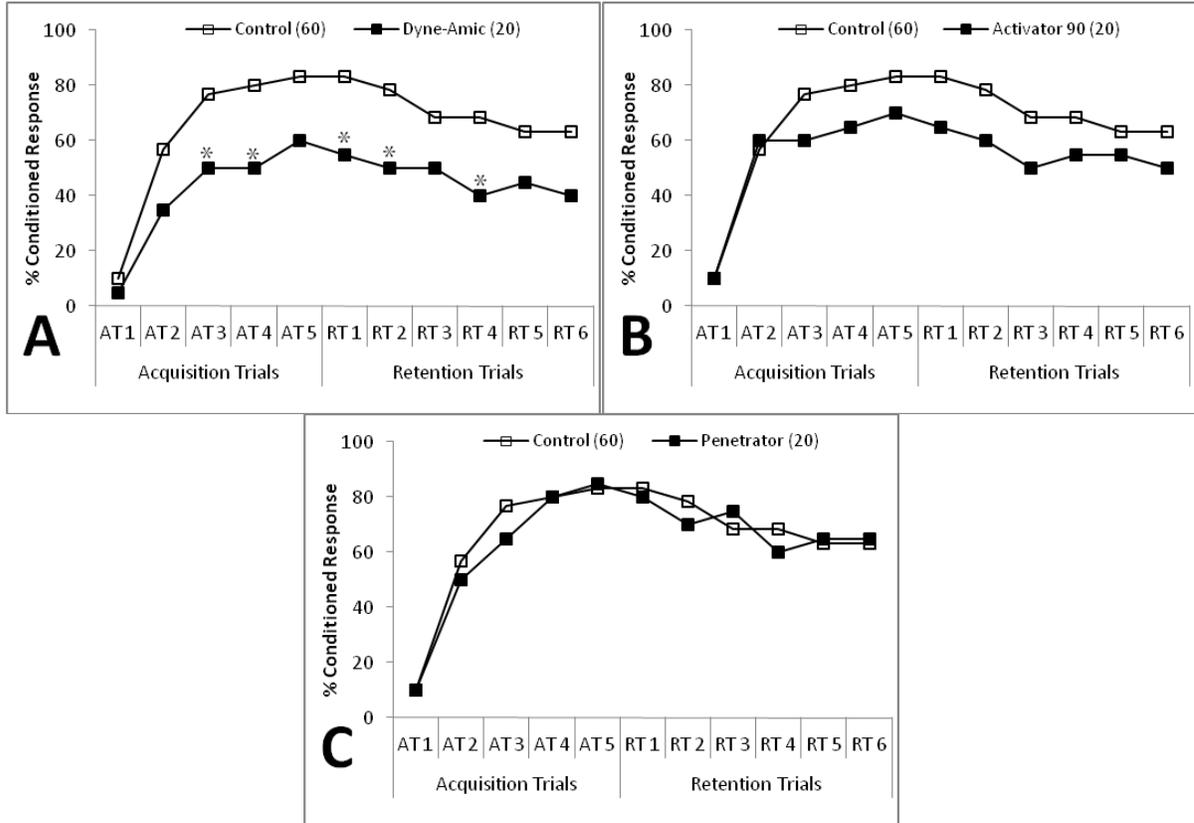
Figure 13. Learning performances of honey bees 5 minutes after oral ingestion of Dyne-Amic (**A**), Activator 90 (**B**), and Penetrator (**C**) (1% v/v for each adjuvant). Percent conditioned response refers to the percentage of bees in each group that gave positive responses at each trial. Number of subjects in each group is indicated in parentheses. * $P < 0.05$ (Fisher's exact test).
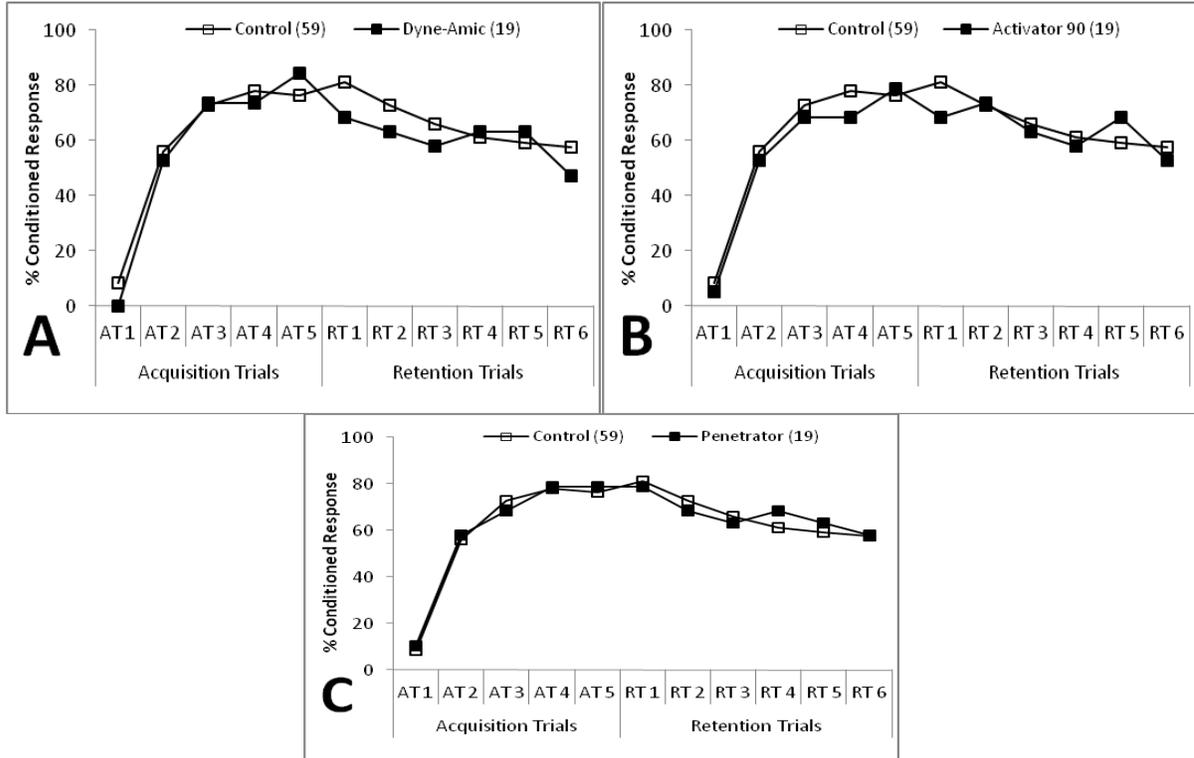
Figure 14. Learning performances of honey bees 5 minutes after antennal contact with Dyne-Amic (**A**), Activator 90 (**B**), and Penetrator (**C**) (1% v/v for each adjuvant). Percent conditioned response refers to the percentage of bees in each group that gave positive responses at each trial. Number of subjects in each group is indicated in parentheses. * *P* < 0.05 (Fisher's exact test).
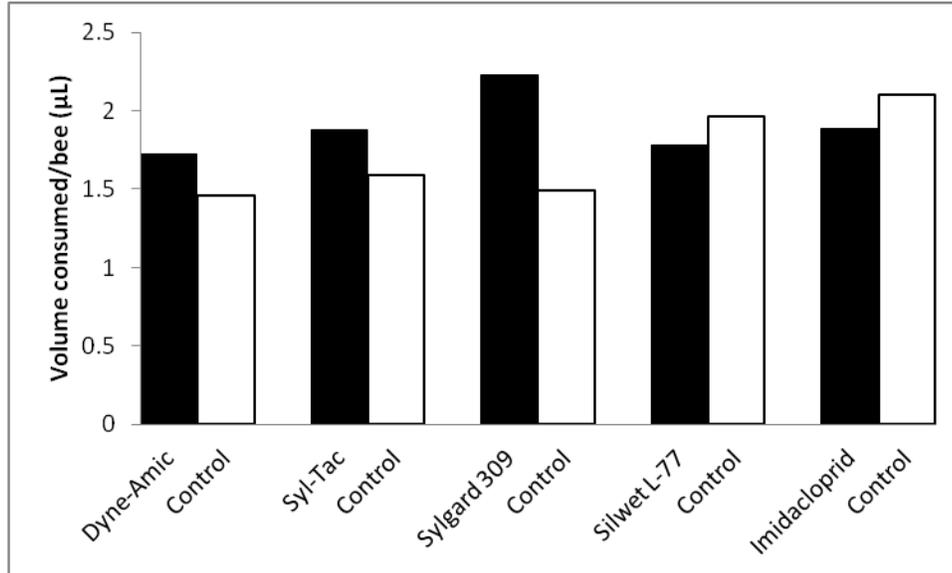
**Figure 15.** Amounts of 50% (w/v) sucrose consumed per bee 5 min prior to AT1. Treatment solutions containing organosilicone adjuvants at 1% (and imidacloprid at 6.25 mg L^{-1}) denoted by black bars. Open bars denote control groups for each experiment. All solutions were applied first to the antennae and then to the extended proboscis for ~2 s. Imidacloprid control solution contained 1% acetone (v/v).
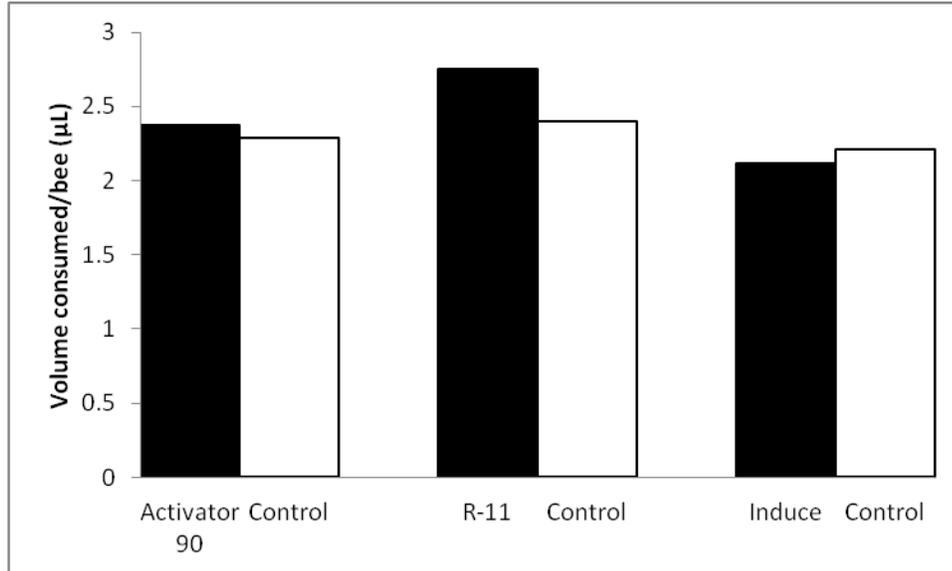
Figure 16. Amounts of 50% (w/v) sucrose consumed per bee 5 min prior to AT1. Treatment solutions containing nonionic surfactants at 1% denoted by black bars. Open bars denote control groups for each experiment. All solutions were applied first to the antennae and then to the extended proboscis for ~2 s.
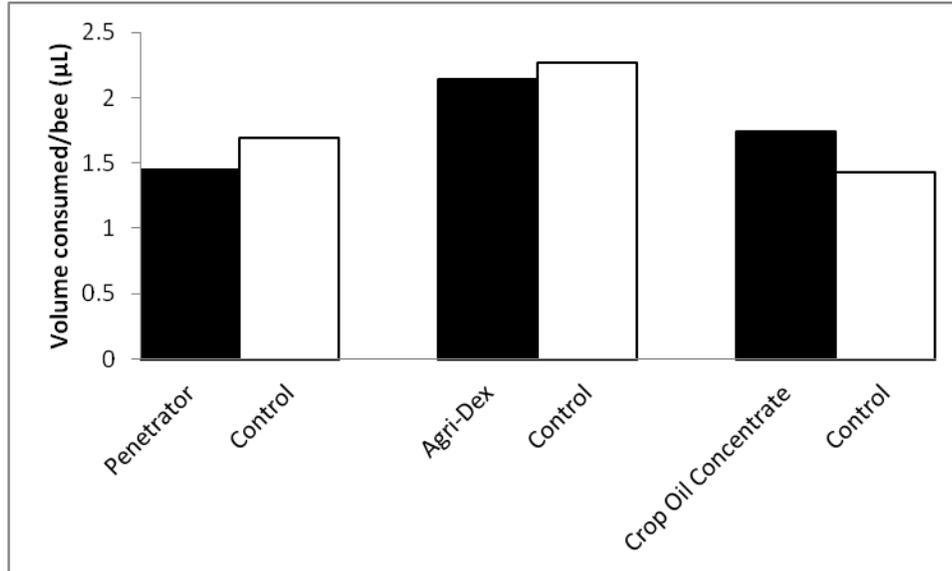
Figure 17. Amounts of 50% (w/v) sucrose consumed per bee 5 min prior to AT1. Treatment solutions containing crop oil concentrates at 1% denoted by black bars. Open bars denote control groups for each experiment. All solutions were applied first to the antennae and then to the extended proboscis for ~2 s.
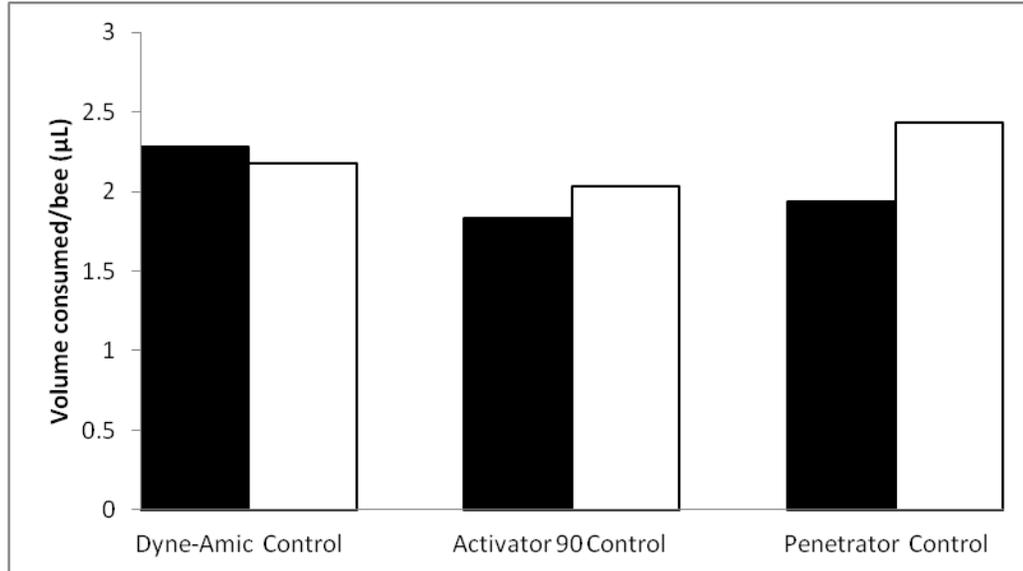
Figure 18. Amounts of 50% (w/v) sucrose consumed per bee 5 min prior to AT1. Treatment solutions containing the top adjuvant from each class at 1% denoted by black bars. Open bars denote control groups for each experiment. All solutions were applied only to the extended proboscis (not antennae) for ~2 s.
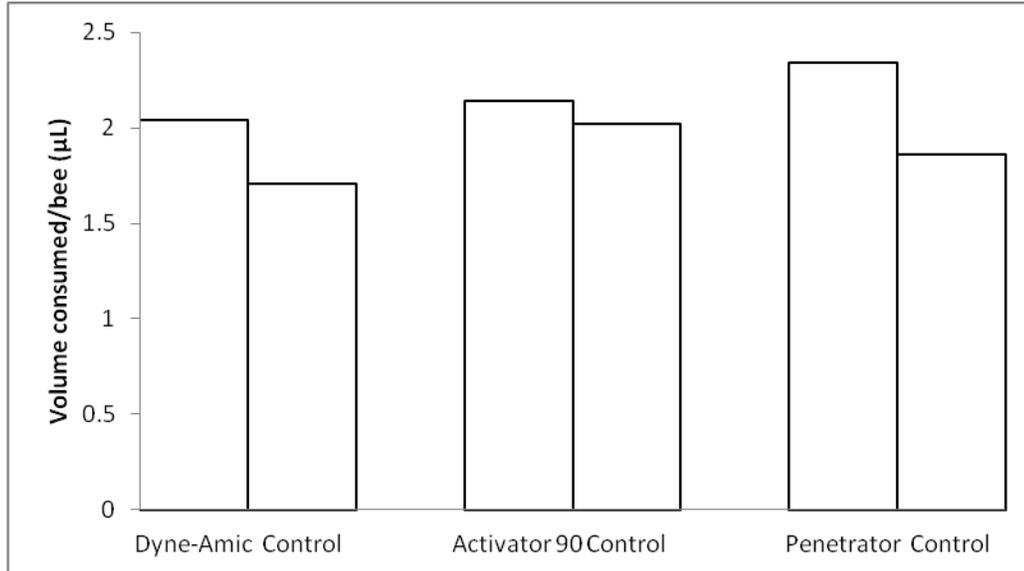
Figure 19. Amounts of 50% (w/v) sucrose consumed per bee 5 min prior to AT1. Treatment solutions containing the top adjuvant from each class at 1% were applied to the antennae only and were not ingested. Open bars denote sucrose solution applied only to the extended proboscis (not antennae) for ~2 s.
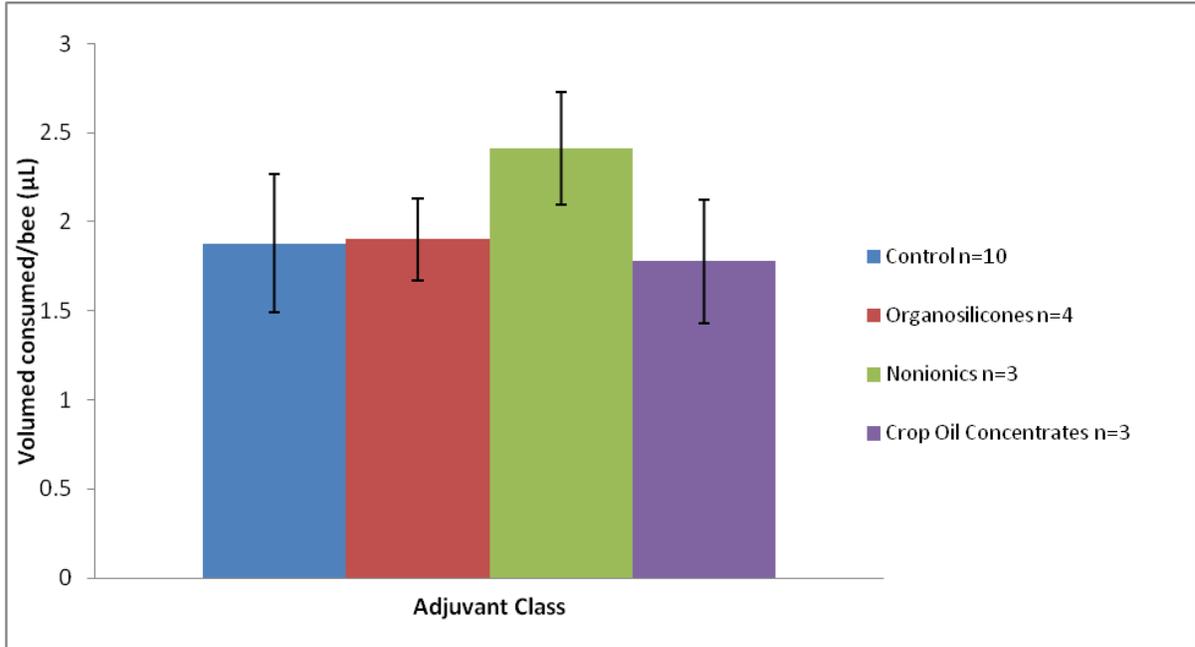
Figure 20. Amounts of treatment solution consumed per bee according to adjuvant class. Data for each individual adjuvant tested in the antennal contact plus oral ingestion experiments were treated as replicates according to class. Mean consumption amounts were 1.88, 1.90, 2.41, and 1.78 μL bee^{-1} for the control, organosilicone, nonionic, and crop oil concentrate classes, respectively. Error bars represent 1 S.D. of the mean. Amounts consumed per bee were not significantly different from the control ($P < 0.05$, Student's t-test).
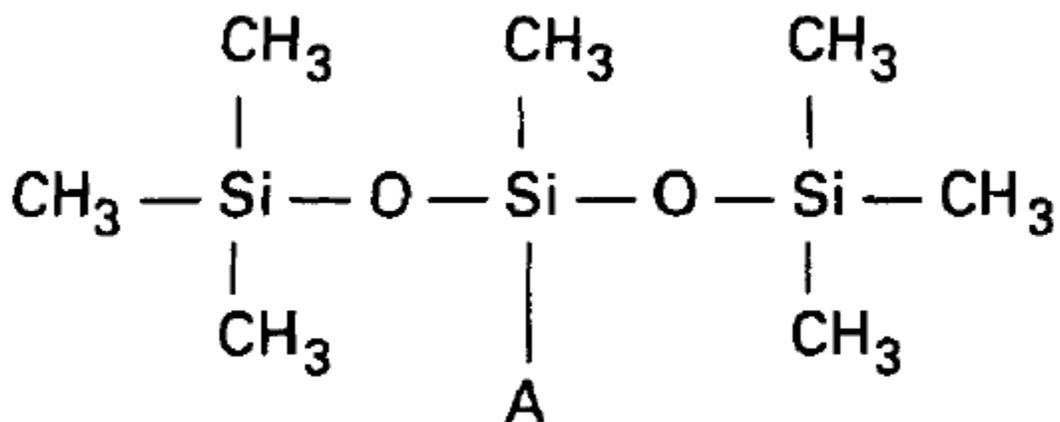
Figure 21. Generalized structure of a trisiloxane organosilicone surfactant. Taken from Stevens et al. (1993).

A= —C_3H_6O—$(C_2H_4O)_x$—CH_3
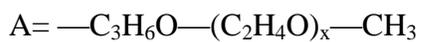
Table 1. Effects of 4 concentrations of Dyne-Amic on learning performances of honey bees.

Concentration	N[a]	% Conditioned Response										
		AT1	AT2	AT3	AT4	AT5	RT1	RT2	RT3	RT4	RT5	RT6
0.1%	19	10.5[b]	57.9	68.4	68.4	73.7	78.9	68.4	63.2	52.6	57.9	57.9
Control	19	10.5	57.9	78.9	78.9	84.2	84.2	73.7	78.9	68.4	68.4	63.2
0.5%	20	15.0	60.0	75.0	85.0	80.0	80.0	65.0	75.0	65.0	60.0	55.0
Control	20	15.0	65.0	75.0	85.0	85.0	80.0	75.0	65.0	65.0	60.0	65.0
1.0%	20	15.0	45.0	50*	55*	50*	50*	40*	35*	40.0	35*	30*
Control	20	15.0	70.0	80.0	85.0	85.0	85.0	80.0	70.0	65.0	65.0	60.0
5.0%	20	5.0	15*	20*	20*	25*	25*	25*	20*	20*	15*	15*
Control	20	10.0	65.0	75.0	80.0	80.0	80.0	75.0	70.0	60.0	55.0	60.0
Aggregate Control	79	12.7	64.6	77.2	82.3	83.5	82.3	75.9	70.9	64.6	62.0	62.0

[a] N, number of bees per treatment group.

[b] Percent conditioned responses for each treatment group were compared to the aggregate control group for each treatment modality using the Fisher's exact test (* $P < 0.05$)

Table 2. Learning performances of honey bees 5 minutes after antennal contact plus oral ingestion of spray adjuvants (1% v/v).

Adjuvant	N[a]	AT1	AT2	AT3	AT4	AT5	RT1	RT2	RT3	RT4	RT5	RT6
							% Conditioned Response					
Dyne-Amic	20	15.0[b]	45.0	50*	55*	50*	50*	40*	35*	40*	35*	30*
Control	20	15.0	70.0	80.0	85.0	85.0	85.0	80.0	70.0	65.0	65.0	60.0
Sylgard 309	20	0.0	40.0	55.0	60.0	50*	65.0	45*	45*	40*	45.0	35*
Control	20	0.0	55.0	75.0	85.0	85.0	95.0	85.0	80.0	70.0	80.0	70.0
Syl-Tac	20	15.0	40.0	50*	45*	45*	50*	45*	45*	40*	35*	35*
Control	20	10.0	45.0	60.0	70.0	80.0	65.0	75.0	65.0	65.0	60.0	60.0
Silwet L-77	20	10.0	35.0	50*	40*	50*	40*	30*	30*	35*	30*	25*
Control	20	5.0	60.0	90.0	85.0	90.0	90.0	75.0	85.0	75.0	70.0	70.0
Activator 90	20	15.0	50.0	60.0	60.0	60*	60*	45*	50*	45*	40*	45.0
Control	20	10.0	60.0	65.0	80.0	85.0	85.0	75.0	75.0	60.0	65.0	65.0
R-11	20	15.0	45.0	65.0	65.0	70.0	65.0	55.0	60.0	45*	55.0	50.0
Control	20	5.0	50.0	70.0	80.0	85.0	85.0	75.0	80.0	70.0	70.0	70.0
Induce	20	5.0	50.0	65.0	70.0	70.0	70.0	60.0	60.0	55.0	50.0	50.0
Control	20	5.0	60.0	80.0	90.0	85.0	85.0	85.0	75.0	80.0	70.0	65.0
Penetrator	20	15.0	55.0	80.0	85.0	80.0	85.0	80.0	65.0	65.0	70.0	65.0
Control	19	15.8	63.2	84.2	78.9	78.9	78.9	78.9	68.4	73.7	63.2	63.2
Agri-Dex	20	5.0	60.0	80.0	85.0	90.0	90.0	80.0	85.0	70.0	70.0	70.0
Control	20	10.0	60.0	85.0	85.0	80.0	90.0	75.0	75.0	70.0	75.0	65.0
Crop Oil Concentrate	20	15.0	50.0	70.0	75.0	85.0	85.0	70.0	75.0	70.0	65.0	65.0
Control	20	10.0	50.0	75.0	70.0	75.0	75.0	65.0	60.0	70.0	60.0	55.0
Imidacloprid	20	5.0	25*	35*	40*	35*	40*	30*	30*	25*	30*	25*
Acetone Control	20	5.0	55.0	70.0	80.0	75.0	75.0	80.0	70.0	65.0	70.0	65.0
Aggregate Control	199	8.5	57.3	76.4	80.9	82.9	83.4	76.9	73.4	69.8	67.8	64.3

[a] N, number of bees per treatment group.
[b] Percent conditioned responses for each treatment group were compared to the aggregate control group for each treatment modality using the Fisher's exact test (* $P < 0.05$).

Table 3. Learning performances of honey bees 5 minutes after oral ingestion of Dyne-Amic, Activator 90, and Penetrator (1% v/v for each adjuvant)

Adjuvant	N[a]	AT1	AT2	AT3	AT4	AT5	RT1	RT2	RT3	RT4	RT5	RT6
						% Conditioned Response						
Dyne-Amic	20	5.0[b]	35.0	50*	50*	60.0	55*	50*	50.0	40*	45.0	40.0
Control	20	5.0	55.0	80.0	75.0	90.0	90.0	85.0	70.0	75.0	70.0	70.0
Activator 90	20	10.0	60.0	60.0	65.0	70.0	65.0	60.0	50.0	55.0	55.0	50.0
Control	20	15.0	60.0	75.0	80.0	75.0	75.0	70.0	60.0	65.0	55.0	60.0
Penetrator	20	10.0	50.0	65.0	80.0	85.0	80.0	70.0	75.0	60.0	65.0	65.0
Control	20	10.0	55.0	75.0	85.0	85.0	85.0	80.0	75.0	65.0	65.0	60.0
Aggregate Control	60	10.0	56.7	76.7	80.0	83.3	83.3	78.3	68.3	68.3	63.3	63.3

[a] N, number of bees per treatment group.

[b] Percent conditioned responses for each treatment group were compared to the aggregate control group for each treatment modality using the Fisher's exact test (* $P < 0.05$).

Table 4. Learning performances of honey bees 5 minutes after antennal contact with
Dyne-Amic, Activator 90, and Penetrator (1% v/v for each adjuvant)

Adjuvant	N[a]	AT1	AT2	AT3	AT4	AT5	RT1	RT2	RT3	RT4	RT5	RT6
						% Conditioned Response						
Dyne-Amic	19	0.0[b]	52.6	73.7	73.7	84.2	68.4	63.2	57.9	63.2	63.2	47.4
Control	20	5.0	55.0	70.0	70.0	75.0	80.0	70.0	50.0	50.0	50.0	45.0
Activator 90	19	5.3	52.6	68.4	68.4	78.9	68.4	73.7	63.2	57.9	68.4	52.6
Control	19	5.3	63.2	73.7	84.2	78.9	78.9	78.9	73.7	68.4	63.2	68.4
Penetrator	19	10.5	57.9	68.4	78.9	78.9	78.9	68.4	63.2	68.4	63.2	57.9
Control	20	15.8	52.6	78.9	84.2	78.9	89.5	73.7	78.9	68.4	68.4	63.2
Aggregate Control	59	8.5	55.9	72.9	78.0	76.3	81.4	72.9	66.1	61.0	59.3	57.6

[a] N, number of bees per treatment group.
[b] Percent conditioned responses for each treatment group were compared to the aggregate control group for each treatment modality using the Fisher's exact test (* $P < 0.05$).

Appendix

Preliminary Silwet L-77 Data

Experiments conducted in October 2010 revealed evidence of learning impairment in foragers treated orally with the organosilicone adjuvant Silwet L-77 in the 'A+O' treatment modality. Experimental protocol was slightly different in that treatment solutions were administered 15 minutes before AT1 for a duration of 5 seconds. A 1% solution of α-pinene in mineral oil (500 μL total volume) was used as the CS instead of cinnamon oil. Foragers collected at the hive entrance were used instead of house bees, meaning that their average age was greater. Two concentrations of Silwet L-77 were investigated according to this protocol: 0.1% and 0.25%. Assuming a per bee consumption rate of 1 $\mu L\ s^{-1}$ (see Chapter 2), each bee received 5 μg Silwet L-77 from a 5 second feeding of the 0.1% solution, and 12.5 μg Silwet L-77 from a 5 second feeding of the 0.25% solution.

Significant reductions in learning ability were observed at AT3 and AT5 in bees treated with 0.25% Silwet L-77 (Figure). A dose response is evident, although no significance was seen in bees treated with 0.1% Silwet L-77. The results obtained in these preliminary experiments prompted a more thorough investigation of the effects of spray adjuvants on learning in honey bees.
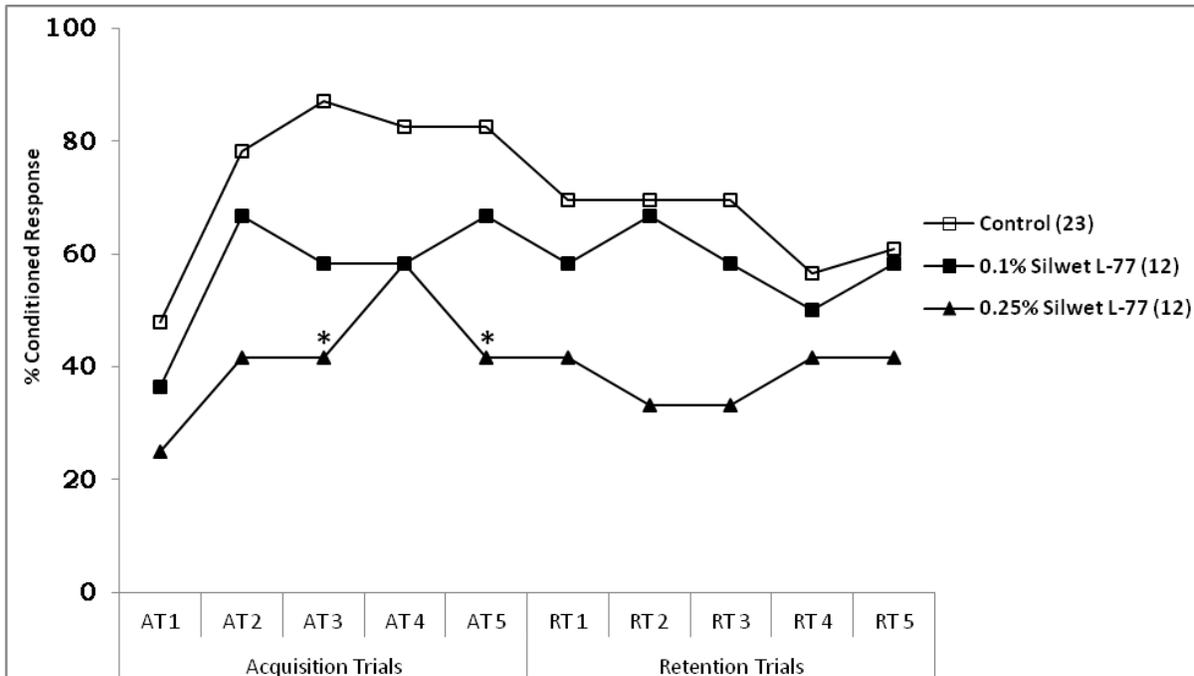
Learning performances of foraging honey bees 15 minutes after antennal contact plus oral ingestion of two concentrations of Silwet L-77 (0.1% and 0.25% v/v). Treatment solutions were administered for 5 seconds. Percent conditioned response refers to the percentage of bees in each group that gave positive responses at each trial. Number of subjects in each group is indicated in parentheses. * $P < 0.05$ (Fisher's exact test) relative to the control.

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