UNIVERSITY OF CALIFORNIA, SAN DIEGO

Effects of imidacloprid on the ability of honey bee foragers to distinguish safe and unsafe food sources

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in

Biology

by

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Abstract of the Thesis

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Pesticides can harm honey bee health and foraging behavior, even when bees are exposed to sublethal doses. We tested the effect of imidacloprid, a common neonicotinoid pesticide, on honey bee avoidance of danger during foraging in indoor and outdoor feeder choice trials. Danger stimuli consisted of honey bee sting gland alarm pheromone or live praying mantises. In outdoor foraging experiments in which bees chose between two feeders, bees fed repeatedly upon sucrose solution containing a high but field-realistic concentration of 40 µg/L imidacloprid. However, there was no effect of pesticide treatment. Neither pesticide nor control bees demonstrated significant avoidance of the live mantis ($P \geq 0.63$ for non pesticide bees and $P \geq 0.42$ for pesticide bees). In contrast, control bees avoided the alarm pheromone feeder in all three consecutive choices ($P \leq 0.01$). Pesticide-treated bees avoided the alarm pheromone feeder only on their second choice ($P = 0.004$). There was no significant

overall effect of pesticide treatment in this experiment. In indoor trials designed to mimic outdoor foraging under controlled conditions, bees exposed to 1 ng or 2.16 ng of imidacloprid spent 16-26% less time on the side of the indoor arena with alarm pheromone as compared to control bees. These trials possibly presented a different context: bees attempting to escape rather than forage. Overall, our results do not provide strong support for the hypothesis that imidacloprid alters bee decision-making about danger in the context of foraging, though the indoor trials suggest a new avenue of investigation into the effects of imidacloprid on honey bee escape behavior.

Introduction

The Food and Agriculture Organization of the United Nations reports that 46 of the top 100 crops used for human consumption depend on insect pollination. Insects such as moths, butterflies, flies, bees, and wasps provide annual pollination services valued at $681 billion (€625 billion) (Gallai *et al.* 2009). Honey bees (*Apis mellifera*), are particularly important because their colonies are transported around the country to pollinate crops (vanEngelsdorp *et al.* 2008; 2010).

Wild and managed honey bee colony populations have been in decline for several decades (Potts *et al.* 2010). In the United States, the number of managed colonies has declined from about 6 million in 1947 to about 2.5 million in 2008, and the population of honey bee colonies is not growing quickly enough to meet global agricultural demands (vanEngelsdorp *et al.* 2010). Although honey bee colony declines, sometimes referred to as colony collapse disorder (CCD) but now more typically discussed as poor colony health, are the result of multiple factors such as disease, parasites, and management practices, the global increase of neonicotinoid insecticide usage since the early 1990s is believed to play a role in reducing honey bee health (Kollmeyer *et al.* 1999; Goulson *et al.* 2013).

Neonicotinoids are popular insecticides because they bind selectively to insect nicotinic acetylcholine receptors (nAChRs) in their central nervous system (Jeschke *et al.* 2008). Because of this selective binding to insect nAChRs, vertebrates are largely not at risk of being affected if they consume the pesticide. Additionally, due to their high solubility in water, neonicotinoids are absorbed by the plant material and are found in phloem and xylem, the vasculature of the plants (Jeschke *et al.* 2008). This makes neonicotinoids effective against pest insects because any insect imbibing or consuming plant material will be exposed to the insecticide. Because of their high specificity and toxicity to many types of agricultural insect

pests, neonicotinoids are a popular choice for pest management (Jeschke *et al.* 2008; Bass *et al.* 2015). Unfortunately, non-target species, such as bees and other pollinators, are also susceptible to this insecticide (Desneux *et al.* 2007).

Although there have been attempts to reduce the impact of neonicotinoids on honey bee health by carefully timing the applications and reducing the pesticide concentration, these applications still interfere with various nervous system pathways and negatively affect honey bee behavior (European Food Safety Authority 2015). Honey and bumble bees prefer nectar containing neonicotinoids, either thiamethoxam or imidacloprid, compared to nectar alone (Kessler *et al.* 2015). Additionally, honey bees are not able to detect the pesticides via their gustatory, or taste-sensing, neurons, which are involved in detection of toxins (Kessler *et al.* 2015). Even when neonicotinoids are applied at sublethal doses, these doses can reach higher bee-ingested levels because honey bees favor nectar that contains neonicotinoids (Kessler *et al.* 2015). Finally, individual honey bee exposure to sublethal doses of neonicotinoids can weaken colonies because mortality rates increase for bees that are exposed to sublethal levels of imidacloprid and its metabolites over time (Suchail *et al.* 2001).

In addition to physically weakening honey bee colonies, neonicotinoids may affect predator-prey relationships and thereby impact food web dynamics (Tan *et al.* 2014). Predators can have non-consumptive impacts that are as significant as consumptive impacts (Preisser *et al.* 2005). For example, fear of an actual or perceived threat can significantly alter prey behavior (Laundre *et al.* 2010). When bumble bee foragers experienced a simulated attack, they consequently reduced their foraging levels, altered the type of flower they foraged at, or both, depending on the floral reward level of the flower (Jones *et al.* 2011). Even when not attacked, honey bees express avoidance behavior of seemingly dangerous flowers that have dead bees or live predators such as spiders, praying mantises, or hornets (Dukas 2001;

Bray and Nieh. 2014; Tan *et al.* 2014). Interestingly, Asian honey bees (*Apis cerana*) exposed to sucrose containing 40 μg/L of imidacloprid lost the ability to distinguish between food sources with and without predators, suggesting that neonicotinoids may reduce predator avoidance (Tan *et al.* 2014). If neonicotinoids reduce honey bee predator avoidance, bees would continue to frequent dangerous food sources, increase their risk of death, and, potentially, increase the number of predators that are drawn to prey in a particular region (Brown *et al.* 1999).

The effects of imidacloprid upon predator avoidance have primarily been studied on the Asian honey bee (*Apis cerana*) (Tan *et al.* 2014). We therefore tested the effect of imidacloprid upon predator avoidance by *Apis mellifera*, a very widespread species and one widely used for agricultural pollination (Southwick and Southwick Jr. 1992; Calderone 2012). We evaluated the ability of bees to choose between two dangerous locations, one of which was dangerous because it contained a live Chinese praying mantis (*Tenodera sinensis*) and the other, alarm pheromone only. We tested bee choices at paired outdoor feeders. We hypothesized that imidacloprid would reduce forager avoidance of the dangerous feeder, as compared to control bees.

Developing an indoor avoidance assay would be beneficial since it would allow testing of predator avoidance in a highly controlled setting. We therefore also built an enclosed arena in which each bee was given a choice between two arms, one with sting gland alarm pheromone. We hypothesized that control foragers would avoid the alarm pheromone, but that imidacloprid would reduce this avoidance.

Materials and Methods

Study site and colonies

We conducted outdoor trials at the Biology Field Station and indoor trials in the Muir Biology Building, both at University of California, San Diego in California, USA. Outdoor trials were conducted from April-October 2016, while indoor trials were run from January-June 2016. In total, we used bees from nine different colonies.

Sublethal doses

Field realistic levels of imidacloprid vary greatly based on application type, sampling time after application, application site, and plant species. We focused on nectar concentrations of imidacloprid since our experiments involved nectar foragers and they were exposed via sucrose. When imidacloprid is applied as a seed dressing, its concentration in nectar ranges from 0.9 to 8.6 μg/L (EFSA 2012). Soil drenching applications resulted in nectar concentrations of 10 and 18 μg/L for squash and pumpkin respectively (Stoner and Eitzer 2012; Dively and Kamel 2012). Soil injections, another common treatment method, result in some of the highest nectar imidacloprid concentrations, with one study showing 660 μg/L in *Eucalyptus* (Paine *et al.* 2011). In citrus trees treated with imidacloprid and grown within an enclosure, residues of 3–39 μg/L were detected (Byrne *et al.* 2013). The concentration we used, 40 μg/L, is therefore a high but field-realistic concentration.

The acute oral toxicity Lethal Dose LD_{50} (dose that resulted in 50% of bees dying within 24 hours) of imidacloprid was found to be 118.74 ng/bee (Laurino *et al.* 2013), which is more than 50-fold higher than the highest dose we used (2.16 ng/bee) in the indoor trials. Rortais et al. (2005) estimated that bees collecting nectar could receive imidacloprid doses of 1.1–4.3 ng/bee weekly, while the European Food Safety Authority (2012) estimated that bees

foraging on nectar containing 10% sugar would ingest 0.065-0.097 ng of imidacloprid per hour flying and 0.259-1.037 ng of imidacloprid per day. Thus, our lowest dose for the indoor trials is on the high end of field-realistic levels, while the highest dose we used is not a field-realistic level.

Experiment 1: Outdoor foraging trials

Training bees

We trained bees from their colonies to the experiment site using a yellow petri dish with about 5 ml of 2.5 M sucrose placed on a tripod at first directly in front of their colony. We then moved the tripod incrementally to the experimental site, which was about 8-10 meters from any given colony. Once we had trained the bees to the experimental site, we applied a small dot of paint on the thorax to individually identify the bee. We then recorded the color and time the paint was applied, as well as any time that bee returned over the next hour. On days in which we ran a pesticide trial, the 2.5 M sucrose with 40 µg/L of imidacloprid was introduced into the feeder once the bees were trained to the experimental site. We tested bees that returned at least five times during this hour. Any bees that were not painted or did not return at least five times were removed with an aspirator before beginning the trial. After about 50 minutes of exposure to the pesticide solution, we gently captured painted bees that had returned at least five times and set them to the side to prevent interference while we set up the new tripods for the experiment. This capture was designed to provide a controlled number of visits of returning bees, to give us time to clean and set up the feeders between choices, and to insure independent choices by preventing multiple bees from visiting the feeder at the same time.

Testing bees

We used clean tripods with identical white sheets of paper clipped to the top and fresh yellow feeders with about 1 ml of 2.5 M sucrose. Control bees were fed pure sucrose solution without pesticide. Bees treated with imidacloprid were fed 40 μg/L imidacloprid in 2.5 M sucrose solution and therefore were exposed to imidacloprid during training. Control trials were run on separate days from imidacloprid trials.

We replaced the feeder and paper whenever a bee landed on them to remove potential odor marks that could attract foragers. There was at least a one-minute delay between releases. When a bee landed on the control, or safe feeder, we allowed her to feed for three seconds then gently captured her in a clean vial, changed the petri dish feeder and paper as needed, then released the bee at least a minute later. If another bee was in the vicinity, we would wait to release any captured bees to prevent simultaneous visits from multiple bees. This ensured that each bee made an independent choice.

When a bee landed on the experimental, or dangerous feeder, she was attacked either by the live mantis or researcher-operated tweezers, depending on the experiment type. These bees always departed the feeder and thus were not captured before making their next decision.

We recorded the time that each bee made a decision for up to three consecutive choices. Sometimes, bees would not come back the full three times. If a bee did not return in 20 min, we no longer continued to test its subsequent choices and scored it as abandoning the food source. To control for potential site bias, each five minutes we swapped tripod positions. To reduce potential orientation of bees to observers, researchers sat to one side and equidistant from both feeders.

Experiment 1a: Live mantis avoidance

In trials with the live mantis, we used Chinese mantises, *Tenodera sinensis*, that were at least 7th instar. At the start of each trial, one mantis was placed on the experimental tripod and was given the freedom to walk around the tripod and attack and kill bees at will. In a few cases, the mantis attempted to fly away and was recaptured and returned to the tripod. However, in most cases, the mantis remained on the tripod. The control tripod was left empty.

Experiment 1b: Alarm pheromone avoidance

In separate trials, we tested bee avoidance to sting alarm pheromone, because this elicited a very strong aversive response. We dissected the alarm pheromone from bees beforehand by dissecting out the sting glands of captured bees, verifying that the gland was not ruptured (ruptured glands release a strong, characteristic alarm pheromone odor), and placing the sting gland into a clean, sealed glass vial. To each sting gland, we added 100 µl of hexane (Fisher Scientific, cat# H303-4). We left the vial on a shaker overnight at room temperature to extract the pheromone. During an alarm pheromone trial, we placed 100 µl of this alarm pheromone solution (one bee equivalent) onto a filter paper under the feeder on the experimental tripod. On the control tripod, we placed 100 µl of pure hexane (blank control) onto a filter paper under the feeder. In all experiments with alarm pheromone, we replenished the alarm pheromone and the blank control each five minutes because each trial generally lasted for 20 min.

Experiment 2: Indoor trials

The indoor arena was a 45 X 31.5 cm wooden box that was divided into three identical partitions (45 X 10.5 cm) to enable us to test three bees at the same time. The three rectangles were divided by an 8 cm high wall that prevented activity in one rectangle from

being seen by a bee in another. We equally spaced three small plastic cups into each partition. Each cup was connected to the other via a clear 10 cm long arm that allowed the bee to move from the center cup towards the left and right cups, but a mesh screen prevented the bee from entering either side cup. We marked the halfway point on each arm and defined a choice as a bee crossing this halfway point. These choice arenas were evenly and equally illuminated on both sides by a strip of white light emitting diodes (LED, Fig. 1A). The trial was always run in a dark room, and thus the only illumination came from these lights.

At the end of each arm we pipetted a drop of 10 µl of pure 2.0 M sucrose that always contained no pesticide. This sucrose solution provided a food source that was intended to simulate the choices that bees had when choosing between two outdoor food sources. Both cups contained a 1 cm diameter circle of filter paper. Before each trial, we randomly selected which side was the experimental side. The experimental side was always the same for all three partitions in any given trial. The experimental side contained a dissected honey bee sting gland that was crushed at the start of the trial (see above for sting gland dissection) to release the alarm pheromone.

To collect the foragers to be used in the trial, we set out yellow petri dish feeders with 2.5 M sucrose near the opening of the colony then captured the bees that came to feed from the dishes in small vials, then placed them together in small observation cages and left them in an incubator (33°C and 40% humidity) overnight.

Before running the trial, we collected individual bees in small vials from the cage. We fed them 10 µl of 1.5 M sucrose, and then waited an hour before starting the trial. If we ran the trial with imidacloprid, the bees would have been exposed to either 1.0 or 2.16 ng of imidacloprid in the 10µl of 1.5 M sucrose. After 55 minutes, we put the bees into the center cup in the arena, which had dividers inserted until the trial began to prevent the bee from

moving to either arm. We allowed the bees to rest for 5 minutes, and then started the trials once we had crushed the sting gland in some filter paper on the experimental side. We then removed the dividers, observed the bees for 10 minutes, and recorded times they entered and left either side as well as if and for how long they fed from the sucrose at the end of either arm.

Statistics

For the indoor trials, we calculated the proportion of time that each bee spent in each arm, per minute and applied the arcsine square root transform, to normalize the data. We then used a Repeated-Measures Analysis of Covariance (ANCOVA) with bee identity nested within treatment (ordinal variable), time (continuous variable), and the interaction treatment*time. We used a two-tailed non-parametric test, the Wilcoxon Signed-Rank test, to determine if the mean amount of time that a bee spent in the experimental arm differed from 0.5. We used Tukey Honestly Significant Difference (HSD) tests to compare between treatments.

To test bee avoidance of the dangerous feeder in the outdoor experiments, we used a Repeated-Measures Nominal Logistic regression with bee feeder choice (control or experimental) as the dependent variable, bee identity nested within pesticide treatment (nominal variable), choice number (ordinal variable) and the interaction pesticide treatment*choice number. We used Fisher's Exact tests to analyze bee survival and choices.

All statistical analyses were conducted with JMP v13 software. We report mean±1 standard error.

Results

Experiment 1a: Foragers did not avoid the live mantis and there was no effect of pesticide

We tested 38 bees exposed to 40 µg/L of imidacloprid and 45 that were not exposed to pesticide from nine different colonies.

The mantises had a 9.2% success rate at catching and killing bees that landed on the dangerous feeder (including data that had to be excluded from analysis of pesticide versus non pesticide bees). There was no significance of pesticide treatment on honey bee success escaping the mantis if they chose the dangerous feeder (P=0.6588). However, bees did not avoid the mantises in any of the three decisions, including the first, for both control and pesticide bees (Fisher's Exact tests: $P \geq 0.6324$ for control and $P \geq 0.4182$ for pesticide).

There were no significant effects of pesticide treatment (L-R Chi-square=0.0002, 1 df, P=0.996, Fig. 1A), choice number (L-R Chi-square=1.68, 2 df, P=0.43), or the interaction treatment*choice (L-R Chi-square=1.71, 2 df, P=0.43). There was no significant effect of using the vials to capture bees: bees did not switch to a different feeder in a subsequent visit (Fisher's Exact tests, $P \geq 0.05$, Fig. 1A).

Experiment 1b: Foragers avoid honey bee alarm pheromone, but there was a weak effect of pesticide

We tested 43 bees exposed to imidacloprid and 42 that were not exposed to pesticide, all from eight different colonies. Control foragers avoided the alarm pheromone feeder in all three choices (Fisher's Exact test, $P \leq 0.0106$). Pesticide-treated bees only avoided the alarm pheromone during their second choice (Fisher's Exact test, P=0.004). Pesticide-treated bees

did not avoid the alarm pheromone on their first (Fisher's Exact test, P=0.08 or third choices (Fisher's Exact test, *P*=0.13).

However, there were no significant effects of pesticide treatment (L-R Chi-square=0.0002, 1 df, *P*=0.996, Fig. 1B), choice number (L-R Chi-square=1.08, 2 df, *P*=0.58), or the interaction treatment*choice (L-R Chi-square=0.14, 2 df, *P*=0.93, Fig. 1B).

Thus, pesticide-treated bees may have reduced their avoidance of alarm pheromone as compared to control bees, but the effect is not strong.

Experiment 2: Pesticide treated bees are less attracted to alarm pheromone in indoor choice trials

We tested 67 bees from five different colonies, 24 without pesticide (0 ng), 24 with 1 ng imidacloprid per bee, and 19 with 2.16 ng imidacloprid per bee. Bees fed on average for 14.1±2.2 seconds at the sucrose solution provided inside the arms of the choice apparatus. There was no effect of pesticide treatment on feeding duration ($F_{2,67}$=2.35, *P*=0.10).

There was a significant effect of treatment ($F_{2,615}$=9.81, *P*<0.0001), but no significant effects of time ($F_{1,615}$=1.00, *P*=0.32) or the interaction treatment*time ($F_{2,615}$=2.95, *P*=0.05). Although the interaction was close to significant, visual inspection of the data did not reveal strong differences between the different treatments over time.

Somewhat surprisingly, control bees did not avoid the arm with the alarm pheromone, they spent an average of 50±3% of their time in the arm with the alarm pheromone (no significant preference, Wilcoxon Signed-Rank test, *W*=85, *P*=0.93, Fig. 2B). However, bees that received 1 ng or 2.16 ng of imidacloprid spent significantly less time in the arm with alarm pheromone as compared to control bees (Tukey HSD test, *P*<0.05, Fig. 1). Bees that received 1 ng or 2.16 ng of imidacloprid/bee respectively spent 40±3% and 36±3% of their

time in the arm with alarm pheromone (significant preference for the control arm, Wilcoxon Signed-Rank test, $W \geq 3888$, $P < 0.0001$).

Thus, control bees (0 ng/bee) did not avoid the dangerous side with alarm pheromone, but bees that received either pesticide dose significantly avoided the arm with alarm pheromone.

Discussion

We therefore found weak support for the hypothesis that imidacloprid reduces forager avoidance of danger. In the outdoor foraging experiments, control bees avoided the "dangerous" feeder with alarm pheromone in all choices. However, the pesticide-treated bees only avoided the alarm pheromone feeder on their second choice. The repeated-measures analysis showed no significant overall effect of pesticide treatment in the alarm pheromone trials. In the indoor trials, which were designed to simulate a controlled foraging situation, we also found a somewhat surprising result. Control foragers did not avoid the arm with alarm pheromone, but foragers that received an acute dose of either 1 ng/bee or 2.16 ng/bee significantly avoided the alarm pheromone by spending more time in the "safer" arm without alarm pheromone.

Although foragers exhibited strong avoidance of sting alarm pheromone, as expected based upon prior studies, they did not show avoidance of live mantis predators, an unexpected result that may arise from a key experimental difference between our study and a previous one that demonstrated bee avoidance of mantis predators (Bray and Nieh 2014). Our results may differ from those of Bray and Nieh (2014) because we tended to train our bees over a longer period of time. Like Bray and Nieh (2014), we trained bees for a minimum of 5 trips before testing them, but we often trained bees for many more trips and for a longer period of time because the most frequent foragers would continue to return while the less frequent visitors were being trained. Thus, bees may have come to more strongly trust this highly rewarding food source than in Bray and Nieh (2014).

In addition, we wished to document multiple trips because we hypothesized that bees would learn to show even stronger avoidance of the threat over time. However, to do this, we had to allow bees to imbibe a small amount of food reward each time they made a choice.

Otherwise, foragers would not return. Bray and Nieh (2014) captured each bee as soon as it landed, before it could imbibe a reward. This small amount of food reward that each bee received, regardless of which feeder it choose, may have reduced feeder discrimination, at least in the second and third return trips. However, it is true that even in the first trip, bees overall had was no significant preference for the control feeder (P=0.83).

In the outdoor foraging experiments, there were no overall significant effects of pesticide. Unlike control bees, pesticide-treated bees showed no avoidance of alarm pheromone in their first and third choices (Fisher's Exact tests). However, the repeated-measures model that accounted for repeated visits by the same individual found no significant effects of pesticide. It is quite possible that *A. mellifera* responds differently to imidacloprid than *A. cerana* in the context of foraging danger.

It is also possible that our method of capturing bees to space out their choices, although conducted as gently as possible, may have influenced their perception of danger. For example, even a gentle capture may be aversive. However, in both the mantis and alarm pheromone experiments, there was no change in forager preference after the first choice, suggesting that they were not being influenced by the vial captures. Fisher's exact tests showed no evidence of switching ($P \geq 0.05$). Thus, bees were evidently not simply responding to vial capture by visiting a feeder different from the one at which they were captured at.

In the indoor trials, we expected that control bees would spend more time on the "safer" side of the arena without alarm pheromone. This did not occur. Over the 10 min trial, control bees spent, on average, 50% of their time in both sides of the arena. However, there was a significant effect of pesticide in these trials. Bees given an acute dose of imidacloprid exhibited aversion to the alarm pheromone and therefore spent less time in the potentially "dangerous" arm. An important question is the true context of these trials. We tried to set up a

foraging scenario by offering a small drop of sucrose solution in the arm and bees would feed, but they often seemed more intent upon escaping the setup or exploring it than actually foraging. Based upon our results, we speculate that context may play a role in how imidacloprid acts. If the bees were attempting to escape, control bees may have disregarded the danger signal provided by the alarm pheromone and thus spent equal time in arena arms. Imidacloprid-treated bees may have become more risk-averse in this escape context.

It is therefore unclear if neonicotinoids alter predator avoidance. Imidacloprid may alter bee decision-making about risk in *A. cerana* (Tan *et al*. 2014), but we do not have strong evidence that this occurs in *A. mellifera*. More data and experiments are needed. However, it is reasonable to expect that imidacloprid can alter bee decision making. Neonicotinoids target receptors throughout the bee brain and can interfere with communication and learning (Deglise *et al.* 2002; Barbara *et al.* 2008; Eiri *et al.* 2012; Gauthier 2010). We should therefore proceed with caution when applying imidacloprid or other neonicotinoids to crops to limit the adverse effects of these chemicals on honey bees and other non-target species.

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Figures

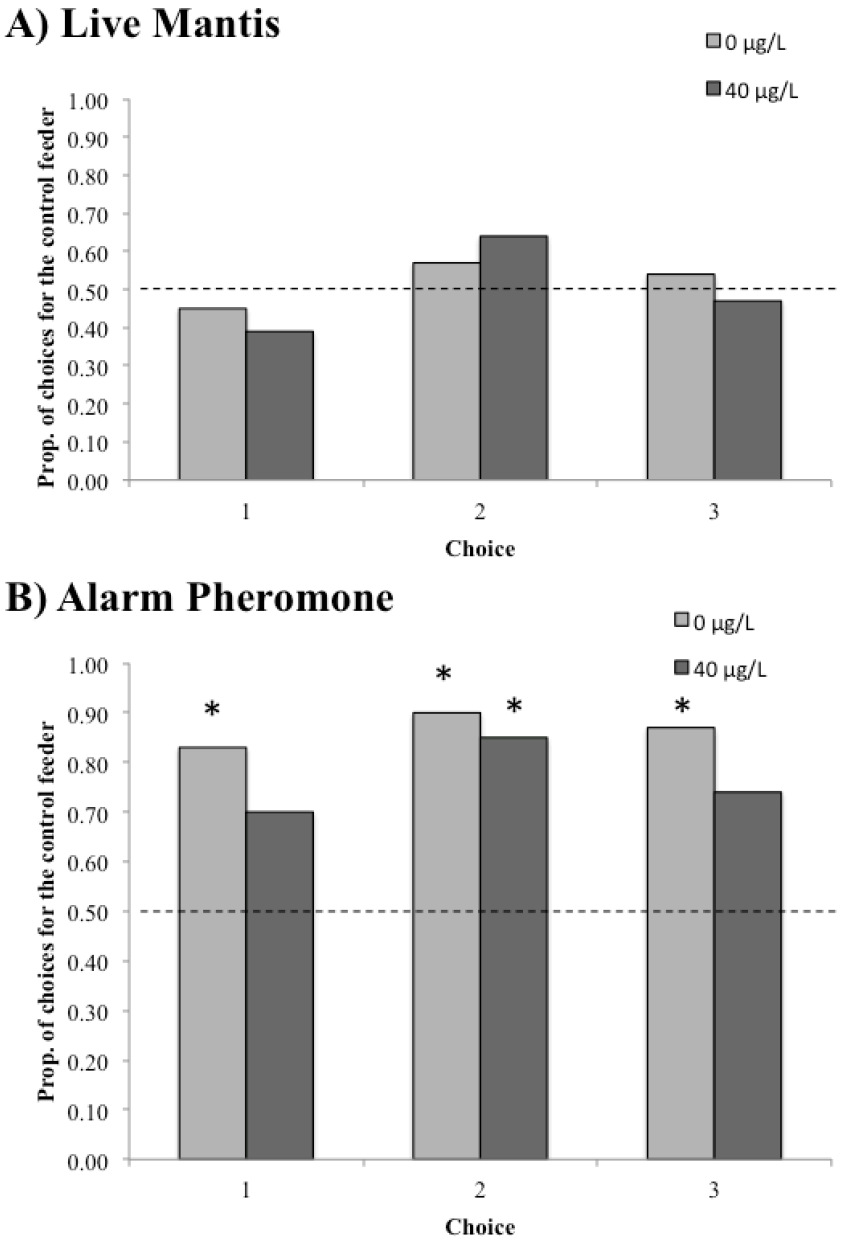

Figure 1. Effect of imidacloprid on honey bee decision making in outdoor trials. A) In the *live mantis* trials, bees did not exhibit any preferences. The dashed line indicates the null hypothesis expectation of no choice. B) Bees in the *alarm pheromone* trials had a much stronger preference for the control feeder than the alarm pheromone feeder. For all choices, control bees (0 µg/L) chose the safe feeder without alarm pheromone (Fisher's Exact test, **P*<0.05). Pesticide-treated bees (40 µg/L) only significantly avoided the dangerous feeder in the 2nd choice (Fisher's Exact test, *P*=0.004).

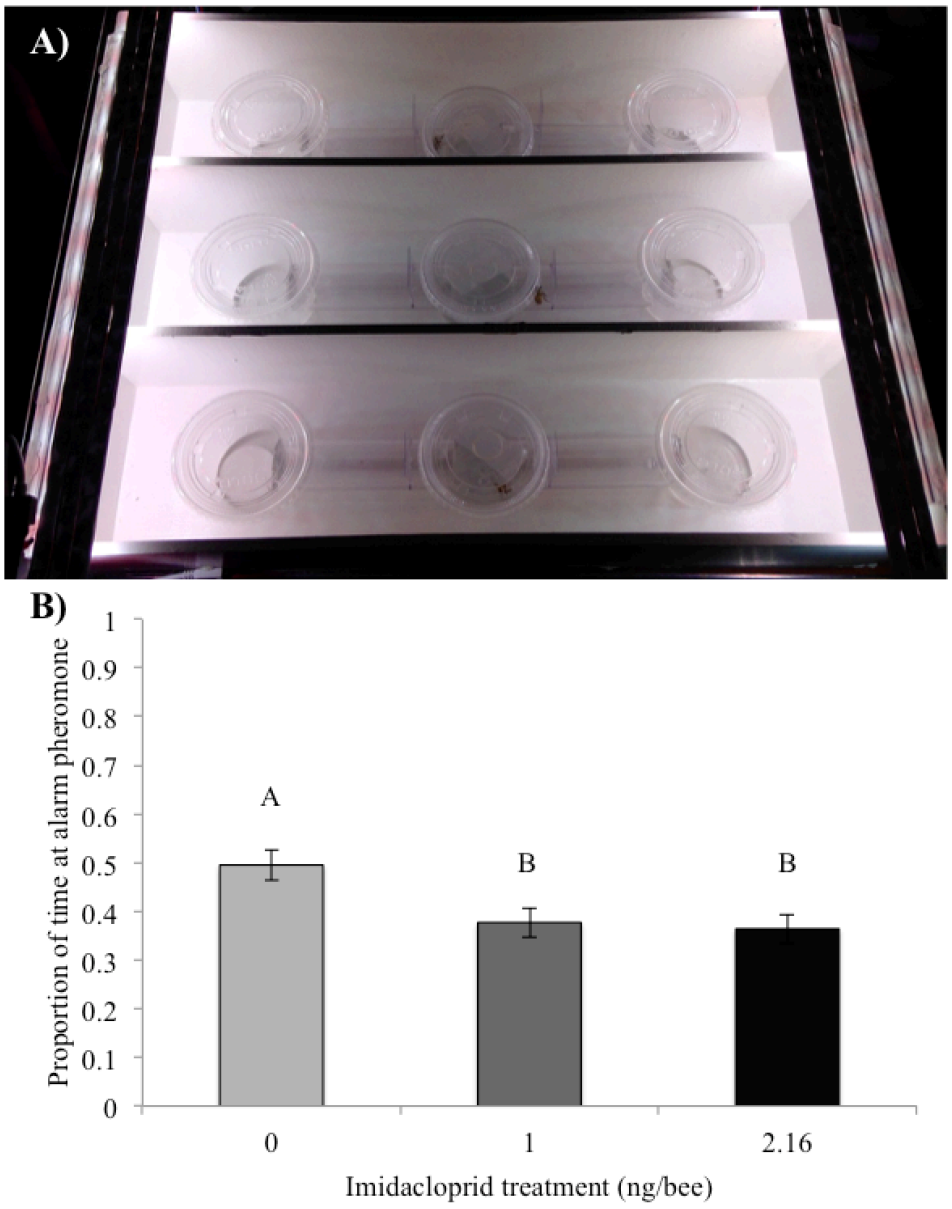

Figure 2. Effect of imidacloprid on bee choices in the indoor trials. (A) Experimental setup for indoor trials with alarm pheromone. Bees are placed in the center cup, and dividers are located in each arm to prevent bee from moving until trial began. (B) Mean proportion of time spent for each pesticide treatment at the alarm pheromone side of the arena over the entire 10 min trial. Different letters indicate significant differences (Tukey HSD test, $P<0.05$).

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