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RESEARCH ARTICLE

The neonicotinoid imidacloprid impairs honey bee aversive learning of simulated predation

Erica Zhang* and James C. Nieh

ABSTRACT

Neonicotinoid insecticides can impair bee learning and memory – cognitive features that play a key role in colony fitness because they facilitate foraging. For example, the commonly used neonicotinoid imidacloprid reduces honey bee olfactory learning. However, no studies have previously determined whether imidacloprid can impair aversive associative learning, although such learning should enhance bee survival by allowing bees to avoid dangerous foraging sites. To mimic attempted predation of foragers, we developed an electro-mechanical predator that consistently attacked foragers with a pinching bite at a fixed force and elicited aversive olfactory learning in a sting extension response (SER) assay. We show that chronic exposure to a sublethal concentration of imidacloprid (25.6 µg l^{-1}=20.8 ppb) over 4 days (mean of 1.5 µg per bee day^{-1}), significantly impaired aversive short-term learning and memory retention. Imidacloprid treatment reduced short-term learning by 87% and memory retention by 85% in comparison with control bees. Imidacloprid therefore impairs the ability of honey bees to associate a naturalistic predation stimulus – biting – with floral odor compounds. Such learning should enhance bee survival, suggesting that xenobiotics could alter more complex ecological interactions such as predator–prey relationships.

KEY WORDS: Sting extension response, SER, Olfactory learning, Classical conditioning, Pesticides, Xenobiotic, *Apis mellifera*, Bee health

INTRODUCTION

Honey bees (*Apis mellifera*) provide valuable pollination services to commercial crops (Klatt et al., 2013; Klein et al., 2007) and make important contributions to human nutrition (Chaplin-Kramer et al., 2014; Ellis et al., 2015). However, the number of managed honey bee colonies has declined in Europe and the United States (Dainat et al., 2012a; vanEngelsdorp et al., 2011) because of multiple factors (Dainat et al., 2012b; van Lexmond et al., 2015). Recently, attention has focused on neonicotinoid pesticides like imidacloprid, which can impair individual and colony fitness at even sublethal doses (Desneux et al., 2007; Goulson, 2013; Henry et al., 2012; Sanchez-Bayo, 2014). Imidacloprid is a systemic insecticide that spreads throughout all plant tissues and is found in nectar and pollen at concentrations up to 50 ppb (Goulson, 2013). Imidacloprid can also be found in water: 8% of samples from diverse sites contained 7–131 ppm (Johnson and Pettis, 2014). In the United States, imidacloprid residues are widespread in pollen sampled from honey bee hives (Krupke et al., 2012; Mullin et al., 2010). Imidacloprid can have delayed toxic effects in bees, particularly in cases of chronic exposure (Rondeau et al., 2014). In addition, the degradation products of imidacloprid from insect metabolism and environmental decay are also toxic to bees (Goulson, 2013). Imidacloprid and its byproducts can linger in the soil and subsequently be incorporated in plants that are not treated, initiating a new cycle of pollinator exposure (Goulson, 2013; Sanchez-Bayo, 2014).

Imidacloprid is insecticidal because it is an agonist of insect nicotinic acetylcholine receptors (nAChRs) (Gauthier, 2010), which are found in honey bees (Jones et al., 2006) and play a role in bee learning (Dacher and Gauthier, 2008). Honey bee nAChRs play an important role in cholinergic neural signaling, and bees fed sublethal doses of imidacloprid downregulate nAChRs in their brains (Zhou et al., 2014). Not surprisingly, imidacloprid has a wide variety of effects: brain cell death (Wu et al., 2014), decreased food uptake (Ramirez-Romero et al., 2005), reduced motor function (Lambin et al., 2001; Williamson et al., 2014), diminished hive entrance activity (Decourtye et al., 2004), impaired foraging (Schneider et al., 2012), impaired visual learning (Han et al., 2010), decreased predator avoidance (Tan et al., 2014), impaired navigation to the nest (Fischer et al., 2014; Henry et al., 2012) and deficient rewarded olfactory learning (Cresswell, 2011; Williamson and Wright, 2013; Yang et al., 2012).

Olfactory learning is crucial because foragers learn to associate floral odors with rewarding nectar. Olfactory learning therefore facilitates foraging (Giurfa and Sandoz, 2012) and floral constancy, which is important for efficient pollination (Wright and Schiestl, 2009). However, sublethal doses of imidacloprid (Decourtye et al., 2004; Tan et al., 2015; Williamson and Wright, 2013; Williamson et al., 2013; Yang et al., 2012) or a primary metabolic byproduct (Decourtye et al., 2003) significantly impair short-term and longer-term rewarded olfactory learning. Synergistic effects increase this problem. For example, cellular mechanisms for detoxifying neonicotinoids are also impaired by an acaricide commonly used to kill *Varroa* mites (Johnson et al., 2009). Combined exposure to sublethal doses of these compounds can therefore significantly reduce olfactory learning (Williamson et al., 2013).

Somewhat surprisingly, no studies have previously examined the effect of neonicotinoids on aversive appetitive learning. Aversive learning is important because learning to avoid predators should enhance bee survival. Only 9–11% of spider attacks on bees at flowers end in successful predation (Dukas and Morse, 2003; Morse, 1986). An attacked bee therefore has a high probability of surviving and should benefit from learning to avoid a dangerous resource.

Honey bee aversive olfactory learning studies (Carcaud et al., 2009) typically examine the sting extension reflex (SER), a defensive reflex of a bee extending its stinger in response to a noxious stimulus. SER studies have made important contributions

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to our understanding of bee aversive learning (Tedjakumala et al., 2014; Vergoz et al., 2007), but all prior SER studies have used electric shock as the aversive stimulus (Tedjakumala and Giurfa, 2013). Electric shock is a reliably aversive stimulus, but it does not have a direct natural analog. The extremely weak electric field that bees may sense when entering some flowers does not provide a shock or elicit SER. In fact, it can be associated with food reward, eliciting proboscis extension (Clarke et al., 2013).

Electric shock may elicit a strong sensory neural response that is partially similar to a predator's bite. Is it possible to simulate a predator bite to provide a more natural aversive stimulus to study appetitive learning? Predators such as crab spiders often bite bees when attacking them on flowers (Dukas and Morse, 2003; Morse and Nowogrodzki, 1990; Reader et al., 2006). Not surprisingly, bees can learn to avoid food patches with such predators (Dukas and Morse, 2003) and imidacloprid can impair the ability of honey bees (*Apis cerana*) to avoid predators (Tan et al., 2014). However, it is unclear whether imidacloprid affects honey bee aversive learning. We therefore tested if a sublethal dose of imidacloprid could impair olfactory learning and developed an electro-mechanical 'robo-predator' that approximates a natural predator attack.

MATERIALS AND METHODS

Pesticide concentration

Oral or topical application of imidacloprid can impair honey bee learning (Dai et al., 2013). We simulated bee oral exposure to imidacloprid in nectar over 4 days of foraging, following Williamson and Wright (2013) who chronically fed bees 10 nmol l^{-1}, 100 nmol l^{-1}, or 1000 nmol l^{-1} of imidacloprid in 1.0 mol l^{-1} sucrose and then tested rewarded olfactory conditioning (PER). We provided bees with 100 nmol l^{-1} imidacloprid (25.6 µg l^{-1}, Sigma-Aldrich PS2086 analytical standard), but in a 1.8 mol l^{-1} sucrose solution because this higher concentration enhanced survival in our experimental setup. The density of 1.8 mol l^{-1} sucrose solution at room temperature is 1.2296 kg l^{-1} (Bubník et al., 1995), and thus our 100 nmol l^{-1} solution is equivalent to 20.8 µg kg^{-1} (20.8 ppb). This is a sublethal concentration (see Results). Only imidacloprid concentrations ≥1000 nmol l^{-1} increase mortality (Williamson and Wright, 2013).

In bee-pollinated squash plants, imidacloprid occurred at 18 ppb in pumpkin nectar (Dively and Kamel, 2012) and at 10±3 ppb in *Cucurbita pepo* nectar (Stoner and Eitzer, 2012). Soil injection to treat trees resulted in levels of 30–99 ppb in maple and horse chestnut flowers (Scholer and Krischik, 2014). Byrne et al. (2014) measured imidacloprid levels in treated citrus trees grown within an enclosure, and detected residues of 3–39 µg l^{-1} in nectar. Field-realistic concentrations of imidacloprid from a variety of crops and studies are 0.7–10 µg l^{-1} (Cresswell, 2011). This is lower than the 25.6 µg l^{-1} used in our study. 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 Byrne et al. (2014).

Preparing bees

We used 17 *Apis mellifera ligustica* Spinola 1806 colonies at the University of California San Diego Biology Field Station in La Jolla, California, USA. The natural context for our study is predator attacks on foragers, and we therefore tested aversive learning in foragers, who also have stronger aversive learning than younger bees (Roussel et al., 2009). We captured nectar foragers in plastic vials while they fed on unscented 1.8 mol l^{-1} pure sucrose solution (50% sucrose w/w) in grooved plate sucrose feeders (von Frisch, 1967) at the entrance of each colony, allowing us to determine forager colony identity. All pure sucrose solutions consisted of reagent-grade sucrose in double-distilled water and contained no imidacloprid. The captured bees were then divided into two groups (usually five bees per cage) and placed into a ventilated plastic cage (12×8×12 cm) with a 5 ml syringe containing the treatment. We also placed 10 g of pollen in a 1.5 ml tube on the floor of each cage. Bees fed *ad libitum* from the treatments, which were either pure 1.8 mol l^{-1} sucrose solution or 1.8 mol l^{-1} sucrose solution with 100 nmol l^{-1} imidacloprid, each treatment in a separate cage. Cages were placed in an incubator at 30°C and 70% humidity (Medrzycki et al., 2013). To determine consumption, we recorded the initial mass of each sucrose-filled syringe. After 4 days (96 h), we recorded the number of living and dead bees and re-weighed the syringes. We then calculated the average amount of solution (and imidacloprid, as appropriate) ingested by each bee by dividing the mass consumed by the number of living bees. Each syringe had a 2 mm diameter opening through which bees fed. To check for potential evaporation, we incubated 10 filled syringes in 10 cages under identical conditions, but without bees, for 4 days. After 4 days, we measured 1.25±0.03% evaporative loss in weight and corrected our consumption values accordingly. In 11% of our cage trials, mortality exceeded 20% in the control cages. We therefore excluded both control and pesticide-treated cage data from these high-mortality trials. Furthermore, we excluded bees from these trials (0.7% of all bees) from our SER analyses.

We used cold anesthesia to prepare bees for SER testing, placing them for approximately 3 min at 0°C until their movements slowed. To increase the visibility of sting extension, each bee was placed with its thorax facing down (standard SER protocol) (Vergoz et al., 2007) and was held in place with a thin (1.5 mm wide) strip of cloth tape over the ventral side of its thorax on a 3.5-cm-high and 8-mm-diameter stainless steel tube stand. All of the bee's legs and antennae were free. After harnessing, we fed each bee with 0.5 µl of unscented pure 1.8 mol l^{-1} sucrose solution (the only source of food during all testing) and then allowed it to recover in the incubator for 20 min.

Odor apparatus

We presented odors by pumping air (Active Aqua air pump, Hydrofarm Model AAPA25L) through PFTE-lined silicon tubing (6 mm inner diameter) connected to two different odor capsules whose output was controlled via momentary switches activating two separate solenoid valves. We adjusted separate valves to equalize output pressures and used a digital manometer (HT-1890 Digital Manometer) to set the exit airflow at 71±13 mbar. The output tubes were separated by 5 mm from the antennae of the harnessed bee. A fan behind the bee drew odors away.

Pure odorants that occur in the volatiles of flowers visited by honey bees were used – geraniol and thymol (Knudsen et al., 2006). We chose this natural odor pair because two major chemotypes (G and T) of *T. vulgaris* are respectively rich in geraniol and thymol (Linhart et al., 2005) and *T. vulgaris* is 96% pollinated by *A. mellifera* (Assouad et al., 1978). Thymol is sometimes used in relatively large quantities as a miticide (Carayon et al., 2014), but none of our colonies were treated with thymol, and thus bees were not exposed to it before the trials. Half of bees were conditioned with thymol as the punished odor and geraniol as the unpunished odor. For the remaining bees, geraniol was the punished odor and thymol was the unpunished odor. Roussel et al. (2012) also successfully used geraniol for aversive conditioning.

We pipetted 10 µl of pure geraniol (MP Biomedical, cat. no. 157184, lot R2558) or 10 µl of pure thymol (Sigma-Aldrich, cat. no. 89-83-8, lot SZB82760) after heating the thymol to its melting point of 51°C onto a 2.5-cm-diameter filter paper inserted inside a clean plastic capsule connected with tubing between the air input and solenoid-controlled output. We wore latex gloves and used clean tweezers to handle each filter paper, changing these items when handling a new odor to avoid cross contamination.

Testing bee learning

We used differential conditioning (Bitterman et al., 1983) with two trial types, A (punished) and B (not punished) in the order ABBABABAABBA. We followed the recommendations of Giurfa et al. (2009) who demonstrated that optimal aversive SER learning occurs with forwarding conditioning in which the CS (odor) is presented 3 s before the US (aversive stimulus) and is followed by a 10 min intertrial interval. In the punished trials (A), we allowed the bee to adjust to the apparatus for 3 s (before odor), then exposed it to odor A for 3 s, and then punished it (pinching it on its right metathoracic leg at the basitarsus with the robo-predator) while continuing to expose it to odor

Journal of Experimental Biology

Journal of Experimental Biology (2015) 218, 3199-3205 doi:10.1242/jeb.127472

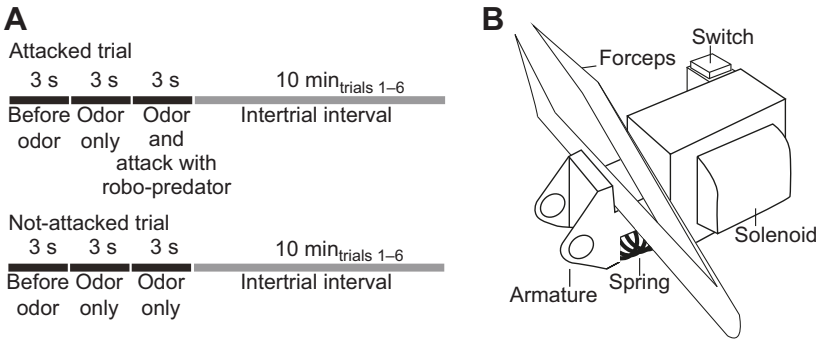

Fig. 1. Design of the learning experiment. (A) The timeline of each trial type (bars are not to scale). The retention tests occur 1 h after the last trial. (B) Details of the robo-predator. The momentary switch allows current to flow through the solenoid, drawing in the armature, which in turn closes the forceps. The spring returns the forceps to the open position when there is no current. Pinching force is proportional to current, which is controlled by a rheostat (not shown) and calibrated (see Materials and methods).

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, defining extension as 0 (no sting visible) or 1 (sting visible). Bees that did not extend their stinger while being pinched were not used (4.9% of 567 bees) and bees that spontaneously extended their stinger before the first learning trial or before odor presentation were also not used (10.9%). Each bee was aversively conditioned over six trials. After the last conditioning trial, we waited 1 h to test memory retention (Carcaud et al., 2009; Vergoz et al., 2007), exposing each bee to the punished odor (A) for 3 s and 10 min later to the unpunished odor (B) for 3 s. This 1 h period corresponds to associative mid-term memory formation (Giurfa and Sandoz, 2012). We tested 10 bees at a time. After the experiment, control-treated bees were unharnessed from the stands, painted with permanent enamel paint on their abdomens, and released into the field. Pesticide-treated bees were frozen and safely discarded.

Robo-predator

To mimic predator biting, we used a custom-modified electro-mechanical CKD AS-0RN solenoid coil (CKD Corp., Komaki, Aichi, Japan) that drew 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 (Fig. 1B). We calibrated this device by using the forceps tips to pinch a 1-mm-wide flat metal bar (matching the width of where we pinched the bee's metathoracic basitarsus) 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 measured sensor voltage with a digital oscilloscope. To determine the 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 vibration calibrator (both items by Brüel and Kjaer, Norcross, GA, 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 scissors lift. Raising the lift applied more force from the vibrational calibrator 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.

The average bite force of a predator attacking a honey bee has not been measured, but the force exerted by leaf-cutting ant mandibles is 10–20 $mN_{pk–pk}$ for soft leaves and 50–100 $mN_{pk–pk}$ for tough leaves (Tautz et al., 1995). Sclerotized insect cuticle, such as a bee leg, is likely to be harder and tougher than most leaves. We therefore set our robo-predator to provide average forces of 150±29 $mN_{pk–pk}$, the minimum force required to elicit sting extension in most (97%) of bees. No bees pinched with this level of force suffered evident harm.

Statistics

We used a chi-square test to test for an effect of treatment upon mortality (using the total number of dead bees in each treatment). To simplify our learning analyses, we separately analyzed bee responses to each odor (geraniol or thymol) and trial type (attacked or not). All of our data met parametric assumptions as determined through residuals analysis. To determine the effect of pesticide on short-term learning, we used repeated-measures analysis of variance (ANOVA), REML algorithm. To analyze the effect of treatment on memory retention, we only compared bee SER at the 7th trial and thus performed ANOVA with attacked odor type, treatment and trial type (attacked or not) as fixed effects. To compare the effects of different treatments, we used *post hoc* Tukey's Honestly Significant Difference (HSD) tests. Colony is a random effect in our analyses. We report mean±1 s.d. Comparison data were extracted from graphs in published papers with Plot Digitizer v2.6.6.

RESULTS

Pesticide effects on total mortality and sucrose consumption

We chronically exposed foragers (bees captured while foraging on sucrose solution) to imidacloprid (20.79 ppb imidacloprid in 1.8 mol l^{-1} sucrose solution) in incubated cages over 4 days. Our imidacloprid treatment was sublethal because it did not significantly increase mortality (χ^2_1=0.11, P=0.73): 9 and 11% of control and imidacloprid-treated foragers, respectively, died after 4 days. There was no effect of pesticide treatment on sucrose consumption ($F_{1,123}$=1.08, P=0.30). Colony accounted for 17.3% of model variance. On average, imidacloprid-treated bees consumed 1.5±0.4 ng imidacloprid per day (total of 6.0±1.5 ng over 4 days).

Pesticide effects on short-term learning

In total, we tested SER learning in 299 control bees and 268 pesticide-treated bees from 17 colonies. Pesticide decreased bee learning of both attack odors, but to different degrees. Thus, there was a significant effect of attack odor ($F_{1,84}$=12.91, P=0.0006, Fig. 2A,B). We therefore divided our analyses of short-term learning by odor. With geraniol as the attack odor, there was a significant effect of treatment ($F_{1,297}$=37.99, P<0.0001), trial number ($F_{1,1862}$=9.51, P=0.0002) and the interaction treatment×trial number ($F_{1,1862}$=8.91, P=0.003) because pesticide-treated bees did not exhibit short-term learning (Fig. 2A). Colony accounted for <0.1% of model variance. At the 6th learning trial, control bees exhibited 9.3-fold greater levels of learning compared with pesticide-treated bees.

As expected, bees did not learn the non-attack odor (Fig. 2C). There was no significant effect of treatment ($F_{1,296}$=0.19, P=0.66) or the interaction treatment×trial number ($F_{1,1867}$=1.92, P=0.17). However, there was a significant effect of trial number ($F_{1,1867}$=13.66, P=0.0002) because there was a slight decrease in SER response over time (Fig. 2C). Colony accounted for 2.1% of model variance.

With thymol as the attack odor, there was also a significant effect of treatment ($F_{1,251}$=19.18, P<0.0001), trial number ($F_{1,1541}$=10.24, P=0.001) and the interaction treatment×trial number ($F_{1,1541}$=21.05, P<0.0001) because pesticide-treated bees did not exhibit short-term learning (Fig. 2B). Colony

Journal of Experimental Biology (2015) 218, 3199-3205 doi:10.1242/jeb.127472

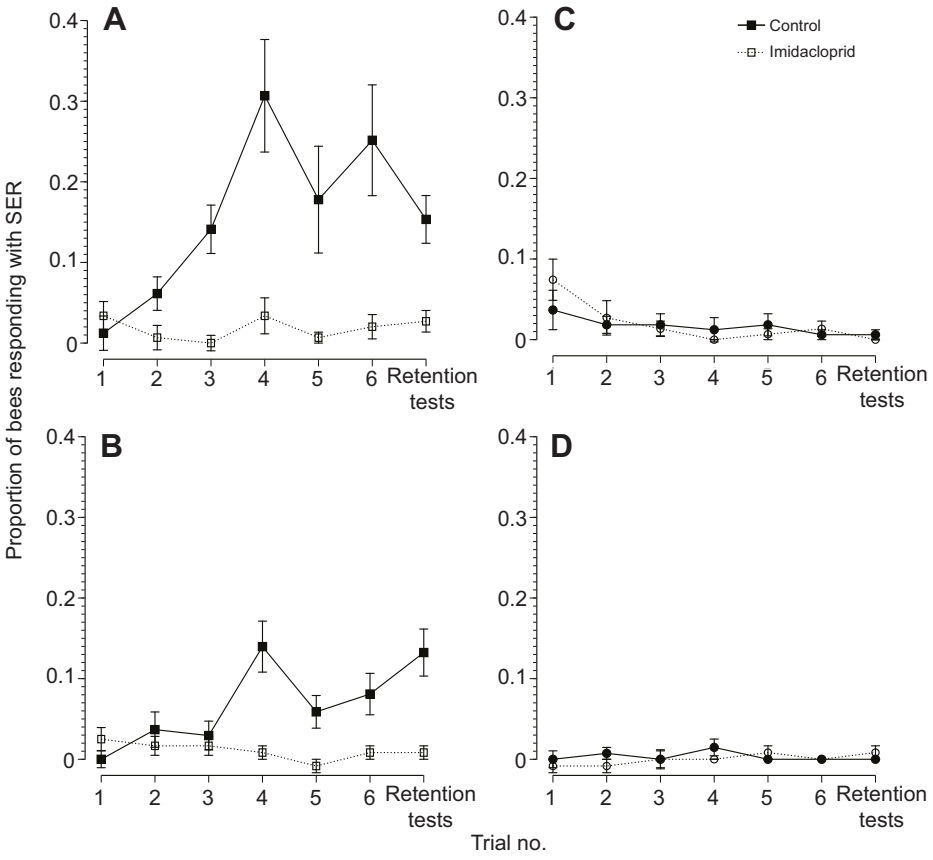

Fig. 2. Effect of imidacloprid on learning in the honey bee *Apis mellifera ligustica*. Bees learned to associate (A) geraniol and (B) thymol with attacks, but to different degrees. Thus, data for each odor type is shown separately. (C,D) Bees did not exhibit SER to odors not associated with attack. Means with standard error bars are shown.

accounted for less than 0.1% of model variance. At the 6th learning trial, control bees exhibited 9.7-fold greater learning than pesticide-treated bees.

Bees did not learn the non-attack odor (Fig. 2D). There was no significant effect of treatment ($F_{1,206}$=0.79, P=0.37), trial number ($F_{1,1511}$=1.11, P=0.29) or the interaction treatment×trial number ($F_{1,1511}$=2.46, P=0.12). Colony accounted for 0.1% of model variance.

Pesticide effects on memory retention

In the memory retention tests (Fig. 3), there was no significant effect of attack odor type on SER learning ($F_{1,196}$=0.70, P=0.40). We therefore pooled the data from both attack odor types for subsequent analyses. There is a significant effect of treatment ($F_{1,1128}$=25.89, P<0.0001), trial type ($F_{1,1117}$=42.64, P<0.0001) and the interaction treatment×trial type ($F_{1,1117}$=27.83, P<0.0001) because pesticide-treated bees did not learn to associate odor with attack (Fig. 3). Only control bees learned to associate odor with attack and exhibited memory retention (Tukey HSD, P<0.05, Fig. 3). Colony accounted for 1.4% of model variance. On average, control bees exhibited 7.7-fold greater levels of memory retention than pesticide-treated bees.

DISCUSSION

We provide the first evidence that the neonicotinoid, imidacloprid, can significantly impair honey bee aversive learning. Our concentration of imidacloprid (20.8 ppb=100 nmol l^{-1}) was sublethal because chronic exposure over 4 days did not alter mortality. Our mortality for control (9%) and imidacloprid-fed (11%) bees after 4 days is also within standard levels (Williams et al., 2013). There was no significant difference in the amount of sucrose and imidacloprid–sucrose solution ingested by bees over the course of 4 days. However, imidacloprid significantly impaired negative short-term learning and memory retention (corresponding

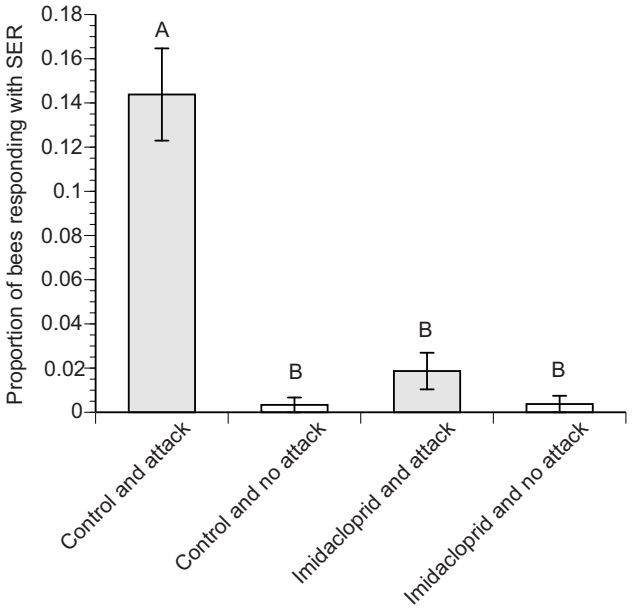

Fig. 3. Effect of imidacloprid on memory retention in the honey bee *Apis mellifera ligustica*. Data from both geraniol and thymol are pooled because there was no significant effect of punished odor type on memory retention. Different letters indicate significant differences (Tukey's HSD tests, P<0.05). Standard error bars are shown.

to mid-term memory). Control bees increased learning over multiple trials, but imidacloprid-treated bees did not. Imidacloprid decreased short-term learning by an average of 87.3% at the 6th trial and memory retention by an average of 85% compared with control bees.

Foragers exhibited no significant difference in memory retention for either conditioned odor. However, they showed higher olfactory learning acquisition for geraniol than thymol (Fig. 2). We used thymol because it is a dominant olfactory component of a common chemotype of *Thymus vulgaris*, a plant that honey bees readily visit (Assouad et al., 1978). A high dose of thymol (10 or 100 ng bee^{-1} applied topically) can reduce olfactory memory retention, but not learning acquisition (Bonnafé et al., 2014). In contrast, we directed a low-concentration thymol odor stream at bee antennae for only 6 s per trial. Early adult exposure to certain odors can decrease subsequent proboscis extension response (PER) learning (Sandoz et al., 2000). However, our colonies were never treated with thymol. The reason for the observed odor-specific learning acquisition difference is thus unclear, but imidacloprid significantly impaired learning acquisition for both odors.

Our biting aversive stimulus elicited weaker SER learning than electric shock, perhaps because it was a weaker aversive stimulus. At the 6th trial, 17% of control bees exhibited SER learning in response to a biting punishment (25% for geraniol and 8% for thymol, Fig. 1C). In contrast, 67% (Bos et al., 2014), 54% (Carcaud et al., 2009), and 44% (Vergoz et al., 2007) of bees exhibited olfactory SER learning at the 6th learning trial when the aversive stimulus was electric shock. Thus, electric shock elicited 2- to 8-fold higher short-term SER learning than our pinching stimulus. The same trend applies to memory retention: 57% (shock punishment) (Vergoz et al., 2007) and 14% (biting punishment) of bees exhibited SER when tested 1 h after the 6th learning trial. Thus, 8-times more bees learned the electric shock as compared with the biting punishment. In general, stronger stimuli are more easily learned (Menzel et al., 1993), and aversive learning may be influenced by bee perceptions of the stimulus (Roussel et al., 2009).

No prior studies have tested the effect of pesticides on honey bee aversive olfactory learning, but we can compare our results with data on how imidacloprid impairs rewarded olfactory PER learning. Imidacloprid reduced PER learning when fed to bees at concentrations of 14.8–29.5 µg l^{-1} (Decourtye et al., 2003, 2004) and 25.6 µg l^{-1} (Williamson and Wright, 2013) (values converted to µg l^{-1}). We chronically exposed foragers to 25.6 µg l^{-1}. Decourtye et al. (2004) chronically exposed workers to imidacloprid (29.5 µg l^{-1}) over 9 days and showed that bees had 2-fold higher PER (at the 2nd learning trial) before they were treated with pesticide. Our experimental conditions are closest to Williamson and Wright (2013), who also chronically exposed workers for 4 days to the same concentration of imidacloprid as our study. Their control bees had 1.3-fold higher short-term PER than pesticide-treated bees at the 6th learning trial and 2.4-fold better retention when tested 24 h later. In contrast, our control bees had 8-fold better short-term SER learning than pesticide-treated bees (Fig. 2A,B) and 7-fold higher retention 1 h later (Fig. 3). Imidacloprid may therefore have stronger effect on SER than on PER learning, but testing this hypothesis will require an experiment in which identically treated bees from the same colonies are kept under the same conditions and then tested for either SER or PER learning.

It is unclear how imidacloprid impairs aversive learning. Dopamine signaling is involved in aversive olfactory learning (Vergoz et al., 2007). Imidacloprid may suppress aversive learning by blocking nicotinic acetylcholine receptors. However, given that imidacloprid also impairs PER and a wide variety of other behaviors (Blacquière et al., 2012), aversive learning impairment may result from memory retrieval deficits (Gauthier, 2010) or non-specific cognitive deficits.

Researchers are increasingly concerned about the ecological effects of neonicotinoids (Goulson, 2013; Sanchez-Bayo, 2014). Our results suggest a need to revise pesticide risk assessment methods because neonicotinoids can affect aversive learning and could thereby influence an important ecological interaction, prey avoidance of predators. Recently, Tan et al. (2014) reported that honey bees (*A. cerana*) foraging on sucrose solution with imidacloprid showed no aversion to a hornet predator they would normally avoid. These researchers did not examine olfactory aversive learning, and thus our results provide a potential mechanism for their results: learning to avoid odors associated with predators can be impaired by imidacloprid. It is unclear if increased risk-taking and decreased aversive learning by bees actually increases their mortality from predation. However, memories of danger and normal predator avoidance behavior should enhance bee survival. This potential effect of neonicotinoids on predator–prey relationships provides a new research direction into how xenobiotics can affect food webs.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.Z. and J.C.N. designed the experiments. E.Z. conducted the study. E.Z. and J.C.N. analyzed the data and wrote the paper.

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