UNIVERSITY OF CALIFORNIA, SAN DIEGO

Imidacloprid impairs shorter-term and longer-term learning in honey bees (*Apis mellifera*)

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Erica Zhang

Committee in charge:

Professor James Nieh, Chair
Professor David Holway
Professor Jonathan Shurin

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The Thesis of Erica Zhang is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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ABSTRACT OF THE THESIS

Imidacloprid impairs shorter-term and longer-term learning in honey bees (*Apis mellifera*)

by

Erica Zhang

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Professor James Nieh, Chair

Even at sublethal doses, neonicotinoids, commonly used insecticides can affect neurons involved in learning and memory, cognitive features that play a key role in colony fitness because they facilitate foraging. The commonly used neonicotinoid,

imidacloprid, impairs the ability of bees to associate floral odors with a nectar reward. However, no studies, to date, have examined how if imidacloprid impairs negative associative learning. Sit-and-wait predators like spiders can attack foraging bees. Because bees can escape such attacks, learning to avoid dangerous foraging sites should enhance their survival. Negative associative learning classically uses electric shock as the punishing stimulus, although this is not natural. To better mimic predation, we developed a robo-predator so we consistently attack foragers with a pinching bite at a fixed force. Also, all prior aversive learning studies have used single, pure odors to test learning. We challenged bees with a more natural task and used the sting extension reflex (SER=the unconditioned response) to show that they can learn to associate an aversive biting stimulus with the complex odors of different *Thymus vulgaris* chemotypes. Bees exhibited a two fold higher SER response to the odors of punished as compared to non-punished chemotypes in short- and longer-term learning tests. Finally, we show that chronic exposure over 4 days to a sublethal concentration (25.56 µg/L=20.79 ppb) significantly impairs negative short-term and longer-term learning. Control bees show 8 fold better short-term learning and longer-term learning than imidacloprid-treated bees. The impact of neonicotinoids on aversive learning and how bees avoid danger should therefore be more widely considered.

Introduction

Honey bees (*Apis mellifera*) provide valuable pollination services to both commercial crops and wild plants. However, the number of managed honey bee colonies has been in decline in Europe and the United States in recent decades (Ellis et al. 2010; Potts et al. 2010). Unusually high winter losses since 2006 in the US and Europe have contributed to colony collapse disorder (CCD), characterized by the rapid loss of adult workers, absence of dead bees within and surrounding affected hives, and the delayed invasion of some hive parasites (vanEngelsdorp et al. 2009). Several factors such as pesticide exposure (even at sublethal doses), *Varroa destructor* mite infestation, pathogens, and interactions between these factors contribute to CCD and poor colony health (Neumann & Carreck 2010). For example, pesticides and infection by the pathogen *Nosema cerana* reduce colony health, and honey bees exposed to sublethal pesticide doses had significantly higher *Nosema* infection levels (Pettis et al. 2012).

In the past two decades, pesticide use has shifted from organophosphates and carbamates to neonicotinoid compounds such as imidacloprid. Imidacloprid is a systemic insecticide commonly given as a seed treatment. As the plant grows, imidacloprid spreads throughout all of the plant tissues, including nectar and pollen. In the United States, insecticide residues are widespread in pollen sampled from honey bee hives (Mullin et al. 2010). In addition, the degradation products of imidacloprid, both following insect metabolism and from environmental decay, are just as toxic to bees (Goulson 2013). Imidacloprid can linger in the soil and therefore be incorporated in plants that were not initially treated.

Imidacloprid is an agonist of insect nicotinic acetylcholine receptors (nAChRs, Buckingham et al. 1997; Elbert et al, 2008; Ihara et al, 2006). Honey bee nAChRs play an important role in the cholinergic neural signaling involved olfactory learning and memory, and both acute (one-time) and chronic (multi-dose) ingestion of imidacloprid impairs honey bee foraging behavior (Gauthier, 2010). Synergistic effects increase the problem. The main honey bee mechanisms for detoxifying neonicotinoids, *p*-glycoprotein xenobiotic efflux transporters and cytochrome P450 mono-oxygenase enzymes, are also impaired by the organophosphates used to kill *Varroa* mites, one of the most common and deadly honey bee parasites (Johnson et al, 2009). Thus, combined exposure to even sublethal doses of these xenobiotics can lead to significant learning and memory impairment (Williamson & Wright 2012).

These memory impairment studies have focused on appetitive classical conditioning using the proboscis extension reflex (PER) with odor as the conditioned stimulus. Bees learn to associate floral odors with nectar rewards and this allows them to better recognize rewarding resources during subsequent foraging trips (Menzel et al. 1993). In detail, a honey bee reflexively extends its proboscis to drink when its antennae are stimulated with a sufficiently concentrated sugar solution. If the bee simultaneously experiences an odor, it learns to associate this odor with the sugar reward (Bitterman et al. 1983; Giurfa and Sandoz, 2012). After a single learning trial, many bees will learn and extend their proboscis upon sensing the rewarded odor alone. Such olfactory conditioning studies have provided important insights into the anatomical, neural, and molecular bases of insect learning and memory (Matsumoto et al. 2012).

Sublethal, field-realistic doses of imidacloprid significantly impair olfactory learning as assayed by PER, even 9 days after the end of the imidacloprid treatment (Decourtye et al. 2003). The metabolic byproducts of imidacloprid also impair learning (Decourtye et al. 2003). Memory is impaired by an acute treatment (bees fed a single dosage of syrup containing imidacloprid) and by a chronic treatment (bees have access to syrup containing imidacloprid for at least 6 days, Cresswell 2010). Imidacloprid impairs both short term and long term olfactory memory (Williamson & Wright 2013).

Surprisingly, no studies, to date, have examined the effect of neonicotinoids on aversive memory formation. Aversive learning is important because learning to avoid predators should enhance bee survival. Only 9.4-10.8% of spider attacks on bees at flowers end in successful predation (Dukas & Morse 2003; Morse 1986). An attacked bee therefore has a high probability of surviving, and would benefit by learning to avoid a dangerous resource. Such learning would also be useful in other contexts. Olfactory learning is important for enemy identification by bees defending the colony (Breed et al 2004).

In general, our knowledge of honey bee aversive learning is more limited, largely because rewarded olfactory learning is a more robust phenomenon and because the natural context for the typically used aversive learning stimulus (electric shock) is weak. In honey bees, the sting extension response (SER) is a defensive reflex of bees extending their stingers in response to noxious stimuli such as being attacked. Aversive olfactory learning experiments test the ability of bees to learn odor associated with a relatively strong electric shock (Balderrama et al., 2002; Núñez et

al., 1998; Núnez et al., 1983; Vergoz 2007). Once bees have learned to associate and odor with the punishing electric shock, they will extend their stinger upon detecting the odor alone. Aversive learning can be stored as stable long term memory that helps a forager avoid food at which it had a negative experience and to enhance colony defense by remembering predator odors (Tedjakumala & Giurfa 2013).

However, such aversive learning has a lower response rate, takes longer to learn, and is weaker than rewarded PER learning (Tedjakumala & Giurfa 2013). Moreover, electric shock does not have a strong natural analog. The very weak electric field that bees may sense when entering some flowers does not elicit SER and can be associated with food reward, eliciting PER (Clark et al. 2013). At best, the natural analog for electric shock is an extremely strong sensory neural response arising from a wound inflicted by a predator. The logic is that bees will enter a defensive mode and prepare to sting once they smell the predator.

Electroshock does not naturally occur, but predators often bite when attacking bees on floral resources (Morse and Nowogrodzki 1990). For example, a crab spider attack involves the spider attempting to position itself around the bee, holding the bee with the spider's legs, and then biting (Dukas & Morse 2003; Reader et al 2006). We therefore used a pinching stimulus to reliably and consistently simulate a predator attack.

Our goals were to test the following hypotheses centered on using more natural stimuli to test the ability of foragers to learn odors associated with attacks. First, we hypothesized (H1) that honey bees can show olfactory aversive learning (SER) of whole plant odors in response to simulated biting. All prior SER experiments have

only tested learning of pure single odors, although bees typically experience a complex bouquet of plant odors when foraging. We took advantage of the *Thymus vulgaris* (thyme) system, in which genetically distinct strains of the same species have distinct olfactory phenotypes (chemotypes) that share some of the same odor compounds, but also have distinct major compounds (Linhart et al. 2005). Second, we hypothesized (H2) that aversive olfactory learning will be impaired by sublethal, field-realistic doses of imidacloprid. To test this second hypothesis, we used pure odors that represent the major distinguishing odor compound in each of our plant chemotypes because the learning of pure odors is faster and more robust.

Materials & Methods

General methods

We used 25 *A. mellifera* colonies (8 for the plant chemotype experiment, 17 for the pesticide experiment) located at the University of California, Biology Field Station in La Jolla, California, USA. To ensure that we tested forager responses, we used plastic vials to capture bees foraging on unscented 1.8 M sucrose solutions (50% sucrose w/w) in grooved plate sucrose feeders (based upon design of von Frisch, 1967) at the entrance of each colony. This placement limited foraging to bees from the same colony, allowing us to easily determine colony identity.

To harness bees, we used cold anesthesia and placed them for approximately 3 min at 0°C until their movements have slowed or stopped. To make sting extension easily visible, bees were then placed with their thorax facing down (standard SER protocol, Vergoz 2007) and were held with a thin (3 cm wide) strip of cloth tape over the ventral side of their thorax on a 3.5 cm high and 8 mm diameter stainless steel tube stand (ensuring that all their legs and antennae were free). Each stand was labeled with a unique bee identification number. After harnessing, the bees were then fed 0.5 µl of unscented pure1.8 M sucrose solution (prepared with reagent-grade sucrose and distilled water and never containing pesticide) before they were placed in the incubator for 20 minutes to revive and adjust to the harness.

Odor chamber setup

We presented odors by pumping air from an air pump (Active Aqua by Hydrofarm Model AAPA25L) through PFTE lined silicon tubing (6 mm) into two

clear chambers (modified 17 L polycarbonate cylindrical containers with lids: Thunder Group PLRFT018PC). On the outside of each clear lid, we affixed a 400 nm LED plant growth bulb (200 lumens, used when a plant was placed inside the chamber to keep it in good condition over a 3.5 hr period). We placed a digital thermometer inside the chamber. Using the LED light did not appreciably alter the chamber temperature (22.3±3.4°C). Once we attached and sealed the lid with a silicone gasket, all air pumped into this odor chamber exited through an attached solenoid valve (Evolutionary Concepts ITT, P/N 623-238) controlled by a momentary switch into output tubing. We used two chambers, one for each odor type. The output tubes of both chambers terminated at a platform so that there were at the same height and separated by 5 mm from the antennae of the harnessed bee. We adjusted valves to equalize output pressures and used a digital manometer (HT-1890 Digital Manometer) to measure the terminal airflow (71±13 mbar). A 10 cm diameter fan positioned 15 cm away from the bee drew odors away.

For the *whole plant odor experiment*, we reared two chemotypes of *T. vulgaris*: G (major odor component is geraniol) and T (major odor component is thymol) from seeds sorted into chemotypes. *Thymus vulgaris* and *A. mellifera* are both native Mediterranean species (Linhart et al. 2005; Michener 2000). We used GC-MS analysis to confirm that the plants were correctly chemotype identified. Plants were grown inside a screened-in greenhouse for at least 1 month before use so that no pollinators could visit and deposit potential odor marks on the plants. Blooming plants of equal size (in 10 cm diameters pots) were then used for the experiment. In total, we used 27 G-chemotypes and 27 T-chemotypes.

For the *pesticide experiment*, we used pure odors (geraniol or thymol) presented as follows. We pipetted 10 µl of pure geraniol (MP Biomedical, CAT #157184, LOT #R2558) or 10 µl of pure thymol (Sigma Aldrich, CAS #89-83-8, LOT #SZB82760) onto a 2.5 cm diameter filter paper that we inserted inside a clean plastic capsule connected with tubing between the air input and solenoid-controlled output of the odor chamber (see above). Thymol is a solid at room temperature, so we briefly liquefied it in a sealed vial at 60°C to enable pipetting. During handling of odors, we wore latex gloves and used clean tweezers to handle the filter paper.

Testing bee learning

We typically tested 10 bees at a time. We lined up these bees near the odor presentation setup. We used a pseudorandom differential conditioning design (Bitterman 1985) with two trial types, A (punished) and B (not punished) in the order ABBABABAABBA. In trial type A, the bee was observed for 3 s, exposed to the odor from chamber A for 3 s, and then punished being pinched on the right metathoracic leg at the basitarsus with the robo-predator while still being exposed to odor A for another 3 s (Fig. 1a). For trial type B, we followed the same timeline, but the bee was not punished (Fig. 1a). We recorded if the bee extended its stinger in each of these 3 s intervals. Bees that did not extend their stinger while being pinched were discarded. We repeated each trial, with a 10 min intertrial interval, for a total of six trials to test short-term learning. We chose 10 min because this interval leads to both good memory acquisition and higher retention in honey bees (Menzel et al. 2001) and matches the procedure of Williamson and Wright (2013). After the 6th trial, we waited 1 hr to test

memory retention, exposing each bee to odor A for 3 s and 10 min later to odor B for 3 s. This 1 hr wait tests a pre-cursor to long-term memory (Menzel 2001). After the experiment, the bees were unharnessed from the stands, painted blue on their abdomen, and released into the field.

Robo-predator

To mimic predator biting, we used a custom-modified electro-mechanical CKD AS-0RN solenoid coil (CKD Corp., Komaki, Aichi, Japan) that draws an arm in, squeezing forceps to exert a bite force proportional to the current supplied (modulated with a rheostat). A momentary switch allowed the operator to activate the robotic predator. We calibrated this device by using the forceps tips to pinch a 1 mm wide flat metal bar (matching the width of a bee's metathoracic basitarsus) glued across the center of an Arduino piezoelectric ceramic disc sensor (20 mm diameter). We generated a calibration curve by adjusting the rheostat to give varying levels of force and measuring the sensor voltage with a digital oscilloscope (TDS2024B, Tektronix Inc., Beaverton, Oregon, USA). To determine the actual force levels, we modified the procedure of Tautz et al. (1995). We measured force with a type 8001 impedance head attached to a type 4294 vibrational calibrator (Brüel & Kjaer, Norcross, Georgia, USA). The impedance head was pressed against the center of the metal bar on the sensor disc, which in turn rested on a soft foam base attached to a lab jack. As the jack was raised, more force from the vibrational calibrator was applied to the disc sensor via the impedance head. We could then calibrate the sensor disc voltages with actual

force levels measured by the impedance head. This allowed us to determine the force levels applied by the robo-predator forceps at different rheostat settings.

Tautz et al. (1995) reported that leaf cutting ant mandibles applied different levels of force, depending upon the type of leaf being cut (10-20 mN_{pk-pk} for soft leaves and 50-100 mN_{pk-pk} for tough leaves). We set our robo-predator to provide average forces of 150±29 mN_{pk-pk}. Sclerotized insect cuticle, such as bee legs, is likely much harder and tougher than most leaves. No bees pinched with this level of force suffered evident harm. They continued to walk normally on the feeder and could waggle dance inside the nest, a task that requires complex motor coordination of leg movements (Landgraf et al. 2011). To attack bees, we applied the forceps around the right metathoracic leg at the basitarsus.

Bee incubation for pesticide experiment

Right after capture, bees were each fed 5 µl of 1.8 M sucrose with a micropipette to ensure that they all immediately had some food. The captured bees were then divided into two groups (usually 5 bee per cage) and placed into either pesticide or control ventilated clear plastic cage (12 cm x 8 cm x 12 cm, with a 5 ml syringe containing the treatment in 1.8 M sucrose solution). As a protein source, we placed 10 g of pollen in a 1.5 ml centrifuge tube on the floor of each cage. Treatments were either pure 1.8 M sucrose solution or this solution with 100 nM imidacloprid (justification below), approximately 3 ml per cage. We recorded the initial mass of each sucrose-filled syringe. Cages were incubated at 30ºC and 70% humidity. After four days (96 hrs), we removed the cages from the incubator and recorded the number

of living and dead bees (if any) and re-weighed the syringes. We calculated the average amount of solution (and pesticide, as appropriate) ingested by each bee was calculated by dividing the mass consumed by the number of living bees and applying appropriate conversions (see below).

Pesticide concentration

We simulated bee exposure to imidacloprid in nectar over four days of foraging, following the design of Williamson and Wright (2013) who used sublethal doses of imidacloprid (10 nM, 100 nM, and 1000 nM imidacloprid dissolved in 1 M sucrose solution) and then tested PER reward learning. We provided bees with 100 nM imidacloprid in 1.8 M sucrose solution (equal to 25.56 µg/L=20.79 µg/Kg=20.79 ppb). The density of 1.8 M sucrose solution (50% sucrose w/w) at room temperature is 1.2296 Kg/L (Bubnik et al. 1995). Byrne et al. (2013) measured imidacloprid levels in treated citrus trees grown within an enclosure, and detected residues of 3-39 µg/L in nectar, with an average of 16.4 µg/L from floral nectar and 15.3 µg/L from the crops of bees foraging inside a tunnel to collect this nectar (Byrne et al 2013). In open field studies, residues of imidacloprid were 5.0 µg/L in floral nectar and 3.5 µg/L in bee crops (Bryne et al. 2013). Field realistic concentrations of imidacloprid from a variety of crops and studies are 0.7-10 µg/L (Cresswell 2010). However, Goulson (2013) reported that imidacloprid concentrations can range up to 50 ppb in the nectar and pollen of different crop species. Our 20.79 ppb imidacloprid treatment therefore falls within this range: it is 42% of the maximum concentration reported by Goulson (2013) and 66% of the maximum concentration reported by Bryne et al. (2013).

Statistics

To simplify our analyses, we separately analyzed bee responses to each odor type (G or T) and trial type (attacked or not). All of our data met parametric assumptions. To determine the effect of pesticide on shorter-term learning, we used Repeated-Measures Analysis of Variance (ANOVA). Trial (consecutive 10 min intervals) was a continuous variable. To analyze the effect of treatment on longer-term learning, we only compared bee SER at the 7^{th} trial and thus performed ANOVA with attacked odor type, treatment, and trial type (attacked or not) as fixed effects and colony as a random effect. To compare between the effects of different treatments, we used Tukey Honestly Significant Difference (HSD) tests. We report means±1 standard deviation

Results

Shorter-term learning of whole plant odors

There is no significant effect of plant chemotype on SER learning ($F_{1,260}$=0.05, P=0.82). However, there is a significant effect of trial number ($F_{1,2921}$=14.80, P<0.0001), trial type ($F_{1,2917}$=24.82, P<0.0001), and the interaction trial number*trial type ($F_{1,2917}$=28.99, P<0.0001) because bees only learned the odor associated with attack (Fig. 1b). Colony accounted for 14.9% of model variance. On average, 20.7±41.5% of attacked bees exhibited shorter-term learning by trial 6.

Longer-term learning of whole plant odors

There is no significant effect of plant chemotype on SER learning ($F_{1,510}$=1.16, P=0.28). However, there is a significant effect of trial type ($F_{1,519}$=13.81, P=0.0002, Fig. 1c). The interaction chemotype*trial type ($F_{1,518}$=2.21, P=0.14) is not significant. Colony accounts for 18.2% of model variance. On average, 21.5±41.1% of attacked bees exhibited longer-term learning (SER).

Pesticide effects on sucrose consumption

There is no effect of pesticide treatment on sucrose consumption ($F_{1,233}$=2.49, P=0.12, Fig. 2). Colony accounts for 5.0% of model variance. On average, imidacloprid-treated bees consumed 1.9±0.5 ng imidacloprid per day (total of 7.5±2.2 ng over four days).

Pesticide effects shorter-term learning

With geraniol (G) as the attack odor, there is a significant effect of treatment ($F_{1,301}$=32.47, P<0.0001), trial number ($F_{1,1571}$=13.81, P=0.0002), and the interaction treatment*trial number ($F_{1,1571}$=14.92, P=0.0001) because pesticide-treated bees did not exhibit shorter-term learning (Fig. 3). Colony accounted for 0.1% of model variance. In contrast, bees did not show learning for the non-attack odor. There is no significant effect of treatment ($F_{1,300}$=0.16, P=0.69) or the interaction treatment*trial number ($F_{1,1576}$=1.55, P=0.21). However, there is a significant effect of trial number ($F_{1,1576}$=9.93, P=0.0017) because there was a slight decrease in SER response over time (Fig. 3). Colony accounted for 2.1% of model variance.

With thymol (T) as the attack odor, there is a significant effect of treatment ($F_{1,224}$=10.46, P=0.0014), trial number ($F_{1,1282}$=4.37, P=0.0367), and the interaction treatment*trial number ($F_{1,1282}$=14.24, P=0.0002) because pesticide-treated bees did not exhibit shorter-term learning (Fig. 3). Colony accounted for less than 0.1% of model variance. In contrast, bees did not show learning for the non-attack odor. There is no significant effect of treatment ($F_{1,198}$=1.72, P=0.19), trial number ($F_{1,1251}$=0.80, P=0.37), or the interaction treatment*trial number ($F_{1,1251}$=1.10, P=0.29) (Fig. 3). Colony accounted for 0.2% of model variance.

Pesticide effects on longer-term learning

There is no significant effect of attack odor type on SER learning ($F_{1,204}$=0.71, P=0.40). However, there is a significant effect of treatment ($F_{1,1136}$=26.31, P<0.0001),

trial type ($F_{1,1125}$=43.16, P<0.0001), and the interaction treatment*trial type ($F_{1,1125}$=28.35, P<0.0001) because pesticide-treated bees did exhibit longer-term learning. Specifically, only control bees learned the attack odor (Tukey HSD, P<0.05, Fig. 4). Colony accounted for 1.4% of model variance. On average, 14.4±0.4% of control bees exhibited longer-term learning (SER).

Discussion

We provide the first evidence bees can learn to associate whole plant odors with aversive stimuli. We also provide the first evidence that sublethal exposure to the neonicotinoid, imidacloprid, can significantly impair honey bee aversive learning. This result has broader implications because all previous studies on learning impairment induced by neonicotinoids have only examined their impact on rewarded learning.

Distinguishing between chemotypes

One of our goals was to test aversive learning under more realistic conditions. Honey bees are attacked by a variety of sit-and-wait predators while foraging for nectar and pollen (Tan et al. 2013). They are therefore exposed to aversive stimuli, often biting, while smelling the entire olfactory headspace provided by a flowering plant. This is a far more complex stimulus than the single-odors typically used to test learning. We therefore used a custom-built robo-predator to administer a consistent biting stimulus and tested the ability of bees to distinguish between whole plant odors from two different chemotypes (*G* and *T*) of *T. vulgaris*. Bees were able to distinguish between these different chemotypes. Bees showed increasing SER to the punished odor, but not to the unpunished odor, with multiple learning trials (Fig. 2). Ultimately, bees exhibited a two fold higher SER response to the odors of punished as compared to non-punished chemotypes in short- (6^{th} trial) and longer-term learning tests. These results were independent of the chemotype associated with punishment.

Although bees can perform this task, they do not learn the biting punishment associated with whole plant odor as well as an electric shock punishment associated with a single pure odor. The average SER learning in the biting punishment associated with whole plant odors (thymol and geraniol) is about 17%. Previous SER learning experiments have used an electric shock of 7.5V associated with pure odors (1-hexanol and eugenol) to obtain an average SER learning of 35%. Other SER learning experiments have also achieved up to 43% SER learning using pure odors (1-hexanol and 1-nonanol) that have been proven to have high discrimination and induce low generalization in honey bees (Carcaud et al. 2009; Guerrieri et al. 2005).

There are several reasons why the biting punishment associated with whole plant odors was not as effectively learned. First, the biting punishment is likely a weaker aversive stimulus than a whole-body electric shock. In general, stronger stimuli are more easily learned (Menzel et al. 1993). Second, although the *T. vulgaris* system provides us with the opportunity of using plants with consistent, genetically inherited olfactory phenotypes (chemotypes), some olfactory variation still exists even in plants with the same chemotype (Linhart et al. 2005). In contrast, there is essentially no variation in the chemically pure single-odor compounds that are more typically used to test olfactory learning.

For our pesticide-effect experiment, we therefore simplified the olfactory stimuli, testing the ability of bees to associate the biting punishment with a single, pure odor. Although this design is somewhat less natural, it provides greater experimental repeatability. Nonetheless, we feel that the approach of using the entire

floral headspace to test honey bee olfactory learning is valuable and will yield more insights into bee learning of more natural stimuli.

Effects of imidacloprid

We show that chronic exposure over 4 days to a sublethal concentration (25.56 µg/L=20.79 ppb, 1.9±0.5 ng imidacloprid per day) significantly impairs negative short-term and longer-term learning. Control bee learning increased with multiple learning trials, but imidacloprid bee learning did not. Control bees ultimately exhibited 8 fold better short-term learning (comparing the 6^{th} trials) and longer-term learning (tested 1 hr after the 6^{th} trial) than imidacloprid-treated bees.

The imidacloprid concentrations that we used (39.1-156.5 nmol/L) were sublethal (Williamson and Wright 2013). In *A. mellifera,* only imidacloprid concentrations ≥1000 nmol/L increased mortality: 10 and 100 nmol/L did not alter mortality (Williamson and Wright 2013). The acute 48-hr oral LD_{50} for imidacloprid ranges widely and depends upon a variety of factors that include bee physiological condition and season (Decourtye et al. 2003, Belzunces et al. 2012). Values range between 3.7 ng/bee (Schmuck et al. 2001) and >81 ng/bee for *A. mellifera* (Nauen et al. 2001).

There was no significant difference in the amount of sucrose and sucrose-pesticide solution ingested by the control and experimental bees over the course of four days. This impaired learning in pesticide-treated bees was independent of the odor used (Fig. 3). Control bees learned over time as shown by the increased response in trial numbers while the pesticide bees did not exhibit such learning.

Although no prior published studies have examined the effect of pesticides on honey bee aversive learning, we can make comparisons with rewarded learning (PER) studies. These PER experiments have shown that sub-lethal exposure to imidacloprid decreases honey bee rewarded learning. For example, Decourtye et al. (2004) chronically exposed *A. mellifera* workers imidacloprid (24 µg/kg=116 nM) over 9 days and tested PER rewarded learning (20 minutes intertrial intervals) and found that control bees had 7 fold higher PER than pesticide-treated bees (70% vs. 10%) by the second learning trial.

The conditions used by Williamson and Wright (2013) were most similar to our experiment: they chronically exposed *A. mellifera* workers to 100 nM of imidacloprid over 4 days and then tested per learning over 6 trials with 10 min intertrial intervals. Likewise, in our SER experiment, we chronically exposed bees to 100 nM of imidacloprid over 4 days and tested their short-term learning in 6 trials with 10 min intertrial intervals. Williamson and Wright (2013) found a 3.5 fold increase in PER response in control as compared to pesticide-treated bees (70% vs. 20%) by the 2nd learning trial. In our SER experiment, control-treated bees exposed showed a 3.5 fold increase in SER response (5.25% vs. 1.5%) by the 2nd trial (average of geraniol and thymol punished odors) as compared to the pesticide-treated bees. The PER response rate of control bees in trials 3-6 was approximately 1.2 fold greater (80% vs. 65%) than the PER response of pesticide-treated bees. In our SER experiment, control-treated bees exposed showed an 8.4 fold increase in SER response (16.7% vs. 2%) in trials 3-6 (average of both punished odors) as compared to the pesticide-treated bee

Imidacloprid also impairs longer-term memory. Williamson and Wright showed control bees had 3 fold better longer-term memory (after 24 hrs) than bees chronically exposed to 100 nM imidacloprid over 4 days (60% vs. 20%). In contrast, we tested longer-term memory only after 1 hr, but likewise found that control bees had a 7 fold higher SER response (14% vs 2%) compared to pesticide-treated bees.

Summary

Our results expand our understanding of the detrimental sublethal effects of neonicotinoids honey bee foraging. Recently, Tan et al. (2014) showed that bees foraging on nectar with imidacloprid (40 µg/L=34 ppb) showed no aversion to a hornet predator that would normally avoid. Thus, in the context of foraging danger, imidacloprid can decrease aversive learning (Fig. 3) and detrimentally affect decision making.

Imidacloprid affects other aspects of foraging. Sublethal doses decrease the ability of bees to return to the nest and decrease overall foraging (Bortolotti et al. 2003). Oral ingestion of thiamethoxam at 3 ng per bee resulted in a 23% decrease of associative learning between a visual landmark and a reward, thereby harming honey bee orientation (Decourtye & Devillers 2010). Neonicotinoids can also generally alter bee mobility by increasing the rate of uncoordinated movements, falling, tremors, and hyperactivity (Lambin et al. 2001; Nauen et al.2001; Suchail et al. 2001; Medrzycki et al. 2003; Colin et al. 2004). Subsequent adult learning can also be impaired in bees exposed to imidacloprid as larvae to a very small dose of 0.4 ng/larva (Yang et al. 2012).

It would be beneficial for future studies to test the seasonal effects of imidacloprid on aversive learning. Honey bees are typically more resistant to the acute and chronic effects of imidacloprid on rewarded learning in the winter than during the summer (Decourtye et al. 2003). In addition, it would be useful to study the sublethal effects of other neonicotinoids such as clothianidin and acetamiprid on bee learning because these compounds are not banned by the European Union (Gross 2013), and therefore may become more widely used. Finally, a better understanding of the exact neural mechanisms underlying how neonicotinoids retard bee learning would be valuable.

Figures

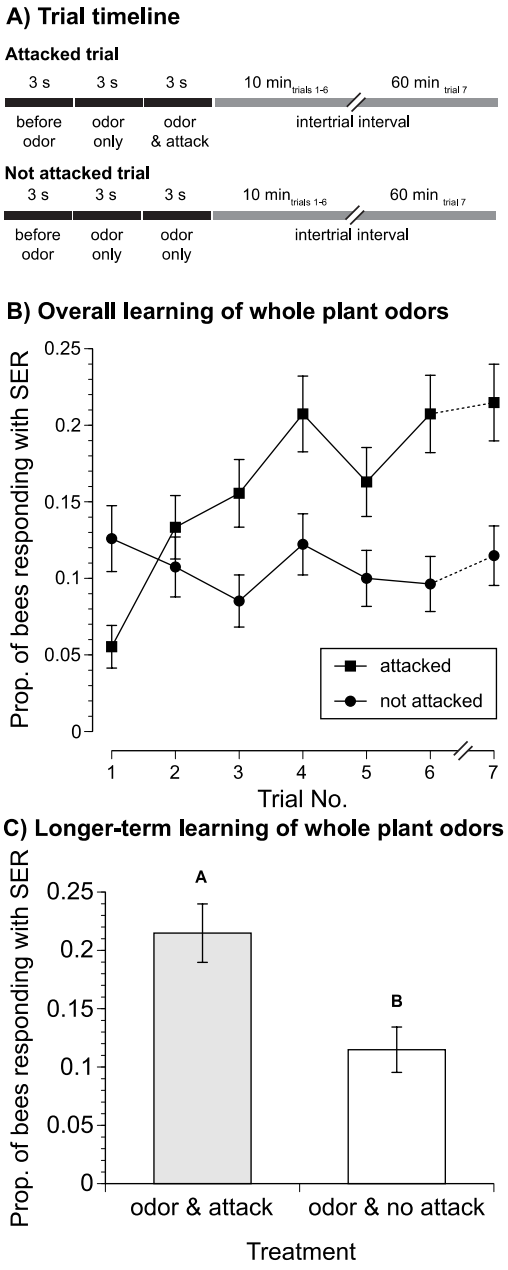

Figure 1. Honey bees can learn to associate whole plant odors with attack. a) The timeline of each trial type is shown (bars are not to scale). In the SER results, data are pooled from both plant chemotypes because there is no significant effect of chemotype on learning. Means with standard error bars are shown. b) Overall learning (10 min intervals between trials 1-6) including the 7th trial (1 hr interval), which tests longer-term learning. This time break is shown by the dashed lines and the hatch marks on the x-axis. c) Longer-term learning showing the 7th trial only.

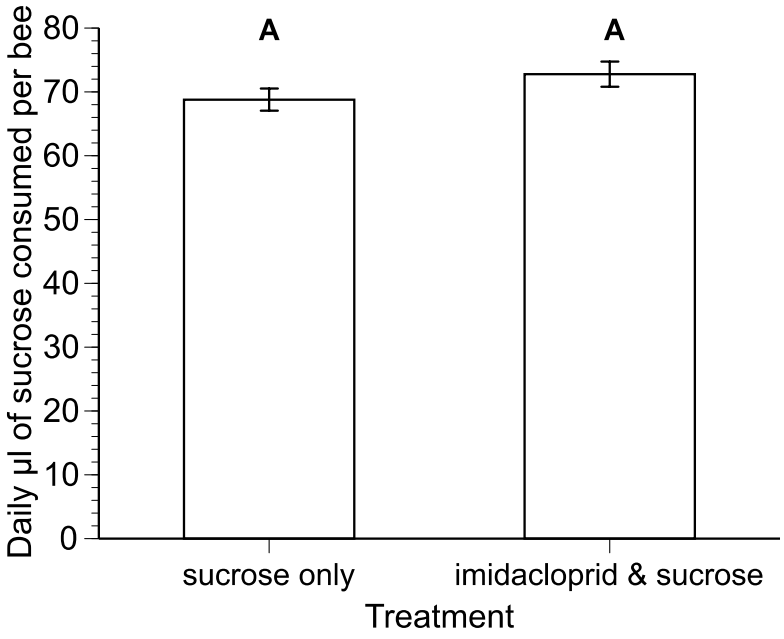

Figure 2. No effect of 100 mM imidacloprid on caged bee consumption of 1.8 M sucrose solution. On average, imidacloprid-treated bees consumed 1.9±0.5 ng imidacloprid per day (total of 7.5±2.2 ng over four days). Bees that only received sucrose (controls) consumed no imidacloprid. The letters show that consumption is not significantly different between treatments. Standard error bars are shown.

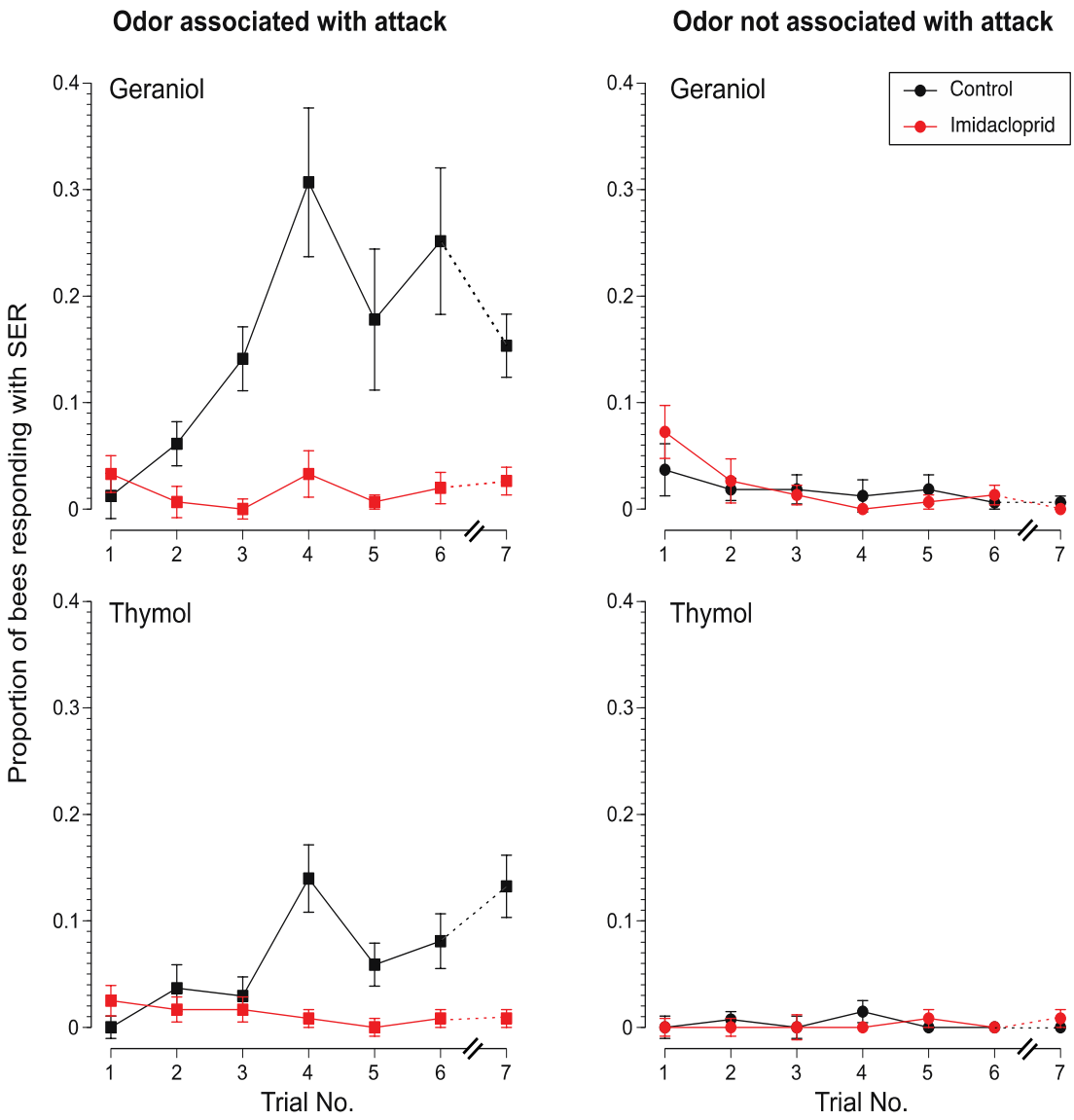

Figure 3. Effect of imidacloprid on overall learning. Bees learned to associate geraniol and thymol with attacks, but to different degrees. Thus, data for each odor is type is shown separately. Means with standard error bars are shown. For each trial type, there is a 10 min intertrial interval for trials 1-6, but the 7th trial (testing longer-term learning) occurs 1 hr after the 6th trial. This time break is shown by the dashed lines and the hatch marks on the x-axis of each plot.

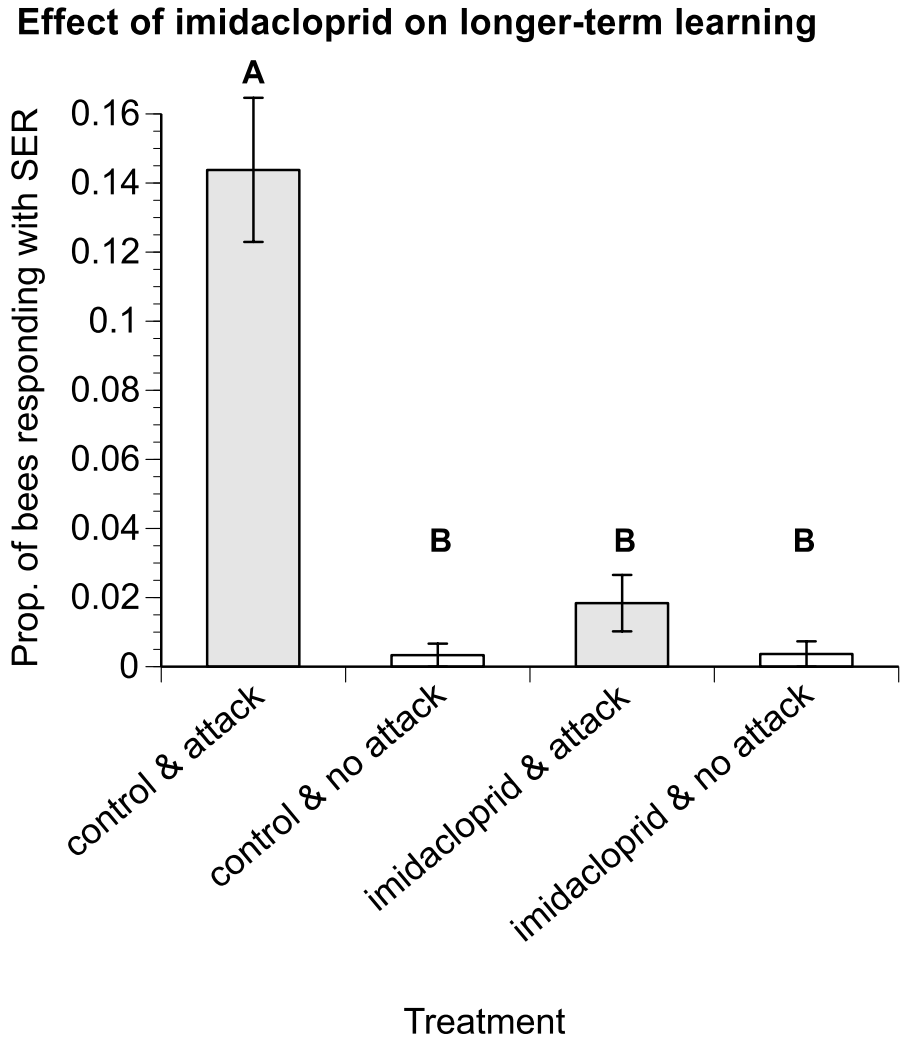

Figure 4. Observed SER responses for bees under different treatment types in different trial types. Data from the different odors are pooled because bees were able to learn to associate attacks with geraniol or thymol. Different letters indicate significant differences. Standard error bars are shown.

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